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사람과 가축에서 분리한 Non-Typhoidal *Salmonella* 균주의 Quinolone 저도 내성: Nalidixic Acid 내성검사의 유용성

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Prevalence and Mechanisms of Low Level Quinolone Resistance among Non-Typhoidal *Salmonella* Isolates from Human and poultry/Livestock in Korea: Usefulness of Nalidixic Acid Resistance Test

Background: Non-typhoidal *Salmonella* (NTS) are important commensal microorganisms. We intended to investigate the prevalence and mechanisms of nalidixic acid resistance among NTS isolated from human and poultry/livestock.

Methods: A total of 151 *Salmonella* isolates (36 human and 115 livestock isolates, respectively) was tested for the Minimum inhibitory concentrations (MICs) of nalidixic acid, together with serotyping. As for the nalidixic acid resistant isolates, further studies were taken: MICs of ciprofloxacin, mutation analysis of *gyrA* and *parC* genes, and organic solvent tolerance test.

Results: Eighty-four isolates of 151 human and livestock isolates were resistant to nalidixic acid. The prevalence of nalidixic acid resistance and was 13.9% (5 of 36 isolates) in human isolates and 68.7% (79 of 151 isolates), in the livestock isolates respectively. Among 84 nalidixic acid-resistant isolates, the The prevalence of ciprofloxacin resistance in livestock isolates was 24.1% (1 resistant and 18 intermediate of 79 strains), but no ciprofloxacin resistance was found in 5 human isolates. Among 65 nalidixic acid resistant, ciprofloxacin-susceptible isolates, 3 (60%, of 5 human isolates) and 60 (100%, all livestock isolates) showed low level fluoroquinolone resistance (ciprofloxacin MIC, 0.125-1.0 μg/μL). Six types of point mutations were found in the analysis of DNA sequencing of the *gyrA* gene in the 84 isolates; 75 isolates showed point mutations on amino acid Ser 83 and/or Asp 87. On the other hand, no point mutation was found from the *parC* genes. Forty-seven nalidixic acid resistant isolates showed tolerance to organic solvents.

Conclusions: Nalidixic acid resistance was a good marker of low level fluoroquinolone resistance. As for the severe NTS infection, MIC test for nalidixic acid would be required.

Key Words: Non-typhoidal *Salmonella*, Nalidixic acid, Low level fluoroquinolone resistance

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Introduction

Salmonellosis remains a major public health problem worldwide. Non-typhoidal *Salmonella* (NTS) is a major pathogen of gastroenteritis, and multiple antibiotic resistant strains have been found among specimens from poultry/livestock in recent years [1]. Nowadays, fluoroquinolones are widely used for treating salmonellosis since they have a potent activity against *Salmonella enterica*. In treatment failure cases with fluoroquinolones, however, the ciprofloxacin Minimum inhibitory concentrations (MICs) for these strains were reported to be ≥0.125 μg/mL [2, 3]. Of note, antibiotic-resistant NTS have been increased in the ten years in Spain and other European countries, as well as in the United States of America [4-10].

In Gram-negative bacteria, the principal target of quinolone activity is type II topoisomerase, which is a DNA gyrase [1]. Less frequently, quinolone resistance is associated with point mutations in the type IV topoisomerase. Usually, high-level quinolone resistance has been associated with mutations in the quinolone resistance-determining region (QRDR) of *gyrA* in *Salmonella,* with the most commonly described mutations being Ser-83-to-Phe (TCC→TTC), Ser-83-to-Tyr (TCC→TAC), Asp-87-to-Tyr (GAC→TAC), Asp-87-to-Gly (GAC→GGC) and Asp-87-to-Asn (GAC→AAC) [3, 11-14].

We investigated the prevalence of nalidixic acid resistance among NTS isolates from human and livestock in Korea. We also evaluated the mechanisms of nalidixic acid resistance among them.

