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# Analysis of the Fluoroquinolone Antibiotic Resistance Mechanism of *Salmonella enterica* Isolates

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Quinolone-resistant *Salmonella* strains were isolated from patient samples, and several quinolone-sensitive strains were used to analyze mutations in the quinolone resistance-determining region (QRDR) of *gyr*A, *gyr*B, *par*C, and *par*E and to screen for plasmid-mediated quinolone resistance. Among the 21 strains that showed resistance to nalidixic acid and ciprofloxacin (MIC 0.125–2.0 μg/ml), 17 strains had a mutation in QRDR codon 87 of *gyr*A, and 3 strains had a single mutation (Ser83 → Phe). Another cause of resistance, efflux pump regulation, was studied by examining the expression of *acr*B, *ram*A, *mar*A, and *sox*S. Five strains, including Sal-KH1 and Sal-KH2, showed no increase in relative expression in an analysis using the qRT-PCR method ($p < 0.05$). In order to determine the genes involved in the resistance, the Sal-9 isolate that showed decreased susceptibility and did not contain a mutation in the *gyr*A QRDR was used to make the STM (MIC 8 μg/ml) and STH (MIC 16 μg/ml) ciprofloxacin-resistant mutants. The *gyr*A QRDR Asp87 → Gly mutation was identified in both the STM and STH mutants by mutation analysis. qRT-PCR analysis of the efflux transporter *acr*B of the AcrAB-TolC efflux system showed increased expression levels in both the STM (1.79-fold) and STH (2.0-fold) mutants. In addition, the expression of the transcriptional regulator *mar*A was increased in both the STM (6.35-fold) and STH (21.73-fold) mutants. Moreover, the expression of *sox*S was increased in the STM (3.41-fold) and STH (10.05-fold) mutants ($p < 0.05$). Therefore, these results indicate that AcrAB-TolC efflux pump activity and the target site mutation in *gyr*A are involved in quinolone resistance.

**Keyword:** *Salmonella enterica*, quinolone resistance, QRDR, AcrAB-TolC, *mar*A

## Introduction

*Salmonella enterica* is a well-adapted microbial pathogen that can cause various illness symptoms ranging from common food poisoning to the more severe typhoid fever [10, 14].

Fluoroquinolones, such as ciprofloxacin, are antimicrobial agents with broad-spectrum usage for treating invasive gastrointestinal infections, including salmonellosis [12]. In fact, fluoroquinolones are the primary treatments clinically in use for salmonellosis [33]. However, *Salmonella* strains recently isolated from humans and animals were reported to have nalidixic acid resistance and exhibit significantly reduced susceptibility to fluoroquinolones [6, 27].

Developing adequate therapeutic options for prevention or medicines for multidrug-resistant (MDR) bacteria is challenging, making infectious diseases caused by MDR bacteria the most serious public health problem worldwide [16, 28, 37]. Recently, the primary mechanism of quinolone resistance in *Salmonella* was found to involve the results of amino acid changes in the quinolone-resistance determining region (QRDR), such as mutations in DNA gyrase (*gyrA* and *gyr*B) [8, 9] and topoisomerase IV (*par*C and *parE*) [9]. However, recently, other mechanisms related to resistance were identified regarding the production of Qnr protein by

plasmid-mediated quinolone resistance (PMQR) genes and the overexpression of multidrug efflux pumps such as AcrAB-TolC [1, 20].

Studies on several gram-negative pathogens, including *Escherichia coli* and *Salmonella enterica*, showed that these mechanisms are responsible for the MDR phenotype [2]. AcrAB-TolC is a member of the resistance-nodulation-division family and consists of three functional domains: a membrane fusion protein (AcrA), a drug efflux transporter (AcrB), and an outer membrane channel protein (TolC) [25, 34]. Efflux pump expression is regulated in *Salmonella* by various transcriptional regulatory genes. AcrAB expression is adjusted by a local repressor, AcrR, and by the global regulators SoxS, MarA, RamA, and Rob [19, 21, 29]. The expression of AcrAB is controlled by a specific regulator, RamA in *Salmonella* [29] and Rob in *E. coli* [36].

