Study on the drug resistance in *Salmonella* and counter measure for it

(サルモネラ菌の薬剤耐性とその対策に関する研究)

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ABBREVIATIONS

ABSSSIs	Acute Bacterial Skin and Skin Structure Infections
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
CC_{25}	Concentration of quinolones that induce 25% of maximum DNA cleavage
CDC	Centers for Disease Control and Prevention
Cl	Chlorine
CLSI	Clinical and Laboratory Standards Institute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENA	European Nucleotide Archive
F	Fluorine
FQs	Fluoroquinolones
GLASS	Global Antimicrobial Resistance and Use Surveillance System
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B
IC_{50}	Concentration of quinolones that inhibit DNA gyrase activity by 50%
IPTG	Isopropyl beta-D-1-thiogalactopyranoside
LB	Luria-Bertani
MDR	Multidrug-resistant
MHA	Mueller–Hinton agar
MIC	Minimum Inhibitory Concentration
MSRV	Modified Semi-Solid Rappaport-Vassiliadis
N	Nitrogen
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
PMQR	Plasmid-Mediated Quinolone Resistance
QRDRs	Quinolone-Resistance Determining Regions
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SNP	Single-nucleotide polymorphism
STs	Sequence types

TAE	Tris-acetate-EDTA
US FDA	US Food and Drug Administration
VFDB	Virulence factor database

NOTES

1. **Toyting J**, Miura N, Utrarachkij F, Tanomsridachchai W, Belotindos LP, Suwanthada P, Kapalamula TF, Kongsoi S, Koide K, Kim K, Thapa J, Nakajima C, Suzuki Y. Exploration of the novel fluoroquinolones with high inhibitory effect against quinolone-resistant DNA gyrase of *Salmonella* Typhimurium, Microbiol Spectr 2023 Oct 5:e0133023. doi: 10.1128/spectrum.01330-23.

2. **Toyting J**, Nuanmuang N, Utrarachkij F, Supha N, Thongpanich Y, Leekitcharoenphon P, Aarestrup FM, Sato T, Thapa J, Nakajima C, Suzuki Y. Genomic analysis of *Salmonella* isolated from canal water in Bangkok, Thailand. Microbiol Spectr 2024 May 12:e04216-23. doi: 10.1128/spectrum.04216-23.

3. **Toyting J**, Supha N, Thongpanich Y, Thapa J, Nakajima C, Suzuki Y, Utrarachkij F. Wide distribution of plasmid mediated quinolone resistance gene, *qnrS*, among *Salmonella* spp. isolated from canal water in Thailand. J Appl Microbiol 2024, In press.

PREFACE

Salmonella enterica is a Gram-negative, rod shape bacteria that is a significant pathogen for gastroenteritis and invasive infection. *Salmonella enterica* serovars Typhi, Paratyphi A, Paratyphi B, and Paratyphi C are collectively known as typhoidal *Salmonella*, while other serovars are classified as nontyphoidal *Salmonella* (1). Typhoidal *Salmonella* strains are organisms restricted to infecting humans, leading to illnesses like typhoid fever and paratyphoid fever, mutually known as enteric fever. Nontyphoidal *Salmonella* strains, on the other hand, may either have a broad host range, infecting or colonizing various vertebrate animals, or may be adapted to particular nonhuman animal species (2).

Nontyphoidal *Salmonella* is one of the leading causative agents of acute gastroenteritis. The Global Burden of Diseases, Injuries, and Risk Factors Study estimated that nontyphoidal *Salmonella* caused 95.1 million cases of gastroenteritis with 61,600 deaths worldwide (3, 4). In Southeast Asia, nontyphoidal *Salmonella* is also one of the top 10 causes of gastroenteritis, contributing to nearly 16 million cases with about 16,000 deaths yearly (5). In addition to diarrhea disease, non-typhoidal *Salmonella* infections can invade normal sterile sites, resulting in bacteremia, meningitis, and other focal infections that refer to invasive non-typhoidal *salmonella* disease. Infants suffering from malnutrition, elderly individuals, and those with conditions such as sickle-cell disease, Human Immunodeficiency Virus (HIV) infection, as well as those dealing with acute or recent malaria are at particular risk (2, 6). It was estimated that 593,900 cases of invasive non-typhoidal *Salmonella* disease and 34,900 deaths occurred globally in 2019, with the highest incidence in sub-Saharan Africa. In Thailand, there were approximately 2,000 cases of invasive non-typhoidal *Salmonella* disease with 136 deaths (Figure 1) (4). Among more than 2,500 serotypes of *S. enterica*, *S.* Typhimurium and *S.* Enteritidis are the most common serotypes associated with foodborne illness (7, 8). Animal products and produce contaminated with animal feces are the main sources of nontyphoidal *Salmonella*, while contact with animals, such as reptiles, and with animal environments are important sources of nontyphoidal *Salmonella* infection not associated with food in developed countries (9, 10). Modes of nontyphoidal *Salmonella* transmission in low- and middle-income countries are less well understood than in developed countries. Even though transmission by food contaminated with animal feces must be considered, significant roles for waterborne transmission, transmission directly from animals and their environments, and transmission between people and nonhuman animal reservoirs have been hypothesized in developing countries (1). Specifically, 85% of cases were estimated as foodborne infections, but

contaminated drinking water and recreational water can be the risk of *Salmonella* infection (5, 11).

Antibiotics are often used as a countermeasure for *Salmonella* infection. Drugs of choice for *Salmonella* infection include fluoroquinolones (FQs), trimethoprim-sulfamethoxazole, ampicillin, chloramphenicol, and third-generation cephalosporins. Particularly, FQs and third-generation cephalosporins are currently recommended treatments for invasive *Salmonella* infections or patients at risk of developing an invasive infection, while macrolide antibiotics can be used as an alternative (1, 12).

Quinolones act as antibacterial agents by disturbing bacterial DNA synthesis and inhibiting the replication pathway (13, 14). The intracellular targets of quinolones are two type II DNA topoisomerases: DNA gyrase and DNA topoisomerase IV. DNA gyrase encompasses two pairs of GyrA/GyrB subunits that take part in bacterial DNA synthesis (15), while DNA topoisomerase IV consists of two pairs of ParC/ParE subunits. Quinolones bind to DNA-DNA gyrase complex in a non-covalent manner at the cleavage-ligation active site and act as a physical block to prevent the ligation of bacterial DNA, resulting in replication process inhibition, DNA fragmentation, and cell death. The interactions between quinolones and DNA gyrase are mediated by a water-metal ion bridge, which is formed through essential contacts contributed by the carbonyl substituents at R_3 and R_4. This bridge comprises a noncatalytic Mg^{2+} ion coordinated with four water molecules and carbonyl oxygens at R_3 and R_4 of the quinolones, forming an octahedral complex. Two water molecules interact with enzyme residues Ser83 and Asp87 in GyrA, thus completing the bridge structure (16, 17). Nonetheless, the emergence of quinolone resistance has limited the use of these antibiotics. The mutations occurring in DNA topoisomerase encoding genes (*gyrA*, *gyrB*, *parC*, and *parE*) at quinolone-binding sites named quinolone-resistance determining regions (QRDRs) are recognized as primary causes of quinolone resistance, specifically, mutation as amino acid substitution in the residues anchoring the bridge. Amino acid substitutions in Ser83 and Asp87 of GyrA subunit, including Ser83Phe, Ser83Tyr, Asp87Gly, Asp87Asn, and Asp87Tyr, are the most common mutations associated with quinolone resistance in *Salmonella*. In addition, Ser83Ile and Ser87Phe-Asp87Asn confer high resistance levels against FQs were also reported (18-24). Figure 2 illustrates the contrasting structural models of the wild-type (WT) and mutated DNA gyrase. Additionally, plasmid-mediated quinolone resistance (PMQR) determinants have become significant mechanisms in the growing global challenge of quinolone resistance. Despite providing low-level resistance on their own, PMQR genes create a conducive environment for selecting additional resistance mechanisms, such as chromosomal mutations,

leading to the development of significant levels of resistance (25). What sets PMQR determinants apart is the capacity for horizontal transfer, allowing them to traverse between different bacterial species. This ability facilitates the rapid dispersion of resistant bacteria across diverse environments, making PMQR determinants the potent mechanism of resistance dissemination. The categorization of PMQR genes based on their mode of action sheds light on the various tactics employed by bacteria to resist quinolones. The *qnr* genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrE*, *qnrS*, and *qnrVC*) protect the quinolone's target site from drug action. Other genes like *oqxAB* and *qepA* function as antibiotic efflux pumps, actively expelling quinolones from bacterial cells. Additionally, enzymes like *aac-(6')-Ib-cr* modify quinolones, rendering them ineffective against the bacteria (26). These genes, particularly *qnr* genes, have been reported in over 90 countries worldwide (Figure 3, Table S1) and are mainly detected in *Escherichia coli*, *Enterobacter* spp., *Klebsiella* spp., and *Salmonella* spp. (27).

Quinolone-resistant *Salmonella* is increasingly reported in various regions (28), contributing to the high burden of *Salmonella* infections globally. The large European surveillance in early 2000 that included over 27,000 clinical isolates from 10 countries revealed low-level fluoroquinolone resistance (resistance to nalidixic acid coupled with a decreased susceptibility to ciprofloxacin) in 13% of *S.* Typhimurium, 8% of *S.* Enteritidis, 53% of *S.* Virchow, and 57% of *S.* Hadar isolates (29). In 2009, a high prevalence of *Salmonella* strains with reduced susceptibility to ciprofloxacin was observed in the Philippines (15%), Singapore (25%), and Thailand (46.2%) (30). Later, in 2011, 31% of *Salmonella* isolated from pork and humans in Thailand exhibited ciprofloxacin resistant phenotype (31). A subsequent study of *S. Choleraesuis* isolated from patients with systemic salmonellosis in Thailand reported that all isolates were resistant to nalidixic acid, and 14.6% were resistant to ciprofloxacin (32). Alarmingly, the resistance rate of ciprofloxacin in nontyphoidal *Salmonella* isolated from patients in Central Thailand increased from 16.6% in 2011 – 2015 to 39.5% in 2016 – 2020 (33). A similar trend was also observed in the United States, in which the resistance to ciprofloxacin of nontyphoidal *Salmonella* continued to rise from 2016 and approached 10% in 2019 (Figure 4) according to Centers for Disease Control and Prevention (CDC) (34). The recent World Health Organization (WHO) Global Antimicrobial Resistance and Use Surveillance System (GLASS) report in 2022 revealed the low rates of resistance to both carbapenems and third-generation cephalosporins (≤ 5%), but ciprofloxacin resistance rate was above 10% for *Salmonella* spp. causing gastrointestinal infections (Figure 5A). A comparable pattern was noted in *Salmonella* spp. causing bloodstream infections, where the resistance rates to ciprofloxacin and levofloxacin neared 20%, surpassing the resistance rates observed for

other antibiotics (Figure 5B) (35). The high resistance rate to fluoroquinolones of *Salmonella* spp. is of concern.

FQs are chemically stable, and their residuals are frequently detected in wastewater that ends up in natural surface water (36), leading to the increase of antimicrobial-resistant bacteria in the aquatic environment. Multidrug-resistant (MDR) *Salmonella* spp. has been identified in various water sources worldwide. The study in Southeastern United States showed that 16 *S.* Newport exhibited MDR-AmpC phenotype, which presented resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, and to the 1st, 2nd, and 3rd generations of cephalosporins (cephalothin, amoxicillin-clavulanic acid, and ceftriaxone) (37). A subsequent investigation conducted in Maryland Eastern Shore found that 8% of the tested *Salmonella* isolates exhibited multidrug resistance. These isolates manifested two resistance patterns: (1) resistance to ampicillin, sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, cefoxitin, and ceftriaxone, and (2) resistance to sulfisoxazole and tetracycline (38). The Canadian study indicated that 16% of *Salmonella* isolates displayed resistance to at least one antimicrobial agent. Among these, three *Salmonella* isolates exhibited resistance to Category I antimicrobials (amoxicillin-clavulanic acid, ceftriaxone, and ceftiofur), which are categorized as critically important for treating severe bacterial infections in humans (39). The examination conducted in artificial waterways in China unveiled elevated levels of antimicrobial resistance among *Salmonella* isolates, particularly notable resistance to β-lactams and quinolones. Among these isolates, 25% exhibited multidrug resistance. Notably, a subset of these isolates showed concurrent resistance to ciprofloxacin and production of Extended-Spectrum β-Lactamase (ESBL) (40). Research conducted in two canals in Jordan revealed that 20.7% of *S.* Typhimurium isolates were classified as MDR. All the MDR isolates exhibited resistance to tetracycline and nalidixic acid. Besides, some of these isolates also displayed resistance to trimethoprim–sulfamethoxazole, ciprofloxacin, and ampicillin (41). The literature previously mentioned suggests that surface water, such as lakes, rivers, and canals, could serve as a potential reservoir for antimicrobial-resistant *Salmonella* strains.

Since environmental water could serve as a potential reservoir for antimicrobial-resistant *Salmonella* strains, it is vital to conduct surveillance of antimicrobial resistance in *Salmonella* spp. originating from environmental sources. This step is crucial in reinforcing the comprehensive approach to addressing the issue of antimicrobial resistance (AMR) in alignment with the One Health concept. In addition, molecular epidemiological analysis is crucial for understanding the genomic profile and genetic relationship of *Salmonella* isolated from environmental water.

Based on criteria set by WHO, fluoroquinolone-resistant *Salmonella* rank high for (1) prevalence in the community, (2) transmissibility and zoonotic potential, (3) length of hospitalization after infection, and (4) unlikeliness of development of alternative antibiotics in the nearby future. As such, WHO precisely ranked fluoroquinolone-resistant *Salmonella* as a high-priority pathogen for the research and development of new antibiotics since 2017 (42).

Quinolones have undergone continuous development, leading to the introduction of new antibiotics within this drug class. These antibiotics are utilized in the treatment of bacterial infections, including those caused by *Salmonella* and resistant bacterial strains. Quinolones constitute a class of synthetic antimicrobial agents featuring a bicyclic core structure employed in the treatment of bacterial infections (15, 43). Nalidixic acid was the first quinolone to be developed, which presents antimicrobial activity against Gram-negative bacteria, excluding *Pseudomonas* species (44). It was generally used for urinary tract infection treatment caused by *E. coli* and *K. pneumoniae* (45). However, its application in complicated infections was limited due to poor bioavailability, short half-life, and low potency (46). To extend their activity spectrum and improve pharmacokinetics, various modifications have been processed to the quinolone core structure (Figure 6, 7). FQs are the major improvement of quinolones by adding fluorine (F) atom at R_6 position of bicyclic core structure (47), contributing to increased activity against Gram-negative bacteria, volume of distribution, and tissue penetration.

