Interaction of the plasmid-encoded quinolone resistance protein QnrB19 with *Salmonella* Typhimurium DNA gyrase

Ruttana Pachanon[1], Kentaro Koide[1], Siriporn Kongsoi[2], Chie Nakajima[1, 3], Thoko Flav Kapalamula[1], Orasa Suthienkul[4,5], Yasuhiko Suzuki[1, 3]

[1]Division of Bioresources, Research Center for Zoonosis Control, Hokkaido University, Japan

[2]Faculty of Veterinary Medicine, Kasetsart University, Thailand

[3]Global Station for Zoonosis Control, Hokkaido university, Japan

[4]Center of Education and Research in EcoHealth and OneHealth, Faculty of Public Health, Thammasat University, Thailand

[5]Center of Excellence in Global Health, Faculty of Public Health, Thammasat University, Thailand

Corresponding author: Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Kita 20 Nishi 10, Kita-ku, Sapporo, Hokkaido 001-0020, Japan.

suzuki@czc.hokudai.ac.jp

All authors meet the ICMJE authorship criteria.

Abstract

Background

Plasmid-encoded quinolone resistance protein Qnr is an important factor in bacterial resistance to quinolones. Qnr interacts with DNA gyrase and reduces susceptibility to quinolones. The gene *qnr* likely spreads rapidly among Enterobacteriaceae via horizontal gene transfer. Though the vast amounts of epidemiological data are available, molecular details of the contribution of QnrB19, the predominant Qnr in *Salmonella* spp., to the acquisition of quinolone resistance has not yet been understood well.

Objectives

We aimed to examine the role of QnrB19 in quinolone resistance acquisition using recombinant *Salmonella* Typhimurium DNA gyrases and QnrB19.

Materials and Methods

Recombinant QnrB19 was expressed in *E. coli* and purified by Ni-NTA agarose column chromatography. DNA supercoiling activities of recombinant *Salmonella* Typhimurium DNA gyrase were assessed with or without QnrB19 under the existence of three quinolones to measure IC_{50}s, the concentration of each quinolone required for 50% inhibition in vitro.

Results

The IC_{50}s of norfloxacin, ciprofloxacin and nalidixic acid against DNA gyrases were measured to be 0.30, 0.16 and 17.7 μg/mL, respectively. The addition of QnrB19 increased the IC_{50}s of norfloxacin and ciprofloxacin to be 0.81 and 0.48 μg/mL, respectively, where no effect of QnrB19 was observed on the IC_{50} of nalidixic acid.

Conclusion

QnrB19 was shown for the first time *in vitro* to have ability to grant non-classical quinolone resistance to *S.* Typhimurium DNA gyrase. Structural insight on quinolones in this study may

contribute to investigate drugs useful for preventing the spread of plasmid carrying PMQR along with other factors associating with antimicrobial resistance in *S.* Typhimurium and other bacteria.

Keywords: PMQR, quinolone resistance, QnrB19, *Salmonella* Typhimurium, DNA gyrase

Introduction

Non-typhoidal *Salmonella* (NTS) is the cause of approximately 153 million gastroenteritis cases in humans resulting in 57,000 deaths each year worldwide [1]. In most cases, *Salmonella enterica* Enteritidis and *S.* Typhimurium are the major serotypes causing foodborne diseases [2]. *Salmonella* gastroenteritis is mostly self-limiting except for severe cases such as immune compromised patients, young children and the elderly. In such cases, the use of antimicrobials is indicated [2–4]. First-line drugs such as ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole were traditionally used for treating invasive NTS. However, the development of resistance to these drugs by bacteria led to the introduction of fluoroquinolones such as ciprofloxacin [5]. Fluoroquinolones have since been used as the drugs of choice for the treatment of NTS in humans and animals. Recently, however, the World Health Organization (WHO) published a list of high-priority fluoroquinolone-resistant *Salmonella* spp., for which new drugs are urgently required [6].

Fluoroquinolones target DNA gyrase, an important enzyme responsible for DNA replication and transcription, by binding to target sites in the enzyme-DNA complexes, which cause bacterial cell death [7]. Fluoroquinolone resistance usually develops after chromosomally mediated mutations, which cause amino acid substitutions at the quinolone-resistant determining regions (QRDR) of the DNA gyrase subunits (GyrA and GyrB) in Gram-negative bacteria, and the DNA topoisomerase IV (*parC* and *parE*) in Gram-positive bacteria. Plasmid-mediated quinolone resistance (PMQR) is also an important mechanism in quinolone resistance [8,9].

PMQR has indeed become a threat to the effective therapeutic use of quinolones for the treatment of infections in humans and animals, because it is believed that the plasmid can rapidly spread among Enterobacteriaceae through horizontal gene transfer [9]. Plasmid-encoded quinolone resistance protein Qnr among PMQR includes seven Qnr families: QnrA, QnrB, QnrC, QnrD, QnrS, QnrE *and* QnrVC pentapeptide repeat proteins, which interact with DNA gyrases and reduce susceptibility to quinolones. In addition, efflux systems (*oqx*AB, *qep*A and *qaq*BIII) and *aac(6')-Ib-cr* (an aminoglycoside-, ciprofloxacin- and norfloxacin-inactivating acetyl-transferase) are also reported to be responsible for PMQR [9,10].