The emergence of MDR bacteria is the result of the excess use of antibiotics, and the resistance gene is easily transferred to other bacteria to spread antibiotic resistance. New resistant microorganisms cause infections that often fail to respond to traditional treatments, thus increasing the duration of illness for individuals, health care expenditures for society, and, in some cases, risk of death. Recently, the rate of fluoroquinolone antibiotic resistance in Korean clinical *Salmonella* strains has increased, and as a result, we investigated the resistance mechanism against quinolones of *Salmonella* strains isolated from Korean patients' clinical samples and some quinolone-resistant strains.

## Materials and Methods

### Bacterial Isolates

Forty-two *Salmonella* strains were used in this study. The *Salmonella* strains were isolated from Korean patients with diarrhea and identified through 16S rRNA sequencing. Quinolone-resistant *Salmonella enterica* serovar Typhimurium (Sal-KH1) and *S. enteritidis* (Sal-KH2) were obtained from the collection of the Korea National Institute of Health (Seoul, Korea). The oligonucleotide primers used in this study are listed in Table 1.

### Antimicrobial Susceptibility Test

The antimicrobial susceptibility was determined by the disk diffusion method using ampicillin, tetracycline, chloramphenicol, ofloxacin, nalidixic acid, and ciprofloxacin. Mueller-Hinton medium (Difco Inc., USA) was used, and the interpretation of susceptibility followed the guidelines of the Clinical Laboratory Standards Institute (CLSI 2014) (Table 2). MICs for quinolones (ciprofloxacin and nalidixic acid) were determined by E-test (Oxoid Inc., UK) and by the broth dilution method, respectively. The *E. coli* ATCC 25922 strain was used for quality control in the susceptibility tests.

### DNA Sequence Analysis of the QRDR

The QRDRs of the DNA gyrase (*gyr*A and *gyr*B) and topoisomerase IV (*par*C and *par*E) genes were amplified by PCR under the following cycling conditions: 5 min for predenaturation at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at an annealing temperature of 57°C, 1 min extension at 72°C, and a final extension step of 5 min at 72°C.

The PCR products were purified from agarose gels using an MG Gel SV gel DNA extraction kit (Macrogen Inc., Korea), and nucleotide sequencing was performed by Macrogen Inc. The sequence was aligned using the NCBI BLAST results for *Salmonella enterica* serovar Typhimurium (GenBank Accession No. AE006468).

### Amplification of PMQR Genes

In order to confirm the presence of PMQR genes (*qnr*A, *qnr*B, *qnr*S, *aac(6')-lb–cr*, and *qep*A) in the isolated strains, the genomic DNA was used as a template for PCR.

PCRs were performed under the following conditions: one cycle of predenaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec; annealing at 53°C for *qnr*A, *qnr*B, and *aac(6')-lb–cr*, 58°C for *qnr*S, and 55°C for *qep*A for 30 sec; and 72°C for 30 sec, and a final extension at 72°C for 5 min.

### In Vitro Selection of Ciprofloxacin-Resistant Mutants

Mutants, obtained by treating $10^9$ CFU/ml of the parental strain (Sal-9) with different concentrations of ciprofloxacin ranging from 0.5 to 16 μg/ml, and overnight cultures were plated on Mueller-Hinton agar supplemented with different concentrations of ciprofloxacin. After 72–96 h of incubation, selected colonies were incubated on media supplemented with the same concentration of ciprofloxacin used to screen the mutants for fluoroquinolone resistance. The phenotype of ciprofloxacin resistance was confirmed by the disk diffusion and broth dilution methods. All of the selected mutants were subcultured on antimicrobial-free medium five more times to obtain a stable resistance phenotype.