The modifications at the R_1 position significantly enhance the drug's overall potency. For instance, the addition of a cyclopropyl group in ciprofloxacin and 2,4-difluorophenyl group in trovafloxacin enhance the activity of the compounds (48, 49).

The modification at the R_5 position with $–NH_2$, –OH, and $–CH_3$ groups were revealed to increase the activity against Gram-positive pathogens (50). The modifications at the R_7 position contribute to the activity against both gram-negative and Gram-positive bacteria. Specifically, the piperazine ring in ciprofloxacin enhances Gram-negative potency (51). On the other hand, other modifications enhance activity against Gram-positive bacteria: alkylated piperazine in ofloxacin and gatifloxacin, pyrrolidinyl groups in clinafloxacin (52), and azabicyclic group in moxifloxacin (47). Additionally, the incorporation of an amino-azepinyl group at this position enables dual inhibition of both target enzymes, DNA gyrase and DNA topoisomerase IV, as observed in besifloxacin (53). Alternatively, the inclusion of a pyrrolo-oxazinyl moiety at the R_7 in finafloxacin is a distinctive feature of the molecule, aiding in avoiding detection by efflux transporters. This is a critical factor in reducing the development of bacterial resistance (54).

The modifications at the R_8 position enhance the activity against Gram-positive bacteria and anaerobes. Specifically, the presence of a nitrogen (N) atom at the R_8 position contributes to improved activity against anaerobes (48). Additionally, the addition of a -OCH_2 group in ofloxacin, a –OCH_3 group in moxifloxacin, and chlorine (Cl) in clinafloxacin at the R_8 position improves Gram-positive activity (50, 55). A summary of the quinolone development process is presented in Table 1.

This thesis consists of three chapters, CHAPTER I: Wide distribution of plasmid mediated quinolone resistance gene, *qnrS*, among *Salmonella* spp. isolated from canal water in Thailand, CHAPTER II: Genomic analysis of *Salmonella* isolated from canal water in Bangkok, Thailand, and CHAPTER III: Exploration of the novel fluoroquinolones with high inhibitory effect against quinolone-resistant DNA gyrase of *Salmonella* Typhimurium. The first and second chapters examined *Salmonella* strains from canal water samples in Bangkok, Thailand. The first chapter investigated the prevalence of PMQR determinants and antimicrobial susceptibility, while the second chapter delved into genomic characterization. Finally, as per the initiative of WHO for research and development of a new treatment targeting fluoroquinolone-resistant *Salmonella*, the third chapter assessed the inhibitory effect and antimicrobial activity of two novel fluoroquinolones, WQ-3034 and WQ-3154. These efforts aimed to provide scientific evidence to bolster comprehensive approaches addressing AMR and safeguarding public health in Thailand and globally.

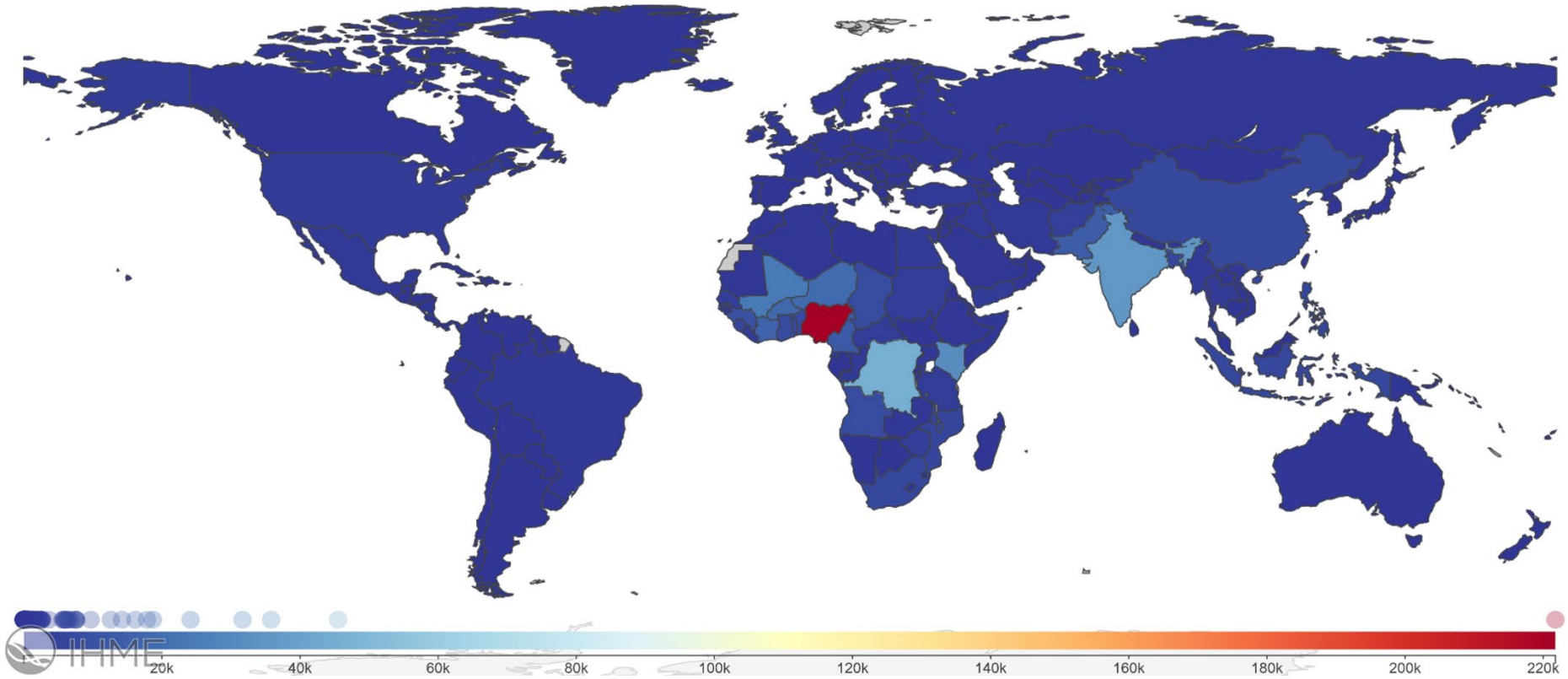


Figure 1 Invasive non-typhoidal *Salmonella* disease incidence in 2019 by country. The colors depict the number of new cases. This figure was obtained from (56).

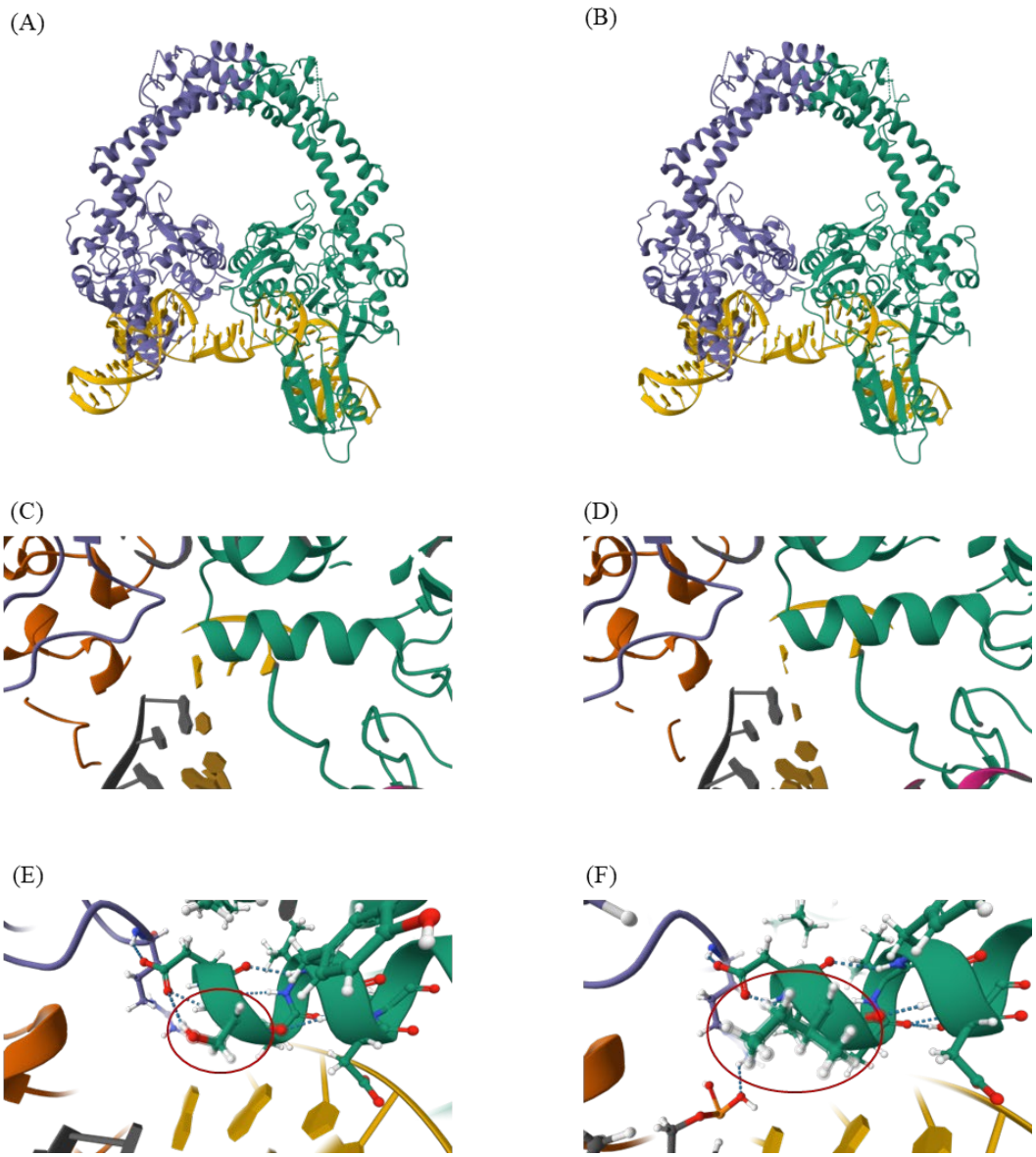


Figure 2 The 3D structures of *Salmonella* Typhimurium DNA gyrases, created using homology-based methods from *E. coli*. The figure highlights the DNA gyrase A subunits (A, B), the structure of its alpha helix (C, D), and the structure of codon 83 in both the WT and mutant Ser83Ile (E, F). The WT structures are represented in panels A, C, and E, while the mutant structures are shown in panels B, D, and F. The purple and green ribbons represent DNA gyrase A subunits. The double helix structure of DNA is shown in yellow and the white, red, and blue balls represent hydrogen, oxygen, and nitrogen atom, respectively.

Figure 3 Global distribution of *qnr* genes. The metadata of all registered isolates in the NCBI gene database isolates browser was retrieved on 21 April 2023. The colors in each circle represent each *qnr* gene, and the circles represent the number of isolates. Figure was created with Microreact (https://microreact.org) and BioRender (https://BioRender.com).

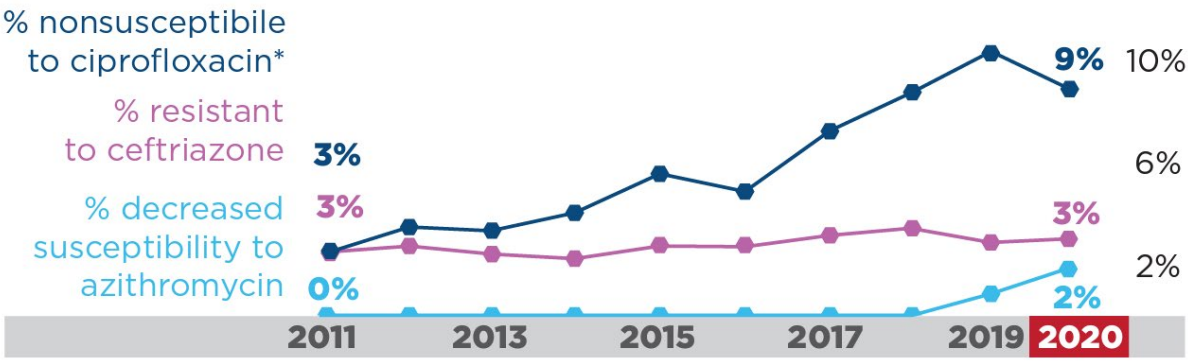


Figure 4 Percentage resistance to antimicrobials of nontyphoidal *Salmonella* in the United States reported by CDC. * Fully or partially resistant to ciprofloxacin. The figure was modified from (34).