Materials and Methods

1. Bacterial strains

A total of 36 human NTS isolates were collected from patients with clinical NTS infection who visited Korea University Guro Hospital between October 2002 and August 2003. All of isolates were the first isolates from patients suffering from salmonellosis. The sources of 36 human NTS isolates by clinical specimens consisted of 32 stool isolates, 1 urine isolate and 3 blood isolates. We obtained 115 livestock NTS isolates from three slaughterhouses and one butcher's shop located in Gyeong-gi province and Gang-won province, Korea between June 2000 and July 2003. The sources of 115 livestock isolates consisted of 112 chicken isolates and 3 pork isolates. NTS livestock isolates were cultured from the flesh and intestine of livestock.

2. Antimicrobial susceptibility testing

Antimicrobial susceptibility test was done with 36 human isolates and 115 livestock isolates against 2 antibiotics (ciprofloxacin and nalidixic acid). Minimal inhibitory concentrations (MIC) of all isolates were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. *Salmonella* of 1×10^4 CFU (colony forming unit) were inoculated at the Mueller-Hinton agar, and the resulting colonies were incubated at 35℃ for 20 hours according to the CLSI guidelines [15]. *Escherichia coli* ATCC 25922 was used for quality control of the test.

To detect nalidixic acid resistant NTS among ciprofloxacin susceptible isolates, we also measured nalidixic acid MICs according to CLSI guidelines [15].

3. Serotyping

Serotyping was done in accordance with the Kauffmann-White scheme, using the slide and tube agglutination method, at the Korea Institute of Health and Enviroment, Seoul, Korea [16].

4. Polymerase chain reaction (PCR) and mutation analysis of *gyrA* and *parC* gene

1) Chromosomal DNA extraction

Two or three colonies from each *Salmonella* isolates having the resistance to nalidixic acid and ciprofloxacin in antimicrobial susceptibility test were scraped from the surface of the agar plates and resuspended in 100 μL of sterile distilled water. The cell suspension was vortexed and heated for 5 min at 100℃ and then was cooled at 4℃ for 10 min to break the cell wall. This was followed by centrifugation at 13,000 rpm for 30 sec. The supernatant containing DNA was used for PCR.

2) PCR for *gyrA* and *parC* gene

We used sal/*gyrA*-F (5′-TAG-AAC-CGA-AGT-TAC-CCT-GA-3′) and sal/*gyrA*-R (5′-AAT-GAC-TGG-AAC-AAA-GCC-TA-3′) as a *gyrA* gene amplification primer (GenBank accession No. NC003197) and the product size was a 166-bp fragment. The QRDR from *parC* was amplified in a 270-bp fragment (Tyr47 to Leu133) with primers; stmparC1 (5′-CTA-TGC-GAT-GTC-AGA-GCT-GG-3′) and stmparC2 (5′-TAA-CAG-CAG-CTC-GGC-GTA-TT-3′) were used as *parC* gene amplification primer [13]. Each PCR mixture (50 μL) consisted of template DNA 100 ng, 10x buffer solution 5 μL, dNTPs (10 mM) 1 μL, Taq polymerase (BIOTOOLS, Madrid, Spain) 0.5 μL, each primer (10 pmol) 0.5 μL and sterile distilled water 40.5 μL. DNA amplification was carried out in GeneAmp PCR System 2700 (Applied Biosystems), with the

following thermal cycling profile; an initial pre-denaturation step of 5 min at 95℃ was followed by 30 cycles of amplification each consisting of denaturation of 60 sec at 95℃, annealing of 60 sec at 49℃ for *gyrA* and 62℃ for *parC* gene, extension of 60 sec at 72℃, ending with a final extension step of 10 min at 72℃. Obtained PCR products were purified by QIAEX II kit (QIAGEN GmbH, Hilden, Germany).

