Journal of Bacteriology and Virology 2012. Vol. 42, No. 3 p.203 – 210
http://dx.doi.org/10.4167/jbv.2012.42.3.203

Original Article

Mutations in *gyrA* and *parC* Genes and Plasmid-Mediated Quinolone Resistance in Non-typhoid *Salmonella* Isolated from Pediatric Patients with Diarrhea in Seoul

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A total of 91 non-typhoid *Salmonella* isolated from pediatric patients with diarrhea in Seoul from 2003 to 2009 was tested for antimicrobial susceptibility of nalidixic acid (NA). Forty strains of NA resistance or intermediate susceptible non-typhoid *Salmonella* were identified and their minimum inhibitory concentrations (MICs) of NA, ciprofloxacin (CIP), and norfloxacin (NOR) were determined. Of the 40 isolates, 26 were resistant to NA (MIC >256 μg/ml). Only one isolate was high-level resistant to CIP (12 μg/ml) and NOR (48 μg/ml). Mutations in *gyrA* and *parC* genes were studied by PCR and sequencing. All NA-resistant isolates carried point mutations in the gyrA quinolone resistance determining regions (QRDR) at codon 83 or 87 (MICs of NA, >256 μg/ml; MICs of CIP, 0.047~0.25 μg/ml; MICs of NOR, 0.38~1.5 μg/ml). A double change in GyrA was found in one *Salmonella* Enteritidis (MIC of CIP, 12 μg/ml; MIC of NOR, 48 μg/ml). In respect of the ParC protein, a single change at Thr57→Ser was found in 3 isolates (MICs of NA, >256 μg/ml; MICs of CIP, 0.19~0.25 μg/ml; MICs of NOR, 1 μg/ml). At the same time, these strains changed from Ser83 to Tyr in the gyrA. The result of the investigation for the prevalence of plasmid-mediated quinolone resistance (PMQR) genes, 14 isolates harbored *qnr* gene among 40 isolates. All of 14 isolates showed decreased susceptibility at NA (MICs 4~16 μg/ml) and except one strain, all of *qnr* genes were identified as *qnrB*. Mutations in the *gyrA* gene and production of PMQR determinants were critical for quinolone resistance and decreased susceptibility to fluoroquinolone in these isolates.

Key Words: Non-typhoid *Salmonella*, Nalidixic acid, Mutation, PMQR

INTRODUCTION

Salmonella enterica is widespread in humans and animals worldwide and is of increasing public health concern as causative pathogens of food poisoning (1). While approximately 2000 serotypes of *Salmonella* have been associated with enterocolitis, *Salmonella* Typhimurium and *Salmonella* Enteritidis are two major etiologic agents of food-borne salmonellosis in human (2). Non-typhoidal salmonellosis in humans is usually a self-limiting disease confined to the gastrointestinal tract, therefore antibiotic treatment is not recommended. However, antibiotic therapy may be required in immunocopromised patients, in cases of extraintestinal infections, and for infants and elderly peoples (3). In the treatment of the non-typhoid *Salmonella*, the ciprofloxacin is used and recently it is the tendency that the resistance is gradually increased to the all, globally, the quinolone antibiotic (4, 5). In 2011, according to Korea Center for Disease

Received: May 8, 2012/ Revised: June 14, 2012
Accepted: July 5, 2012
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Control and Prevention (KCDC) report, the nalidixic acid (NA) resistance rate in clinical *Salmonella* isolates was 4.8% in 1999 and increased to 62.1% in 2009.

The first quinolone, NA, was introduced into clinical use at the beginning of the 1960s (6). In the 1980s, ciprofloxacin (CIP), a fluoroquinolone with a wide spectrum of *in vitro* antibacterial activity, particularly against gram negative bacteria, first became clinically available (7). However, this high level of use, and to some degree of misuse in the sense of unnecessary use, or use of quinolones with poor activity in some developing countries, has been blamed for the rapid development of bacterial resistance to these agents (4).

Quinolone resistance in Gram-negative bacteria is primarily attributable to mutations in the quinolone resistance determining regions (QRDR) consisting of the *gyrA* and *parC* genes, which are the subunits of the target enzymes of quinolones, DNA gyrase subunit A and topoisomerase IV, respectively (8). Other mechanisms of resistance, such as efflux pump systems or modifications of porins, can decrease susceptibility to quinolone (9). In addition, decreased susceptibility to quinolones can be mediated by plasmid-mediated quinolone resistance genes, named *qnr*, *aac(6')-Ib-cr* and *qepA* (7).