Allelic variants of *qnr* differing by 10% or less are classified in each family, including 7 alleles for *qnrA,* 71 for *qnrB*, 5 for *qnrVC* and 9 for *qnrS* [11]. QnrB is the most prevalent among Qnr families. QnrB is also unique for being controlled by the SOS system, a DNA break produced by ciprofloxacin, which induces expression of QnrB via the binding to a LexA recognition site upstream of this gene. In contrast, no LexA binding site is found upstream of *qnrA1* and *qnrS1*[12,13]. Despite recent research advances, little information is known about the predominant Qnr types in *Salmonella* spp. Therefore, we examined the existing information of the prevalence of Qnr in 692 reports published from 2008 to 2017. Our search showed evidence of 4,459 Enterobacteriaceae isolates with Qnrs, (1,917 QnrB, 1,545 QnrS, 498 QnrA, 459 QnrD, 27 QnrVC, 12 QnrC and 1 QnrE) and QnrB19 to account for the more than 80% of Qnr reported in *Salmonella* spp. Previous studies indicated that QnrB4 and QnrB1 protein inhibited *E. coli* DNA gyrase supercoiling activity, which in turn conferred reduced susceptibility to ciprofloxacin [14,15]. It has also been reported that wildtype and mutant QnrS1 confers to *E. coli* DNA gyrase a low-level resistance to ciprofloxacin [16]. However, the role of QnrB19, the predominant Qnr in *Salmonella* spp., in the acquisition of quinolone resistance has not yet been understood.

Hence, the present study aimed to explore *in vitro* the role of QnrB19 in quinolone resistance acquisition utilizing recombinant *Salmonella* Typhimurium DNA gyrases and QnrB19.

Materials and Methods

Reagents

In the guideline "Expert Rules v 3.2" (https://www.eucast.org/fileadmin/src/media/ PDFs/ EUCAST_files/Expert_Rules/2019/Salmonella_ExpertRules_V3.2_20190613.pdf) of The European Committee on Antimicrobial Susceptibility Testing (EUCAST), ciprofloxacin is used as the indicator agents for MIC against Salmonella species. Hence, we selected ciprofloxacin for one target. In addition, a structurally related new quinolone, norfloxacin, and a structurally related old quinolone, nalidixic acid, were used for comparison. Ciprofloxacin was purchased from LKT Laboratories, Inc. (St Paul, MN). Nalidixic acid and norfloxacin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The chemical structures of quinolone Nalidixic acid and fluoroquinolones Norfloxacin and Ciprofloxacin are shown in Figure 1. Kanamycin was purchased from Wako Pure Chemical Industries, Ltd. Ni-nitrilotriacetic acid (Ni-NTA) protein purification kits were obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA). Relaxed pBR322 DNA and supercoiled pBR322 DNA were purchased from John Innes Enterprises Ltd (Norwich, UK), and complete™, Mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Applied Science (Mannheim, Germany).

Bacterial strains and plasmids

Synthetic target gene *qnrB19* of *S.* Typhimurium, accession No. NC_012916.1, in pUC57 plasmid was purchased from Genscript (Piscataway, NJ). Vector plasmid pET-28b(+) was purchased from Merck KGaA (Darmstadt, Germany) and used to construct expression plasmids for GyrA, GyrB and QnrB19. *E. coli* DH5 α (Takara Bio Inc., Shiga, Japan) was used as the cloning host. *E. coli*

BL-21 (DE3) was purchased from Merck KGaA and used for expression of GyrB. *E. coli* BL-21 (DE3)/pLysS was purchased from Merck KGaA and used for expression of GyrA and QnrB19.

Construction of *Salmonella* Typhimurium QnrB19 expression vector

Target gene *qnrB19* of *S.* Typhimurium in pUC57 was cut out with *Nco* I and *Xho* I and ligated into pET-28b(+) plasmid, which was the digested by these enzymes, and transformed into *E. coli* DH5α, as per the manufacturer's instructions. The sequence of the *qnrB19* gene in pET-28b(+) was confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI PRISM 3130*x* automated genetic analyzer (Applied Biosystems).

Expression and purification of QnrB19

Purification of the QnrB19 protein [16] was conducted as follows. *E. coli* BL21 (DE3)/pLysS carrying *qnrB19* in plasmid vector pET-28b(+) was cultured at 37 °C in 500 mL of Luria-Bertani (LB) broth (Wako Pure Chemical Industries Ltd.) with kanamycin (50 mg/L), and within an OD_{590} range of 0.4-0.6. Expression of QnrB19 was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd.). The bacteria were further incubated for 24 h at 18 °C. *E. coli* cells were retrieved by centrifugation, washed with cold buffer A [50 mM Tris, pH 8.0; 200 mM $(NH_4)_2SO_4$; 10% glycerol; and 20 mM imidazole], resuspended in buffer A supplemented with 1 mg/mL of lysozyme and 0.5% Triton X-100, and kept overnight at 4 °C. Using a Sonifier 250 (Branson, Danbury, CT), the lyzed suspension was sonicated on ice for 4 min in 10 cycles as follows: 40 s of sonication, 40 s of cooling, duty cycle at 30 % and output at 4%. To remove DNA and obtain a clean supernatant, DNase was added to the lysate at a final concentration of 0.1 mg/mL, further incubated on ice for 15 min and centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant containing soluble proteins was then filtered with a 0.2-μm pored membrane and loaded onto the column with Ni-NTA agarose resin to trap the histidine-

tagged protein. Trapped proteins were eluted with different imidazole concentrations (20, 30, 40, 50, 100, and 200 mM) in buffer A containing an EDTA-free protease-inhibiting cocktail and collected in 1 mL fractions. Eluted fractions in 100 mM and 200 mM imidazole-containing buffer A were loaded onto a PD-10 column and eluted with buffer B [20 mM Tris, pH 8.0, 10% glycerol and 50 mM arginine] as previously described [17], and concentrated using Millipore Amicon Ultra tubes (Merck Millipore Ltd., Burlington, MA). After purification, the eluted fractions were combined using glycerol to a concentration of 40% (wt/vol) and kept at -80 °C until further use. The quality and quantity of purified protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE: Wako Pure Chemical Industries Ltd.).

Expression and purification of *S.* Typhimurium DNA gyrase subunits

S. Typhimurium DNA gyrase subunits (GyrA and GyrB) were expressed and purified according to the previous procedures using *E. coli* BL21 (DE3)/pLysS carrying *gyrA* in plasmid vector pET-28b(+) and *E. coli* BL21 (DE3) carrying *gyrB* in the same plasmid [18–21]. The quality and quantity of purified proteins were analyzed by SDS-PAGE.