5. Molecular characterization of *gyrA* and *parC*

The PCR products were sequenced in an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and the primers for *gyrA* and *parC* gene synthesis were also used as primers for sequencing. The sequences were analyzed using the BLAST 2.0 (Basic Local Alignment Search Tool) software available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

6. Testing for tolerance to organic solvents

Efflux pump activity was evaluated by an organic solvent tolerance test. Cultures freshly grown in LB broth (Difco Laboratories, Detroit, MI, USA) were diluted into LB broth to 2×10^7 cfu/mL. Five microliters were spotted on to LB plates (LB broth with Bacto-Agar 15 g/L, (Difco Laboratories), allowed to dry, and overlaid to a depth of about 3 mm with hexane (H), cyclohexane (CH) (Aldrich Chemicals, Milwaukee, WI, USA), and H-CH mixtures at ratios of 3:1. Plates were sealed with petroleum jelly and parafilm to prevent evaporation of solvents, and incubated at 37℃. After 24 h, spots were scored for confluent growth. All experiments were done on duplicate plates and performed at least twice. All wild-type strains were inhibited by cyclohexane, while strains with increased organic solvent tolerance grew in the presence of cyclohexane [17, 18].

Results

1. Serotypes of NTS isolates

We identified serotypes of 36 human isolates. The most frequently identified serotype was *Salmonella enterica* subsp. *enterica* serotype *Enteriditis*, accounting for 47.2% (17 of 36 isolates) of all human tested strains followed by *Salmonella enterica* subsp. *enterica* serotype Typhimurium (8 of 36 isolates, 22.2%) (Table 1). As for the 115 livestock isolates, the most frequently identified serotypes were *Salmonella* enterica subsp. enterica serotype Yovokome, accounting for 37.4% (43 of 115 isolates) of all livestock tested isolates followed by serotype *S.* Typhimurium and *S.* Enteritidis (Table 1).

2. Susceptibility of NTS isolates

1) Resistance to nalidixic acid of the isolates

Results of ciprofloxacin and nalidixic acid MIC test were as follows; only one ciprofloxacin resistant isolate was identified among livestock, but 5 (13.9%) isolates among 36 human isolates and 79 (68.7%) isolates among 115 livestock isolates were resistant to nalidixic acid (Table 2).

2) Susceptibility test for the ciprofloxacin among nalidixic acid resistant NTS isolates

Of the 84 isolates which were resistant to nalidixic acid, 1 isolate among 5 human isolates had nalidixic acid MIC value of 32 μg/mL, and the remaining 83 isolates had nalidixic acid MIC of 128 μg/mL or above. Ciprofloxacin MIC values for the isolates resistant to nalidixic acid were between 0.06 μg/mL and 4 μg/mL. According to CLSI guideline, all 5 (100%) human isolates and 60 (75.9%) isolates among 79 livestock isolates were susceptible to ciprofloxacin with MIC value of ≤1 μg/mL; among ciprofloxacin susceptible isolates, 3 (60%) human isolates and 60 (100%) livestock isolates showed low level quinolone resistance (ciprofloxacin MIC range 0.125 to 1.0 μg/mL) (Table 2). Eighteen livestock isolates (22.8%) showed intermediate resistance to ciprofloxacin with MIC value of 2 μg/mL. Only 1 (1.3%) isolate

Table 1. Serotype Distribution of Human and Livestock among Non-Typhoidal *Salmonella* Isolates

Serotypes	No. of human isolate (%)	No. of livestock isolate (%)
Salmonella Enteritidis	17 (47.2)	15 (13.1)
Salmonella Typhimurium	8 (22.2)	16 (13.9)
Salmonella Brandenburg	1 (2.8)	
Salmonella Heidelberg	1 (2.8)	
Salmonella Istanbul	1 (2.8)	
Salmonella Kambole	1 (2.8)	
Salmonella London	1 (2.8)	
Salmonella Montevideo	1 (2.8)	6 (5.2)
Salmonella Newport	1 (2.8)	
Salmonella Ohio	1 (2.8)	
Salmonella Rissen	1 (2.8)	
Salmonella Schwarzengrund	1 (2.8)	
Salmonella Tennessee	1 (2.8)	13 (11.3)
Salmonella Bovismorbificans		3 (2.6)
Salmonella Bsilla		2 (1.7)
Salmonella Kisii		1 (0.9)
Salmonella Salamae		9 (7.8)
Salmonella Virginia		4 (3.5)
Salmonella Yovokome		43 (37.4)
Non-identifed		3 (2.6)
Total	36 (100)	115 (100)