### Total RNA Extraction and cDNA Synthesis

The Hybrid-R (GeneAll, Korea) kit was used to extract total RNA. Once the ciprofloxacin MIC was determined, each strain was cultivated for 2 h in Luria-Bertani media (Difco Inc., USA), and ciprofloxacin was added to cultures at a concentration corresponding to half of the ciprofloxacin MIC for each strain. After 30 min of exposure to ciprofloxacin at 37°C, each culture strain was used for RNA extraction.

The concentration of RNA was adjusted to 1 μg/μl, and then cDNA synthesis was performed in a final volume of 20 μl containing 1 μg of total RNA and 4 μg All-In-One RT Master Mix (Applied Biological Materials Inc., USA). cDNA synthesis was performed at 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min.

### Expression of the Efflux Transporter Gene *acr*B and Transcriptional Regulator Genes

The expression levels of the multidrug efflux transporter gene

**Table 1.** Primer sequences used in this study.

| Primers | Nucleotide sequences (5′ → 3′) | Size (bp) | References |
|---|---|---|---|
| *gyrA*-F | CGTTGGTGACGTAATCGGTA | 251 bp | This study |
| *gyrA*-R | CCGTACCGTCATAGTTATCC | | |
| *gyrB*-F | GTGATCAGCGTCGCCACT | 235 bp | This study |
| *gyrB*-R | GCGCGGTGATCAGCGTC | | |
| *parC*-F | GACGGCCTGAAGCCGGT | 284 bp | This study |
| *parC*-R | CTCGGCGTATTTGGACAGG | | |
| *parE*-F | TATCAGGCGATCATGCCGC | 300 bp | This study |
| *parE*-R | CTTTACCCAAATCGATACGGT | | |
| *qnrA*-F | ATTTCTCACGCCAGGATTTG | 516 bp | [35] |
| *qnrA*-R | GATCGGCAAAGGTTAGGTCA | | |
| *qnrB*-F | GATCGTGAAAGCCAGAAAGG | 469 bp | [12] |
| *qnrB*-R | ACGATGCCTGGTAGTTGTCC | | |
| *qnrS*-F | ACTGCAAGTTCATTGAACAG | 431 bp | [12] |
| *qnrS*-R | GATCTAAACCGTCGAGTTCG | | |
| *aac(6′)-lb -cr*-F | TTGCGATGCTCTATGAGTGGCTA | 482 bp | [20] |
| *aac(6′)-lb -cr*-R | CTCGAATGCCTGGCGTGTTT | | |
| *qepA*-F | AACTGCTTGAGCCCGTAGAT | 596 bp | [20] |
| *qepA*-R | GTCTACGCCATGGACCTCAC | | |
| *acrB*-F | CAATATCCGACGATTGCGC | 121 bp | This study |
| *acrB*-R | TATCGATACCGTTCATATTCTGT | | |
| *marA*-F | GACCCGGACGTTCAAAAACTA | 68 bp | [33] |
| *marA*-R | TCGCCATGCATATTGGTGATC | | |
| *ramA*-F | GCGTGAACGGAAGCTAAAAC | 167 bp | This study |
| *ramA*-R | GGCCATGCTTTTCTTTACGA | | |
| *soxS*-F | GAGCGCCCGATTTTTGATATC | 70 bp | [33] |
| *soxS*-R | CGGAATACACGCGAGAAGGT | | |
| *16s*-F | CGTGTTGTGAAATGTTGGGTT | 130 bp | This study |
| *16s*-R | GACTTGACGTCATCCCCA | | |