Inhibition of *S.* Typhimurium DNA gyrase by quinolones and protection by QnrB19

The DNA supercoiling activity of *S.* Typhimurium DNA gyrase was assessed as previously described [18–21]. Briefly, the reaction mixture (total volume, 30 μL) consisting of a 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; 6.5% glycerol; 0.1 mg/mL of BSA), 1 mM ATP, relaxed pBR322 DNA (0.3 μg), 18 nM DNA gyrase subunits (with/without quinolones), 50 μg/mL of NAL, 1 μg/mL of NOR and 0.5 μg/mL of CIP, and various concentrations of QnrB19 (6 to 4,374 nM; 3-fold serial increases). Reactions were run at 35 °C for 60 min and stopped by adding 8 μL of 5X dye mix (5% SDS, 25% glycerol and 0.25 mg/mL of bromophenol blue). Total reaction mixtures were diluted 4 times

with a 1× DNA sample buffer (0.1% SDS; 0.05% Bromo Phenol Blue; 10% glycerol; and 100 mM Tris-HCl, pH 8.0) and analyzed by electrophoresis [1% agarose gel in Tris-borate-EDTA (TBE) buffer]. The gels were run for 96 min at 50 mA and stained with 0.5 μg/mL of GelRed (Wako Pure Chemical Industries Ltd.). Supercoiling activity was evaluated by tracing the brightness of the bands with software ImageJ (http://rsbweb.nih.gov/ij). To confirm reproducibility, the supercoiling activity assay was conducted at least three times.

Impact of QnrB19 on IC_{50} of quinolones

To assess the ability of QnrB19 to confer quinolone resistance to DNA gyrase, the DNA supercoiling activity of *S.* Typhimurium DNA gyrase under the presence of QnrB19 and quinolones was examined as previously described [18–21]. The reaction mixture (total volume, 30 μL) consisted of a 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; 6.5% glycerol; and 0.1 mg/mL of BSA), 1 mM ATP, relaxed pBR322 DNA (0.3 mg) with 18 nM DNA gyrase subunit, quinolones (3-fold serial concentration increases); 3.2-1,000 μg/mL of nalidixic acid, 0.032-10 μg/mL of norfloxacin, and 0.01-3.2 μg/mL of ciprofloxacin, in the absence or presence of 18 nM QnrB19. After reaction and analysis of the reaction products by agarose gel electrophoresis as in above section, the supercoiling activity was evaluated as described above. And the inhibitory effects of nalidixic acid, norfloxacin and ciprofloxacin on DNA gyrase were assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s), using the calculator at AAT Bioquest (https://www.aatbio.com/tools/ic50-calculator). To confirm reproducibility, the supercoiling activity assay was carried out at least three times.

Result

Expression and purification of recombinant QnrB19

Purified recombinant QnrB19 protein was obtained from the supernatant with a His-tag at approximately 25-kDa, by eluting it from Ni-NTA agarose column with the buffer containing 200 mM imidazole (Figure 2). Approximately 1.4 mg of recombinant QnrB19 protein with high purity (>95%; Figure 3, lanes 3) was obtained from 500 mL *E. coli* culture.

Expression and purification of recombinant *S.* Typhimurium DNA gyrase

Purified recombinant *S.* Typhimurium DNA GyrA and GyrB were obtained from supernatants with His-tags at approximately 97-kDa and 89-kDa, respectively (Figure 3, lanes 1 and 2). Approximately 0.12 mg and 0.23 mg of GyrA and GyrB, respectively, with high purity (>95%) were obtained from 500 mL *E. coli* culture.

Impact of QnrB19 on *S.* Typhimurium DNA gyrase supercoiling activity in the presence or absence of quinolones

Figure 4 shows the interaction between QnrB19, *S.* Typhimurium DNA gyrase and quinolones. At concentrations of 6 - 18 nM, QnrB19 showed little or less impact on DNA supercoiling activity of DNA gyrase. QnrB19 exhibited a concentration-dependent inhibitory activity against DNA gyrase at concentrations higher than 54 nM, while it completely inhibited the DNA gyrase activity at the highest concentration of 4,374 nM. On the other hand, QnrB19 protected DNA gyrase from the impact of nalidixic acid, norfloxacin and ciprofloxacin. Nalidixic acid at the concentration of 50 μg/mL (215 μM), norfloxacin at 1 μg/mL (3 μM) and ciprofloxacin at 0.5 μg/mL (1.5 μM) reduced the DNA gyrase activity to 23.4, 17.6 and 8.7 %, respectively. QnrB19 at the concentration of 6 - 54 nM restored the DNA gyrase activity in dose dependent manner. DNA gyrase activities reduced by nalidixic acid, norfloxacin and ciprofloxacin were increased by addition of 54 nM QnrB19 up to approximately 1.8 times (From 23.4% to 42.4% of original DNA

gyrase activity; Fig. 4D), 3.6 times (from 17.6 % to 62.8 %; Fig. 4D) and 7.1 times (from 8.7% to 62.0%; Fig. 4F), respectively.

Effect of QnrB19 on IC_{50}s of quinolones

In a concentration-dependent manner, nalidixic acid, norfloxacin and ciprofloxacin inhibited *S.* Typhimurium DNA gyrase activity. Addition of 18 nM QnrB19, the same concentration to DNA gyrase, to the DNA supercoiling assay caused little effect on the inhibitory properties of nalidixic acid (Fig. 5A and D). In contrast, addition of same concentration of QnrB19 to those with norfloxacin and ciprofloxacin reduced the inhibitory effect of these quinolones on the DNA gyrase activity (norfloxacin; Fig. 5B and E, ciprofloxacin; Fig. 5C and F). Table 1 shows the effect of QnrB19 on IC_{50}s of quinolones against *S.* Typhimurium DNA gyrase. While the IC_{50} of nalidixic acid was not affected by QnrB19, the those of norfloxacin and ciprofloxacin had been increase to 2.7- and 3-fold, respectively.

