学位論文要約

Studies on *Salmonella* Typhimurium DNA gyrase and the impact of *gyrA* mutations to quinolone susceptibility

（*Salmonella* Typhimuriumが有するDNAジャイレースの性状とAサブユニット遺伝子上の変異のキノロン剤感受性への影響）

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CONTENT

ABBREVIATIONS

GyrA	subunit A of DNA gyrase
GyrB	subunit B of DNA gyrase
ATP	Adenosine triphosphate
QRDRs	Quinolone resistance-determining regions
QBP	Quinolone binding pocket
WT	Wild-type
Ser	Serine
Phe	Phenylalanine
Asp	Aspartic acid
Asn	Asparagine
Gly	Glycine
Tyr	Tyrosine
S83F	An amino acid substitution from serine to phenylalanine at position 83
D87N	An amino acid substitution from aspartic acid to asparagine at position 87
D87G	An amino acid substitution from aspartic acid to glycine at position 87
D87Y	An amino acid substitution from aspartic acid to tyrosine at position 87
S83F-D87N	An amino acid substitution from serine to phenylalanine at position 83 and an amino acid substitution from aspartic acid to asparagine at position 87

FQs	Fluoroquinolones
CIP	Ciprofloxacin
ENR	Enrofloxacin
GAT	Gatifloxacin
LVX	Levofloxacin
OFX	Ofloxacin
SPX	Sparfloxacin
OXO	Oxolinic acid
NAL	Nalidixic acid
MXF	Moxifloxacin
SIT	Sitafloxacin
PCR	Polymerase Chain Reaction
LB	Luria-Bertani
TSB	Tris-borate-EDTA
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
R	Relaxed pBR322 DNA
SC	Supercoiled pBR322 DNA
IC_{50}	The half of maximum inhibitory concentration
CC_{25}	The concentrations of drugs required for the conversion of 25% of the relaxed form plasmid DNA to the linear form
MICs	Minimum inhibitory concentrations
Ni-NTA	Ni-nitrilotriacetic acid

PREFACE

Non-typhoidal *Salmonella* is the primary foodborne zoonotic agent of salmonellosis in many countries. The global impact of non-typhoidal *Salmonella* on human health is high, with an estimated 94 million cases and 155,000 deaths each year, of which 80 million are believed to be foodborne (1). According to the World Health Organization, in most countries participating in the Global Foodborne Infections Network, *Salmonella* serovar Enteritidis and *Salmonella* serovar Typhimurium are the most frequently isolated serotypes of *Salmonella* (2). Gastroenteritis caused by *Salmonella* is generally a self-limited illness. However, antimicrobials may be required to treat invasive cases and susceptible groups such as young children, the elderly and immunocompromised patients (3). In general, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole are used to treat salmonellosis, but resistance to these drugs has increased significantly in recent years (4–6). In the case of invasive and systemic salmonellosis in humans and animals, quinolones are the preferred drugs for treatment.

To date, a number of quinolone antimicrobial agents have been synthesized. The development of quinolones was alteration of the quinolone nucleus through the changes of different substituents at the N-1, C-6, C-7, and C-8 positions (Figure 1). First generation quinolones (i.e., nalidixic acid, oxolinic acid and flumequine) have been used less today. This generation was excellent against aerobic and Gram negative bacteria and restricted to the treatment of uncomplicated urinary tract infections. The second generation quinolones were introduced when norfloxacin was synthesized by adding a fluorine atom at position C-6, which enlarged DNA gyrase inhibitory activity and enhanced penetration into the bacterial cell and norfloxacin was the first of the “fluoroquinolones”. Other second generation

quinolones include ciprofloxacin, ofloxacin and levofloxacin. Third generation quinolones, including gatifloxacin, sparfloxacin and pazufloxacin, these agents are divided into a third class because of their extended activity against Gram positive organisms. Fourth quinolones generation (i.e., sitafloxacin, moxifloxacin and gemifloxacin), they had potent activity against anaerobes while maintaining the Gram positive and Gram negative activity of the third generation quinolones (7).

The enzyme targets for quinolones are type II topoisomerases, a group of enzymes that catalyze the interconversion of topoisomers of the DNA and whose inhibition by interaction with quinolones and leads to the inhibition of replication and transcription. Type II topoisomerases (DNA gyrase and topoisomerase IV) are heterotetramers of two A subunits (encoded by *gyrA* in DNA gyrase or *parC* in topoisomerase IV) and two B subunits (encoded by *gyrB* in DNA gyrase or *parE* in topoisomerase IV) (8). The major activities of DNA gyrase and topoisomerase IV and the action of quinolones were shown in Figure 2 (figure modified from previous study (9)).

The mechanisms responsible for quinolone resistance are mainly a result of chromosomal mutations in gene encoding target enzymes, DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). Mutations result in structural changes at the target site of the enzyme tetramer, which lead to a reduced affinity for quinolone-based drugs and consequent resistance against these agents (10). Mutations are commonly located within small regions of these genes, the quinolone resistance-determining regions (QRDRs) (9, 11–13). In *Salmonella*, mutations at codon 83; serine to phenylalanine (S83F) and codon 87; aspartic acid to asparagine (D87N), glycine (D87G), or tyrosine (D87Y) in subunit A of DNA gyrase (GyrA) (Figure 3) are frequently observed (6, 14–19). Isolates with double mutation at both codons 83 and 87 (S83F-D87N) have previously been documented to associate with high-level fluoroquinolones (FQs) resistance (20). Although, the main target of quinolones is

known to be bacterial DNA gyrase, the molecular details of quinolone-gyrase interaction are not yet fully understood in *Salmonella*. Investigating the contributions of amino acid substitutions by an *in vitro* assay may help gain a comprehensive understanding of the mechanism by which quinolones resistant *Salmonella* emerges.

This thesis consists of two Chapters. In the Chapter I, I have verified the inhibitory effect of 10 quinolones with recombinant wild-type (WT) DNA gyrases by using *in vitro* quinolones-inhibited DNA supercoiling assay and quinolone-mediated DNA cleavage assay in the presence or absence quinolones. In the Chapter II, to reveal the significance of amino acid substitutions at position 83 and 87 in *S.* Typhimurium, I conducted the quinolones-inhibited DNA supercoiling assay utilizing WT and five mutant recombinant gyrases tested with four quinolones.