Research on the mechanisms of resistance to quinolones of *Salmonella* at the early 2000's was reported by several authors in Korea, but there are few reports on the latest strains. In the present study, we investigated the mutations in the QRDR and the prevalence of PMQR determinants of NA resistance or intermediate *Salmonella* isolated from pediatric patients with diarrhea in order to figure out their distribution and significance.

MATERIALS AND METHODS

Isolation and identification of *Salmonella*

From 2003 to 2009, a total 91 non-typhoid *Salmonella* strains were isolated from pediatric patients with diarrhea at the Seoul Metropolitan Government Research Institute of Public Health and Environment (SIHE) in Seoul, Korea. After performing the biochemical identification by API 20E Kit (BioMerieux Co. Mo, USA) about K/A and H_2S formation cell group among the colony growing in MacConkey agar plate into the colorless, the *Salmonella* serotypes were determined by slide agglutination according to the Kauffmann-White scheme using O and H-antisera (Difco Co., Detroit, USA). All isolates were stored at -70℃.

Antimicrobial susceptibility testing and minimal inhibitory concentration (MIC) determination

NA susceptibility of *Salmonella* isolates was determined using a disc diffusion method, according to the guidelines of the Clinical Laboratories Standards Institute (CLSI). Determination of MIC was performed using E-tests (AB Biodisk, Solna, Sweden) on Mueller Hinton plates following the manufacturer's recommendations. The antimicrobial agents tested were NA, CIP, and norfloxacin (NOR).

DNA Sequencing analysis of *gyrA* and *parC* genes

The QRDRs of *gyrA* and *parC* genes were amplified by PCR from the DNA of 26 NA-resistant isolates. In brief, colonies were suspended in 200 μl of distilled water and boiled to prepare DNA templates. The primer set used was those described by Giraud *et al*. (10) (Table 1). The PCR conditions were 3 min at 94℃ and 30 cycles of 94℃ for 1 min, 55℃ for 1 min (*parC* 52℃ 1 min), and 72℃ for 1 min. Positive PCR products were purified using a PCR purification kit (Cosmo Inc., Seoul, Korea), and DNA sequencing was performed by Cosmo Inc. (Cosmo Inc., Seoul, Korea).

Detection of the quinolone resistance (PMQR) gene containing plasmids

All of the isolates showing resistance and intermediate susceptibility to NA were tested for the presence of PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA*) by PCR using the primers listed in Table 1 (11~13).

RESULTS

Isolation and identification of *Salmonella*

Among 91 isolates, a total of 40 strains of NA resistance or intermediate susceptible non-typhoid *Salmonella* were

Table 1. Primers used in this study

Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference
STGYRA1	TGTCCGAGATGGCCTGAAGC	450	(10)
STGYRA12	CGTTGATGACTTCCGTCAG		
STPARC1	ATGAGCGATATGGCAGAGCG	400	(10)
STPARC2	TGACCGAGTTCGCTTAACAG		
qnrA F	ATTTCTCACGCCAGGATTTG	516	(11)
qnrA R	GATCGGCAAAGGTTAGGTCA		
qnrB F	GATCGTGAAAGCCAGAAAGG	469	(11)
qnrB R	ACGATGCCTGGTAGTTGTCC		
qnrS F	ACGACATTCGTCAACTGCAA	417	(11)
qnrS R	TAAATTGGCACCCTGTAGGC		
aac(6')-Ib-cr F	TTG CGA TGC TCT ATG AGT GGC TA	482	(12)
aac(6')-Ib-cr R	CTC GAA TGC CTG GCG TGT TT		
qepA F	GGA CAT CTA CGG CTT CTT CG	720	(13)
qepA R	AGC TGC AGG TAC TGC GTC AT		

Table 2. Antimicrobial susceptibility against nalidixic acid of 91 nontyphoid *Salmonella* isolated in Seoul from 2003 to 2009

Antimicrobial susceptibility results[a]	No. of strains isolated in							No. of isolates
	2003	2004	2005	2006	2007	2008	2009	
Resistance	1	1	1	9	6	5	5	28
Intermediate susceptibility	0	2	1	8	1	0	0	12
susceptibility	10	6	9	9	11	4	2	51
Total	11	9	11	26	18	9	7	91

[a]determined by disc diffusion method.

obtained from pediatric patients with diarrhea from 2003 to 2009. The most frequently identified serotypes were *Salmonella* Enteritidis, accounting for 37.5% (15 isolates) followed by Montevideo (27.5%, 11 isolates), Hillingon (12.5%, 5 isolates), and Typhimurium (7.5%, 3 isolates). The remaining 6 isolates belonged to other serotypes.