Discussion

Fluoroquinolones are the drugs of choice for the treatment of invasive NTS; however, resistance to fluoroquinolones has significantly increased in bacteria. In the present study, we focused on the QnrB19, the predominant Qnr among *Salmonella* spp. Recombinant QnrB19 and DNA gyrase subunits were expressed in *E. coli* host cells and purified by Ni-NTA agarose column chromatography with high purity. Utilizing recombinant proteins, DNA supercoiling assays were conducted with three quinolones in the presence or absence of QnrB19 to access the property (Figure 4A - C). Results showed that QnrB19 had little impact on DNA supercoiling activity of DNA gyrase (18 nM GyrA and 18 nM GyrB) at the concentration of 6 - 18 nM; however, increasing amount of QnrB19 exerted the ability to reduce the activity of DNA gyrase in concentration dependent manner. On the contrary, QnrB19 showed the ability to protect DNA

gyrase from the effect of nalidixic acid (215 μM; 50 μg/mL), norfloxacin (3 μM; 1 μg/mL) and ciprofloxacin (1.5 μM; 0.5 μg/mL). At the concentration of 54 nM, QnrB19 exhibited the maximum restoring ability on the nalidixic acid, norfloxacin and ciprofloxacin inhibited DNA supercoiling activity of DNA gyrase by approximately 1.8-, 3.6- and 7.1-fold, respectively (Figure 4D - F). Indeed, our analyses showed that QnrB19 protected DNA gyrase from the impact of quinolones at certain extent depending on quinolones used. This agreed with the previous epidemiological information reported. It is noteworthy that QnrB19 totally abolished DNA supercoiling activity at the highest concentration of 4,374 nM even without quinolones. QnrB19 at very high concentration might compete with the substrates of DNA gyrase such as positively supercoiled or relaxed DNA and inhibit the supercoiling activity of DNA gyrase even in the absence of quinolones because of the similar three dimensional structure of QnrB19 and DNA double strand. Our results are similar to those previously reported by *in vitro* studies [14,16,22,23,24]. For example, previous work showed that at 2.17, 0.725 and 0.242 μM, QnrA1 protected *E. coli* DNA gyrase from inhibition (96%, 75%, 31%, respectively) by ciprofloxacin (1.5 μM; 0.5 μg/mL) [22]. Nonetheless, at 81, 27, 9 and 3 nM, QnrA1 exerted no protection for ciprofloxacin (1.5 μM; 0.5 μg/mL) inhibited DNA gyrase. It is worth noting that a simple concentration of 2.01 μM QnrA1 seemed to be enough to fully restore the activity of DNA gyrase, which had been inhibited by 0.75 μM ciprofloxacin [22]. Nevertheless, 2.01 μM QnrA1 is progressively less effective against the inhibitory activity of 3.0 μM or 6.0 μM ciprofloxacin [22]. A separate study [14] reported that little protection of *E. coli* DNA gyrases occurred if concentrations of QnrB1 were low in relation to ciprofloxacin (2 μg/mL; 6 μM). However, the protection increased to 50% when ciprofloxacin was 0.5 nM and to 100% when ciprofloxacin was 5 pM [14]. Nonetheless, a concentration of 25 μM QnrB1 was observed to completely inhibit DNA gyrase activity [14]. Separately, purified QnrS1 (0.05 nM) was shown to fully protect *E. coli* DNA gyrase supercoiling activity from the effect of 0.6 μg/ml (1.8 mM) of ciprofloxacin,

and a 100-fold lower concentration (0.5 pM) of QnrS1 could still provide half protection [16]. *Efs*Qnr, the other chromosomally encoded pentapeptide repeat protein in *Enterococcus faecalis*, was also shown to partially protect *E. coli* DNA gyrase from ciprofloxacin inhibition, even at concentrations as low as 20 nM [23]. Thus, from all the above evidence, it can be inferred that a very delicate interaction takes place between quinolones, Qnr and DNA gyrases, and that Qnr protection is inversely proportional to the concentrations of quinolones.

Figure 5 and Table 1 showed that IC_{50} nalidixic acid was not affected by the presence of 18 nM QnrB19. In contrast, to be effective, the IC_{50}s of norfloxacin and ciprofloxacin had been increased to 2- to 3-fold. The IC_{50} of ciprofloxacin for *E. coli* DNA gyrase in the absence of QnrB4 was reported to be 1 μM, but it had to be increased 5-fold if the concentration of QnrB4 was 0.5 μM [15]. Similarly, in the absence of *Efs*Qnr, the IC_{50} of ciprofloxacin for *E. faecalis* DNA gyrase was about 0.25 μM, but in the presence of 0.2 μM *Efs*Qnr, the IC_{50} had to be increased to 1.4 μM [23,24]. Those studies seemed to suggest that DNA gyrase activity was protected from quinolone inhibition by Qnr in Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*E. faecalis*). In the present work, QnrB19 was shown to protect *S.* Typhimurium DNA gyrase from the inhibitory effects of norfloxacin and ciprofloxacin. Other studies also showed that QnrA1, QnrB1, QnrB4, QnrS1 and *Efs*Qnr protected *E. coli* DNA gyrase from ciprofloxacin[14,15,16,22,23,24].