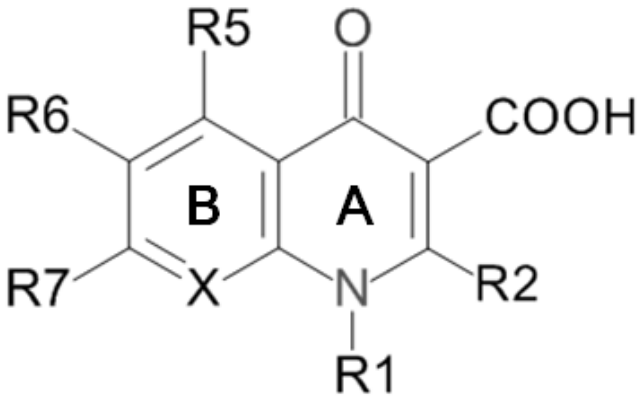


Figure 1. Structure of the quinolone molecule using the accepted numbering scheme for positions on the molecule. Basic structure of these compounds is a bicyclic structure, which contains a ring type A 4-piridinona, combined with aromatic or heteroaromatic ring B. The ring type A 4-piridinona is a ring with absolute necessity: an unsaturation in position 2-3, a free acid function in position 3 and a substituent at nitrogen (N) in position 1. An R indicates possible sites for structural modifications. X is CH or C-R8

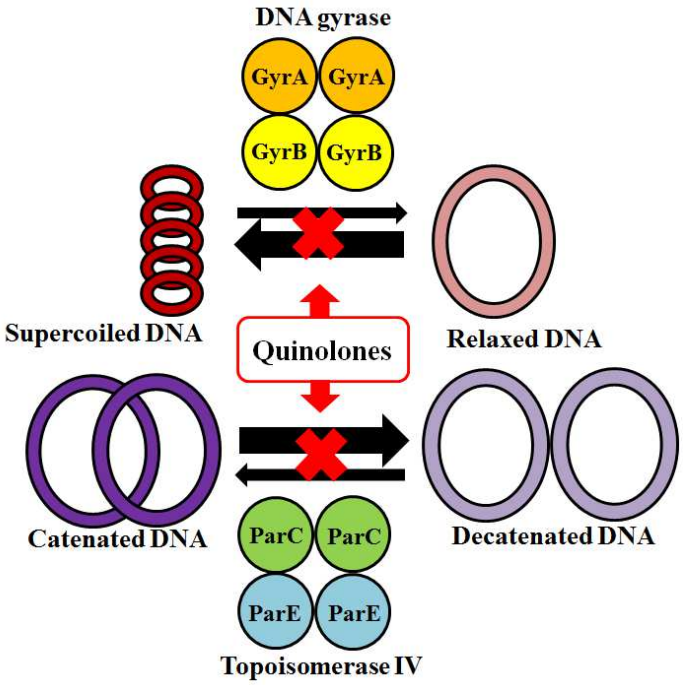


Figure 2. Major activities of DNA gyrase and topoisomerase IV. Quinolones block these activities by stabilizing an enzyme-DNA complex, which also functions as a barrier to the movement of other proteins such as DNA polymerase and RNA polymerase along the DNA.

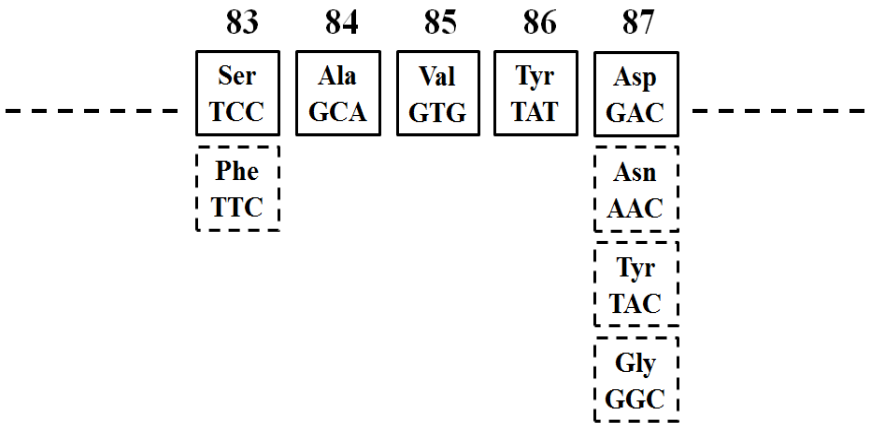


Figure 3. Amino acid substitutions found within the QRDR of GyrA in FQ-resistant *S.* Typhimurium. A nucleotide sequence encoding the QRDR of WT S. Typhimurium GyrA was aligned with the amino acid sequence at the corresponding positions indicated by the numbers. Altered amino acids and corresponding nucleotide substitutions of *S.* Typhimurium are shown below WT sequence and marked by a dashed box.

CHAPTER I

CHARACTERIZATION OF *SALMONELLA* TYPHIMURIUM DNA GYRASE AS A TARGET OF QUINOLONES

INTRODUCTION

Nowadays, resistant *Salmonella* are frequently encountered in most of the world and the rates of resistance have increased remarkably. As a result, quinolones, especially FQs, have become the drugs of choice for the treatment of severe *Salmonella* infections. Accordingly, minimizing the occurrence of resistance to quinolone is a public health priority, and these drugs have been categorised by the World Health Organization as critically important antimicrobials (21). To date, several reports have described the presence of resistance to quinolones (3–5, 22, 23).

DNA gyrases and DNA topoisomerase IV are essential enzymes for the maintenance of chromosomal metabolism in bacteria, and DNA gyrase is known to be the primary target of quinolones in salmonellae. This is due to most amino acid substitutions conferring quinolone resistance being found in DNA gyrase, but not in topoisomerase IV (24–26). It has been demonstrated that the quinolone-binding pocket (QBP) is a site enclosed by the QRDR of both GyrA and GyrB enzymes (13, 27).

The aim of this work was to further investigate the *in vitro* antibacterial activity of quinolones against *Salmonella* DNA gyrases and to establish a more complete model of the WT gyrase-quinolone interaction. To that end, we assessed the potency of 10 quinolones with quinolone-inhibited DNA supercoiling assays and quinolone-mediated DNA cleavage assays.

MATERIALS AND METHODS

Reagents and kits. Ciprofloxacin (CIP), enrofloxacin (ENR), gatifloxacin (GAT), levofloxacin (LVX), ofloxacin (OFX) and sparfloxacin (SPX) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Oxolinic acid (OXO) and nalidixic acid (NAL) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Moxifloxacin (MXF) was obtained from Toronto Research Chemical Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichi-Sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Oligonucleotide primers were synthesized by Life Technologies (Carlsbad, CA, USA). TaKaRa Mighty (Blunt End) Cloning Reagent Set (pUC118) and Ni-nitrilotriacetic acid (Ni-NTA) protein purification kits were purchased from Life Technologies. Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA, USA). Relaxed pBR322 DNA was purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom). Protease inhibitor cocktail (Complete Mini, EDTA-free) was purchased from Roche Applied Science (Mannheim, Germany).

Bacterial strains and plasmids. *S.* Typhimurium NBRC 13245 was purchased from NITE Biological Resource Center (Chiba, Japan). *Escherichia coli* strain TOP-10 (Life Technologies) was used as the host for cloning purposes. *E. coli* strain BL21(DE3)/pLysS was purchased from Merck KGaA (Darmstadt, Germany) and used for protein expression. The vector plasmid pET-20b(+) was used to construct expression plasmids for the *S.* Typhimurium proteins, GyrA and GyrB.

Determination of minimum inhibitory concentration (MICs). *S.* Typhimurium NBRC 13245 was analyzed for antimicrobial resistance patterns using the broth microdilution

method based on guidelines established by the US National Committee on Clinical Laboratory Standards (NCCLS) (28). An adjusted bacterial inoculum (10^6 CFU/10 μl) was added to each well of a sterile U-based microtitre plate containing the test concentrations of quinolones (90 μl/well). Consequently, a 5×10^5 CFU/ml concentration from the last inoculum was obtained in each well, and this plate was incubated for 18 hrs at 37ºC. The MIC was defined as the lowest antibiotic concentrations that inhibited visible bacterial growth after incubation. The panel of antimicrobial agents was CIP, ENR, GAT, LVX, OFX, SPX, OXO, NAL, MXF and SIT.