**Table 2.** Zone diameter and minimal inhibitory concentration (MIC) interpretive standards for *Salmonella enterica*.

| Antibiotics | S | I | R |
|---|---|---|---|
| Ofloxacin | ≥16 mm | 13–15 mm | ≤12 mm |
| Tetracycline | ≥15 mm | 12–14 mm | ≤11 mm |
| Ciprofloxacin | ≥31 mm | 21–30 mm | ≤20 mm |
| (MIC) | ≤0.06 μg/ml | 0.125–0.50 μg/ml | ≥1 μg/ml |
| Ampicillin | ≥17 mm | 14–16 mm | ≤13 mm |
| Nalidixic acid | ≥19 mm | 14–18 mm | ≤13 mm |
| (MIC) | ≤16 μg/ml | - | ≥32 μg/ml |
| Chloramphenicol | ≥18 mm | 13–17 mm | ≤12 mm |

*acr*B and transcriptional regulator genes *ram*A, *mar*A, and *sox*S were analyzed by quantitative real-time PCR (qRT-PCR) using the StepOne Plus real-time PCR kit (Life Technologies, USA). The 16S rRNA gene was used as a housekeeping control gene.

The qRT-PCR was performed in a final volume of 20 μl containing 1 μl of 10 pmol each primer, 7 μl of nuclease-free water,

1 μl of cDNA, and 10 μl of SYBR Green HiPi Real-Time 2× MasterMix-ROX (Elpis, Korea).

The qRT-PCR cycling conditions were 40 cycles at 95°C for 10 min, 95°C for 15 sec, and annealing at 60°C for 1 min. To compare expression levels between reference and target genes and between wild and resistant strains, the ddCt method was used. All tests were repeated three times, and the relative gene expression values indicated are the average of these tests.

### Statistical Analysis

Gene expression data were analyzed using Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

## Results

### Resistance Phenotypes

The results from the disk diffusion method showed that 53% of *Salmonella enterica* serovar Typhimurium strains were resistant against more than two types of antibiotics among the six serotypes; specifically, AM-TE (ampicillin, tetracycline) resistance was the highest (47%). On the other hand, 76.5% of *Salmonella enterica* serovar Enteritidis strains were resistant against three types of antibiotics; specifically, AM-C-NA (ampicillin, chloramphenicol, nalidixic acid) resistance was the highest (70%). Among the 42 *Salmonella* strains, 26 showed resistance against ampicillin: 11 strains of *Salmonella enterica* serovar Typhimurium (42.3%), 13 strains of *Salmonella enterica* serovar Enteritidis (50%), and 2 strains of *Salmonella enterica* serovar Abony (7.7%).

For nalidixic acid, 3 strains of *Salmonella enterica* serovar Typhimurium (14.3%), 16 strains of *Salmonella enterica* serovar Enteritidis (76.2%), and 2 strains of *Salmonella enterica* serovar Abony (9.5%) showed resistance. In addition, 2 strains of *Salmonella enterica* serovar Typhimurium, 15 strains of *Salmonella enterica* serovar Enteritidis, and 2 strains of *Salmonella enterica* serovar Abony showed resistance to ciprofloxacin. As a result, most of the antibiotic resistance shown by *Salmonella enterica* serovar Enteritidis was to ampicillin, nalidixic acid, and ciprofloxacin.

When 19 intermediately sensitive strains and 2 resistant strains were cultured on media containing nalidixic acid and ciprofloxacin, the MIC for all of them was over 64 μg/ml, which is a high level of resistance to nalidixic acid.