To the best of our knowledge, this is the first report that compared the effect of QnrB19 on *S.* Typhimurium DNA gyrases by specific features of quinolones at positions R1, R6, R7 or R8 (Table 1). Position R1 forms part of the enzyme-DNA-quinolone complex and has a hydrophobic interaction with a major grove in DNA [25]. This position in quinolones inhibits DNA supercoiling activity but could also be correlated with the protection conferred by QnrB19. Ciprofloxacin with cyclopropyl substituents at this position have showed the highest increase in IC_{50}s against DNA supercoiling activity (7.2-fold) in the presence of QnrB19. However, nalidixic

acid and norfloxacin with an ethyl group show low (1.8-fold) and moderate (3.6-fold) increases in IC_{50}s against DNA supercoiling activity, respectively. Furthermore, other positions in ciprofloxacin and norfloxacin such as R6 with fluorine or R7 with piperazine directly interacted with DNA gyrases, and position R8 with hydrogen substituents alters drug access to the enzyme or DNA binding sites [25]. Based on these data, it can be theorized that in the present work, the aforementioned positions may have also caused the increases in the IC_{50}s of norfloxacin (2.7-fold) and ciprofloxacin (3-fold), but not in that of nalidixic acid (Table 1).

Conclusion

In the present study, QnrB19 was shown to have ability to protect *S.* Typhimurium DNA gyrase from the inhibitory effects of norfloxacin and ciprofloxacin but have less protective ability from nalidixic acid. This is the first direct *in vitro* evidence of QnrB19 to be the cause of non-classical quinolone resistance. Results in the present study also showed the correlation between specific structural features of quinolones and their increased IC_{50}s driven by QnrB19. These observations may contribute to investigate drugs, which are unaffected by QnrB19 and useful for preventing the spread of plasmid carrying PMQR along with other factors associating with antimicrobial resistance in *S.* Typhimurium and other bacteria.

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Figure legends

Figure 1. Chemical structures of quinolones used in the present study

Figure 2. Expression and purification of recombinant QnrB19 by using SDS-PAGE.

The gene encoding QnrB19 was introduced into *Escherichia coli* expression vector pET28b. Recombinant QnrB19 was expressed in *E. coli* and purified by Ni-NTA agarose column chromatography. Two- μL of each sample was loaded on a SuperSep™ Ace 5-20% gradient gel. lane M: markers; lane 1: supernatant of *E. coli* lysate after centrifuge; lane 2: resuspended pellet of centrifuge in the original volume; lane 3: pass-though of Ni-NTA agarose column; lanes 4 – 14: eluted fraction by 20, 30, 40, 50, 100, 200 (fraction 1), 200 (fraction 2), 300 (fraction 1), 300 (fraction 2), 300 (fraction 3) and 300 (fraction 4) mM imidazole. "*" denotes recombinant QnrB19 with the MW of ~25 kDa.

Figure 3. Purified recombinant DNA gyrase subunits of *S.* Typhimurium and QnrB19 used in this study.

Recombinant DNA gyrase of *S.* Typhimurium and QnrB19 expressed and purified as in Materials and Methods were examined their purity by SDS-PAGE. Each protein sample (500 ng) was loaded on a SuperSep™ Ace 5-20% gradient gel. lane M: protein size markers, lane 1: GyrA, lane 2: GyrB, lane 3: QnrB19. "†" denotes GyrA with the MW of ~97 kDa, "‡" denotes GyrB with the MW of ~89 kDa, "*" denotes recombinant QnrB19 with the MW of ~25 kDa.

Figure 4. Inhibition of wild type *S.* Typhimurium DNA gyrase by quinolones and protection by QnrB19

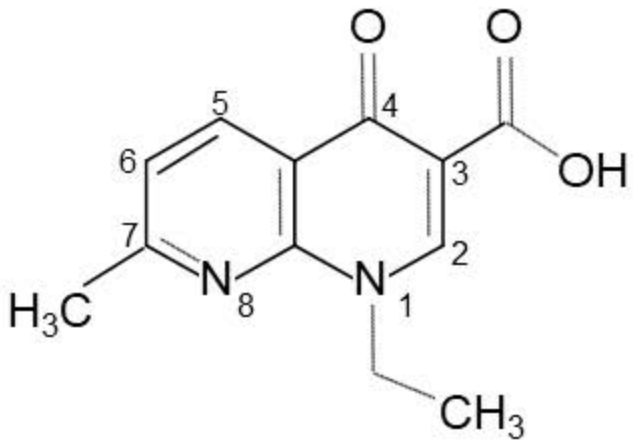
Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. lanes 2 - 16: contained 0.3 μg relaxed plasmid

pBR322, reaction buffer and 18 nM DNA gyrase subunit. Lane 1 – 8: with the absence of nalidixic acid/norfloxacin/ciprofloxacin; lane 9 – 16: with the presence of 50 μg/mL nalidixic acid (A) 1 μg/mL norfloxacin (B) 0.5 μg/mL ciprofloxacin (C). Lanes 2 and 10: with 6 nM QnrB19; lanes 3 and 11: with 18 nM QnrB19; lanes 4 and 12: with 54 nM QnrB19; lanes 5 and 13: with 162 nM QnrB19; lanes 6 and 14: with 486 nM QnrB19; lanes 7 and 15: with 1458 nM QnrB19; lanes 8 and 16: with 4374 nM QnrB19; lane 17: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, liner and nicked form of pBR322 DNA, respectively. In panel D - F, dotted lines with filled triangles denote the effect of QnrB19 on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without QnrB19). Solid lines with filled squares denote those with the presence of 50 μg/mL nalidixic acid (D) 1 μg/mL norfloxacin (E) 0.5 μg/mL ciprofloxacin (F).