Construction of WT DNA gyrase expression vectors. DNA fragments, including *gyrA* and *gyrB*, were amplified from *S.* Typhimurium NBRC 13245 DNA by polymerase chain reaction (PCR) using the primers listed in Table 1. Two pairs of primers, ST1/ST2 and ST8/ST9, were used to amplify the complete *gyrA* and *gyrB* genes, respectively. *Nde*I restriction sites (CATATG) were included as overlaps of the ATG initiation codons for *gyrA* (ST1) and *gyrB* (ST8) primers, and the *Xho*I site (CTCGAG) was included after the stop codon for the *gyrA* (ST2) and *gyrB* (ST9) primers. PCR products corresponding to the 2.6-kb *gyrA* and 2.4-kb *gyrB* genes were ligated into the blunt-ended cloning plasmid, transformed into *E. coli* TOP-10, and plated on Luria-Bertani (LB) agar containing ampicillin (100 μg/ml). The *gyrA* and *gyrB* cassettes were digested with *Nde*I and *Xho*I, ligated into pET-20b(+) digested with the same restriction endonucleases, and transformed into *E. coli* TOP-10 to obtain GyrA and GyrB expression plasmids. Recombinant clones were selected from the resistant colonies on LB agar plates containing ampicillin (100 μg/ml).

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described (29–32). Expression plasmids carrying the WT *gyrA* and WT

gyrB genes of *S.* Typhimurium were transformed into *E. coli* BL21(DE3)/pLysS. GyrA and GyrB expression was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 18°C for 13 hrs. Recombinant DNA gyrase subunits in the supernatant of the sonicated lysate (by Sonifier 250; Branson, Danbury, CT, USA) were purified by Ni-NTA agarose resin column chromatography (Life Technologies Corp.). Protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After purification, the protein fractions were added by glycerol to yield 50% (wt/vol) and stored at -80°C until use.

DNA supercoiling activities and inhibition by quinolones. ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described (29–32) with the following modifications. The reaction mixture (total volume, 30 μl) consisted of DNA gyrase assay buffer (35 mM Tris-HCl pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol and 0.1 mg/ml BSA), relaxed pBR322 DNA (0.3 μg), and GyrA and GyrB proteins (30 ng each). Reactions were run at 35°C for 20 min and stopped by the addition of 30 μl of chloroform-isoamyl alcohol (24:1 mixture, v/v) and 3 μl of 10x DNA loading dye. The total reaction mixtures were subjected to electrophoresis using a 1% agarose gel in 0.5x Tris-borate-EDTA (TBE) buffer. The gels were run for 60 min at 80 mA and stained with ethidium bromide (0.7 μg/ml). Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). The inhibitory effect of FQs on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s) in the absence or presence of 2-fold serial dilutions in the concentrations of 10 quinolones. To allow direct comparisons, all incubations with DNA gyrase were carried out and processed in

parallel on the same day under identical conditions. All enzyme assays were performed at least three times to confirm reproducibility.

Quinolone-mediated DNA cleavage assay. Quinolone-mediated DNA cleavage assays were carried out as previously described (29–32). Supercoiled, rather than relaxed, pBR322 DNA was used as the substrate for cleavage assays. The reaction mixture (total volume, 30 μl) contained DNA gyrase assay buffer, purified GyrA and GyrB (30 ng each), supercoiled pBR322 DNA (0.3 μg) and 2-fold serially diluted concentrations of 10 quinolones. After incubation for 20 min at 35°C, 3 μl of 2% SDS and 3 μl of proteinase K (1 mg/ml) were added to the reaction mixture. After additional incubation for 30 min at 35°C, reactions were stopped to allow a relaxation activity by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isoamyl alcohol (24:1 mixture, v/v) and 3 μl of 10x DNA loading dye. The plasmid pBR322 linearized by *Bam*HI digestion was used as a marker for cleaved DNA. The total reaction mixtures were subjected to electrophoresis using 0.8% agarose gels in 0.5x TBE buffer. The gels were run for 60 min at 80 mA, stained with ethidium bromide (0.7 μg/ml) and photographed under UV transillumination. The extent of DNA cleavage was quantified with the Molecular Analyst software ImageJ (http://rsbweb.nih.gov/ij). The quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined for the 10 quinolones.

Correlation between MICs, IC_{50}s and CC_{25}s against *S.* Typhimurium gyrases. The relationships between the MICs, IC_{50}s and CC_{25}s were converted to log10 and assessed by estimating a linear regression between two components. The strength of this relationship was quantified by the R coefficient and displayed graphically by the regression line and the two curves defining the 95% confidence interval for this regression.

RESULTS

Quinolone susceptibility patterns. The MICs of quinolones and fluoroquinolones were determined against *S.* Typhimurium NBRC 13245 (Table 2). The MICs of fluoroquinolones (GAT, SPX, LVX, MXF, ENR, OFX, CIP and SIT) were 64- to 512-fold lower than those of quinolones (OXO and NAL).

Expression and purification of recombinant GyrA and GyrB proteins. Ni-NTA affinity purification from 200 ml cultures resulted in 16 and 1.6 mg of soluble His-tagged 97-kDa and 89-kDa proteins, respectively, corresponding to GyrA and GyrB (Figure 4). Both recombinant gyrase subunits were obtained at high purity (>95%).

DNA supercoiling activity of recombinant DNA gyrase. Combinations of GyrA and GyrB were examined for DNA supercoiling activity. A combination of GyrA and GyrB at 30 ng each in the presence of ATP was sufficient for the conversion of 100% of 0.3 μg of relaxed plasmid pBR322 DNA to its supercoiled form and was used for all DNA supercoiling experiments. No subunit alone exhibited DNA supercoiling activity in the presence of 1 mM ATP, and no supercoiling activity was observed when ATP was absent from the reaction mixture, which indicated the lack of or low concentration of *E. coli*-derived DNA gyrase subunits (Figure 5).

IC_{50}s of quinolones. The inhibitory effects of quinolones including CIP, ENR, GAT, LVX, OFX, SPX, MXF, SIT, OXO and NAL on the *S.* Typhimurium gyrase were elucidated by the quinolone-inhibited DNA supercoiling assay. A set of representative results showing the inhibitory effect of CIP and NAL is shown in Figure 6 (IC_{50} values were exhibited by the red

arrows). Each quinolone showed dose-dependent inhibition, with IC_{50}s ranging from 0.22 to 65.1 µg/ml (Table 2). The inhibitory effects of quinolones against the recombinant gyrase are presented as IC_{50}s in ascending order in Table 2. A good correlation was found between the IC_{50} values and the corresponding MICs, as shown in Figure 7 (correlation coefficient values, R = 0.999).

CC_{25}s of quinolones. To examine the effects of quinolones on the cleavable-complex formation by the recombinant DNA gyrases, quinolone-mediated DNA cleavage assays were carried out in which supercoiled pBR322 was incubated with recombinant GyrA and GyrB in the presence or absence of quinolones. Figure 8 shows the results of a representative quinolone-mediated DNA cleavage assay using CIP and NAL (CC_{25}s were exhibited by red arrows). Table 2 presents the CC_{25}s of the ten quinolones, in which each quinolone showed dose-dependent inhibition, with CC_{25}s ranging from 0.24 to 8.64 µg/ml (Table 2). A good correlation was found between the CC_{25} values and the corresponding MICs, as shown in Figure 9 (correlation coefficient values, R = 0.969).

