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Increase of multidrug efflux pump expression in fluoroquinolone-resistant *Salmonella* mutants induced by ciprofloxacin selective pressure

Hyun-Wol Kang, Gun-Jo Woo *

Laboratory of Food Safety and Evaluation, Department of Food Bioscience and Technology, Korea University, Anam-dong 5-ga, Seongbuk-gu, Seoul 136-713, Republic of Korea

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ABSTRACT

Multidrug-resistant foodborne pathogens are a leading public health concern, as antimicrobial resistance can lead to therapeutic failure. In this study, a ciprofloxacin-susceptible *Salmonella* Istanbul (Sal10-FC-KU12) was isolated from chicken meat obtained from a market in Korea to induce ciprofloxacin-resistant mutants (SalML, SalMM, and SalMH). Minimum inhibitory concentrations (MICs) of 12 antibiotics were measured in the presence or absence of an efflux pump inhibitor. Expression levels of efflux pump-related genes (*acrB*, *acrF*, *marA*, *ramA*, *rob*, and *soxS*) were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Elevated MICs for the derived mutants were shown to result from the action of the efflux pump, with increased expression of *marA*, *ramA*, and *acrB* compared with the wild-type strain. The results of this study suggest that continued use of ciprofloxacin might induce the emergence of *Salmonella* mutants resistant not only to fluoroquinolones, but also to several other classes of antimicrobials.

1. Introduction

Salmonella is a major pathogen in foodborne diseases generally acquired by the consumption of contaminated food (Mead et al., 1999). In Korea, non-typhoid *Salmonella* is reported as the second most common cause of human food poisoning (Lee et al., 2001). Fluoroquinolones are the main antibiotics used to treat human salmonellosis because of their broad antimicrobial spectrum (Giraud et al., 2006). However, the emergence of less fluoroquinolone-susceptible strains (minimum inhibitory concentration [MIC] 0.125–0.5 μg/ml) and the failure of ciprofloxacin treatment against *Salmonella* strains (MIC, 0.06–0.38 μg/ml) have been reported in the past decade (Aarestrup et al., 2003; Choi et al., 2005). In addition, recent studies have reported high isolation rates of quinolone- and fluoroquinolone-resistant *Salmonella* strains from poultry samples (Miranda et al., 2009).

Previous studies have reported that quinolone resistance is caused by mutations in chromosomal genes; however, recently, certain transferable resistance plasmids represent novel mechanisms for the emergence and spread of quinolone resistance and/or of multiresistance respectively (Tran and Jacoby, 2001). Chromosomal resistance to fluoroquinolones results from the interplay of different mechanisms such as mutational modifications in the quinolone resistance determining region (QRDR), DNA gyrase (*gyrA* and *gyrB*), and topoisomerase IV (*parC* and *parE*), and decreased accumulation of fluoroquinolone chemicals in resistant strains. Overexpression of the AcrAB-TolC efflux pump also increases resistance to fluoroquinolones. Studies of several Gram-negative pathogens, including *Escherichia coli* and *Salmonella enterica*, showed this system to be responsible for the multidrug-resistant (MDR) phenotype (Baucheron et al., 2004). AcrAB-TolC is a member of the resistance nodulation-division (RND) family and is composed of three functional domains: a cytoplasmic membrane component (AcrB), a membrane fusion protein (AcrA), and an outer membrane channel protein (TolC) (Li and Nikaido, 2009; Quinn et al., 2006). In the cytoplasmic membrane component, AcrB is part of the multidrug efflux transporter, which extrudes chemicals such as drugs, dyes, disinfectants, and detergents from the cell (Blair and Piddock, 2009; Sun et al., 2011).

AcrAB expression is modulated by a local repressor, AcrR, and by the global regulators SoxS, MarA, RamA, and Rob (Kehrenberg et al., 2009; Kern et al., 2000; Nikaido et al., 2008). RamA plays a key role in increasing the expression level of the efflux pump by binding to the upstream promoter region of *acrAB* and *tolC* (Nikaido et al., 2008), and can be regulated by the local repressor RamR. In addition, over expression of MarA and SoxS is also associated with over expression of the MDR efflux pump system, which is regulated by MarR and SoxS (Kern et al., 2000; Linde et al., 2002; Oethinger et al., 1998).

* Corresponding author. Tel.: +82 2 3290 3021; fax: +82 2 3290 4984.
E-mail address: visionkorea@korea.ac.kr (G-J. Woo).

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In this study, we obtained ciprofloxacin-resistant mutants via *in vitro* exposure of ciprofloxacin-susceptible *S. enterica* strains grown on tryptic soy agar (TSA; Bacto, Sparks, MD) to gradually increasing concentrations of ciprofloxacin. Phenotypic and genotypic changes of the wild-type strain and derived mutants were characterized and examined to determine the expression levels of efflux pump-related genes, changes in QRDR sequence, and MICs of fluoroquinolones and other classes of antimicrobials. We performed an efflux pump AcrAB-TolC inhibition test to confirm the contribution of efflux pump in the development of the multidrug-resistant (MDR) phenotype.

2. Materials and methods

2.1. *Bacterial strains,* in vitro *selection of mutants, and culture conditions*

A *Salmonella* Istanbul isolate (Sal10-FC-KU12) recovered from chicken meat, which showed high resistance to nalidixic acid (MIC > 4096 μg/ml) and reduced susceptibility to ciprofloxacin (MIC ≥ 0.125 μg/ml), was used in this study. Three fluoroquinolone-resistant mutants (SalML, SalMM, and SalMH) were derived from wild-type *Salmonella* Istanbul strain Sal10-FC-KU12 after six serial passages (ranging from 0.5 to 16 μg/ml) on tryptic soy agar (TSA) containing ciprofloxacin (Bayer Korea, Seoul, Korea). The selected mutants were incubated in broth containing ciprofloxacin to confirm their ciprofloxacin resistance phenotype. Subsequently, subcultivation of all the selected colonies on antimicrobial-free medium was carried out (for five passages) to stabilize the resistant phenotype. The wild-type strain and three mutants were stored at –80 °C in 10% skim milk (BD-Diagnostic Systems) until use.