**Table 3.** The MIC and mutations in the QRDR of the *gyr*A, *gyr*B, *par*C, and *par*E genes of *Salmonella enterica*.

| No. of strains | Serovar | MIC (μg/ml) | | Substitutions in QRDR amino acid | | | |
|---|---|---|---|---|---|---|---|
| | | NA | Cip | *gyr*A | *par*C | *gyr*B | *par*E |
| ATCC 13311 | *Typhimurium* | 8 | 0.030 | wt | wt | wt | wt |
| Sal-6 | *Abony* | > 64 | 0.125 | Ser83 → Phe | wt | wt | wt |
| Sal-7 | *Enteritidis* | > 64 | 0.125 | Asp87 → Gly | wt | wt | wt |
| Sal-9 | *Typhimurium* | > 64 | 0.125 | wt | Thr57 → Ser | wt | wt |
| Sal-10 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-13 | *Enteritidis* | > 64 | 0.125 | Asp87 → Asn | wt | wt | wt |
| Sal-19 | *Enteritidis* | > 64 | 0.125 | Asp87 → Gly | wt | wt | wt |
| Sal-21 | *Typhimurium* | > 64 | 0.125 | Asp87 → Tyr | Thr57 → Ser | wt | wt |
| Sal-22 | *Enteritidis* | > 64 | 0.125 | Asp87 → Gly | wt | wt | wt |
| Sal-23 | *Abony* | > 64 | 0.250 | Ser83 → Phe | wt | wt | wt |
| Sal-27 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-28 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-31 | *Enteritidis* | > 64 | 0.125 | Asp87 → Gly | wt | wt | wt |
| Sal-32 | *Enteritidis* | > 64 | 0.125 | Asp87 → Asn | wt | wt | wt |
| Sal-39 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-41 | *Enteritidis* | > 64 | 0.250 | Asp87 → Ser | wt | wt | wt |
| Sal-42 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-43 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-44 | *Enteritidis* | > 64 | 0.250 | Asp87 → Gly | wt | wt | wt |
| Sal-45 | *Enteritidis* | > 64 | 0.250 | Ser83 → Phe | wt | wt | wt |
| Sal-KH1 | *Typhimurium* | > 64 | 2 | Asp87 → Gly | wt | Thr469 → Ala | wt |
| Sal-KH2 | *Enteritidis* | > 64 | 2 | Asp87 → Asn | wt | wt | wt |

Intermediately sensitive strains to ciprofloxacin showed MIC values of 0.125–0.25 μg/ml, and resistant strains (Sal-KH1 and Sal-KH2) showed MIC values of 2 μg/ml (Table 3).

**Analysis of QRDR Mutations**

The sequences of *gyr*A, *gyr*B, *par*C, and *par*E of 19 strains that were resistant to nalidixic acid and intermediately sensitive to ciprofloxacin and 2 resistant strains were used to investigate mutations in the QRDR of DNA gyrase (topoisomerase II) and topoisomerase IV.

Twenty strains had a mutation in the QRDR of the *gyr*A gene. Among them, the mutation at codon 87 (Asp87→Gly) was the most frequent ($n$ = 12), followed by the mutations Ser83→Phe (n = 3), Asp87→Asn ($n$ = 3), Asp87→Ser ($n$ = 1), and Asp87 → Tyr ($n$ = 1).

Two strains (Sal-9, Sal-21) had a mutation at codon 57 (Thr57→Ser) of *par*C, and Sal-KH1 had a mutation at codon 469 (Thr469 → Ala) of *gyr*B. No mutation in the QRDR of *par*E was found (Table 3), whereas many silent mutations were found in the QRDR of *gyr*A, *gyr*B, *par*C, and *par*E.

Sal-21 had simultaneous mutations in the QRDRs of *gyr*A and *par*C. Sal-KH1 had a single mutation in the QRDRs of *gyr*A and *gyr*B.

**Detection of PMQR genes**

The existence of PMQR genes such as *qnr*A, *qnr*B, *qnr*S, *aac(6')-lb–cr*, and *qep*A was examined using PCR. The 40 clinical strains did not show PCR products for PMQR genes, whereas the Sal-KH1 strain, which had an MIC for ciprofloxacin of 2 μg/ml, showed a PCR product for the *qnr*A gene.

**Antimicrobial Resistance Phenotype and Genotype Determination of Ciprofloxacin-Resistant Mutants**

Two ciprofloxacin-resistant mutants derived from the clinical strain *Salmonella enterica* serovar Typhimurium Sal-9 that can grow in medium containing 8 μg/ml and 16 μg/ml ciprofloxacin were isolated and designated as STM and STH, respectively. For the phenotypic and genotypic characterization of these mutants, antimicrobial resistance tests and MIC determination for ciprofloxacin were performed, and the nucleotide sequences of the QRDR of *gyr*A and *par*C were determined.