Table 2. Ciprofloxacin MIC of the Nalidixic Acid-Resistant Non-Typhoidal *Salmonella* Isolates

	Human isolates (n=5/36, 13.9%)			Livestock isolates (n=79/115, 68.7%)		
No. of nalidixic acid resistant isolates (n=84)	5			79		
MIC range (μg/mL)	32 - >128			128 - >128		
Classification based on ciprofloxacin	S	I	R	S	I	R
No. (%)	5 (100)	0 (0)	0 (0)	60 (75.9)	18 (22.8)	1 (1.3)
MIC range (μg/mL)	0.06 - 1	-	-	0.125 - 1	2	4
No. of low level quinolone resistance (%)	3 (60)	-	-	60 (100)	-	-

MIC, Minimal inhibitory concentration; S, Susceptible; I, Intermediate; R, Resistant
Ciprofloxacin MIC value: Susceptible (≤1 μg/mL), Intermediate (2 μg/mL), Resistance (≥4 μg/mL).
Nalidixic acid MIC value: Susceptible (≤16 μg/mL), Intermediate (-), Resistance (≥32 μg/mL).
Low level quinolone resistance MIC range 0.125 to 1.0 μg/mL.

Table 3. Sequence of a Portion of QRDR in *gyrA* and *parC* of the Nalidixic Acid-Resistant Non-Typhoidal *Salmonella*

gyrA mutation		*parC* mutation	No. of isolates (%)	MIC range (μg/mL)	
Codon 83	Codon 87	Codon 57, 66, 80		NA	CIP
Ser83 (TCG) → Phe (TTC)	Wild type	Wild type	59 (70.2)	128 - >128	0.125 - 4
Ser83 (TCG) → Phe (TTC or TAC)	Wild type	Wild type	5 (6.0)	>128	0.5 - 2
Ser83 (TCG) → Phe (TTC or TAC)	Asp87 (GAC) → Glu (GAA or GAG)	Wild type	6 (7.1)	128 - >128	0.25 - 2
Ser83 (TCG) → Phe (TTC)	Asp87 (GAC) → Arg (CGA)	Wild type	1 (1.2)	>128	2
Ser83 (TCG) → Phe (TTC)	Asp87 (GAC) → Tyr (TAC)	Wild type	2 (2.4)	128	1
Ser83 (TCG) → ?	Wild type	Wild type.	1 (1.2)	128	1
Wild type	Asp87 (GAC) → Asn (AAC)	Wild type	1 (1.2)	>128	0.06
Wild type	Wild type	Wild type.	9 (10.7)	32 - >128	0.06 - 1

A total of 84 isolates was examined.
CIP, Ciprofloxacin; NA, Nalidixic acid

from livestock isolates showed ciprofloxacin resistance (MIC value ≥4 μg/mL) (Table 2).

3. PCR and mutation analysis of *gyrA* and *parC* gene

PCR of *gyrA* and *parC* genes were performed on a total of 84 nalidixic acid resistant isolates (5 human isolates, 79 livestock isolates) and DNA sequencing of the PCR products was carried out. Of the 84 isolates, DNA sequencing of the *gyrA* and *parC* genes were successful in 83 and 84 isolates. Six types of point mutations were found in the analysis of DNA sequencing of the *gyrA* gene in the 84 isolates. Among them, 74 isolates showed amino acid substitutions: 59 isolates of Ser 83 (TCG) → Phe (TTC), 5 isolates of Ser 83 (TCG) → Phe (TTC or TAC), 6 isolates of Ser 83 (TCG) → Phe (TTC or TAC) and Asp 87 (GAC) → Glu (GAA or GAG), 2 isolates of Ser 83 (TCG) → Phe (TTC) and Asp 87 (GAC) → Tyr (TAC), 1 isolate of Ser 83 (TCG) → Phe (TTC) and Asp 87 (GAC) → Arg (CGA), and 1 isolate of Asp 87 (GAC) → Asn (AAC). In comparison, 9 isolates showed no point mutation on amino acid Ser 83 and/or Asp 87. A and One isolate was confirmed to have no point mutation on *gyrA* amino acid 87, but no confirmation was made at position 83. No point mutation was found in the 84 isolates of *parC* gene (Table 3).