2.2. *Antimicrobial susceptibility test*

The MICs of the parental strain and derived mutants for four fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, and norfloxacin) and other antibiotics (nalidixic acid, ampicillin, streptomycin, cephalothin, cefoxitin, cefotaxime, chloramphenicol, and tetracycline) were determined by E-test on Mueller-Hinton agar (MHA; BD-Diagnostic Systems, Heidelberg, Germany) according to the manufacturer's instructions (AB-Biodisk, Solna, Sweden) and by agar dilution according to the Clinical and Laboratory Standards Institute (CLSI)-recommended test method (Clinical and Laboratory Standards Institute, 2011). *E. coli* ATCC 25922 and 35218 were used as quality control strains in antimicrobial susceptibility testing.

2.3. *Efflux pump inhibition test*

To confirm that increased multidrug resistance levels in mutants results from efflux pumps, antibiotic susceptibility tests were performed with Phe-Arg β-naphtylamide (PAβN, Sigma), an efflux pump inhibitor. The MICs of strains against ciprofloxacin, ofloxacin, levofloxacin, and norfloxacin were tested by the E-test on MHA containing 20 μg/ml PAβN. The agar dilution method was used to assess the MICs against the eight remaining antibiotics. MHA plates in the absence of the PAβN were used as negative controls.

2.4. *Expression analysis of transporter genes and transcriptional regulator genes*

The expression levels of *acrB*, *acrF*, *ramA*, *rob*, *marA*, and *soxS* were determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR). We used 16S rRNA as a reference gene. Primer sequences for each target gene used in this study have been described previously (O'Regan et al., 2009; Zheng et al., 2009). Briefly, overnight cultures of five strains were diluted 100-fold in 10 ml of Luria-Bertani broth and incubated at 37 °C until the exponential phase of bacterial growth was achieved (OD_{600} = 0.5–1). Cells were collected by centrifugation (12000× *g*, 3 min), and total RNA was purified using the GeneJET RNA purification kit according to the manufacturer's instructions (Fermentas, Canada). The RNA was quantified using a Nanodrop 2000 spectrophotometer to measure the OD at 260 nm (Thermo Scientific, Waltham, MA, USA). Total RNA was treated with DNase I (Takara, Ohtsu, Japan). cDNA was synthesized using 1 μg of template RNA with the Maxima First Strand cDNA synthesis kit for qRT-PCR (Fermentas). qRT-PCR was carried out in an iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad). PCR was conducted with an initial denaturation step of 3 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, annealing temperature for 30 s, and 72 °C for 30 s. The standard curves of each gene were individually generated. The $2^{-\Delta\Delta Ct}$ method was used to calculate changes in transcription of target genes in mutants compared to the parental strain (Sal10-FC-KU12).

2.5. *Sequence analysis of QRDR region*

Chromosomal DNA from the four strains was used as template for PCR. Primer sequences used in the PCR amplification were used as previously described (O'Regan et al., 2009). After a 3 min predenaturation step at 95 °C, amplification was performed with 35 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature, 30 s at 72 °C, and a final extension step of 10 min at 72 °C by using a Thermal iCycler system (Bio-Rad, Hercules, CA). Positive PCR products were purified from agarose gels by using a commercial purification kit (Bionics, Seoul, Korea) and forwarded for nucleotide sequencing to Macrogen Inc. (Seoul, Korea). The sequence was aligned using the National Center for Biotechnology Information (NCBI) BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) and analyzed with Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Multiple sequence alignment using ClustalW (http://www.clustal.org/clustal2/) was also carried out.

3. Results

3.1. *Resistance phenotypes and QRDR mutations of the* Salmonella *isolate and corresponding mutants*

Three derived mutants (SalML, SalMM, and SalMH) exhibiting intermediate (MIC = 2 μg/ml) and high resistance (MIC ≥ 4 μg/ml) to ciprofloxacin were obtained from the parental strain, Sal10-FC-KU12, after passage into stepwise increasing concentrations of ciprofloxacin. The MICs of 12 antibiotics, including four fluoroquinolones, are shown in Table 1. All three mutants, like the parental strain, showed no mutations on *gyrA* and *parE*. Four strains showed extremely high MICs against nalidixic acid (≥4096 μg/ml). SalML, with a mutation in *parC* (T57S), exhibited increased MICs against ciprofloxacin (6-fold), ofloxacin (3-fold), levofloxacin (3-fold), and norfloxacin (6-fold). SalML also showed decreased susceptibility to ampicillin (4-fold), streptomycin (4-fold), cephalothin (4-fold), cefoxitin (4-fold), cefotaxime (4-fold), chloramphenicol (2-fold), and tetracycline (2-fold). SalMM harbored the same mutation on the *parC* region and exhibited significantly increased MICs of ciprofloxacin (12-fold), ofloxacin (24-fold), levofloxacin (11-fold), norfloxacin (24-fold), ampicillin (8-fold), cephalothin (8-fold), cefoxitin (8-fold), cefotaxime (8-fold), chloramphenicol (16-fold), and tetracycline (8-fold). It also showed elevated MIC of streptomycin, with the same fold increase as that observed in SalML. SalMH harboring an additional point mutation (E466D) in *gyrB* exhibited higher MICs than those of SalMM against ciprofloxacin (48-fold), ofloxacin (≥32-fold), levofloxacin (63-fold), and norfloxacin (48-fold). However, the increases in MICs for