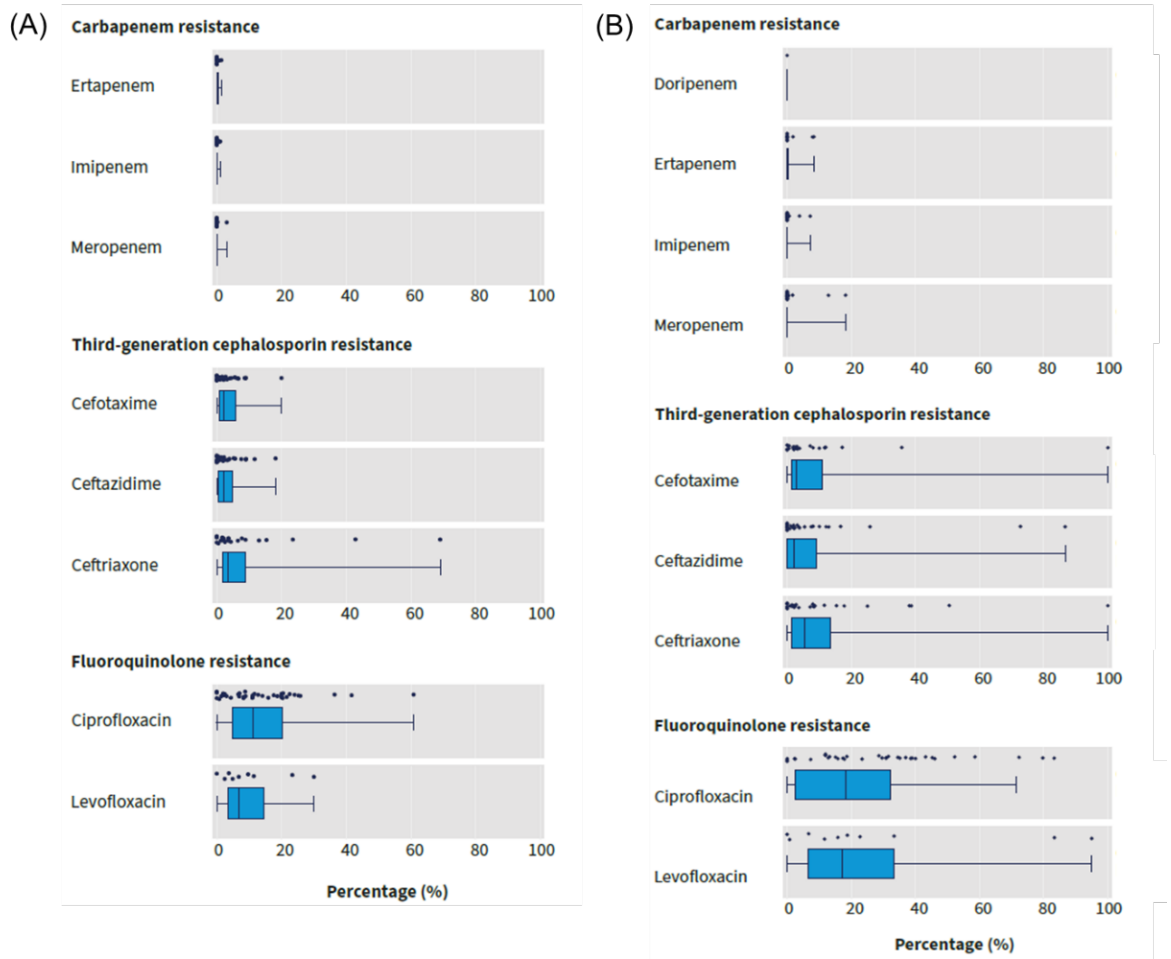


Figure 5 Percentage resistance to antimicrobials under surveillance in *Salmonella* spp. causing (A) gastrointestinal infections and (B) bloodstream infections described in the WHO GLASS report. The figure was modified from (35).

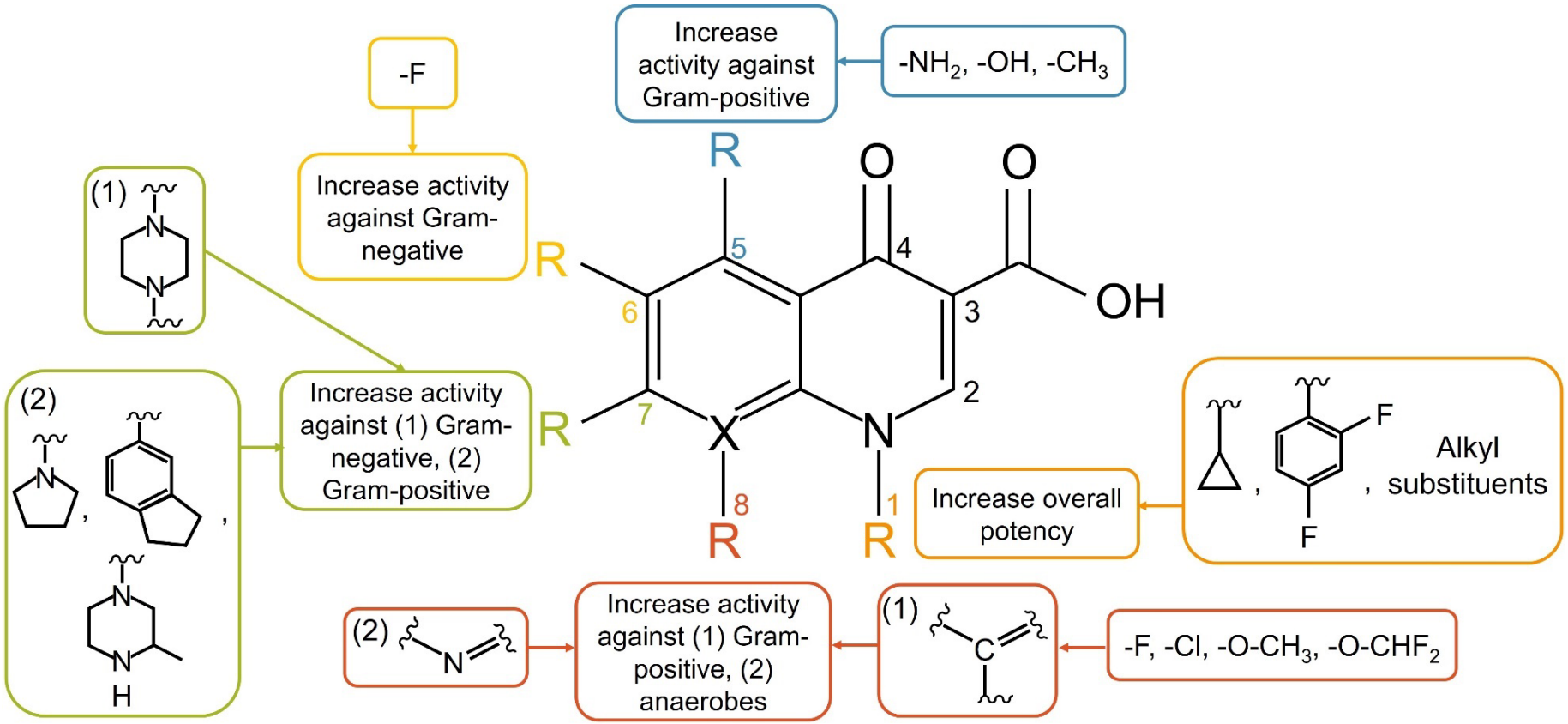


Figure 6 The structure-activity relationship of quinolones. The antibacterial activity of quinolones is improved by modifications of diverse substituents in different positions. The figure was modified from (47).

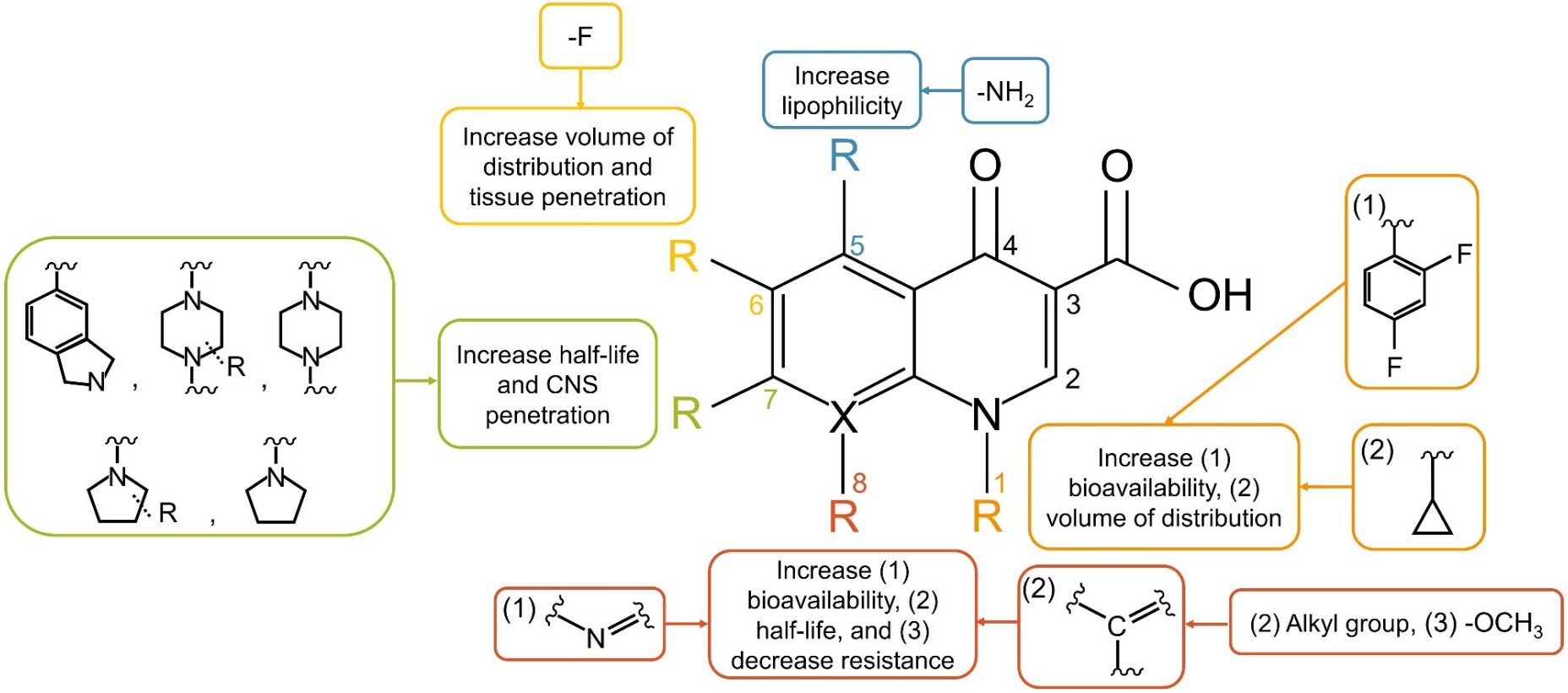


Figure 7 The structure-pharmacokinetic relationship of quinolones. The pharmacokinetic of quinolones is improved by modifications of various substituents in different positions. The figure was modified from (47).

Table 1 Overview of the development of quinolone antibiotic generations.

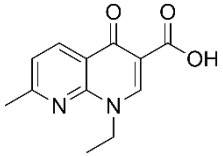
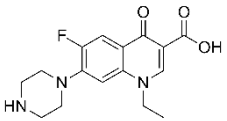
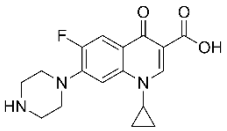
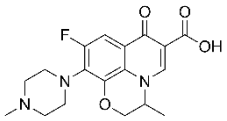
Generation	Name	Structure	Antimicrobial spectrum	Modification	Comment
1	Nalidixic acid		Gram-negative organisms (except *Pseudomonas* species)	N at X_8 = naphthyridone	First quinolone to be developed
	Norfloxacin		All Gram-negative pathogens and some atypical pathogens (including *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*)	Addition of (1) piperazine to R_7, (2) –F to R_6	(1) Improves bioavailability and side effects. (2) Improves activity against Gram-negative organisms (inhibits the efflux mechanism)
2	Ciprofloxacin			Addition of (1) piperazine to R_7, (2) –F to R_6, (3) cyclopropyl group at the R_1	(1) Improves anti-Gram-negative activity (2) Increases potency
	Ofloxacin		All Gram-negative pathogens and some Gram-positive bacteria (including S*taphylococcus aureus*), and some atypical organisms	Addition of (1) methylated piperazine to R_7, (2) $–OCH_2$ at R_8	(1) Increases anti-Gram-positive activity (2) Increases anti-Gram-positive activity, tissue penetration, half-life

Table 1 Overview of the development of quinolone antibiotic generations.

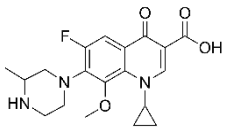
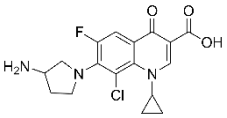
Generation	Name	Structure	Antimicrobial spectrum	Modification	Comment
3	Gatifloxacin		Maintains the activity of second-generation drugs and expands Gram-positive coverage (penicillin-sensitive and penicillin-resistant *S. pneumoniae*) and improved activity against atypical pathogens	Addition of (1) methylated piperazine group to R_7, (2) methoxy group at R_8, and (3) cyclopropyl group at R_1	(1) Improves anti-Gram-positive activity (2) Improves anti-Gram-positive activity, tissue penetration, half-life (3) Improves potency of the drug
	Clinafloxacin			Addition of (1) 3-aminopyrrolidin-1-yl group to R_7, (2) –Cl at R_8, (3) cyclopropyl group at R_1	(1) Improves anti-Gram-positive activity and overcomes physical drawbacks (2) Improve anti-Gram-positive activity, tissue penetration, half-life (3) Improves potency of the drug

Table 1 Overview of the development of quinolone antibiotic generations.

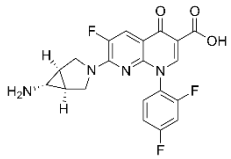
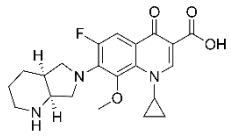
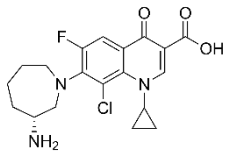
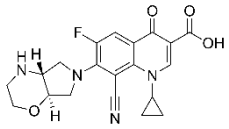
Generation	Name	Structure	Antimicrobial spectrum	Modification	Comment
	Trovafloxacin			Addition of (1) amine-substituted bicyclic pyrrolidin-1-yl group to R_7, (2) 2,4-difluorophenyl group at R_1	(1) Improve anti-Gram-positive activity (2) Improves potency and activity against anaerobes
4			Covers all the activities of third-generation drugs and extra anaerobic activity		(1) Improves anti-Gram-positive activity but may cause low water solubility and oral bioavailability (2) Improves anti-Gram-positive activity, tissue penetration, half-life (3) Improves potency of the drug
	Moxifloxacin			Addition of (1) azabicyclo group to R_7, (2) -OCH_3 at R_8, (3) cyclopropyl group at R_1	

Table 1 Overview of the development of quinolone antibiotic generations.

Generation	Name	Structure	Antimicrobial spectrum	Modification	Comment
New	Besifloxacin		Dual inhibition of both target enzymes (DNA gyrase and DNA topoisomerase IV)	Addition of (1) amino-azepinyl group to R_7, (2) -Cl at R_8, (3) cyclopropyl group at R_1	(1) Enables dual action on both target enzymes (2) Increases the antibacterial activity against FQs-resistant mutants (3) Improves potency of the drug
	Finafloxacin			Addition of (1) pyrrolo-oxazinyl group to R_7, (2) cyano-substituent at R_8, (3) cyclopropyl group at R_1	(1) Refers to the molecule's distinctive feature of avoiding detection by efflux transporters (2) Enables dual action on both target enzymes (3) Improves potency of the drug

CHAPTER I

Wide distribution of plasmid mediated quinolone resistance gene, *qnrS,* among *Salmonella* spp. isolated from canal water in Thailand

AMR is a global public health concern rapidly increasing worldwide. The aquatic environment is a neglected exposure route for the spread of AMR, facilitating the emergence and dissemination of novel resistances through contamination with antibiotics, pollutants, and genetic material exchange among different species from various sources (57).