DISCUSSION

The emergence of quinolone-resistant *Salmonella* strains is a serious global public health concern because this class of antibacterial agents constitutes the treatment of choice in cases of acute salmonellosis caused by multidrug-resistant strains. Although the main target of quinolones is known to be DNA gyrase, the molecular details of quinolone-DNA gyrase interactions have not been elucidated in *Salmonella*. Hence, based on previous studies (29–32), we produced the His-tagged GyrA and GyrB of *S.* Typhimurium and obtained a proper amount of functional *S.* Typhimurium DNA gyrase after reconstitution. The ability of reconstituted enzymes to convert the relaxed form pBR322 plasmid DNA to the supercoiled form allowed us to examine and compare the inhibitory effects of 10 quinolones using the quinolone-inhibited DNA supercoiling assay and quinolone-mediated DNA cleavage assay. The quinolone-inhibited DNA supercoiling assay is an estimate of catalytic inhibition, whereas The quinolone-mediated DNA cleavage assay examines the balance between the ternary complex of DNA, gyrase and drug in which the DNA is either cleaved or intact (33, 34).

Quinolones inhibited the DNA supercoiling activity of *S.* Typhimurium DNA gyrase in a dose-dependent manner, as it has been reported for other bacteria (35–39). Among the ten quinolones examined, eight fluoroquinolones exhibited high inhibitory activity against *S.* Typhimurium DNA gyrase with IC_{50}s below 1 μg/ml, in contrast to two quinolones OXO and NAL whose IC_{50}s were 4.93 and 65.1 μg/ml, respectively (Table 2). Similar results were observed in the quinolone-mediated DNA cleavage assay. Eight fluoroquinolones exhibited high activity to mediate DNA cleavage with CC_{25}s below 1 μg/ml, while the two quinolones without fluorine at C-6 had lower activities (Table 2). Analysis of the quinolone structure-activity relationship showed that the eight fluoroquinolones shared certain structural features.

Position 1 is the part of the enzyme-DNA binding complex, and has a hydrophobic interaction with the major grove of DNA (40). A cyclopropyl substituent is now considered the most potent modification here. Another structure at this position is found in OFX and LVX which has a fused ring between position 1 and 8. Position 7 is one of the most influential points on the molecule, and the presence of a five- or six-membered nitrogen heterocycle at this position has been reported to improve the molecule's activity and pharmacokinetic profile (41). The most common heterocycles employed at position 7 are aminopyrrolidines and piparazines. The addition of azabicyclo to position 7 has resulted in MXF with significant anti-Gram-positive activity and marked lipophilicity (42).

DNA gyrase is an essential enzyme for DNA supercoiling, which is required for DNA replication and gene transcription. A domain of the N-terminal part of the A subunit of DNA gyrase is highly conserved among prokaryotes. In addition, the amino acid residues from the positions 67 to 106 of the A subunit in the numbering system used in *E. coli* was defined as the QRDR (12), which is supposed to be the site of interaction between the A subunit of gyrase and quinolones. The *S.* Typhimurium QRDR sequence of GyrA was compared with 4 bacterial species (Figure 10). The results of the quinolone structure-activity relationship analysis based on the *S.* Typhimurium WT QRDR sequence were highly concordant with those based on the *E. coli* WT QRDR sequence, which has a 100% identical QRDR sequence with *S.* Typhimurium (Figure 10). As summarized in Table 3, IC_{50}s of quinolones against *S.* Typhimurium and *E. coli* (43) WT DNA gyrase were lower than those against *Mycobacterium tuberculosis* (38), *Streptococcus pneumoniae* (44) and *Mycoplasma pneumoniae* (39). This result suggested that the interaction of quinolones with *S.* Typhimurium and *E. coli* WT DNA gyrase are stronger than those from other bacterial species. An early study by Guillemin *et al*. (45) proposed that the amino acid residue at position 83 in GyrA played a key role in the intrinsic susceptibility of DNA gyrases to

quinolones in mycobacteria. In their study, the serine residue and alanine residue correlated with low and high MICs, respectively. As shown in Figure 10, the amino acid at position 83 in *S.* Typhimurium and *E. coli* DNA gyrase (exhibited by arrowhead) was serine, while those at equivalent positions in DNA gyrase of *M. tuberculosis*, *S. pneumoniae* and *M. pneumoniae* were alanine, serine and methionine, respectively. The findings by Guillemin *et al*. agree in part with our current observation. The amino acid residue at position 83 may be the cause of the intrinsic susceptibility of WT DNA gyrases to quinolones in *M. tuberculosis* and *M. pneumonia* but not in *S. pneumoniae*. ClastalW analysis demonstrated a strong similarity in the QRDR amino acid sequence QRDR of WT GyrA beyond the genus (Figure 10), a similarity that was a critical point for the wide spectrum of quinolones. However, the amino acids in the QRDR that are distinct between bacterial species may contribute to various intrinsic susceptibilities; nevertheless, amino acid residues outside of QRDR also need to be considered.

The IC_{50}s of the *S.* Typhimurium DNA gyrase correlated strongly with MICs, confirming their ability to inhibit the growth of *S.* Typhimurium (R = 0.999). However, the IC_{50}s and MICs were not proportional; for example, SIT and CIP MICs were about 30-fold higher than in the gyrase assay. This nonproportionality has been noted by others (46) and presumably reflects basic differences in the cell-permeability properties and accumulation of the different quinolones (47). In the quinolone-mediated DNA cleavage assay, the effective quinolone concentrations were slightly different from those inhibiting supercoiling and less correlated with those inhibiting *S.* Typhimurium growth (R = 0.969). The strong correlation between IC_{50}s and MICs observed in other bacterial species (Table 3) corroborated this notion. Although the properties of bacteria to incorporate drugs may have some influence on MICs, the quinolone-inhibited DNA supercoiling assay on recombinant *S.* Typhimurium DNA gyrase could be used as a quick test to screen drugs with promising antibacterial

activities. Quinolones with IC_{50}s below 1 μg/ml can potentially be active against *S.* Typhimurium. In contrast, quinolones with high IC_{50}s in the enzyme assay may not be suitable for further evaluation as antibacterial drugs. Additionally, the high similarity of the amino acid sequences of both the A and B subunits of DNA gyrase between the quinolone-susceptible typhoidal and non-typhoidal *Salmonella* strains enables the usage of recombinant *S.* Typhimurium DNA gyrase for the *in vitro* selection of quinolones not only against *S.* Typhimurium, but also against other *Salmonella* species including highly pathogenic serovers, *S.* Typhi and *S.* Parayphi A.

SUMMARY

Quinolones exhibit good antibacterial activity against *Salmonella* isolates and are often the choice of treatment for life-threatening salmonellosis due to multi-drug resistant strains. To assess the properties of quinolones, we performed an *in vitro* assay to study the antibacterial activities of quinolones against *S.* Typhimurium recombinant DNA gyrase. We expressed the *S.* Typhimurium GyrA and GyrB subunits in *E. coli.* GyrA and GyrB were obtained at high purity (>95%) by Ni-NTA agarose resin column chromatography as His-tagged 97-kDa and 89-kDa proteins, respectively. Both subunits were shown to reconstitute an ATP-dependent DNA supercoiling activity. IC_{50}s or CC_{25}s demonstrated that quinolones highly active against *S.* Typhimurium DNA gyrase share a fluorine atom at C-6. The relationships between MICs, IC_{50}s and CC_{25}s were assessed by estimating a linear regression between two components. MICs measured against *S.* Typhimurium NBRC 13245 correlated better with IC_{50}s ($R = 0.999$) than CC_{25}s ($R = 0.969$). These findings suggest that the quinolone-inhibited DNA supercoiling assay may be a useful screening test to identify quinolones with promising activity against *S.* Typhimurium. The quinolone structure-activity relationship demonstrated here shows that C-8, the C-7 ring, the C-6 fluorine, and N-1 cyclopropyl substituents are desirable structural features in targeting *S.* Typhimurium gyrase.