Antimicrobial susceptibility results

Antimicrobial susceptibility testing by disc method revealed that 28 isolates were resistant to NA, whereas 12 isolates were intermediate susceptible to NA (Table 2). Yearly, the 1 of 11 isolates (9.1%) in 2003, 1 of 9 isolates (11.1%) in 2004, 1 of 11 isolates (9.1%) in 2005, 9 of 26 isolates (34.6%) in 2006, 6 of 18 isolates (33.3%) in 2007, 5 of 9 isolates (55.6%) in 2008, 5 of 7 isolates (71.4%) in 2009 was shown resistant to NA by disc test. The rate of resistance has been increasing since 2006 (Table 2). The MIC values of NA resistance and intermediate susceptible *Salmonella* isolates are shown in Table 2. Of the 28 isolates, 26 were resistant to NA (MIC >256 μg/ml) by MIC test. One *Salmonella* Enteritidis (No. 30) isolated in 2007 exhibited a high level of resistance to both CIP (12 μg/ml) and NOR (48 μg/ml). The remaining isolates for CIP and NOR were 0.047~0.25 μg/ml and 0.25~2 μg/ml, respectively. Among the 40 isolates, 19 (47.5%) showed increased MIC (≥ 0.125 μg/ml) for CIP.

Mutations of *gyrA* and *parC* genes

PCR of *gyrA* and *parC* genes was performed on a total of 26 NA resistant isolates and DNA sequencing of the PCR products was carried out. A single amino acid substitution in the QRDR of GyrA protein was detected in all isolates (MIC >256 μg/ml) (Table 3). All mutations occurred in the Ser83 and Asp87 codon, and the variation observed much in the codon 87. The substitutions were as follows (number of strains): Ser83 → Tyr (3), Ser83 → Leu (2), Ser83 → Phe (1), Asp87 → Asn (11), Asp87 → Gly (7), and Asp87 → Tyr (2). One *Salmonella* Enteritidis isolated in 2007 (No. 30) had the double mutations in gyrA Ser83 → Leu and Asp87 → Tyr. In respect of the ParC protein, a single change at Thr57 → Ser was found in 3 isolates (MICs of NA, >256 μg/ml; MICs of CIP, 0.19~0.25 μg/ml; MICs of NOR, 1 μg/ml). At the same time, these strains changed from Ser83 to Tyr in the gyrA.

Detection of plasmid-mediated quinolone resistance (PMQR) genes

The PMQR genes were detected in 14 (35%) of the 40 *Salmonella* isolates (Table 3). The most commonly observed *qnr*-positive serotypes were Montevideo (N=11), followed by Othmarschen (N=2), and Typhimurium (N=1). The *qnrB* gene was detected in 13 of the 14 *qnr*-positive isolates and the MICs of NA ranged from 4 μg/ml to 16 μg/ml. The *qnrA* gene and *aac(6')-Ib-cr* were found in only 1 strain and MICs of NA, CIP, and NOR were 8 μg/ml, 0.25 μg/ml, and 2 μg/ml, respectively. The *qnrS* and *qepA* genes were not detected in any of the *Salmonella* strains in this study.

DISCUSSION

In the current study, we investigated mechanisms of quinolone resistance of NA-resistance or intermediate susceptibility *Salmonella* isolated from pediatric patients with diarrhea in order to figure out their prevalence and characterization. In Korea, the NA resistance rate in clinical *Salmonella* isolates was 1.8% in 1995-1996, but has increased to 21.8% in 2000-2002 (5). In this study, we found that the resistance rate had increased gradually from 9~11% in 2003-2005 to 34.6~71.4% in 2006-2009. In spite of being the strains isolated from the child, the resistance rate of the NA was increased over time and the injury reached a serious level.