Multidrug-resistant *Salmonella* spp. has been identified in various water sources worldwide (37-41), indicating that surface water such as lakes, rivers, and canals could serve as a potential reservoir for antimicrobial-resistant *Salmonella* strains. Many of the genes that contribute to antimicrobial resistance in *Salmonella* spp. are located on plasmids, and these plasmids often carry multiple resistance genes (58). This implies that *Salmonella* spp. could transfer antimicrobial resistance genes to other pathogenic bacteria, such as *K. pneumoniae* and *E. coli*, through the horizontal gene transfer of plasmids, making it a critical organism that facilitates the spread of antimicrobial resistance in the environment.

PMQR genes have emerged as critical mechanisms in the escalating global challenge of quinolone resistance, as they have become increasingly prevalent in recent times. In Thailand, 22.3% of clinical *E. coli* and 65.3% of *K. pneumoniae* isolates harbored PMQR genes, which mainly comprised of *aac(6')-Ib-cr* and *qnrS,* while 10% of clinical *S*. Enteritidis were positive for *qnrS* (24, 59).

Bangkok, the capital city of Thailand, is home to a network of canals that play various roles, including a drainage system, wastewater and sewage discharge, means of transportation, agricultural resources, tourist attractions, and recreational space (60). Therefore, the canals in Bangkok are not only open spaces for people but also represent the traditional way of life. Although the prevalence of PMQR genes in clinical isolates of specific bacterial strains in Thailand is well-studied, limited data are available on their distribution in environmental isolates, particularly in aquatic environments such as canal water in Bangkok. Therefore, this chapter aimed to investigate the prevalence of PMQR determinants and antimicrobial susceptibility of *Salmonella* spp. isolated from canal water in Bangkok.

Materials & Methods

Sample collection

A total of 333 water samples were collected monthly between 2016 and 2020 from six canals in Bangkok as geographical locations presented in Figure 8. The land-use zones in Bangkok comprise of 10 categories including low-density residential, moderate-density residential, high-density residential, commercial, industrial, warehouse, rural and agricultural conservation, rural and agricultural, Thai cultural conservation, and government organization and public utilities areas. All six canals are located in different land-use zones as follows: Canal A - Low-density residential area, Canal B - Agricultural area, Canal C - Low-density residential area, Canal D - High-density residential area, Canal E - Low to moderate-density residential area, and Canal F - Moderate to high-density residential area (61). The collection process was based on the WHO Water Sampling and Analysis Scheme (62). Briefly, the sterile bottles were immersed in the canal to a depth of approximately 20 cm, and approximately 200 mL of water was filled in each bottle, then capped by leaving a small air space inside. The samples were immediately placed in a lightproof, insulated cool box and transported to the laboratory within a few hours of the collection for bacterial culture.

Isolation, identification, and serogrouping of Salmonella spp.

All 333 canal water samples were first processed in the pre-enrichment step by mixing 50 mL of water with the same volume of buffer peptone water, followed by incubation at 37°C for 24 h. The enriched water samples were processed in the selective enrichment step by being dropped onto the Modified Semi-Solid Rappaport-Vassiliadis (MSRV) agar (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 42°C overnight. Subsequently, the swarming edge on MSRV was picked and streaked on Xylose Lysine Deoxycholate (XLD) agar (Becton, Dickinson and Company), then incubated at 37°C for 24 h. Then, the presumptive three *Salmonella* spp. colonies on XLD agar according to colony characters were randomly selected for biochemical identification, including Triple Sugar Iron Agar and Motility Indole Lysine Medium. After biochemical confirmation of *Salmonella* spp., the serogroup of selected isolates was identified by slide agglutination using O & H-grouping antisera. *Salmonella* spp. isolates were stored in Luria-Bertani (LB) broth (Becton, Dickinson and Company) with 30% glycerol and kept at -80°C for further analysis. The isolates kept for future use were selected based on the serogroup differences (Table S2).

PCR-based screening for PMQR-positive isolates

In total, 399 *Salmonella* isolates from 282 *Salmonella* positive canal water samples between 2016 and 2020 were analyzed for PMQR genes by Polymerase Chain Reaction (PCR). The PCR reaction mixture (20 µL) contained 1X Green GoTaq® Reaction Buffer (Promega, Madison, WI), 1.25 U of Taq DNA polymerase (Promega), 0.2 mM of each dNTP (Takara Bio Inc., Shiga, Japan), 0.5 mM of each primer, and 3.1 µL of DNA template. The primers and thermocycling conditions for PMQR gene determination were listed in Table 2. The PCR products were visualized by gel electrophoresis on 2% agarose gels stained with GelRed (Biotium Inc., Fremont, CA). The PCR product images were captured by Printgraph Classic (ATTO Corp., Tokyo, Japan).

Antimicrobial susceptibility testing

The 399 *Salmonella* spp. isolates recovered from Bangkok canal water samples were tested for antimicrobial susceptibility by disk diffusion method (63) using commercially available antimicrobial disks (Oxoid, England) against quinolones; nalidixic acid (30µg) and ciprofloxacin (5µg). Briefly, the bacterial isolates were emulsified in normal saline solution equal to 0.5 McFarland standard and swabbed on Mueller–Hinton agar (MHA) plates (Merck, Germany). The antimicrobial disks were placed on the surface of MHA plates and incubated at 37°C for 16-18 h. Antimicrobial susceptibility of *Salmonella* isolates was determined according to the zone diameter breakpoints for *Enterobacteriaceae* and interpreted as susceptible, intermediate, and resistant.

Data processing and visualization

The proportion of *Salmonella*-positive water samples, PMQR-positive isolates, quinolones resistant isolates were analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA). The graphical representation of the results was generated using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, MA). To observe the worldwide distribution of *qnr* genes and compare the results from this study to other countries, metadata from all recorded isolates was obtained from the NCBI gene database isolates browser, with isolates lacking location data being omitted (Table S1). The global map visualization was produced utilizing Microreact (https://microreact.org) and BioRender (https://BioRender.com).

Results

Prevalence and serogroup of Salmonella spp. in Thai canal water

Salmonella spp. was positive in 92.2% (n = 307/333) of canal water samples. During the rainy season (June to October), the *Salmonella* detection rate was higher at 95.7% (n = 134/140) compared to 89.6% (n = 173/193) during the dry season (January to May and November to December). A total of 399 *Salmonella* spp. isolates from 282 *Salmonella*-positive water samples were investigated for PMQR genes. Thirty-seven isolates from 25 positive samples were excluded due to a lack of bacterial growth in a re-culture process from bacterial stock culture. The serogroup distribution of *Salmonella* spp. isolated from canal water samples between 2016 and 2020 was presented in Figure 9. In 2016, 2017, and 2019, the most frequent serogroup was serogroup B, representing 43.8% (n = 28/64), 35.3% (n = 30/85), and 40.6% (n = 28/69), respectively. On the other hand, in 2018 and 2020, the most common serogroup was serogroup C, with 39.8% (n = 35/88) and 36.6% (n = 34/93), respectively. The third most common serogroup in all five years was serogroup E. Serogroup G was consistently detected each year but with low prevalence. Other serogroups that were occasionally found include serogroups D, F, I, OMC, OMD, and OME.

Prevalence of PMQR determinants

The prevalence of PMQR-positive *Salmonella* spp. isolates from canal water samples were summarized in Table 2. Of 399 *Salmonella* spp. isolates, 35.8% (n = 143/399) were positive for PMQR genes. Of these, most of the isolates were positive for *qnrS* (97.2%, n = 139/143). Three isolates carrying *qnrB* were found only in 2019 while three isolates carrying *qnrD* were found one each in 2018, 2019, and 2020. None of the isolates was positive for *qnrA* and *qepA*. Six *Salmonella* spp. isolates harbored more than one PMQR gene in the following combinations: *qnrB*, *qnrS,* and *aac(6')-Ib-cr* (n = 2), *qnrS* and *aac(6')-Ib-cr* (n = 2), *qnrS* and *oqxAB* (n = 2). The majority of PMQR positive isolates were serogroup B followed by serogroup C and serogroup E. PMQR genes were also occasionally found in *Salmonella* spp. serogroup G, serogroup I, serogroup OMC, and serogroup OMD (Table 4). Sampling locations-wise, Canal D had the highest prevalence of PMQR-positive *Salmonella* spp. isolates (43.5%, n = 30/69), followed by Canal F (40.5%, n = 32/79). Canal B (37.0%, n = 20/54), Canal E (36.4%, n = 28/77), and Canal C (32.8%, n = 22/67), while Canal A (20.8%, n = 11/53) had the lowest prevalence of PMQR among *Salmonella* spp. isolates as demonstrated in Table 5.

Antimicrobial susceptibility

The proportion of resistant *Salmonella* spp. isolates to nalidixic acid decreased from 12.5% in 2016 to 5.9% in 2017, then increased to 13.0% in 2019 and decreased again to 6.5% in 2020. A similar trend was observed for ciprofloxacin, with the proportion of resistant isolates decreasing from 3.1% in 2016 to 2.4% in 2017, followed by an increase to 11.6% in 2019, and finally a decrease to 1.1% in 2020 (Figure 10). As shown in Table 6, 13.5% (n = 54/399) of *Salmonella* spp. isolates from canal water samples showed reduced susceptibility, while 9.3% (n = 37/399) were fully resistant to nalidixic acid. For ciprofloxacin, 33.8% (n = 135/399) of the isolates showed reduced susceptibility, whereas 3.8% (n = 15/399) were resistant. Among PMQR-positive isolates, 11.9% (n = 17/143) and 7.7% (n = 11/143) were resistant to nalidixic acid and ciprofloxacin, respectively. For PMQR-negative isolates, 7.8% (n = 20/256) were resistant to nalidixic acid, and 1.6% (n = 4/256) were resistant to ciprofloxacin. Fifteen of 399 isolates were resistant to both antimicrobial agents, with 11 of these isolates being PMQR-positive. Two isolates harboring *qnrS* and *aac(6')-Ib-cr* were resistant to nalidixic acid and ciprofloxacin with narrow zone diameters at ≤ 6 mm for both drugs. Two isolates carrying *qnrB*, *qnrS*, and *aac(6')-Ib-cr* were resistant to nalidixic acid and ciprofloxacin with the same zone diameter at ≤ 6 mm for nalidixic acid and slightly different zone diameters for ciprofloxacin at 11 and 14 mm. Two isolates with *qnrS* and *oqxAB* were resistant to nalidixic acid with zone diameters of 11 and 9.5 mm, and also exhibited reduced susceptibility to ciprofloxacin with zone diameters of 23 and 25 mm (Table S1).

Discussion

In the present study, a high prevalence (92.2%) of *Salmonella* spp. was observed from canal water. Similar results were reported in China, where the mean positive detection rate of *Salmonella* spp. in flooded man-made rivers was 90% (40). In contrast, 26%, 29.4%, and 32.2% of *Salmonella* spp. positive environmental water samples were reported in the river in Canada (39), the river in the United States (37), and the canal in Jordan (41), respectively. The high prevalence of *Salmonella* spp. in Bangkok canal water may be attributed to several factors. Firstly, the stagnant condition of the canals in this study, coupled with the densely populated urban setting of Bangkok, may provide favorable conditions for the proliferation of the bacteria. Secondly, inadequate sanitation practices in some households, livestock-based farming, and insufficient treatment measures may facilitate bacterial contamination and distribution. Similar speculations were observed in the river with a high prevalence of

Salmonella spp. in China (40). Furthermore, *Salmonella* detection levels in the watersheds have been shown to be positively correlated with precipitation and water temperature (64, 65). Specifically, the *Salmonella* detection rate was higher during the rainy season compared to the dry season in this study. When compared to other countries like Canada, the United States, and Jordan, Thailand experiences higher levels of precipitation and warmer water temperatures (66, 67). These environmental parameters could potentially contribute to the elevated prevalence of *Salmonella* spp. in canal water in Thailand.

Several previous studies have examined the presence of PMQR genes in *Salmonella* spp. in Thailand. *qnrB* (1.8%) and *qnrS* (4.1%) were found in *Salmonella* spp. isolated from pork, human, and poultry (68). The later studies reported that 10% of clinical *S.* Enteritidis were positive for *qnrS* (24) and 20.5% of clinical *S.* Choleraesuis harbored at least one PMQR gene (32). In contrast, the prevalence of PMQR-positive *Salmonella* spp. isolates from Bangkok canal water elucidated in this study (35.8%) was higher than that reported in any previous study. In Thailand, fluoroquinolones are one of the top antimicrobial agents consumed by both human and food-producing animals (Health Policy and Systems Research on Antimicrobial Resistance Network, 2022). Owing to their chemical stability and low biodegradability, their residues can persist in the environment and are often detected in water sources (36, 69). A previous study conducted in Thailand revealed significant concentrations of nalidixic acid and ciprofloxacin in city canals (70). The presence of fluoroquinolone residues in water bodies exerts selective pressure on bacterial populations, leading to the acquisition of resistance mechanisms such as PMQR genes, which are crucial for their survival and ultimately contribute to the emergence of antibiotic-resistant bacteria.

A significant proportion of *Salmonella* spp. isolated from canal water harbored PMQR genes, predominantly *qnrS*. *qnrS* has been proposed to originate from an aquatic microorganism (26) and is usually carried by small, non-conjugative plasmids that are more likely to be found in *S. enterica* serovars (71). The global distribution of *qnr* genes (Figure 3) demonstrated that *qnrS* is the predominant *qnr* gene in most Southeast Asian countries as well as in China and Middle Eastern countries. Conversely, *qnrB* is the main *qnr* gene in several European countries, as well as North and South American countries. Notably, *qnrS* has demonstrated extensive dispersion throughout Thailand and neighboring countries, suggesting that Thailand may serve as a hotspot for the distribution of *qnrS*. Only three isolates harbored *qnrB,* which agreed well with the global distribution of this PMQR gene. Markedly, *Salmonella* isolates carrying *qnrD* were found for the first time in Thailand, suggesting the possible influx.

qnrA was not found in any isolates in this study, which similarly observed in *E. coli* from water in Bangladesh (72). In contrast, *qnrA* was the only PMQR detected in *E. coli* from Portuguese environmental water (Mendonça et al., 2012). These results confirmed that PMQR gene distribution varies by organisms and geographic areas. The difference in PMQR gene distribution in different geographical locations may be influenced by numerous factors, such as the different antibiotic usage patterns, population dynamics of bacteria, dissimilar evolution of bacteria, and transferability of mobile genetic elements carrying PMQR genes (73-75). In *Enterobacteriaceae*, several plasmids associated with specific resistance determinants are predominant in certain geographic regions, which can replicate in a broad host range, and their dissemination relies on selective pressure from antibiotics (76). Thus, the plasmid typing, gene organization, and genetic relationship of PMQR harboring *Salmonella* spp. will be elucidated in our future study.