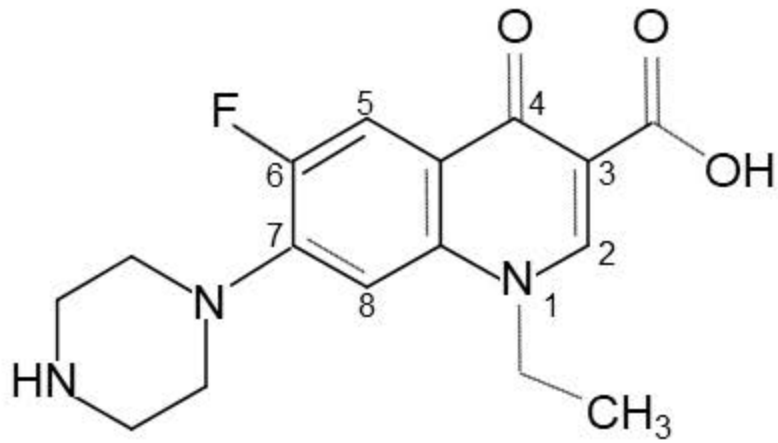
Figure 5. Effect of QnrB19 on IC_{50}s of quinolones

Reaction mixtures were diluted 4 times with 1× DNA sample buffer and 10 μL of them were analyzed by electrophoresis in 1% agarose gels. Lane 1 – 13: Reaction mixture contained 0.3 μg relaxed plasmid pBR322, reaction buffer and 18 nM DNA gyrase subunit; Lanes 2 - 7: with 3.2 - 1,000 μg/mL nalidixic acid (A), with 0.032 - 10 μg/mL norfloxacin (B), with 0.01 - 3.2 μg/mL ciprofloxacin (C) in the absence of QnrB19; lanes 8 - 13: with 3.2-1,000 μg/mL nalidixic acid (A), with 0.032-10 μg/mL norfloxacin (B), with 0.01-3.2 μg/mL ciprofloxacin (C) in the presence of 18 nM QnrB19; lane 14: relaxed plasmid pBR322. R, SC, L and N denote relaxed, supercoiled, liner and nicked form of pBR322 DNA, respectively. Solid lines with filled squares in panels D, E and F denote the impact of nalidixic acid, norfloxacin and ciprofloxacin on the DNA supercoiling activity of DNA gyrase (expressed as % activity comparing to that without quinolones), respectively. Dotted lines with filled triangles denote those in the presence of 18 nM QnrB19.