The results of MICs for quinolone and fluoroquinolone were 26 isolates (28.5%) with high resistance to NA between 2003 and 2009. Except one, all isolates were 0.064~0.25 μg/ml and 0.25~2 μg/ml in MICs for CIP and NOR, respectively, and this means that the resistance of fluoroquinolones is still uncommon. The results are similar to those in previous reports (14~16). Lee *et al*. (17) in Korea has reported that fluoroquinolone resistance of *Salmonella* Haardt isolated from chicken was a little higher than for the humans.

Consistent with previous reports, we found that the main mechanism of NA resistance was single point mutations in the QRDR region of the *gyrA* gene at codons 83 or 87. In this study, Ser83 changed at Tyr, Leu, and Phe and Asp87 changed at Asn, Gly, and Tyr. These results were same with the substitution which had been reported as the most frequent substitution in GyrA of *Salmonella* (5, 14, 16, 18, 19). A previous study by Fabrega *et al*. (14) suggested that the position of the mutations in the *gyrA* gene and the substituting amino acid can be depended on the serovar analysis. In this study, there was the difference in several papers (15, 17) and this reason is considered to be the origin and the serotypes. The occasion mutation of ParC had been reported. Also, the substitution from Thr57 to Ser had been reported as the occasion mutation in ParC (14). In this study, three isolates (two of Haardt, one of Bovismorbificans) showed same substitution form with the substitution from Thr57 to Ser. In Korea, the research of substitution form had been reported by Jeong *et al*. (19) and Kim *et al*. (20) and it showed that the substitution from Thr57 to Ser happens occasionally. In *Salmonella*, given the fact that a single mutation of *gyrA* gene might cause quinolone resistance and reduced fluoroquinolone susceptibility (21), it was interesting that a double mutation of the *gyrA* gene happened in the *parC*. However, there was a discrepancy between Eaves *et al*.'s (22) and Ling *et al*.'s (23) research.

Table 3. The minimum inhibitory concentration and quinolone resistance mechanisms of 40 nontyphoid-*Salmonella* isolates

No. of strains	Serotypes	Isolation year	MIC (μg/ml)			QRDR mutations[a]		PMQR gene
			NA[b]	CIP[c]	NOR[d]	GyrA	ParC	
1	Bovismorbificans	2003	>256	0.19	1	Ser83 → Tyr	Thr57 → Ser	−
2	Othmarschen	2004	12	0.125	0.38	NT[e]	NT	*qnrB*
3	Othmarschen	2004	8	0.125	0.38	NT	NT	*qnrB*
4	Typhimurium	2004	>256	0.25	1.5	Asp87 → Asn	−[f]	−
5	Montevideo	2005	8	0.094	0.25	−	−	*qnrB*
6	Montevideo	2005	4	0.094	0.25	NT	NT	*qnrB*
7	Hillingdon	2006	>256	0.094	0.75	Asp87 → Asn	−	−
8	Hillingdon	2006	>256	0.094	0.75	Asp87 → Asn	−	−
9	Hillingdon	2006	>256	0.19	0.75	Asp87 → Asn	−	−
10	Enteritidis	2006	>256	0.064	0.38	Asp87 → Gly	−	−
11	Montevideo	2006	4	0.094	0.25	NT	NT	*qnrB*
12	Montevideo	2006	6	0.094	0.25	NT	NT	*qnrB*
13	Montevideo	2006	6	0.094	0.25	NT	NT	*qnrB*
14	Montevideo	2006	12	0.125	0.5	NT	NT	*qnrB*
15	Montevideo	2006	16	0.25	0.5	−	−	*qnrB*
16	Montevideo	2006	8	0.094	0.25	NT	NT	*qnrB*
17	Montevideo	2006	6	0.094	0.25	NT	NT	*qnrB*
18	Montevideo	2006	6	0.125	0.38	NT	NT	*qnrB*
19	Haardt	2006	>256	0.19	1	Ser83 → Tyr	Thr57 → Ser	−
20	Typhimurium	2006	8	0.25	2	NT	NT	*qnrA* + *aac(6')-Ib-cr*
21	Hillingdon	2006	>256	0.064	0.38	Asp87 → Gly	−	−
22	Enteritidis	2006	>256	0.094	0.50	Asp87 → Asn	−	−
23	Enteritidis	2006	>256	0.047	0.38	Asp87 → Gly	−	−
24	Haardt	2007	>256	0.25	1	Ser83 → Tyr	Thr57 → Ser	−
25	Enteritidis	2007	>256	0.19	0.75	Asp87 → Asn	−	−
26	Enteritidis	2007	>256	0.19	0.75	Asp87 → Asn	−	−
27	Enteritidis	2007	>256	0.125	0.75	Asp87 → Asn	−	−
28	Montevideo	2007	4	0.094	0.25	NT	NT	*qnrB*
29	Enteritidis	2007	>256	0.094	0.50	Asp87 → Tyr	−	−
30	Enteritidis	2007	>256	12	48	Ser83 → Leu + Asp87 → Tyr	−	−
31	Enteritidis	2008	>256	0.094	0.75	Asp87 → Asn	−	−
32	Enteritidis	2008	>256	0.094	0.5	Asp87 → Asn	−	−
33	I4,[5],12:i:-[g]	2008	>256	0.25	1	Ser83 → Phe	−	−
34	Enteritidis	2008	>256	0.094	0.5	Asp87 → Gly	−	−