The prevalence of PMQR-positive *Salmonella* spp. isolates might be associated with the land use plan of the surrounding areas. Canal D is situated in central Bangkok, where is a high-density residential area. In contrast, Canal A is located in a low-density residential area. The high population density in the area along Canal D may contribute to the high prevalence of PMQR-positive *Salmonella* spp. isolates. The previous study in Thailand also reported the diverse level of antimicrobial resistance gene abundance in the different land-based zones (77). Additionally, the varying degrees and types of human activities within the different land use plans may have played a role in the distinct levels of antimicrobial resistance gene spread (78).

The proportion of ciprofloxacin-resistant *Salmonella* spp. isolated from canal water increased in 2019 and decreased in 2020. A similar trend was observed in non-typhoidal *Salmonella* isolated from blood in Thailand (79). A higher proportion of PMQR-positive isolates were non-susceptible to nalidixic acid and ciprofloxacin compared to PMQR-negative isolates. The concomitant presence of multiple PMQR genes in this study, specifically *qnrS* and *aac(6')-Ib-cr*, showed narrow zone diameters. This result may indicate the synergistic effect of *qnrS* and *aac(6')-Ib-cr*. Previous studies also demonstrated that coharboring of several PMQR genes might increase the final minimum inhibitory concentration without the mutation in QRDRs (80, 81). Moreover, the combination of PMQR genes with the mutation in QRDRs can confer a high level of fluoroquinolone resistance (24, 82). The substantial proportion of *Salmonella* isolates with reduced susceptibility to quinolones in the canals located in highly populated areas of Bangkok during these recent years is concerning, given the frequent use of canals and tributaries as a resource for agriculture, transportation, and recreation.

Figure 8 Sampling locations of 6 canals in Bangkok, Thailand. The yellow area represents Bangkok, and the red pins represent canal locations (A - F). The figure was modified from (83) and created with MapChart (https://www.mapchart.net/) and BioRender (https://BioRender.com).

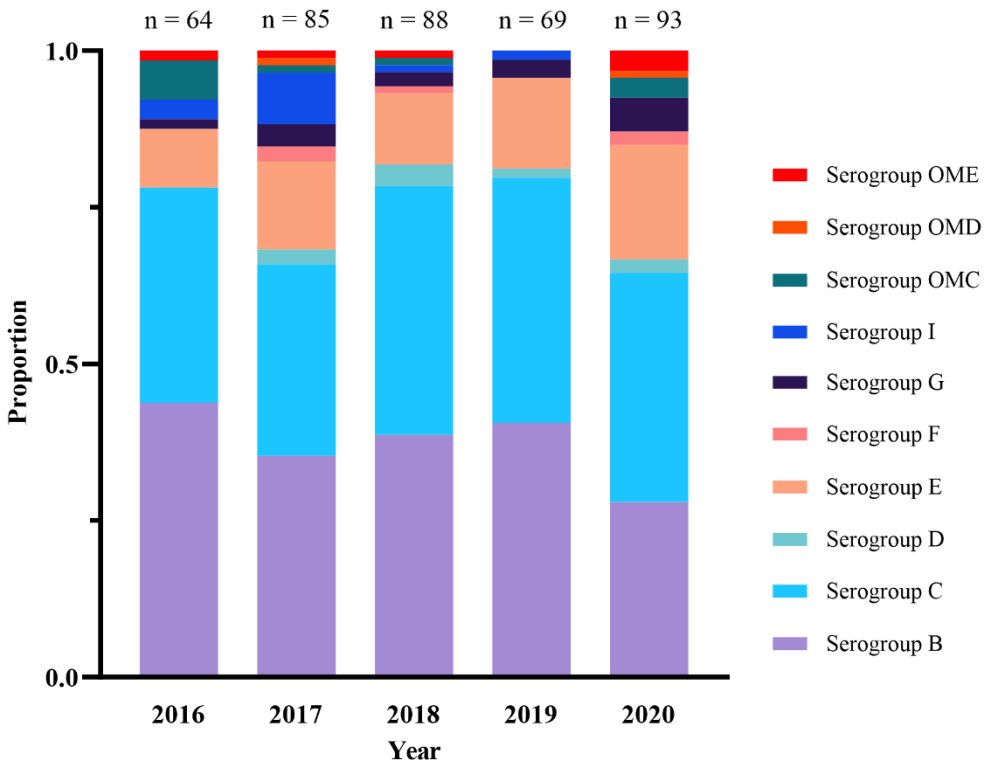


Figure 9 Serogroup distribution of *Salmonella* spp. isolated from canal water samples between 2016 and 2020. The colors in each bar, Y-axis, and X-axis represent serogroup, proportion of each serogroup, and year, respectively.

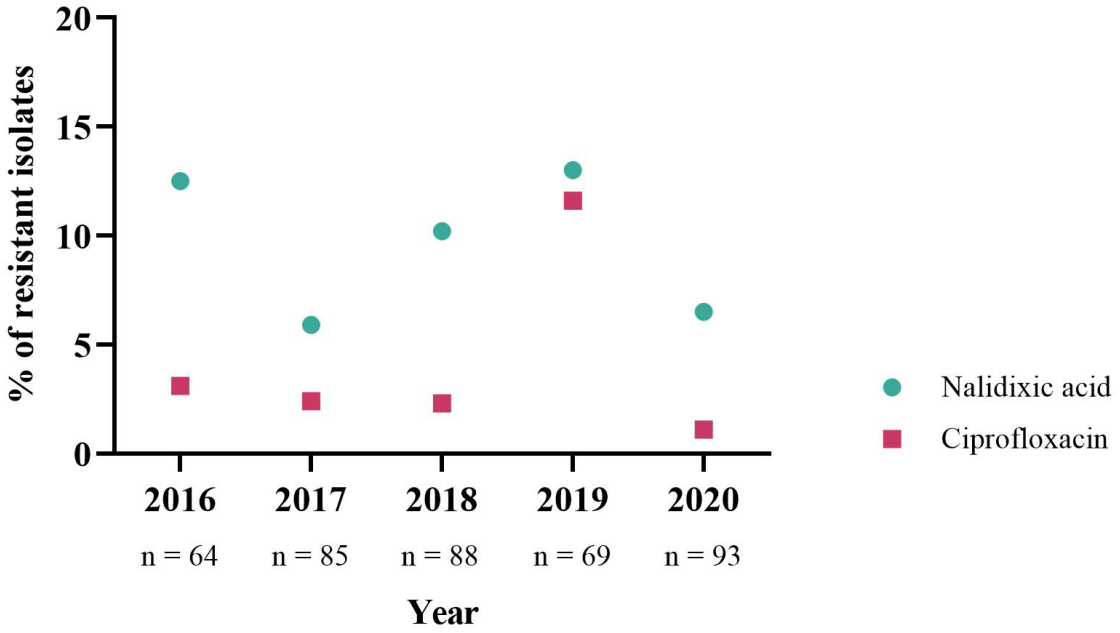


Figure 10 Percentage of nalidixic acid and ciprofloxacin resistant *Salmonella* spp. isolates from canal water samples between 2016 and 2020. The symbol colors, Y-axis, and X-axis represent the antibiotics, percentage of resistant isolates, and year, respectively.

Table 2 List of primer sequences used for PMQR gene determination.

Target gene	Primer sequence 5' - 3'	Product size (bp)	Reference	Amplification (35 cycles)		
				Denaturation	Annealing	Extension
qnrA-F	AGAGGATTTCTCACGCCAGG	580	(84)	98°C	54°C	72°C
qnrA-R	TGCCAGGCACAGATYTTGAC		(84)	5 s	5 s	20 s
qnrB-F	GGMATHGAAATTCGCCACTG	264	(84)	94°C	56°C	72°C
qnrB-R	TTTGCYGYYCGCCAGTCGAA		(84)	30 s	40 s	1 m
qnrC-F	GCGAATTTCCAAGGGGCAAA	135	(85)	98°C	59°C	72°C
qnrC-R	ACCCGTAATGTAAGCAGAGCAA		(85)	5 s	5 s	20 s
qnrD-F	AGGTGTAGCATGTATGGAAAAGC	208	(85)	98°C	59°C	72°C
qnrD-R	GCTAGCGTTTTTTAGTTTGGCTC		This study	5 s	5 s	20 s
qnrS-F	GCAAGTTCATTGAACAGGGT	428	(84)	98°C	56°C	72°C
qnrS-R	TCTAAACCGTCGAGTTCGGCG		(84)	5 s	5 s	20 s
aac(6')-Ib-cr-F	ATGACTGAGCATGACCTTG	477	(86)	95°C	55°C	72°C
aac(6')-Ib-cr-R	AACCATGTACACGGCTGG		(86)	45 s	15 s	45 s
qepA-F	CAAGCAGTTGGCCGAGCATG	207	This study	95°C	57°C	72°C
qepA-R	ATGAAGATGTAGACGCCGAAC		This study	45 s	15 s	45 s
oqxAB-F	GACAGCGTCGCACAGAATG	339	(87)	95°C	57°C	72°C
oqxAB-R	GGAGACGAGGTTGGTATGGA		(87)	45 s	15 s	45 s

Table 3 Prevalence of PMQR-positive *Salmonella* isolates obtained from canal water samples from 2016 to 2020.

Year	PMQR positive (%)	No. (%) of isolates positive for PMQR genes			
		***qnrB* (%)**	***qnrD* (%)**	***qnrS* (%)**	**Other PMQR genes (%)**
2016 (n = 64)	30 (46.9)	0	0	30 (46.9)	2[a] (3.1)
2017 (n = 85)	28 (32.9)	0	0	28 (32.9)	0
2018 (n = 88)	29 (33.0)	0	1 (1.1)	28 (31.8)	0
2019 (n = 69)	30 (43.5)	3 (4.3)	1 (1.4)	28 (40.6)	4[b] (5.8)
2020 (n = 93)	26 (28.0)	0	1 (1.1)	25 (26.9)	0
Total (n = 399)	143 (35.8)	3 (0.8)	3 (0.8)	139 (34.8)	6 (1.5)

[a]: Two isolates carried *qnrS* and *oqxAB*

[b]: Two isolates carried *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and two isolates carried *qnrS*, *aac(6')-Ib-cr*

Table 4 PMQR gene distribution across serogroups of *Salmonella* spp. isolated from canal water samples between 2016 and 2020.

Serogroup	PMQR positive (%)	No. (%) of isolates positive for PMQR genes			
		***qnrB* (%)**	***qnrD* (%)**	***qnrS* (%)**	**Other PMQR genes (%)**
B (n = 146)	82 (56.1)	2 (1.4)	1 (0.7)	80 (54.8)	2[a] (1.4)
C (n = 144)	46 (31.9)	0	2 (1.4)	44 (30.6)	1[b] (0.7)
D (n = 8)	0	0	0	0	0
E (n = 55)	9 (16.4)	1 (1.8)	0	9 (16.4)	2[c] (3.6)
F (n = 5)	0	0	0	0	0
G (n = 13)	2 (15.4)	0	0	2 (15.4)	0
I (n = 11)	1 (9.1)	0	0	1 (9.1)	0
OMC (n = 9)	2 (22.2)	0	0	2 (22.2)	1[d] (11.1)
OMD (n = 2)	1 (50.0)	0	0	1 (50.0)	0
OME (n = 6)	0	0	0	0	0
Total (n = 399)	143 (35.8)	3 (0.8)	3 (0.8)	139 (34.8)	6 (1.5)

[a]: One isolate carried *qnrS* and *oqxAB*, and one carried *qnrB*, *qnrS*, *aac(6')-Ib-cr*

[b]: One isolate carried *qnrS*, *aac(6')-Ib-cr*

[c]: One carried *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and one isolate carried *qnrS*, *aac(6')-Ib-cr*

[d]: One isolate carried *qnrS* and *oqxAB*

Table 5 Prevalence of PMQR-positive *Salmonella* isolated from water samples from 6 canals in Bangkok.

Canal	Land use plan[a]	PMQR positive (%)	No. (%) of isolates positive for PMQR genes			
			qnrB **(%)**	***qnrD*** **(%)**	***qnrS*** **(%)**	**Other PMQR genes (%)**
A (n = 53)	Low-density residential area	11 (20.8)	0	0	11 (20.8)	0
B (n = 54)	Agricultural area	20 (37.0)	0	1 (1.9)	19 (35.2)	4[b] (7.4)
C (n = 67)	Low-density residential area	22 (32.8)	1 (1.5)	1 (1.5)	21 (31.3)	1[c] (1.5)
D (n = 69)	High-density residential area	30 (43.5)	0	0	30 (43.5)	0
E (n = 77)	Low to moderate-density residential area	28 (36.4)	1 (1.3)	0	28 (36.4)	1[c] (1.3)
F (n = 79)	Moderate to high-density residential area	32 (40.5)	1 (1.3)	1 (1.3)	30 (38.0)	0
Total (n = 399)		143 (35.8)	3 (0.8)	3 (0.8)	139 (34.8)	6 (1.5)

[a]: Information was obtained from the Department of City Planning, 2015

[b]: Two isolates carried *qnrS* and *oqxAB*, and two isolates carried *qnrS*, *aac(6')-Ib-cr*

[c]: One isolates carried *qnrB*, *qnrS*, *aac(6')-Ib-cr*

Table 6 Antimicrobial susceptibility against nalidixic acid and ciprofloxacin of *Salmonella* spp. isolated from canal water samples between 2016 and 2020.