Table 1. Oligonucleotide sequences of primers used for PCR

Primer name	Sequence (nucleotide position)[a]	Comment
ST1	5'-ggcatatgagcgaccttgcgagaga-3' (1-20), *Nde*I site	Wild-type *gyrA*
ST2	5'-ggctcgagctcgtcagcgtcatccgc-3' (2617-2634), *Xho*I site	Wild-type *gyrA*
ST8	5'-ggcatatgtcgaattcttatgactc-3' (1-20), *Nde*I site	Wild-type *gyrB*
ST9	5'-ggctcgagaatatcgatattcgctgctttc-3' (2391-2412), *Xho*I site	Wild-type *gyrB*

[a]Restriction enzyme sites are underlined

Table 2. Structural features, minimum inhibitory concentration and concentration of quinolones inhibiting *S*. Typhimurium wild-type gyrase activity

Quinolone	R-1	R-6	R-7	R-8	IC_{50} (µg/ml)	CC_{25} (µg/ml)	MIC (µg/ml)
SIT	fluorinated cyclopropyl	F	pyrrolidine	Cl	0.22 ± 0.04	0.24 ± 0.05	0.0078
CIP	cyclopropyl	F	piperazine	H	0.25 ± 0.05	0.31 ± 0.10	0.0078
GAT	cyclopropyl	F	piperazine	O-CH3	0.30 ± 0.05	0.30 ± 0.03	0.0625
SPX	cyclopropyl	F	piperazine	F	0.42 ± 0.04	0.69 ± 0.14	0.0625
LVX	bridge C1-C8	F	piperazine	bridge C1-C8	0.43 ± 0.03	0.31 ± 0.03	0.0625
MXF	cyclopropyl	F	azabicyclo	O-CH3	0.48 ± 0.01	0.34 ± 0.13	0.0625
ENR	cyclopropyl	F	piperazine	H	0.57 ± 0.03	0.86 ± 0.24	0.0625
OFX	bridge C1-C8	F	piperazine	bridge C1-C8	0.69 ± 0.20	0.67 ± 0.13	0.0625
OXO	ethyl	H	bridge C6-C7	H	4.93 ± 1.54	3.40 ± 0.75	0.5
NAL	ethyl	H	CH3	N	65.1 ± 19.08	8.64 ± 0.38	4

Table 3. IC_{50}s (μg/ml) and MIC_{50}s (μg/ml) for different bacterial species

Quinolone	*S.* Typhimurium[a]		*E. coli*[b]		*M. tuberculosis*[c]		*S. pneumoniae*[d]		*M. pneumoniae*[e]	
	IC_{50}s	**MICs**	**IC_{50}s**	**MICs**	**IC_{50}s**	**MICs**	**IC_{50}s**	**MICs**	**IC_{50}s**	**MICs**
SIT	0.22	0.0078			2.5	0.25				
CIP	0.25	0.0078	0.3	0.007	3.5	0.5	40	1-2		
GAT	0.30	0.0625			3	0.12	20-40	0.25	5.71	0.125
SPX	0.42	0.0625	0.2	0.015	2	0.25				
LVX	0.43	0.0625	0.29	0.015	5	0.5	80	1	47.5	0.5
MXF	0.48	0.0625			4.5	0.5	20	0.25	7.44	0.0625
ENR	0.57	0.0625								
OFX	0.69	0.0625	0.35	0.03	10	1				
OXO	4.93	0.5			300	32				
NAL	65.1	4	50	2	1100	128				

[a]This work

[b]Reference 43

[c]Reference 38

[d]Reference 44

[e]Reference 39

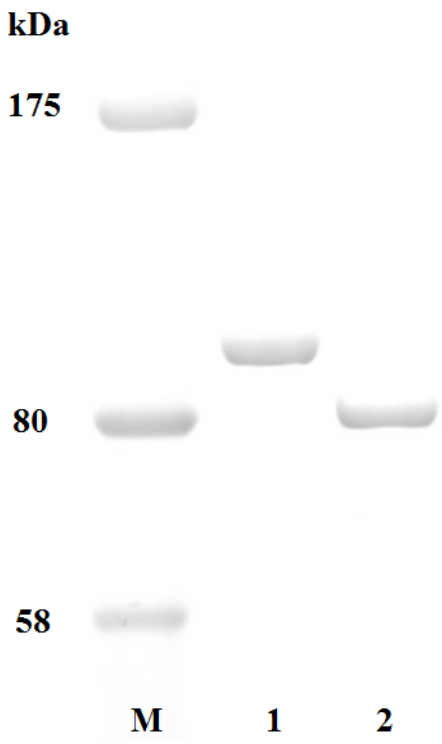
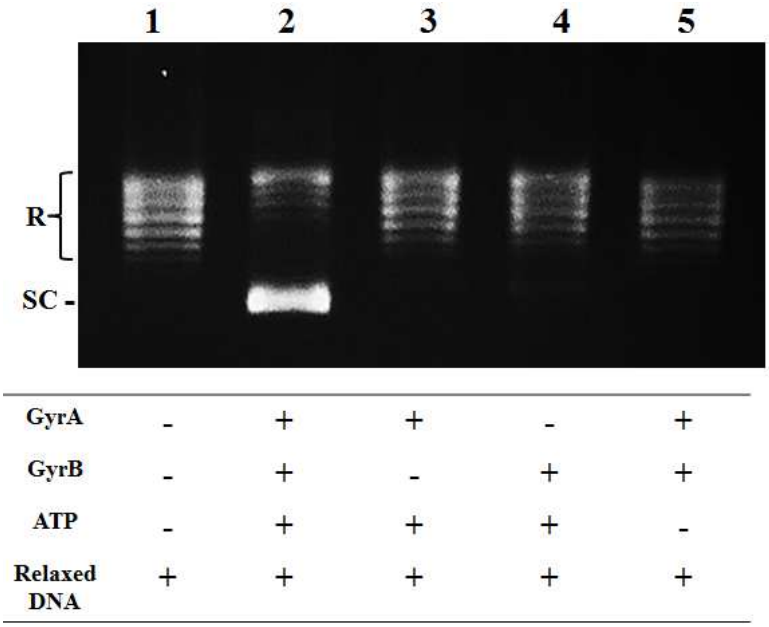


Figure 4. SDS-PAGE analysis of recombinant DNA gyrase subunits of *S.* Typhimurium. His-tagged proteins were overexpressed by an *E. coli* expression system and purified by nickel resin chromatography. Each protein sample (approximately 300 ng) was loaded in a SuperSep^{TM}Ace 5-20% gradient gel. Following electrophoresis, proteins were visualized by Quick CBB staining. Lane M, protein molecular weight markers (sizes are indicated to the left in kilodaltons; kDa); lane 1, WT GyrA subunit; lane 2, WT GyrB subunit.