Sal-9 was sensitive to ofloxacin, whereas the STM mutant was intermediately sensitive and the STH mutant was resistant. All of the strains were resistant to ampicillin, nalidixic acid, and chloramphenicol but were sensitive to tetracycline. The STM mutant showed resistance to four drugs (AM-C-CIP-NA), and the STH mutant was resistant to five drugs (AM-C-CIP-NA-OFX) (Table 4).

The sequence analysis of the QRDR of *gyr*A showed a single amino acid change at codon 87 (Asp87 → Gly) for the STM and STH mutants, whereas Sal-9 did not show any mutation at this codon. No mutation was found in *par*C of the STM and STH mutants (Table 4). The single mutation at *gyr*A is believed to cause resistance to ciprofloxacin.

**Expression of the Transporter Gene *acr*B of the AcrAB-TolC Efflux System and the Transcriptional Regulators *ram*A, *mar*A, and *sox*S**

The expression level of the transporter gene *acr*B of the AcrAB-TolC efflux system in fluoroquinolone-resistant and intermediately resistant *Salmonella enterica* strains was analyzed using qRT-PCR.

The expression level of the transporter gene *acr*B of the AcrAB-TolC efflux system was reduced (0.33–0.94-fold) in five strains (Sal-6, Sal-9, Sal-10, Sal-21, Sal-22) that showed reduced sensitivity to ciprofloxacin and in quinolone-resistant strains (Sal-KH1, Sal-KH2) compared with *Salmonella enterica* serovar Typhimurium ATCC 13311 ($p < 0.05$). In addition, the expression level of the transcriptional regulators increased or decreased less than 2-fold among the strains: *mar*A, 0.39–1.46-fold; *sox*S, 0.35–0.95-fold; and *ram*A, 0.49–1.66-fold. Sal-9 showed the highest increase in *ram*A gene expression (1.66-fold, $p < 0.05$) (Fig. 1).

The ciprofloxacin-resistant mutant strains STM and STH

**Table 4.** Disk diffusion, MIC, and QRDR mutation results for the *Salmonella* Sal-9 strain and derived mutants STM and STH.

| Strains | Disk diffusion (mm) | | | | | | MIC (μg/ml) | Target site of mutations | |
|---|---|---|---|---|---|---|---|---|---|
| | OFX | TE | CIP | AM | NA | C | CIP | *gyr*A | *par*C |
| Sal-9 | 24 (S) | 29 (S) | 27 (I) | 0 (R) | 8 (R) | 8 (R) | 0.125 | wt | Thr57 → Ser |
| STM | 15 (I) | 15 (S) | 18 (R) | 0 (R) | 0 (R) | 0 (R) | 8 | Asp87 → Gly | wt |
| STH | 9 (R) | 19 (S) | 12 (R) | 0 (R) | 0 (R) | 0 (R) | 16 | Asp87 → Gly | wt |

OFX : Ofloxacin; CIP : Ciprofloxacin; NA : Nalidixic acid; AM : Ampicillin; TE : Tetracycline; C : Chloramphenicol. Resistant (R); Intermediate (I); Susceptible (S) (CLSI 2014).