4. Organic solvent test

According to the results of the tolerance test to organic solvents with 84 nalidixic acid resistant isolates, all 5 (100%) human isolates and 42 (53.2%) out of the 79 livestock isolates showed tolerance to organic solvents.

Discussion

NTS usually causes gastroenteritis, which can be improved without antibiotic treatment in most cases. However, antibiotic treatment will be needed when the infection is likely to spread beyond gastrointestinal tracts, or when the cases one immunocompromised patients [19]. Ciprofloxacin is frequently used for the treatment of salmonellosis. However, the incidence of fluoroquinolone resistance or tolerance in *Salmonella* species has increased over the past decade [20, 21]. Moreover, there have been reports of treatments failure with ciprofloxacin, when it showed nalidixic acid resistance [22]. Quinolone resistant NTS obtained through *gyrA* or *gyrB* gene mutations have been reported in Turkey, India, and other areas [23]. In addition, the appearance of 3rd generation cephalosporine resistant NTS also has been reported, along with carbapenem resistant isolates [24, 25]. As

for the status of antibiotic resistance in *Salmonella* isolated in the Republic of Korea (ROK), very few antibiotic resistant *Salmonella* strains were found in the 1970s. Of 535 *Salmonella* strains isolated in 1982, 17 isolates (3.2%) appeared to be resistant to ampicillin, and 15 isolates (2.8%) were resistant to chloramphenicol [26]. The prevalence of multi-drug resistant salmonellosis has increased in recent decades [27]. Recently, Choi et al. reported that 17.6% (46 of 261 strains) of the examined *Salmonella* strains were nalidixic acid resistant [28]. Of the 55 strains collected in 1995-1996, only one strain (1.8%) was nalidixic acid resistant, whereas 21.8% (45 of 206 strains) of the collected strains in 2000-2002 were nalidixic acid resistant [28]. In comparison, 13.9% (5 of 36 strains) of the examined human isolates and 68.7% (79 of 151 strains) of livestock isolates were nalidixic acid resistant in the present study. We could infer that the higher rate of nalidixic acid resistance among livestock isolates compared to human would be related to the widespread use of ciprofloxacin and enrofloxacin, both in the fluoroquinolone family, in commercial farms [29]. Such increase in antibiotic resistant bacteria could pose serious problems [30]. In particular, severe gastroenteritis caused by low level fluoroquinolone resistant NTS (ciprofloxacin MICs, 0.125-1.0 μg/mL), which is currently regarded as a fluoroquinolone sensitive strain according to the CLSI guideline, might not improve with short-term fluoroquinolone treatment [31].

Nalidixic acid resistance in *Salmonella* isolates reflects the low level fluoroquinolone resistance. Currently, such low level fluoroquinolone resistant isolates are regarded as sensitive isolates according to disk diffusion test, but they tend to show MIC approximately 10 times higher than that of wild type strains (MIC 0.03 μg/mL or less). There is a chance that infection caused by these low level fluoroquinolone resistant isolates may not respond well to current quinolone treatment. Therefore, if this problem continues, the ciprofloxacin breakpoint to *Salmonella* strains need to be reconsidered [32].