	1	2	3	4	5
GyrA	-	+	+	-	+
GyrB	-	+	-	+	+
ATP	-	+	+	+	-
Relaxed DNA	+	+	+	+	+

Figure 5. WT GyrA and WT GyrB proteins of *S.* Typhimurium generate ATP-dependent DNA supercoiling activity. Relaxed pBR322 DNA (0.3 μg) was incubated with WT GyrA (30 ng) and WT GyrB (30 ng) in the absence or presence of 1 mM ATP. The reactions were stopped, and the DNA products were separated by electrophoresis in 1% agarose gels. DNA was stained with ethidium bromide and photographed under UV illumination. Lane 1, relaxed pBR322 DNA; lane 2, relaxed pBR322 DNA and both recombinant WT GyrA and WT GyrB proteins; lane 3, relaxed pBR322 DNA and only WT GyrA protein; lane 4, relaxed pBR322 DNA and WT GyrB protein; lane 5, absence of ATP. R and SC are indicated relaxed and supercoiled pBR322 DNA, respectively.

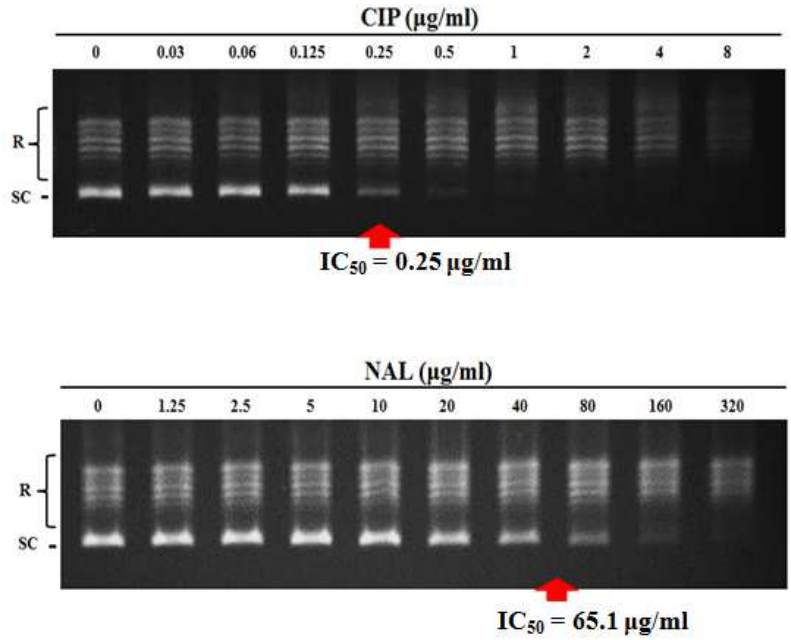


Figure 6. Inhibitory activities of CIP and NAL on the supercoiling activities of *S.* Typhimurium DNA gyrase. Relaxed pBR322 DNA (0.3 μg) was incubated with WT GyrA (30 ng) and WT GyrB (30 ng) in the presence of the indicated amounts (μg/ml) of CIP and NAL. The reactions were stopped, and the DNA products were analyzed by electrophoresis in 1% agarose gels. R and SC denote relaxed and supercoiled pBR322 DNA, respectively. IC_{50} values were exhibited by the red arrows.

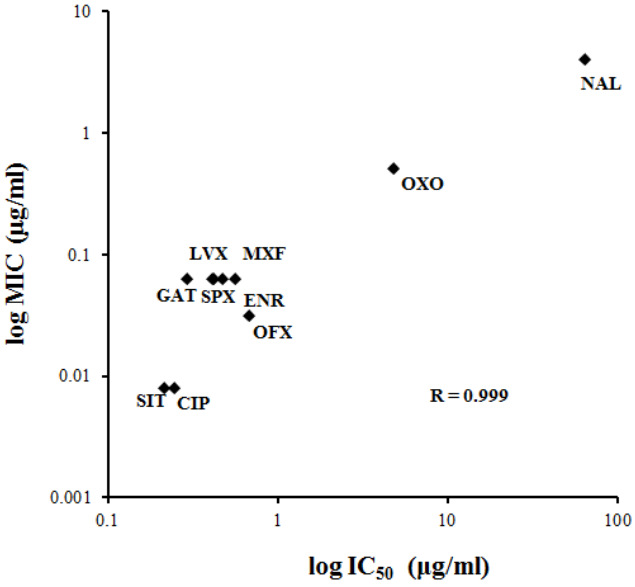


Figure 7. Correlation between the logarithm of the MICs of quinolones and the logarithm of corresponding concentration of quinolones inhibiting the supercoiling activity (IC_{50} values) of DNA gyrases from *S*. Typhimurium (R = correlation coefficient value).

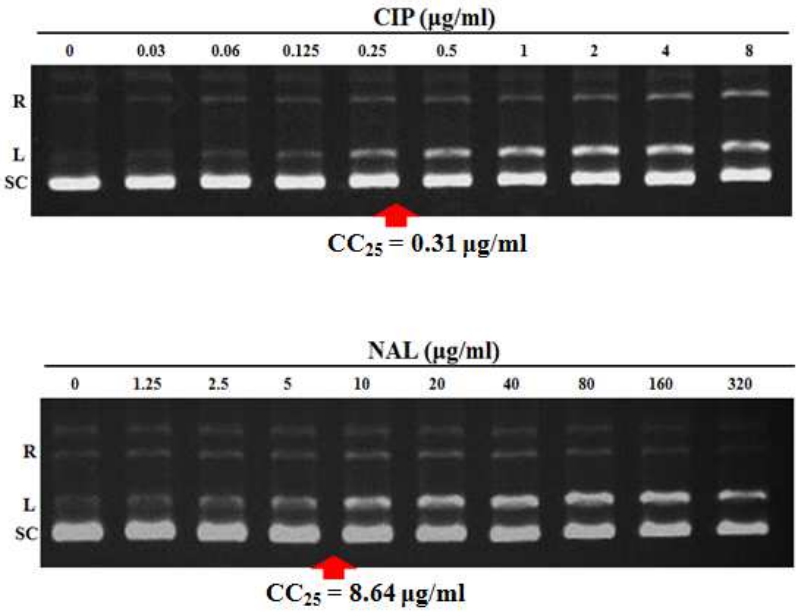


Figure 8. CIP- and NAL-mediated DNA cleavage assay by DNA gyrase of *S.* Typhimurium. Supercoiled pBR322 DNA (0.3 μg) was incubated with WT GyrA (30 ng) and WT GyrB (30 ng) in the presence of the indicated amounts (μg/ml) of CIP and NAL. After the addition of SDS and protease K, the reactions were stopped and the mixtures were analyzed by electrophoresis in 0.8% agarose gels. R, L and SC denote relaxed, *Bam*HI-linearized and supercoiled pBR322 DNA, respectively. The red arrows identify the CC_{25} values.

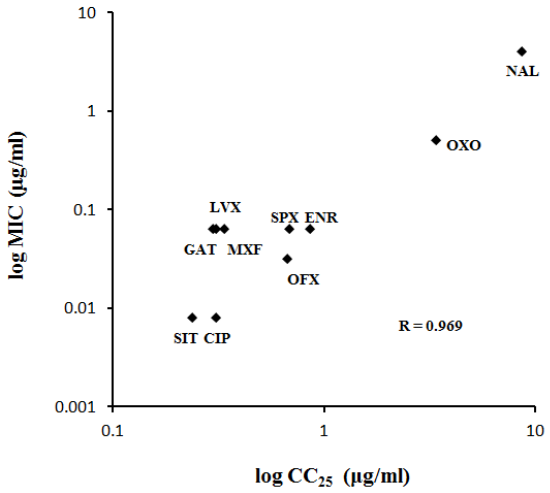


Figure 9. Correlation between the logarithm of the MICs of quinolones and the logarithm of corresponding concentration of quinolones inducing cleavable complex formation (CC_{25} values) of DNA gyrases from *S*. Typhimurium (R = correlation coefficient value).