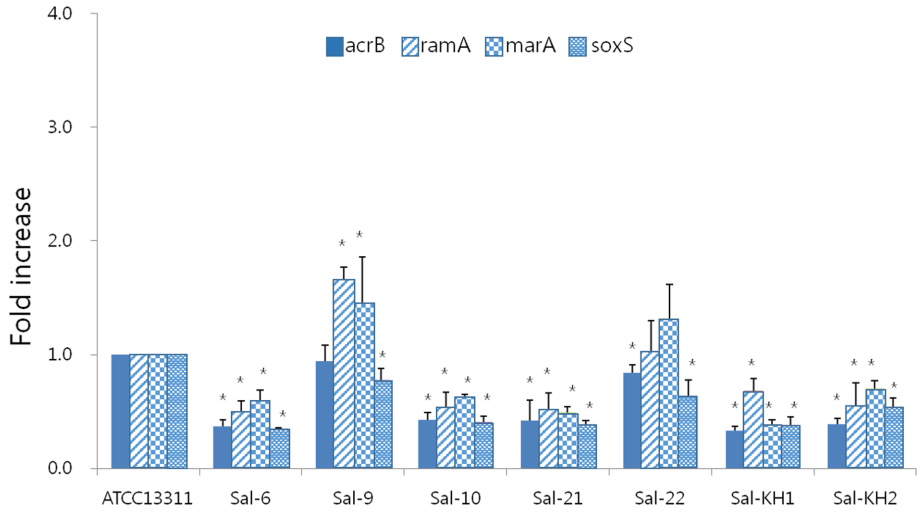


**Fig. 1.** mRNA expression levels of the AcrAB-TolC efflux system-related genes in *Salmonella* isolates.
*$p < 0.05$.

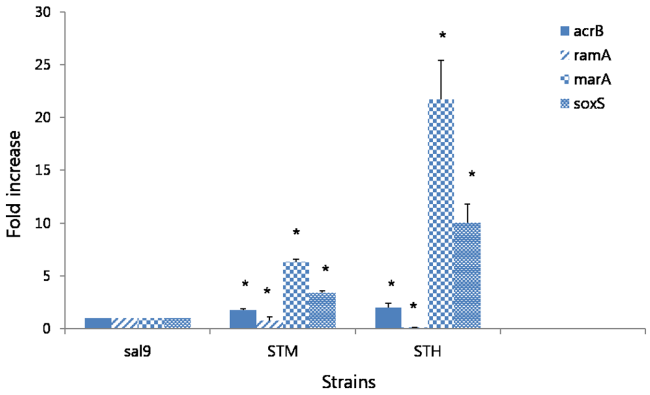


**Fig. 2.** mRNA expression levels of the AcrAB-TolC efflux system-related genes in ciprofloxacin-resistant *Salmonella* mutants.
*$p < 0.05$.

showed 1.79- and 2.0-fold increases in the expression of the *acr*B gene, respectively. They showed 6.35- and 21.73-fold increases in the expression of the transcriptional regulator *mar*A, and 3.41- and 10.05-fold increases in *sox*S, respectively ($p < 0.05$). The STM and STH mutants showed reduced gene expression of *ram*A (0.77- and 0.10-fold, respectively) compared with Sal-9 (Fig. 2).

It is believed that overexpression of the transporter *acr*B and regulators *mar*A and *sox*S of the AcrAB-TolC system is highly correlated with the increase in resistance to fluoroquinolone.

## Discussion

Mutations in the bacterial DNA gyrase (*gyr*A and *gyr*B) and topoisomerase IV (*par*C and *par*E) genes, as well as active efflux genes, are known to mediate bacterial resistance to fluoroquinolones [24]. In *Salmonella*, the most common point mutations associated with resistance to quinolones occur in the *gyr*A gene, resulting from substitutions of Ser-83 with Tyr, Phe, or Ala, and of Asp-87 with Asn, Gly, or Tyr [7]. These residues are close to Tyr-122, which is the active site of DNA gyrase binding to the 5′-end of DNA. Because quinolones bind close to the active site of gyrase, they inhibit the binding of gyrase to DNA and cause reduced gyrase activity [13].