Antimicrobial agents	Phenotype	No. (%) of isolates		
		PMQR-positive isolates (n = 143)	PMQR-negative isolates (n = 256)	Total (n = 399)
Nalidixic acid	Susceptible	75 (52.4)	233 (91.0)	308 (77.2)
	Intermediate	51 (35.7)	3 (1.2)	54 (13.5)
	Resistant	17 (11.9)	20 (7.8)	37 (9.3)
	Average diameter (mm)	18.0	22.7	
Ciprofloxacin	Susceptible	23 (16.1)	226 (88.3)	249 (62.4)
	Intermediate	109 (76.2)	26 (10.1)	135 (33.8)
	Resistant	11 (7.7)	4 (1.6)	15 (3.8)
	Average diameter (mm)	27.1	34.9	

Summary

AMR poses an escalating global public health threat, particularly concerning AMR gene dissemination through the aquatic environment. In Thailand, where canals serve vital agricultural and daily water needs, the presence of resistant bacteria in the canal water is deeply concerning. PMQR represents a crucial resistance mechanism, as it can be transferred through horizontal gene transfer, facilitating the wider dissemination of quinolone-resistant bacteria. Hence, this research focused on the prevalence of PMQR determinants in *Salmonella* strains isolated from Thai canal water.

From 2016 to 2020, 333 water samples were collected from six canals across Bangkok, Thailand. *Salmonella* spp. was isolated, PMQR genes were detected through polymerase chain reactions, and the antimicrobial susceptibility was examined using the disk diffusion method.

The results indicated a 92.2% prevalence of *Salmonella* spp. in canal water, being serogroups B and C the most frequently detected. Overall, 35.8% of isolates harbored PMQR genes, being *qnrS* the most prevalent gene (97.2%, n = 139/143). Other PMQR genes, including *qnrB*, *qnrD*, *oqxAB*, and *aac(6')-Ib-cr*, were detected. Notably, six isolates harbored multiple PMQR genes. Furthermore, 9.3% and 3.8% of overall isolates were resistant to nalidixic acid and ciprofloxacin, respectively. PMQR-positive isolates showed higher rates of non-susceptibility to both nalidixic acid and ciprofloxacin compared to PMQR-negative isolates.

In conclusion, the high prevalence of *Salmonella* spp., significant PMQR-positive, and reduced susceptibility isolates in canal water is of public health concern in Bangkok. This study suggested the urgent need to integrate environmental sampling into comprehensive surveillance programs, providing crucial insights into AMR dissemination in ecosystems.

CHAPTER II

Genomic analysis of *Salmonella* isolated from canal water in Bangkok, Thailand

Introduction

In Thailand, next-generation sequencing technology has been primarily employed in genome characterization of *Salmonella* spp. isolated from humans (88, 89), and pork and poultry production chains (90, 91) for identification of serotypes, sequence types (STs), virulence factors, antibiotic resistance genes (ARGs), and chromosomal-mediated gene mutations. On the other hand, implementation in characterizing *Salmonella* spp. from environmental sources remains notably scarce. Especially, characteristics and dissemination of the antimicrobial-resistant *Salmonella* spp. in the aquatic environment in the urban area have not been well elucidated. Bangkok, the capital city of Thailand, is home to over 11 million people and has an extensive canal network. The canal network is essential for their daily lives with diverse functions (60). This suggests that the molecular epidemiological analysis of *Salmonella* spp. in the canal of Bangkok provides beneficial scientific insights for understanding the dissemination of AMR through the aquatic environment based on public health and One Health Approach.

This chapter aimed to uncover the potential risk of AMR, virulence, and the contribution of urban canals as reservoirs by whole genome sequencing (WGS) of *Salmonella* strains derived from canal water samples in Bangkok. The data obtained from the present study should serve as a basis for public health risk assessment and suggest intervention strategies for AMR challenges in Thailand.

Materials & Methods

Bacterial strains

Thirty *Salmonella* strains were selected based on the difference in collecting years, sampling canals, and the presence of PMQR genes (Table 7) from the *Salmonella* isolate collection in CHAPTER I. Identification of bacterial species was performed by KmerFinder 3.2 as described following section, *In silico species confirmation.*

DNA extraction and whole genome sequencing

The bacterial genomic DNA of selected strains was extracted using Qiagen's QIAamp® DNA mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality and concentration were determined using NanoDrop spectrophotometer and Qubit fluorometer. A total of 1 ng of genomic DNA from each isolate was used to construct sequencing libraries using the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA) following the manufacturer's instructions. Bioanalyzer (Agilent Technologies) was used to check the library size. Selected strains were sequenced as multiplexed libraries on the Illumina MiSeq platform operated per the manufacturer's instructions for 600 cycles to produce paired-end reads of 300 bp in length.

Genome assembly and quality checking

The whole genome sequences of 30 *Salmonella* strains were assembled and checked for quality with FoodQCPipeline (https://bitbucket.org/genomicepidemiology/foodqcpipeline/src/master/) (92). Briefly, raw reads were pre-checked the quality with FastQC version 0.11.5 (93) and trimmed away low quality sequences as well as sequencing adaptors with bbduk2 (part of BBtools version 36.49, https://jgi.doe.gov/data-and-tools/software-tools/bbtools/) based on an internal database. The trimmed raw reads were post-checked for quality with the FastQC version 0.11.5 (93). All QC-passed reads were according to: [1] length of reads was longer or equal to 50 bp, [2] phred score per base was higher or equal to 20 and, [3] adaptors were filtered away. The QC-passed reads were de novo assembled with SPAdes version 3.11.0 (94). Assemblies were assessed the quality by using Quast version 4.5 (95). The assemblies were assessed following to: [1] number of contigs was fewer than 500 (each contig was greater than 500 bp) with an N50 value is preferably larger than 30,000 bp, [2] total number of base pairs was approximately 5,000,000 as size of *Salmonella* genome, [3] base coverage was higher or equal to 30x (base pairs sequenced)/(total base pairs after assembling), and [4] phred score was higher or equal to 20 calculated as a part of trimming process.

In silico species confirmation, serotyping, sequence typing, antimicrobial resistance determinant and virulence factors detection, and plasmid replicon typing

Species confirmation of each stain was performed using k-mer algorithm in KmerFinder 3.2 (https://cge.food.dtu.dk/services/KmerFinder/) (96-98). The strains that were not identified as *Salmonella enterica* subsp. *enterica* were omitted from further analysis.

SeqSero2 was used to predict serotype based on the sequences of the O (*wzx* and *wzy* genes) and H antigens (*fliC* and *fljB* genes) (99). Multilocus sequence typing (MLST) was performed using WGS data on the sequences of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, *thrA*) to determine sequence type (STs) of each strain (https://cge.food.dtu.dk/services/MLST/) (100-103). AMR determinants both acquire resistance genes and chromosomal-mediated gene mutation were detected from assembled genomes by ResFinder (https://cge.food.dtu.dk/services/ResFinder/) (103-105). The pipeline was run with the default parameters with a similarity of 95% and an alignment coverage of 60%. Virulence factors were identified by VFanalyzer based on the information in the virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) (106). Plasmid replicon type was classified by PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/) (103, 107). The pipeline was executed using default settings, including a 95% similarity and 60% alignment coverage threshold.

Phylogenetic analysis of S. Agona

The phylogenetic analysis was conducted on the predominant serotype in this study, *S.* Agona. Nine assembled genomes of *S.* Agona from this study (Strain MU7, MU8, MU9, MU12, MU16, MU22, MU24, MU29, and MU30) and 167 genomes from the global collection retreived from the European Nucleotide Archive (ENA) (Table S3) were used to determine genetic relatedness using CSI Phylogeny version 1.4 (108). *S.* Agona strain 9 was used as a reference strain. The single-nucleotide polymorphisms (SNPs) were determined based on default parameters as criteria: [1] a minimum depth of 10 reads at SNP positions, [2] a minimum relative depth of 10% at SNP positions, [3] a minimum distance of 10 bp between SNPs, [4] a minimum SNP quality of 30, [5] a minimum read mapping quality of 25 and [6] and minimum Z-score of 1.96. The concatenated SNPs were used for constructing maximum-likelihood tree using FastTree version 2.1.11 (109). The tree (Figure 13 and 14) was visualized with iTOL (110).

Results

Species, serotypes, and ST types based on WGS

Species, serotypes, and ST types were summarized in Table 8. Twentynine of 30 strains were confirmed as *Salmonella enterica* subsp. *enterica*. One strain was identified as *Salmonella* sp. SJTUF14170, which is unclassified *Salmonella* and therefore was excluded from

downstream analysis. Serotype prediction showed a high level of serotype diversity. The predominant serotype was Agona (31.0%, n = 9/29), followed by Mbandaka (10.3%, n = 3/29), and Stanley (10.3%, n = 3/29). The most common STs were identical to the serotype.

Antimicrobial resistance determinants in Salmonella strains from Bangkok canal water

As depicted in Figure 11, we identified a total of 35 ARGs by ResFinder and all *Salmonella* strains examined in this study possessed at least one ARG. These genes confer resistance to nine distinct classes of antimicrobials, encompassing aminoglycosides [*aac(3)-IIa*, *aac(3)-IIb*, *aac(6')-Iaa*, *aadA1*, *aadA2*, *aadA2b*, *aph(3')-Ia*, *aph(3')-Ib*, *aph(3')-IIa*, *aph(6)-Id*, and *bleO*], β-lactams (*bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{OXA-1}}$, and *bla*$_{\text{TEM-1B}}$), colistin (polymyxin E) and polymyxin B (*mcr-3.1*), fosfomycin (*fosA7*), phenicols (*catA1*, *catA2*, *catB3*, *cmlA1*, and *floR*), quinolones [*aac(6')-Ib-cr*, *oqxAB*, *qnrB19*, *qnrD1*, and *qnrS1*], sulphonamides (*sul2* and *sul3*), tetracycline [*tet*(A), *tet*(B), and *tet*(M)], and trimethoprim (*dfrA12* and *dfrA14*).

The most prevalent gene across all isolates was *aac(6')-Iaa*. 48.3% of study strains carried *bla*$_{\text{TEM-1B}}$ (n = 14/29) and more than half harbored *tet*(A) (55.2%, n = 16/29). Among PMQR genes, *qnrB19, qnrD1, qnrS1, aac(6')-Ib-cr*, and *oqxAB* were identified in 3, 3, 18, 4, and 2 strains, respectively. Notably, one strain (*S.* Schwarzengrund Strain MU18) carried 20 ARGs that included *mcr-3.1*, which contributes to colistin resistance. Strains from the same canal and year tended to exhibit similar patterns of ARGs.

Through *in silico* analysis, a total of 30 chromosomal-mediated gene mutations (= nonsynonymous mutations) in 7 genes (*acrB*, *gyrA*, *gyrB*, *parC*, *parE*, *pmrA*, and *pmrB*) were identified by ResFinder as illustrated in Figure 11. The most prevalent mutation was in ParC and the amino acid substitution from threonine to serin at amino acid position 57 (T57S) was seen in 28 out of 29 strains (96.5%). Two amino acid substitutions at positions 28 (from phenylalanine to leucine: F28L) and 40 (from leucine to proline: L40P) were also detected in 27 out of 29 strains in AcrB (93.1%). In addition to the substitutions, several well-known mutations associated with quinolone resistance were also detected at positions 83 [from serine to phenylalanine or tyrosine: S83F (20.7%, n = 6/29) or S83Y(6.9%, n = 2/29)] and 87 [from aspartic acid to glycine: D87G (3.4%, n = 1/29)] of GyrA as well as at position 80 of ParC [from serine to arginine: S80R (3.4%, n = 1/29)] and at position 458 of ParE [from serine to proline: S458P (3.4%, n = 1/29)]. Among the 29 strains, 8 strains carried more than one amino acid substitution associated with quinolone resistance, and Strain MU18 harbored 5 amino acid

substitutions in GyrA, ParC, and ParE. Taken together, 22 of the 29 *Salmonella* strains (75.9%) harbored ARGs and chromosomal-mediated gene mutations across the resistance to more than three antimicrobial classes, classifying them as MDR strains (111).

Twenty-one *Salmonella* strains from Bangkok canal harbored both PMQR genes and chromosomal gene mutation linked to fluoroquinolones resistance. There were eight patterns of combination as followed [1] *qnrS1* and ParC T57S (34%, n = 10/29), [2] *aac(6')-Ib-cr, qnrB19, qnrS1,* GyrA S83Y, and ParC T57S (6.9%, n = 2/29), [3] *aac(6')-Ib-cr, qnrS1,* GyrA S83F, and ParC T57S (6.9%, n = 2/29), [4] *qnrD1* and ParC T57S (6.9%, n = 2/29), [5] *qnrD1*, GyrA S83F, and ParC T57S (3.4%, n = 1/29), [6] *qnrB19*, GyrA S83F, and ParC T57S (3.4%, n = 1/29), [7] *qnrS1*, GyrA S83F-D87G, ParC T57S-S80R, and ParE S458P (3.4%, n = 1/29), and [8] *oqxAB*, *qnrS1*, and ParC T57S (6.9%, n = 2/29).

Virulence related genes in Salmonella strains from Bangkok canal water

In total, 206 virulence factors were detected as shown in Figure 12. These virulence factors can be categorized into 9 classes including fimbrial adherence determinants (*csg, bcf, fim, lpf, pef, peg, saf, sta, stb, stc, std, ste, stf, sth, sti, stk,* and *tcf*), nonfimbrial adherence determinants (*misL, ratB, shdA*, and *sinH*), adherence (K88 fimbriae), macrophage inducible genes (*mig-14*), magnesium uptake (Mg^{2+} transporter), regulation (*phoPQ*), secretion system (T3SS-1, T3SS-2, T3SS effectors translocated via both systems, T3SS-1 translocated effectors, and T3SS-2 translocated effectors), stress adaptation (*sodCI*), and toxin (typhoid toxin).

Among 206 virulence factors, 107 genes were present in all *Salmonella* genomes. The invasion gene (*invA*) and colonization gene (*fimA*) were detected in all isolates. All study strains also identified the virulence gene associated with antimicrobial peptide resistance (*mig-14*). The complete *csg* cassette was found in 21 strains. Toxin encoding genes were present in a small number of isolates. Both *cdtB* and *pltA* genes were found in five isolates from serotypes Give (Strains MU5 and MU6), Farmsen or Poona (Strain MU13), Schwarzengrund (Strain MU18), and Panama or Houston (Strain MU19), while serotype Weltevreden (Strain MU3) carried only *pltA* gene. Notably, K88 fimbriae genes were observed in serotypes Give (Strains MU5, MU6), Mbandaka (Strains MU10, MU11, and MU21), Farmsen or Poona (Strain MU13), Kentucky (Strain MU14), and Schwarzengrund (Strain MU18). In contrast, ACE T6SS cassettes were detected in serotypes Weltevreden (Strains MU3 and MU15), and Agona (Strains MU7, MU8, MU9, MU12, MU16, MU22, MU24, MU29, and MU30).