Figure 10. Alignment of amino acid sequences of the QRDR in GyrA for 5 bacterial species. The GyrA QRDR extends from amino acid residues 67 to 106 in the numbering system used for *E. coli*. Arrow identifies the amino acid at position 83.

CHAPTER II

AMINO ACID SUBSTITUTIONS IN GYRA AFFECTS QUINOLONE SUSCEPTIBILITY IN *SALMONELLA* TYPHIMURIUM

The original paper of chapter II had been accepted for publication by journal, however, have not been formally published, yet. Hence, I didn't include the contents of chapter II.

CONCLUSION

Quinolones have a good activity against isolates of *Salmonella* spp. and are often the treatment of choice in cases of life-threatening salmonellosis due to multi-drug resistant strains. Amino acid substitutions conferring resistance to quinolones in *S.* Typhimurium have generally been found within QRDRs of GyrA.

In this regard, Chapter I, based on measurements of the interaction between quinolones and purified *S.* Typhimurium WT DNA gyrase, I carried out the quinolone-inhibited DNA supercoiling assay and the quinolone-mediated DNA cleavage assay with 10 quinolones for investigating the quinolone structure-activity relationship and screening the quinolone derivatives for their anti-*S.* Typhimurium activities. The results showed that IC_{50}s of the *S.* Typimurium WT DNA gyrase correlated strongly with MICs, confirming their ability to inhibit the growth of *S.* Typhimurium (R = 0.999). These finding suggest that quinolones-inhibited DNA supercoiling assays are efficient methods and for the rapid investigation of the quinolone structure-activity relationship to monitor the potency of quinolones for salmonellosis treatment.

As investigating the contributions of amino acid substitutions in GyrA by an *in vitro* assay seemed to help gain a better understanding of quinolones resistant mechanism, therefore, I continued to reveal the significance of amino acid substitutions at position 83 and 87 in GyrA in Chapter II, using the quinolones-inhibited DNA supercoiling assay tested with 4 different quinolones. I clarified the contribution of amino acid substitutions at position 83 and 87 of GyrA and the structural variations at R1 and R8 of quinolones may influence the interaction between DNA gyrases and drugs. Current results also suggest that SIT having halogen atoms at R1 and R8 might be a good choice for the treatment of salmonellosis caused by CIP-resistant *S.* Typhimurium.

Further analysis for clarifying a direct relationship between these amino acid substitutions and various structural quinolones will help to improve appropriate quinolones selection for treatments.

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REFERENCES

1. Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A. and Hoekstra, R.M. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.*, **50**: 882–9.

2. Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M.A., Jensen, A.B., Wegener, H.C. and Aarestrup, F.M. 2011. Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog. Dis.*, **8**: 887–900.

3. Meng, C.Y., Smith, B.L., Bodhidatta, L., Richard, S.A., Vansith, K., Thy, B., Srijan, A., Serichantalergs, O. and Mason, C.J. 2011. Etiology of diarrhea in young children and patterns of antibiotic resistance in Cambodia. *Pediatr. Infect. Dis. J.*, **30**: 331–5.

4. Vo, A.T.T., van Duijkeren, E., Gaastra, W. and Fluit, A.C. 2010. Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolates from Vietnam. *PLoS One*, **5**: e9440.

5. Wannaprasat, W., Padungtod, P. and Chuanchuen, R. 2011. Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. *Int. J. Antimicrob. Agents*, **37**: 457–61.

6. Su, L.H., Chiu, C.H., Chu, C. and Ou, J.T. 2004. Antimicrobial resistance in nontyphoid *Salmonella* serotypes: a global challenge. *Clin. Infect. Dis.*, **39**: 546–51.

7. Andriole, V.T. 2005. The quinolones: past, present, and future. *Clin. Infect. Dis.*, **41**: 113–9.

8. Hooper, D.C. 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. *Clin. Infect. Dis.*, **31**: 24–8.

9. Hooper, D.C. 1999. Mechanisms of fluoroquinolone resistance. *Drug Resist. Updat.*, **2**: 38–55.

10. Piddock, L.J. 1999. Mechanisms of fluoroquinolone resistance: an update 1994-1998. *Drugs*, **58**: 11–8.

11. Friedman, S.M., Lu, T. and Drlica, K. 2001. Mutation in the DNA gyrase A Gene of *Escherichia coli* that expands the quinolone resistance-determining region. *Antimicrob. Agents Chemother.*, **45**: 2378–80.

12. Yoshida, H., Bogaki, M., Nakamura, M. and Nakamura, S. 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.*, **34**: 1271–2.

13. Yoshida, H., Bogaki, M., Nakamura, M., Yamanaka, L.M. and Nakamura, S. 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrob. Agents Chemother.*, **35**: 1647–50.

14. Hansen, H. and Heisig, P. 2003. Topoisomerase IV mutations in quinolone-resistant salmonellae selected *in vitro*. *Microb. Drug Resist.*, **9**: 25–32.

15. Ling, J.M., Chan, E.W., Lam, A.W. and Cheng, A.F. 2003. Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. *Antimicrob. Agents Chemother.*, **47**: 3567–73.

16. Piddock, L.J.V. 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS Microbiol. Rev.,* **26**: 3–16.

17. Levy, S.B. and Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.*, **10**: 122–9.

18. Lindstedt, B.A., Aas, L. and Kapperud, G. 2004. Geographically dependent distribution of *gyrA* gene mutations at codons 83 and 87 in *Salmonella* Hadar, and a novel codon 81 Gly to His mutation in *Salmonella* Enteritidis. *APMIS,* **112**: 165–71.

19. Randall, L.P., Coldham, N.G. and Woodward, M.J. 2005. Detection of mutations in *Salmonella enterica gyrA, gyrB, parC* and *parE* genes by denaturing high performance liquid chromatography (DHPLC) using standard HPLC instrumentation. *J. Antimicrob. Chemother.*, **56**: 619–23.

20. Heisig, P. 1993. High-level fluoroquinolone resistance in a *Salmonella* Typhimurium isolate due to alterations in both *gyrA* and *gyrB* genes. *J. Antimicrob. Chemother.*, **32**: 367–77.

21. Collignon, P., Powers, J.H., Chiller, T.M., Aidara-Kane, A. and Aarestrup, F.M. 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: A critical step for developing risk management strategies for the use of antimicrobials in food production animals. *Clin. Infect. Dis.*, **49**: 132–41.

22. Eaves, D.J., Randall, L., Gray, D.T., Buckley, A., Woodward, M.J., White, A.P. and Piddock, L.J.V. 2004. Prevalence of mutations within the quinolone resistance-determining region of *gyrA, gyrB, parC,* and *parE* and association with antibiotic

resistance in quinolone-resistant *Salmonella enterica*. *Antimicrob. Agents Chemother.*, **48**: 4012–5.

23. Hakanen, A., Kotilainen, P., Huovinen, P., Helenius, H. and Siitonen, A. 2001. Reduced fluoroquinolone susceptibility in *Salmonella enterica* serotypes in travelers returning from Southeast Asia. *Emerg. Infect. Dis.*, **7**: 996–1003.