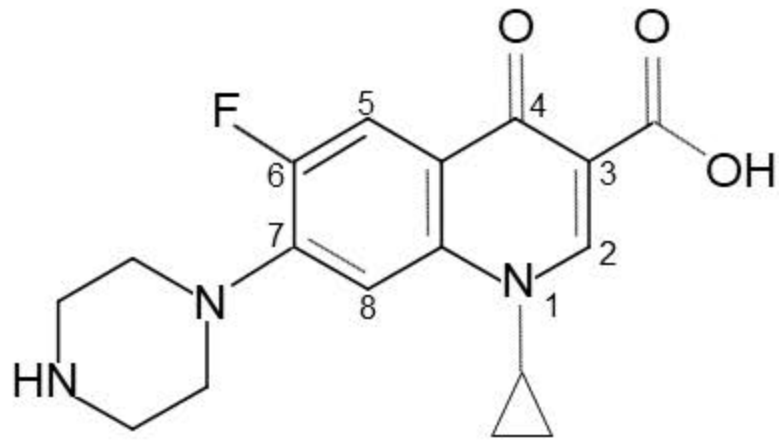
Figure 1



Nalidixic acid



Norfloxacin



Ciprofloxacin

Figure 2

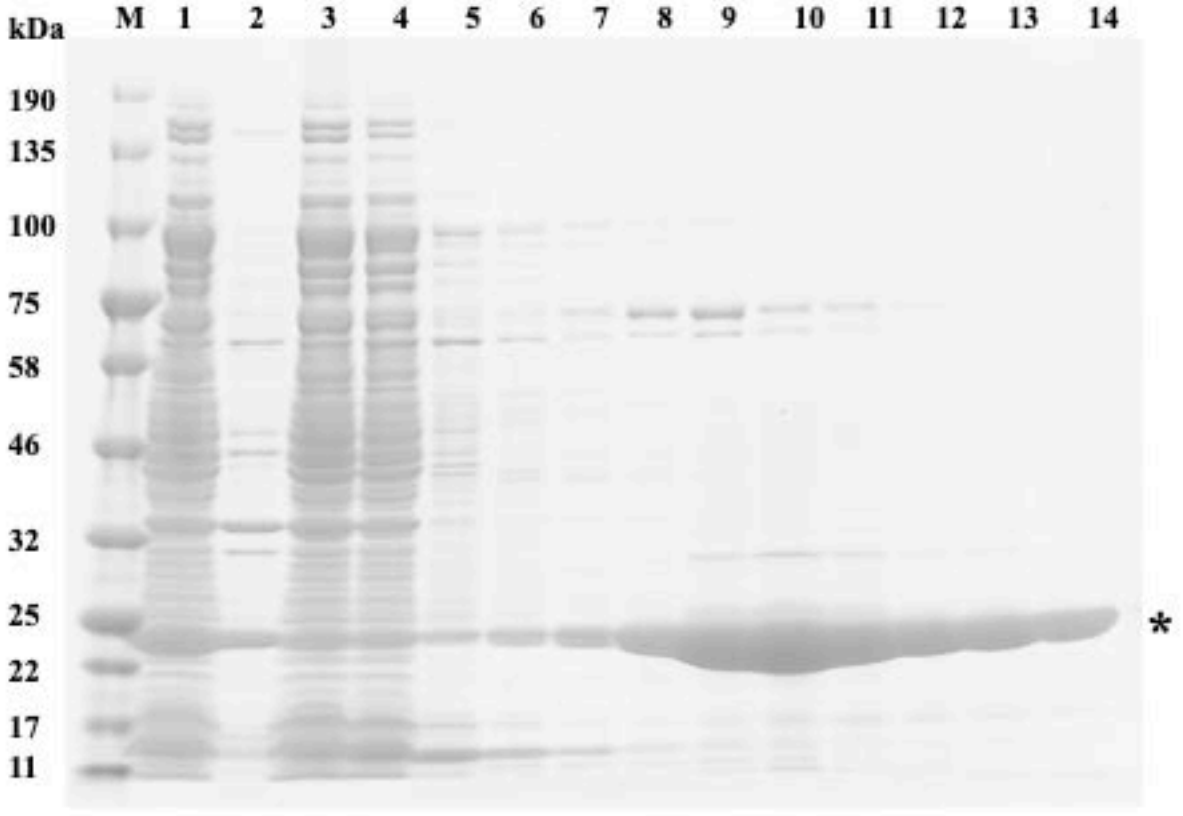


Figure 3

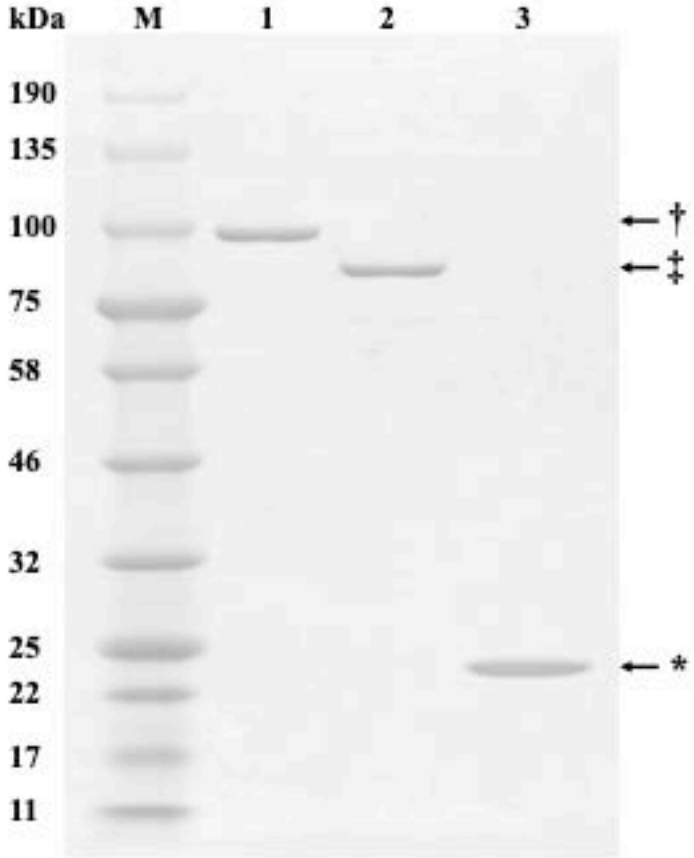


Figure 4

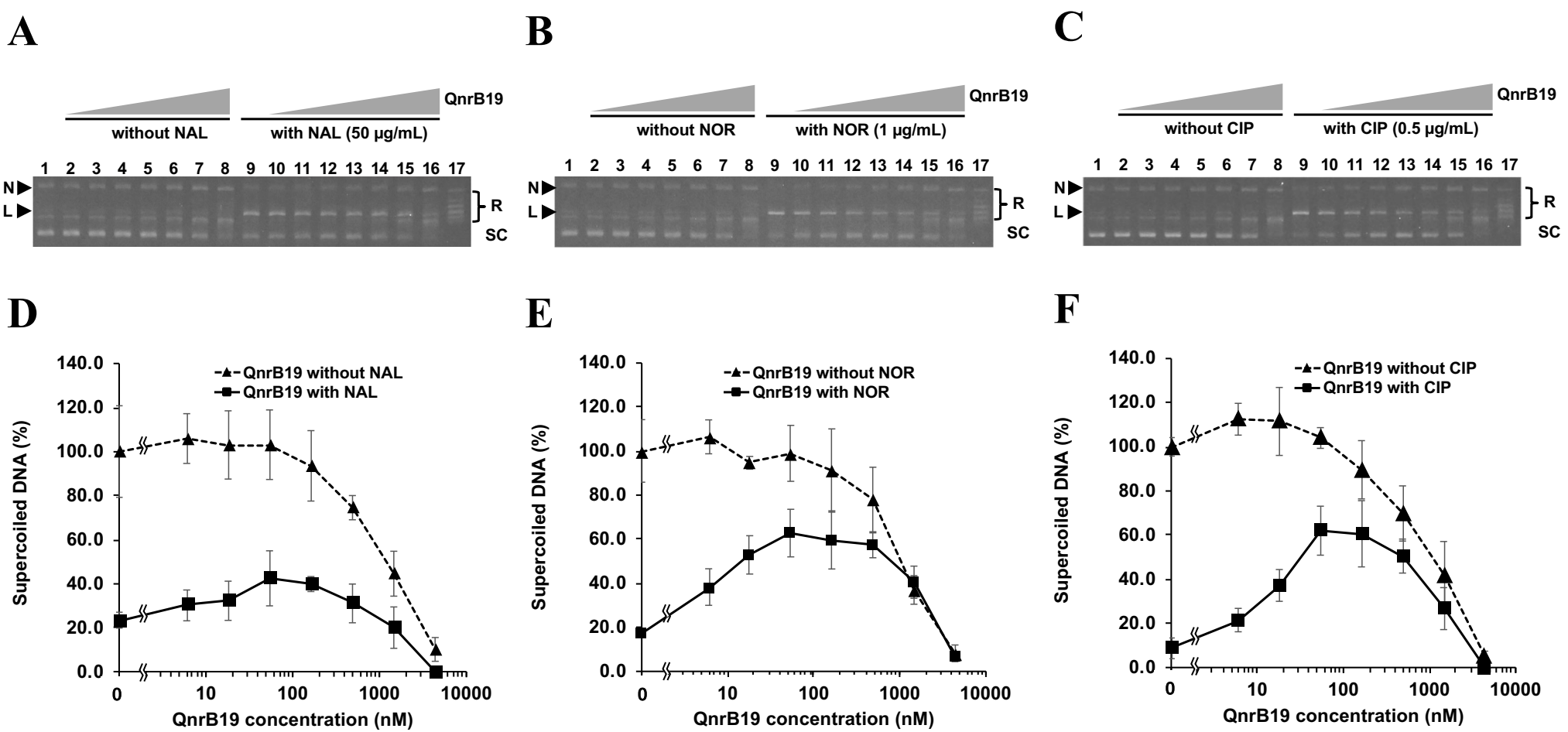

A
B
C
QnrB19
without NAL
with NAL (50 μg/mL)
without NOR
with NOR (1 μg/mL)
without CIP
with CIP (0.5 μg/mL)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
N
L
R
SC
D
E
F
QnrB19 without NAL
QnrB19 with NAL
QnrB19 without NOR
QnrB19 with NOR
QnrB19 without CIP
QnrB19 with CIP
Supercoiled DNA (%)
QnrB19 concentration (nM)
0.0
20.0
40.0
60.0
80.0
100.0
120.0
140.0
0
10
100
1000
10000