Plasmid replicon types in Salmonella strains from Bangkok canal water

The plasmid replicon types of 29 *Salmonella* strains from the Bangkok canal were summarized in Table 9. In total, 15 different plasmid replicon types were detected from study strains, with Col(pHAD28), Col3M, and IncHI2 being the most common replicon types. The strains with plasmids exhibited a range of 1 to 5 plasmid replicon types, in which *S.* Agona Strains MU7 and MU8 carried the highest number of plasmids. Notably, *qnrD1*-carrying strains were all present with Col3M plasmid replicon type, and *qnrB19*-harbouring strains were present with Col(pHAD28) plasmid replicon type. However, among the study strains, 8 strains did not possess any known plasmid replicon types. The strains from the same canal and year tended to display a similar pattern of plasmid replicon types.

Phylogenetic analysis of S. Agona from Bangkok canal water

According to the SNP-based phylogenetic tree analysis, *S.* Agona strains obtained from canal water were categorized into three distinct clades (Figures 13 and 14). Clade 1, depicted in red, consisted of Strains MU12, MU24, MU29, and MU30 isolated from Canals B, D, and F between 2018 and 2020. Meanwhile, clade 2, represented in green, included Strains MU7, MU8, MU9, and MU16 derived from Canals B, C, and F in 2019. Strain MU22, collected from Canal D in 2017, formed an independent clade marked in blue, distinctly separate from the others. Within clade 1, the canal water strains demonstrated relations to isolates sourced from food in Thailand and Vietnam, as well as isolates from animals and the environment in the USA. In clade 2, the study strains were associated with animal strains from the USA and Kenya, along with a food strain from Thailand. Within clade 3, a tested strain was clustered with an environmental strain from the USA, along with food strains from Thailand and China.

Discussion

In this study, *S.* Agona was the most common *Salmonella* serotype isolated from Bangkok canal water. Over the past few years, *S.* Agona has been consistently placed on the list of the top twenty most frequently isolated serotypes in the United States (112) and the tenth most commonly reported serotype in the European Surveillance System (113). In China, *S.* Agona was reported among the top 10 serotypes that cause human diarrheal diseases (114). Moreover, *S.* Agona has been linked to several foodborne outbreaks worldwide in the last few decades and has been detected in a wide range of food and animal products, including infant

milk formula, meat, chicken, and eggs associated with these outbreaks (113, 115). In Europe, *S.* Agona accounted for 13 outbreaks, resulting in 636 reported human cases and 12 hospitalizations with no deaths during 2007 - 2016 (113). Apart from human cases, *S.* Agona was reported as one of the most common serotypes isolated from pigs during the National Animal Health Monitoring Survey in the United States (116) and one of the most common causes of disease in both human and swine populations (117). These results suggest the potential causal risk of salmonellosis in Bangkok canals.

The most frequent ARGs detected in *Salmonella* strains from Bangkok canal water was *aac(6')-Iaa*. This gene appeared consistently in previous studies across all *Salmonella* genomes, regardless of serotype, suggesting its presence in ancestral strains before serotype divergence and subsequent vertical transfer *via* chromosome replication (118). Other research explored that more than 98% of *S. enterica* genomes from ENA contain *aac(6')-Iaa* (119). While ubiquitously found in *Salmonella*, the presence or absence of this gene did not confer aminoglycoside resistance, suggesting it functions as a cryptic gene (120, 121). Resistome Tracker, a tool for AMR exploration based on genome data from the National Center for Biotechnology Information (NCBI), showed that *aph(6)-Id* and *aph(3'')-Ib* were predominant aminoglycoside resistance genes in nontyphoidal *Salmonella* from environmental water (122).

This study identified the ParC T57S substitution as commonly detected in *Salmonella* strains from Bangkok canal water. This mutation alone likely has a low impact on quinolone resistance, providing minimal protection against ciprofloxacin but seemingly associated with enhanced bacterial fitness (123). Resistome Tracker data revealed GyrA D87Y as the predominant mutation in nontyphoidal *Salmonella* from environmental water, followed by GyrA S83F and S83Y (122), although GyrA D87Y was not found in the study strains. Multiple PMQRs were detected in *Salmonella* from Bangkok canal water, predominantly *qnrS1*. However, Resistome Tracker reported *qnrB19* as the most common PMQR gene in nontyphoidal *Salmonella* from environmental water (122). Previous Thai studies indicated a higher prevalence of *qnrS* than *qnrB* in *Salmonella* strains from various sources, including *S.* Enteritidis from humans, *S.* Choleraesuis from patients with systemic salmonellosis, and strains from pig farms and slaughterhouses (24, 32, 90). These findings suggest the circulation of *qnrS* in Thailand, indicating the country may act as a distribution hotspot for this gene.

A prior investigation revealed that *S.* Enteritidis strains co-carrying a PMQR gene and an amino acid substitution in GyrA, and those with double amino acid substitutions in GyrA displayed high levels of fluoroquinolone resistance (24). In the current study, 21 isolates of the

examined strains carried both ARGs and chromosomal-mediated gene mutations. Notably, one strain contained *qnrS1*, double substitutions in GyrA and ParC, and single substitution in ParE. These findings are concerning as these isolates sourced from canal water could potentially confer elevated levels of fluoroquinolone resistance, posing a threat to treating bacterial infections and escalating public health concerns.

One *Salmonella* strain from Bangkok canal water possessed *mcr-3.1* gene that associated with colistin resistance. According to Resistome Tracker, the predominant plasmid-mediated colistin resistance gene was *mcr-1.1*, while *mcr-3.1* was mainly found in nontyphoidal *Salmonella* isolates from humans (122). In Thailand, *mcr-3.1* was recently reported in *S*. Choleraesuis isolated from human blood (89). Colistin is considered a last-resort treatment for bacterial infections. The presence of the colistin resistance gene in *Salmonella* strain from environmental water is alarming and may indicate that canal water could serve as a potential reservoir for AMR gene distribution.

Several isolates harbored resistance genes against β-lactams (except carbapenemase-producing genes), quinolones, and tetracycline. Markedly, one strain carried resistance genes for nearly all drug classes, including colistin. These antimicrobial agents are extensively used in Thailand's human and veterinary medicine (124). The incongruous antimicrobial usage may exert selective pressure, contributing to the widespread distribution of AMR genes. These findings underline that Bangkok canals could serve as passive vectors facilitating the further spread of MDR *Salmonella*. Addressing AMR concerns in Thailand necessitates stringent regulations on antimicrobial usage and comprehensive surveillance on a large scale. Additionally, given that canals receive water from wastewater treatment plants, improving wastewater treatment systems could help tackle the AMR issue. Currently, Bangkok's wastewater treatment plants utilize activated sludge systems, which may not be efficient enough to remove ARGs (125). A previous study demonstrated that the membrane bioreactor was more efficient at removing ARGs than the conventional activated sludge system (126). Thus, the integrated units maximizing the advantages of different technologies, such as the combination of membrane filtration and activated sludge processes, might be a promising strategy. In addition, the local authorities might consider other alternative treatment methods, such as constructed wetlands and tridimensional eco-biological reactors (125).

The finding of invasion, adhesion, and survival genes indicates that the strains from canal water had the potential to colonize and infect humans. Additionally, the detection of K88 fimbriae adhesin and ACE T6SS cassette suggests the probable capacity of several strains from

canal water to acquire new genes and functions by horizontal gene transfer. The *mig-14* gene, found in all study strains, facilitates *Salmonella* survival by blocking antimicrobial peptides (127). Alarmingly, a few *Salmonella* strains from canal water harbored genes associated with typhoid toxins like *cdtB* and *pltA*. The virulence gene identification of *Salmonella* strains assists in depicting their pathogenic potential and comprehending their occurrence and persistence in the environment.

Eight study strains did not contain any known plasmid replicon types, while some of them carried plasmid-mediated resistance genes. This may be due to the tool used in the present study is designed to detect replicons sharing a minimum of 80% nucleotide identity with the cataloged types (107). Plasmid diversity beyond this scope might be missed. Another factor to consider is the ongoing rearrangements and mutations in plasmids that potentially alter typing regions, leading to unclassifiable new types (76). Further investigation using long-read sequencing is needed for comprehensive plasmid insights in *Salmonella* from Bangkok canal water.

Phylogenetic analysis of *S.* Agona revealed that the strains from Bangkok canal water segregated into 3 clades. Each clade shared a common feature: the strains from canal water in this study were grouped with strains derived from food in Thailand. These findings suggest that *S.* Agona might have been circulating between environmental water and food sources in Thailand. Nonetheless, genomic data of *S.* Agona isolated from humans, animals, and environments in Thailand available in public databases is limited due to the lack of comprehensive surveillance and the infrequent use of whole genome sequencing in both human and animal hospitals, slaughterhouses, water treatment plants, and other related sectors. Thus, additional genomic information is needed to establish a comprehensive genetic relationship between environmentally isolated *Salmonella* and clinically obtained *Salmonella* and understand its transmission through One Health perspective. Additionally, the clustering of *S.* Agona from Bangkok canals with strains from foreign countries suggests the possible introduction of antimicrobial-resistant bacteria to Thailand or vice versa through international transportation. The observed relatedness among MDR *Salmonella* strains from canal water in Thailand and other countries emphasizes that MDR *Salmonella* is not solely a national but rather a global concern. This ultimately underscores the necessity for sustainable interventions to address AMR issues and safeguard public health on a global scale.

Figure 11 Heatmap of acquired antimicrobial resistance-related genes and chromosomal-mediated antimicrobial resistance-related gene mutations presence in 29 *Salmonella* spp. isolated from Bangkok canal water. Present genes and gene mutations are shown in red and absent genes and gene mutations are shown in pink. Antimicrobial classes, antimicrobial resistance-related genes, genes, and gene mutations (amino acid substitutions) are indicated next to the heatmap.

Figure 12 Heatmap of virulence factors in 29 *Salmonella* spp. isolated from Bangkok canal water. Present and absent genes are shown in red and pink, respectively. Virulence genes and the virulence category labels are indicated next to the heatmap.

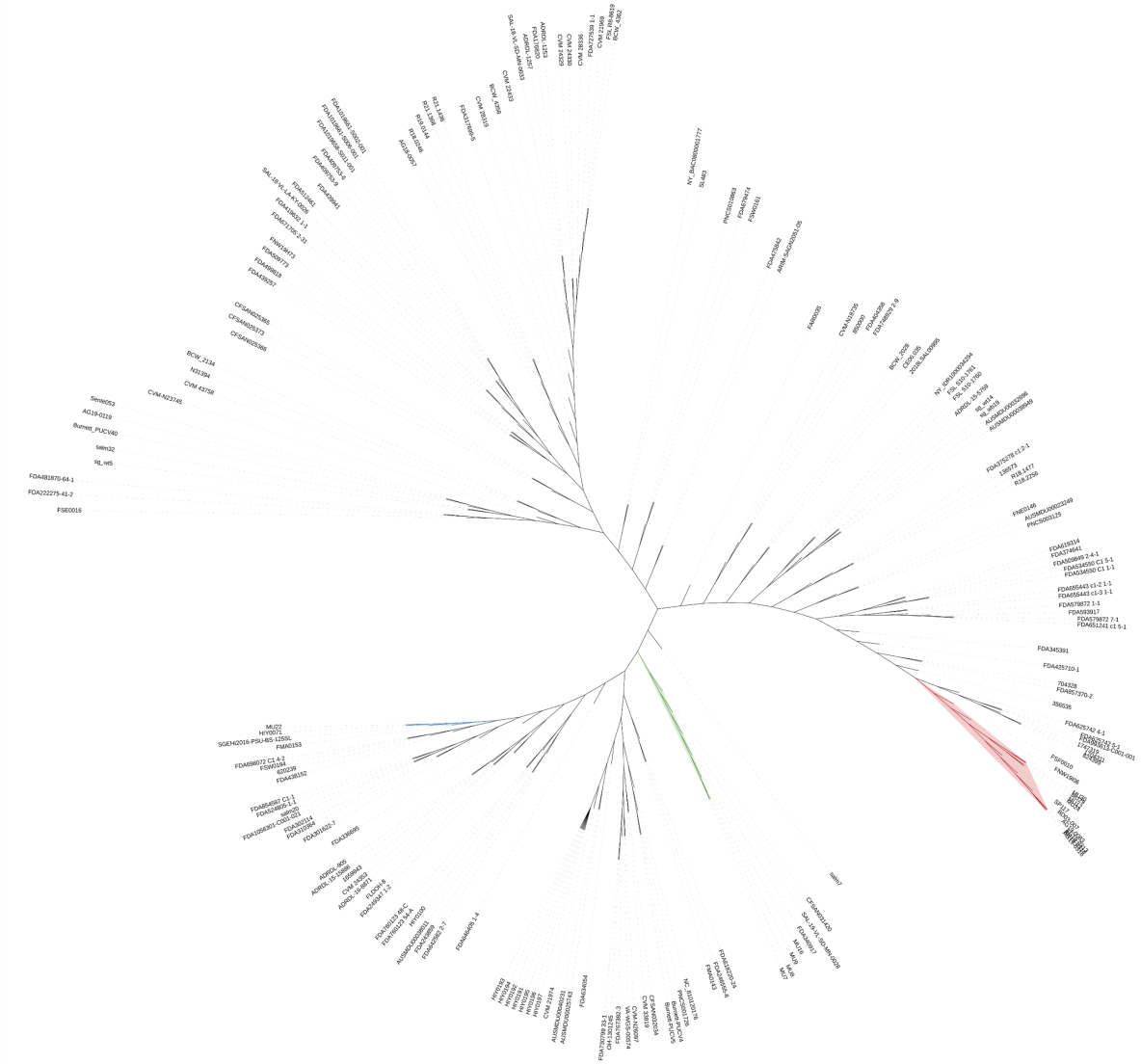


Figure 13 Unrooted SNP-based phylogenetic tree of 9 *S.* Agona strains from Bangkok canal water and 167 strains from the global collection. The tree illustrates the segregation of 9 *S.* Agona strains from Bangkok canal into clades 1, 2, and 3, indicated by red, green, and blue labels, respectively.