24. Hopkins, K.L., Davies, R.H. and Threlfall, E.J. 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. *Int. J. Antimicrob. Agents*, **25**: 358–73.

25. Champoux, J.J. 2001. DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.*, **70**: 369–413.

26. Cloeckaert, A. and Chaslus-Dancla, E. 2001. Mechanisms of quinolone resistance in Salmonella. *Vet. Res.*, **32**: 291–300.

27. Heddle, J. and Maxwell, A. 2002. Quinolone-binding pocket of DNA gyrase: role of GyrB. *Antimicrob. Agents Chemother.*, **46**: 1805–15.

28. Clinical and Laboratory Standards Institute 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A9 9th ed. Wayne, PA.

29. Matrat, S., Petrella, S., Cambau, E., Sougakoff, W., Jarlier, V. and Aubry, A. 2007. Expression and purification of an active form of the *Mycobacterium leprae* DNA gyrase and its inhibition by quinolones. *Antimicrob. Agents Chemother.*, **51**: 1643–8.

30. Kim, H., Nakajima, C., Yokoyama, K., Rahim, Z., Kim, Y.U., Oguri, H. and Suzuki, Y. 2011. Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. *Antimicrob. Agents Chemother.*, **55**: 3661–7.

31. Yokoyama, K., Kim, H., Mukai, T., Matsuoka, M., Nakajima, C. and Suzuki, Y. 2012. Amino acid substitutions at position 95 in GyrA can add fluoroquinolone resistance to *Mycobacterium leprae*. *Antimicrob. Agents Chemother.*, **56**: 697–702.

32. Kongsoi, S., Yokoyama, K., Suprasert, A., Utrarachkij, F., Nakajima, C., Suthienkul, O. and Suzuki, Y. 2014. Characterization of *Salmonella* Typhimurium DNA gyrase as a target of quinolones. *Drug Test. Anal.*, DOI 10.1002/dta.1744.

33. Scheirer, K.E. and Higgins, N.P. 1997. The DNA cleavage reaction of DNA gyrase. Comparison of stable ternary complexes formed with enoxacin and CcdB protein. *J. Biol. Chem.*, **272**: 27202–9.

34. Barrett, J.F., Bernstein, J.I., Krause, H.M., Hilliard, J.J. and Ohemeng, K.A. 1993. Testing potential gyrase inhibitors of bacterial DNA gyrase: a comparison of the supercoiling inhibition assay and ‘cleavable complex’ assay. *Anal. Biochem.*, **214**: 313–7.

35. Blanche, F., Cameron, B., Bernard, F.X., Maton, L., Manse, B., Ferrero, L., Ratet, N., Lecoq, C., Goniot, A., Bisch, D. and Crouzet, J. 1996. Differential behaviors of *Staphylococcus aureus* and *Escherichia coli* type II DNA topoisomerases. *Antimicrob. Agents Chemother.*, **40**: 2714–20.

36. Pan, X.S. and Fisher, L.M. 1997. Targeting of DNA gyrase in *Streptococcus pneumoniae* by sparfloxacin: selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrob. Agents Chemother.*, **41**: 471–4.

37. Han, J., Wang, Y., Sahin, O., Shen, Z., Guo, B., Shen, J. and Zhang, Q. 2012. A fluoroquinolone resistance associated mutation in *gyrA* Affects DNA supercoiling in *Campylobacter jejuni*. *Front. Cell. Infect. Microbiol.*, **2**: 21.

38. Aubry, A., Pan, X.S., Fisher, L.M., Jarlier, V. and Cambau, E. 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. *Antimicrob. Agents Chemother.*, **48**: 1281–8.

39. Nakatani, M., Mizunaga, S., Takahata, M. and Nomura, N. 2012. Inhibitory activity of garenoxacin against DNA gyrase of *Mycoplasma pneumoniae*. *J. Antimicrob. Chemother.*, **67**: 1850–2.

40. Peterson, L.R. 2001. Quinolone molecular structure-activity relationships: what we have learned about improving antimicrobial activity. *Clin. Infect. Dis.*, **33**: 180–6.

41. Tillotson, G.S. 1996. Quinolones: structure-activity relationships and future predictions. *J. Med. Microbiol.*, **44**: 320–4.

42. Emami, S., Shafiee, A. and Foroumadi, A. 2005. Quinolones: Recent Structural and Clinical Developments. *Iran. J. Pharm. Res.*, **3**: 123–136.

43. Guillemin, I., Sougakoff, W., Cambau, E., Revel-Viravau, V., Moreau, N. and Jarlier, V. 1999. Purification and inhibition by quinolones of DNA gyrases from *Mycobacterium avium*, *Mycobacterium smegmatis* and *Mycobacterium fortuitum bv. peregrinum*. *Microbiology*, **145**: 2527–32.

44. Yague, G., Morris, J.E., Pan, X.S., Gould, K.A. and Fisher, L.M. 2002. Cleavable-complex formation by wild-type and quinolone-resistant *Streptococcus pneumoniae* type II topoisomerases mediated by gemifloxacin and other fluoroquinolones. *Antimicrob. Agents Chemother.*, **46**: 413–9.

45. Guillemin, I., Cambau, E. and Jarlier, V. 1995. Sequences of conserved region in the A subunit of DNA gyrase from nine species of the genus *Mycobacterium*: phylogenetic analysis and implication for intrinsic susceptibility to quinolones. *Antimicrob. Agents Chemother.*, **39**: 2145–9.

46. Walton, L. and Elwell, L.P. 1988. *In vitro* cleavable-complex assay to monitor antimicrobial potency of quinolones. *Antimicrob. Agents Chemother.*, **32**: 1086–9.

47. Jarlier, V. and Nikaido, H. 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol. Lett.*, **123**: 11–8.

48. Piton, J., Petrella, S., Delarue, M., André-Leroux, G., Jarlier, V., Aubry, A. and Mayer, C. 2010. Structural insights into the quinolone resistance mechanism of *Mycobacterium tuberculosis* DNA gyrase. *PLoS One*, **5**: e12245.

49. Barnard, F.M. and Maxwell, A. 2001. Interaction between DNA gyrase and quinolones: effects of alanine mutations at GyrA subunit residues Ser(83) and Asp(87). *Antimicrob. Agents Chemother.*, **45**: 1994–2000.

50. Lee, L.A., Puhr, N.D., Maloney, E.K., Bean, N.H. and Tauxe, R.V. 1994. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. *J. Infect. Dis.*, **170**: 128–34.

51. Casin, I., Breuil, J., Darchis, J.P., Guelpa, C. and Collatz, E. 2003. Fluoroquinolone resistance linked to GyrA, GyrB, and ParC mutations in *Salmonella enterica* typhimurium isolates in humans. *Emerg. Infect. Dis.*, **9**: 1455–7.

52. Nakaya, H., Yasuhara, A., Yoshimura, K., Oshihoi, Y., Izumiya, H. and Watanabe, H. 2003. Life-threatening infantile diarrhea from fluoroquinolone-resistant *Salmonella enterica* Typhimurium with mutations in both *gyrA* and *parC*. *Emerg. Infect. Dis.*, **9**: 255–7.

53. Keating, G.M. 2011. Sitafloxacin: in bacterial infections. *Drugs*, **71**: 731–44.