Table 3. Continued

No. of strains	Serotypes	Isolation year	MIC (μg/ml)			QRDR mutations[a]		PMQR gene
			NA[b]	CIP[c]	NOR[d]	GyrA	ParC	
35	Typhimurium	2008	>256	0.25	1.5	Asp87 → Tyr	−	−
36	Enteritidis	2009	>256	0.125	0.5	Asp87 → Gly	−	−
37	Enteritidis	2009	>256	0.125	0.75	Asp87 → Asn	−	−
38	Hillingdon	2009	>256	0.094	0.5	Asp87 → Gly	−	−
39	Enteritidis	2009	>256	0.064	0.38	Asp87 → Gly	−	−
40	Enteritidis	2009	>256	0.094	0.25	Ser83 → Leu	−	−

[a]A total of 26 nalidixic acid- resistant isolates were examined
[b]NA, nalidixic acid; MIC value: susceptible (≤16 μg/ml), intermediate (−), resistance (≥32 μg/ml)
[c]CIP, ciprofloxacin; MIC value: susceptible (≤1 μg/ml), intermediate (2 μg/ml), resistance (≥4 μg/ml)
[d]NOR, norfloxacin; MIC value: susceptible (≤4 μg/ml), intermediate (8 μg/ml), resistance (≥16 μg/ml)
[e]NT indicate not tested
[f](−) indicate no mutation or no PMQR genes.
[g]The variant of the *S.* Typhimurium, presently, the serotype is not named.

Eaves *et al*. suggested that Thr57-Ser substitution in ParC made isolates more sensitive to CIP and formed a hypothesis that the mutation at codon 57 of *parC* could be a naturally occurring compensatory mutation. In this study, we had the same view on the substitution with Eaves *et al*.'s research due to our result in which the substitution did not cause an increase of MICs of CIP in ParC.

PMQR mechanisms, including *qnr* genes, *aac(6')-Ib-cr*, and *qepA*, were found to confer decreased susceptibility to fluoroquinolones (24). In this study, the *qnr* genes had been detected in 14 isolates (35%) that all of the strains showed the decreased susceptibility to NA (MICs 4~16 μg/ml). In particular, one isolate (*S.* Typhimurium) in which the *qnrA* and *aac(6')-Ib-cr* is detected, showed the reduced susceptibility to the quinolone and fluoroquinolones. It could be confirmed to contribute to reduced susceptibility as the production of PMQR determinants. The PMQR genes detected in *Salmonella* are rarely found in Korea (5, 20). Recently, in Korea, Jeong *et al*. (19) reported the PMQR detection in *Salmonella*. However, there was a distinct from our study in that Jeong *et al*. detected 6 *qnr*-positive clinical isolates among 284 samples (2.1%) and these had high level resistance to NA. This study was limited to pediatric patients with diarrhea in Seoul and it is interesting that the *qnr* gene rate is higher. Moreover, this is the first report of the *qnrA* gene in *Salmonella* from Korea. The *qnr* types of the present study correspond well with those of the earlier study (25) which reported that *qnr*-positive Enterobacteriaceae is had *qnrB*. In China (26), USA (27), and Japan (28), there were reported *aac(6')-Ib-cr* and *qnr* genes detected in *Salmonella*. Among those researches, there were differences in the prevalence and variant types of PMQR genes. We are now unsure whether the reason is serotypes or geographic region. We will conduct further research on the reason for the difference.

Due to the continuous increase in quinolone resistance of non-typhoid *Salmonella* in Korea, continued resistance research monitoring is under consideration.

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