Quinolone resistance of NTS develops through combination of several resistance mechanism: mutation in the DNA gyrase, changes in cell membrane porin protein or the active elimination of antibiotics by using energy [33]. Point mutations of *gyrA* gene, which provoke antibiotic resistance to quinolone, occur at a quinolone resistance determining region (QRDR), a well preserved gene at a N-terminal area of A small subunit. Mostly, serine at the position 83 is replaced by phenylalanine, leucine, tyrosine or tryptophan, and aspartate at the position 87 is substituted by tyrosine, glycine, arginine or asparagine. From the results of *gyrA* sequencing analysis of NTS in the present study, point mutations from Ser-83 to Phe, and Asp-87 to Tyr, Glu, Arg and Asn were observed with nalidixic acid resistance.

The mechanism of fluroquinolone resistant *Salmonella* have been studied [1, 28, 32, 34]. According to previous reports, various degree of additional mutations was found in the *gyrB* and *parC* genes, along with more than two point mutations in the *gyrA* gene. In the present study, 1 livestock isolate showed resistance to ciprofloxacin at MIC of 4 μg/mL, and the analysis of DNA sequencing of *gyrA* gene of this isolate showed that Ser 83 (TCG) was replaced by Phe (TTC), which was found to have tolerance to organic solvents. Eighteen livestock isolates showed intermediate resistant to ciprofloxacin at MIC of 2 μg/mL, and three types of point mutations were found in the analysis of DNA sequencing of *gyrA* gene of these isolates: 5 isolates of Ser83 (TCG) were replaced by Phe (TTC or TAC), 6 isolates of Ser83 (TCG) were replaced by Phe (TTC or TAC) and Asp87 (GAC) were replaced by Glu (GAA or GAG), 1 isolates of Ser83 (TCG) was replaced by Phe (TTC) and Asp87 (GAC) was replaced by Arg (CGA). Of 18 livestock isolates, 9 isolates showed tolerance to organic solvents.

In order to perform the test of active transport pump of the cell membrane, which is one of the mechanism for antibiotic resistance of Gram negative bacteria, tolerance test to organic solvents of 84 nalidixic acid resistant isolates were done. As a result, 100% (5 of 5 strains) of human isolates and 53.2% (42 of 79 strains) of livestock isolates showed tolerance to organic solvents. This active transport pump is known to excrete various drugs to outside the cytoplasm which explains one of the mechanisms of multi-drug resistance. It has been considered that the active transport pump of the cell membrane is involved in quinolone resistance of NTS, but MICs for ciprofloxacin and nalidixic acid in this study was not different irrespective of tolerance to organic solvents. The role of efflux pump for the nalidixic acid resistance was not clear, and further studies would be required in the future including inoculum effect and inducible ciprofloxacin resistance.

It is likely that quinolone resistant NTS has increased because of the increasing use of quinolone for the febrile human diseases, along its abuse as a growth promoting agent in livestock [29, 35]. In case of the gastrointestinal tract infections by the strains showing low level fluoroquinolone resistance, the commonly applied 3 day-treatment with oral quinolone is likely to result in failure, and failure cases have been reported [31]. According to the result of this study, 65 (77.4%) isolates among 84 of nalidixic acid resistant isolates was susceptible to ciprofloxacin. Among them, 63 (96.9%) isolates showed low level fluoroquinolone resistance to NTS (ciprofloxacin MICs, 0.125-1.0 μg/mL), while two isolates did not (Table 2). These data suggest that it may not be enough to test just for the ciprofloxacin resistance in NTS.

The limitations of this study are as follows: (i) human isolates were collected from only one hospital of Seoul in Korea; (ii) the number of human isolates compared to livestock was quite small; and (iii) DNA fingerprintings were not compared by PFGE between human and livestock isolates.

In conclusion, it would be essentially required to test the nalidixic acid MIC as a marker of nascent ciprofloxacin resistance in NTS. As for the severe NTS infection, MIC test for nalidixic acid would be required. The prevalence of nalidixic acid resistance in livestock isolates (79 of 115 strains, 68.7%) was significantly higher than those from human isolates (5 of 36 strains, 13.9%) in this study. Prospective, well-designed studies are required to evaluate significance of low level ciprofloxacin and nalidixic acid resistance in relation to clinical outcome.

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