In this study, we identified mutations at codon 83 (Ser83 → Phe, $n = 3$) and codon 87 (Asp87 → Gly, $n = 12$; Asp87 → Thr, $n = 1$; Asp87 → Asp, $n = 3$; Asp87 → Ser, $n = 1$) of the *gyr*A gene in the QRDR. For *gyr*B, only the resistant strain Sal-KH1 had a mutation at Thr469 → Ala. In addition, two strains contained a mutation at codon 57 of *par*C.

Although the difference in MIC values between resistant and intermediately sensitive strains was relatively high, all of them showed only a single mutation in the *gyr*A gene. A double mutation in *gyrA* (83Ser and 87Asp) was reported to occur with resistance to ciprofloxacin at an MIC of 32 μg/ml [5]. Sal-21, which has a double mutation at *gyr*A and *par*C, did not have a higher MIC for ciprofloxacin (0.125 μg/ml) than that of the mutant with a single mutation. However, Sal-KH1, which has a double mutation at *gyr*A and *gyr*B, had an MIC of 2 μg/ml, indicating resistance. In accordance with previous studies, multiple mutants generally had higher MIC values than mutants with a single mutation in the QRDR [15, 17, 26].

Previously, the *qnr* (*qnr*A, *qnr*B, *qnr*S, and *qnr*D) and *aac*

*(6′)-Ib-cr* plasmid-mediated resistance genes were reported in quinolone-resistant, non-typhoidal *Salmonella* [11, 38]. The Qnr protein induces resistance to nalidixic acid, thereby decreasing or limiting susceptibility to fluoroquinolone resistance [30]. In this study, only the Sal-KH1 strain was shown to have the *qnrA* gene in the PMQR, and its MIC was 64 μg/ml and 2 μg/ml to nalidixic acid and ciprofloxacin, respectively. The *qnr*A and *aac(6′)-Ib-cr* genes have been reported to be widely distributed among intestinal microorganisms [22, 32].

The efflux pump system is a well-known resistance mechanism to quinolones [3]. The expression levels of the transporter *acr*B gene and transcriptional regulator *ram*A, *mar*A, and *sox*S genes of the AcrAB-TolC efflux pump system were estimated, but the isolated strains did not show more than a 2-fold difference in the expression levels of the *acr*B, *ram*A, *mar*A, and *sox*S genes.

Two ciprofloxacin-resistant mutant strains (STM and STH) derived from Sal-9 were studied to elucidate the mechanism of resistance to fluoroquinolones. Corresponding to the increase in the MIC for ciprofloxacin, the expression of the global regulators *mar*A and *sox*S in the STM and STH mutants increased 6.35- and 21.73-fold (*mar*A) and 3.41- and 10.05-fold (*sox*S), respectively.

The expression of the transporter gene *acr*B also increased 1.79-fold in STM and 2.0-fold in STH, respectively. O'Regan *et al*. [31] reported that the deletion of *mar*A and *sox*S resulted in the downregulation of *acr*B gene expression by 10-fold and 4.7-fold, respectively. In previous studies, the increased expression of *ram*A downregulated the expression of *sox*S [29, 31]. In this study, the increase in *sox*S expression downregulated the expression of the *ram*A gene.

When the STH mutant was grown in medium with ciprofloxacin at half the MIC for 3 h, the expression of *mar*A, *sox*S, and *acr*B increased 67.88-fold, 23.0-fold, and 5.32-fold, respectively.

Consequently, the mutations at codon 83 and codon 87 in *gyr*A in the clinical strains and mutant strains and the overexpression of the AcrAB-TolC system regulators *mar*A and *sox*S are strongly believed to be related to increased resistance to fluoroquinolones.

The incidence of MDR *Salmonella* strains has increased globally [23, 35] and domestically, especially after 2008, and *Salmonella* strains with multidrug resistance, including against ampicillin and nalidixic acid, were reported to have sharply increased [4, 18]. Similarly, in this study, the ratio of resistance to ampicillin and nalidixic acid was the highest among the test *Salmonella* strains.

These insights into the resistance mechanism will guide the development of proper treatments and countermeasures in the near future.

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