Figure 5

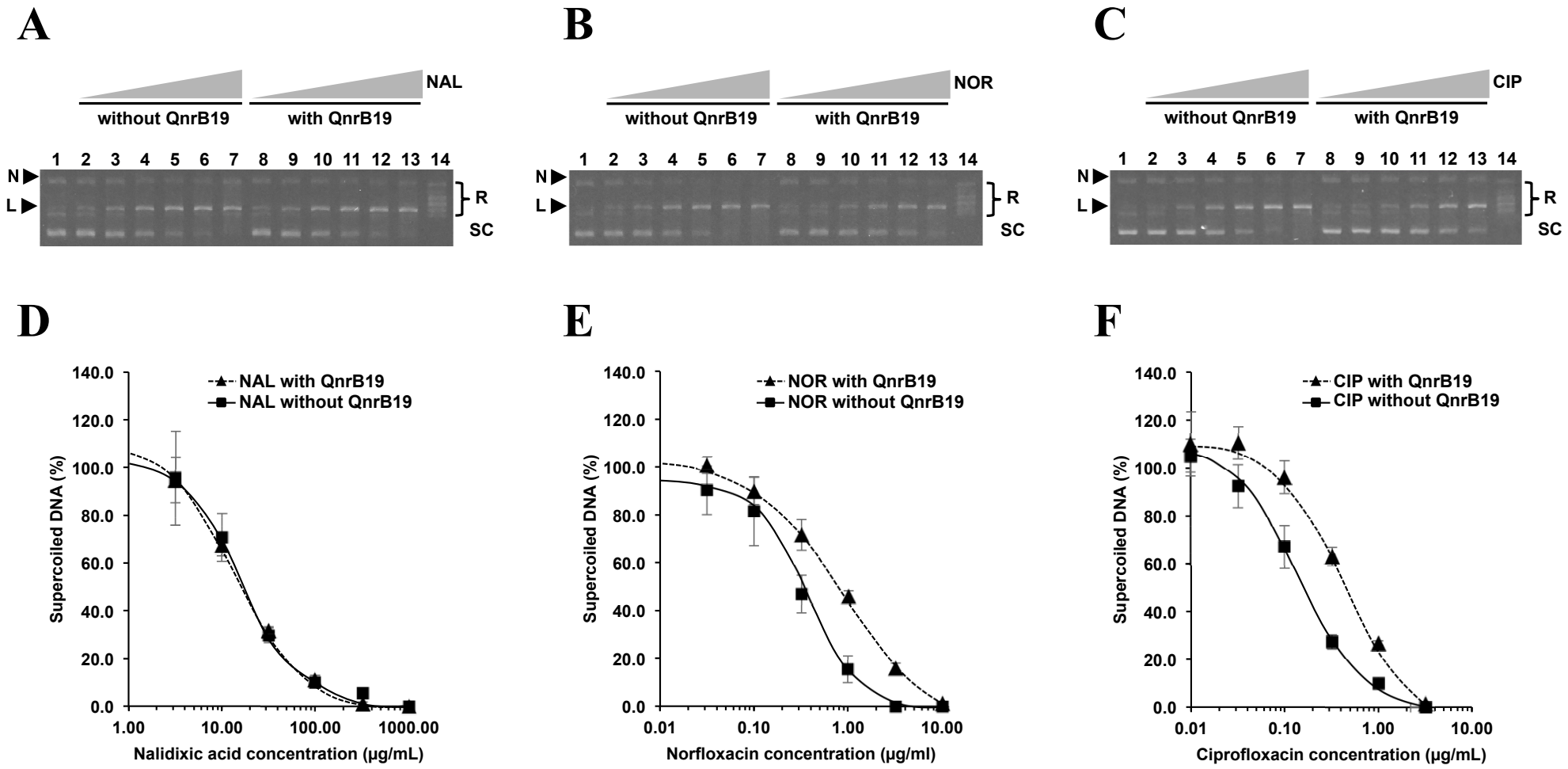

A
B
C
NAL
NOR
CIP
without QnrB19
with QnrB19
1 2 3 4 5 6 7 8 9 10 11 12 13 14
N
L
R
SC
D
E
F
NAL with QnrB19
NAL without QnrB19
NOR with QnrB19
NOR without QnrB19
CIP with QnrB19
CIP without QnrB19
Supercoiled DNA (%)
Nalidixic acid concentration (μg/mL)
Norfloxacin concentration (μg/ml)
Ciprofloxacin concentration (μg/mL)

Table. Effect of the IC_{50}s of quinolones on wild type *S.* Typhimurium DNA gyrase in the presence or absence of QnrB19

Quinolones	R-1[a]	R-6[a]	R-7[a]	R-8[a]	IC_{50}[b] (μg/mL)	
					Without QnrB19	With QnrB19
Nalidixic acid	Ethyl	Hydrogen	Methyl	Nitrogen	17.7 ± 0.35	17.3 ± 0.18
Norfloxacin	Ethyl	Fluorine	Piperazine	Hydrogen	0.30 ± 0.24	0.81 ± 0.09
Ciprofloxacin	Cyclopropyl	Fluorine	Piperazine	Hydrogen	0.16 ± 0.20	0.48 ± 0.17

[a] Position of substituents in the structures of quinolones.

[b] IC_{50}: Concentrations of quinolones to inhibit the supercoiling activity of DNA gyrase by 50%.