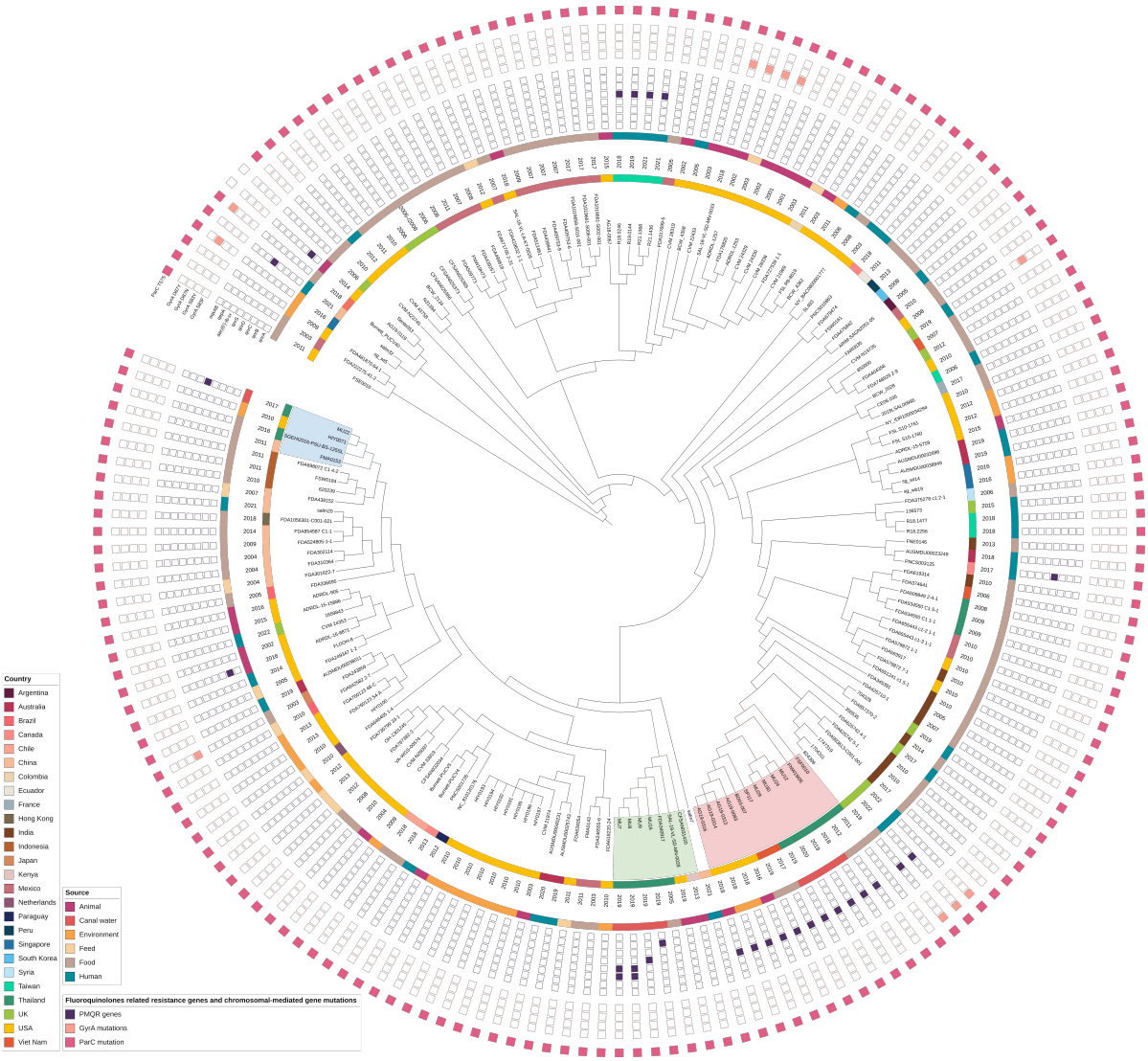


Figure 14 Midpoint rooted SNP-based phylogenetic tree of 9 *S.* Agona strains from Bangkok canal water and 167 strains from the global collection, with *S.* Agona Strain MU9 serving as the reference point. The classification based on the isolates' source and country is represented by different colors, following the figure's scheme from the innermost to the third inner rings. The year of isolation is displayed in the second innermost ring. Additionally, the presence of fluoroquinolones related resistance genes and chromosomal-mediated gene mutations are presented in three outer rings. The clades covering the study strains are depicted in red, green, and blue, corresponding to Figure 13.

Table 7 *Salmonella* strains isolated from Bangkok canal water included in this chapter.

Strain	Canal	Year	PMQR genes							
			qnrA	***qnrB***	***qnrC***	***qnrD***	***qnrS***	***aac(6')-Ib-cr***	***qepA***	***oqxAB***
MU3	A	2016	-	-	-	-	-	-	-	-
MU15	A	2016	-	-	-	-	-	-	-	-
MU1	B	2016	-	-	-	-	+	-	-	+
MU2	B	2016	-	-	-	-	+	-	-	+
MU18	B	2016	-	-	-	-	+	-	-	-
MU19	B	2017	-	-	-	-	-	-	-	-
MU4	B	2018	-	-	-	+	-	-	-	-
MU12	B	2018	-	-	-	-	+	-	-	-
MU7	B	2019	-	-	-	-	+	+	-	-
MU8	B	2019	-	-	-	-	+	+	-	-
MU17	B	2020	-	-	-	-	-	-	-	-
MU20	C	2017	-	-	-	-	+	-	-	-
MU23	C	2018	-	-	-	-	-	-	-	-
MU5	C	2019	-	+	-	-	+	+	-	-
MU9	C	2019	-	-	-	+	-	-	-	-
MU11	D	2016	-	-	-	-	+	-	-	-
MU26	D	2016	-	-	-	-	+	-	-	-
MU21	D	2017	-	-	-	-	+	-	-	-
MU22	D	2017	-	-	-	-	+	-	-	-
MU27	D	2017	-	-	-	-	+	-	-	-
MU13	D	2018	-	-	-	-	-	-	-	-
MU28	D	2018	-	-	-	-	+	-	-	-
MU29	D	2019	-	-	-	-	+	-	-	-
MU14	D	2020	-	-	-	-	+	-	-	-
MU30	D	2020	-	-	-	-	+	-	-	-
MU6	E	2019	-	+	-	-	+	+	-	-
MU25	E	2019	-	-	-	-	-	-	-	-
MU16	F	2019	-	+	-	-	-	-	-	-
MU24	F	2019	-	-	-	-	+	-	-	-
MU10	F	2020	-	-	-	+	-	-	-	-

Table 8 Species, serotypes, and ST types of *Salmonella* spp. isolated from Bangkok canal water.

Strain	Canal	Year	Species	Serotype	ST type
MU3	A	2016	*Salmonella enterica* subsp. *enterica*	Weltevreden	365
MU15	A	2016	*Salmonella enterica* subsp. *enterica*	Weltevreden	365
MU1	B	2016	*Salmonella enterica* subsp. *enterica*	Stanley	29
MU2	B	2016	*Salmonella enterica* subsp. *enterica*	Stanley	29
MU18	B	2016	*Salmonella enterica* subsp. *enterica*	Schwarzengrund	322
MU19	B	2017	*Salmonella enterica* subsp. *enterica*	Panama or Houston	48
MU4	B	2018	*Salmonella enterica* subsp. *enterica*	N/A	22
MU12	B	2018	*Salmonella enterica* subsp. *enterica*	Agona	13
MU7	B	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU8	B	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU17	B	2020	*Salmonella enterica* subsp. *enterica*	II 6,7:z29:[z42] or Tennessee	319
MU20	C	2017	*Salmonella enterica* subsp. *enterica*	potential monophasic variant of Typhimurium (O5-)	34
MU23	C	2018	*Salmonella enterica* subsp. *enterica*	Stanley	29
MU5	C	2019	*Salmonella enterica* subsp. *enterica*	Give	516
MU9	C	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU11	D	2016	*Salmonella enterica* subsp. *enterica*	Mbandaka	413
MU26	D	2016	*Salmonella enterica* subsp. *enterica*	Corvallis or Chailey	1541
MU21	D	2017	*Salmonella enterica* subsp. *enterica*	Mbandaka	413
MU22	D	2017	*Salmonella enterica* subsp. *enterica*	Agona	13
MU27	D	2017	*Salmonella* sp. SJTUF14170	-	-
MU13	D	2018	*Salmonella enterica* subsp. *enterica*	Farmsen or Poona	1069
MU28	D	2018	*Salmonella enterica* subsp. *enterica*	Corvallis or Chailey	1541
MU29	D	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU14	D	2020	*Salmonella enterica* subsp. *enterica*	Kentucky	696
MU30	D	2020	*Salmonella enterica* subsp. *enterica*	Agona	13
MU6	E	2019	*Salmonella enterica* subsp. *enterica*	Give	516
MU25	E	2019	*Salmonella enterica* subsp. *enterica*	Apeyeme	1546
MU16	F	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU24	F	2019	*Salmonella enterica* subsp. *enterica*	Agona	13
MU10	F	2020	*Salmonella enterica* subsp. *enterica*	Mbandaka	413

Table 9 Plasmid replicon types of *Salmonella* spp. isolated from Bangkok canal water.

Strain	Canal	Year	Plasmid replicon
MU3	A	2016	IncFII(S), IncI1-I(Alpha)
MU15	A	2016	IncFII(S)
MU1	B	2016	Col(pHAD28), IncHI2, IncHI2A
MU2	B	2016	Col(pHAD28), IncHI2, IncHI2A
MU18	B	2016	Col(BS512), IncHI2
MU19	B	2017	Col(pHAD28), IncFIA(HI1), IncFIB(K)
MU4	B	2018	Col(pHAD28), Col3M
MU12	B	2018	IncQ1, IncX1
MU7	B	2019	Col3M, IncC, IncHI2, IncHI2A, IncQ1
MU8	B	2019	Col3M, IncC, IncHI2, IncHI2A, IncQ1
MU17	B	2020	No match
MU20	C	2017	IncQ1, p0111
MU23	C	2018	No match
MU5	C	2019	Col(pHAD28)
MU9	C	2019	Col3M, IncC
MU11	D	2016	No match
MU26	D	2016	No match
MU21	D	2017	No match
MU22	D	2017	No match
MU13	D	2018	No match
MU28	D	2018	ColpVC, IncX2
MU29	D	2019	IncX1
MU14	D	2020	IncFIB(K)
MU30	D	2020	ColpVC, IncX1
MU6	E	2019	Col(pHAD28)
MU25	E	2019	No match
MU16	F	2019	Col(pHAD28), IncC
MU24	F	2019	IncX1
MU10	F	2020	Col3M

Summary

AMR poses a rising global public health concern. Canals are essential in Thailand, including the capital city, Bangkok, as agricultural and daily water sources. However, the characteristic and antimicrobial-resistance properties of the bacteria in the urban canals have never been elucidated.

This study employed whole genome sequencing to characterize 30 genomes of a causal pathogenic bacteria, *Salmonella enterica,* isolated from Bangkok canal water between 2016 and 2020.

The dominant serotype was *Salmonella* Agona. In total, 35 antimicrobial resistance genes and 30 chromosomal-mediated gene mutations were identified, in which 21 strains carried both acquired genes and mutations associated with fluoroquinolone resistance. Virulence factors associated with invasion, adhesion, and survival during infection were detected in all study strains. 75.9% of the study stains were multidrug-resistant and all the strains harbored the necessary virulence factors associated with salmonellosis. One strain carried 20 resistance genes, including *mcr-3.1*, mutations in *gyrA*, *parC*, and *parE*, and typhoid toxin-associated genes. Fifteen plasmid replicon types were detected, with Col(pHAD28) being the most common type. Comparative analysis of nine *S.* Agona from Bangkok and 167 from public databases revealed that specific clonal lineages of *S.* Agona might have been circulating between canal water and food sources in Thailand and globally.

These findings provide insight into potential pathogens in the aquatic ecosystem and support the inclusion of environmental samples into comprehensive AMR surveillance initiatives as part of a One Health approach. This approach aids in comprehending the rise and dissemination of AMR and devising sustainable intervention strategies.

CHAPTER III

Exploration of the novel fluoroquinolones with high inhibitory effect against quinolone-resistant DNA gyrase of *Salmonella* Typhimurium

Introduction

FQs are one of the drugs of choice for *Salmonella* infection. One of the most active FQs is ciprofloxacin, which has the addition of a cyclopropyl group to the R_1 position and a piperazine ring to the R_7 position to enhance its activity (16, 47, 128). However, the quinolones resistant *Salmonella* is rising and being reported in various regions of the world, contributing to the high global burden of *Salmonella* infections (28) particularly in *S*. Typhimurium (129, 130). The most prevalent mutations linked to quinolone resistance in *Salmonella* involve amino acid substitutions at Ser83 and Asp87 of the GyrA subunit of DNA gyrase. Hence, developing novel antimicrobial agents showing a high affinity for mutated DNA gyrases is desirable.

WQ-3034 (also known as delafloxacin, ABT-492, RX-3341) is a relatively new synthetic fluoroquinolones, which received US Food and Drug Administration (US FDA) approval for Acute Bacterial Skin and Skin Structure Infections (ABSSSIs) therapy in 2017 (131). WQ-3034 has distinct structures including 6-amino-3,5-difluoropyridine-2-yl at R_1 position, 3-hydroxyazetidinyl at R_7 position, and Cl atom at R_8 position (Table 9 and Figure 14). The previous study revealed that WQ-3034 was the most potent compound tested against methicillin-susceptible and methicillin-resistant *S. aureus*, *S. pneumoniae*, viridans group streptococci, and beta-hemolytic streptococci (132). WQ-3034 also showed more potency against quinolone-susceptible and -resistant gram-positive bacteria, while having similar activity to ciprofloxacin in certain *Enterobacteriaceae* members (133). In contrast to other quinolones, WQ-3034 is considered a double-targeting drug that can bind to DNA gyrase and DNA topoisomerase IV and displays equal affinity to both enzymes (134). With this property, WQ-3034 is expected to have good in vitro activity against both gram-positive and gram-negative bacteria. WQ-3810 possesses 3-isopropylaminoazetizine-1-yl at R_7 position and methyl group at R_8 position (Table 10 and Figure 15). Previous studies revealed potent antimicrobial activity of WQ-3810 against *Acinetobacter baumannii*, *E. coli*, *S. pneumonia*, methicillin-resistant *S. aureus*, and *Neisseria gonorrhoeae* (135). In addition, WQ-3810 showed a high inhibitory effect against DNA gyrase of *S*. Typhimurium (136) and *Mycobacterium leprae* (137). WQ-3154 has a similar basic pharmacophore to WQ-3034 except

for the methyl group instead of Cl atom at R_8 position (Table 10 and Figure 15). Studies on WQ-3034 and *Salmonella* in terms of DNA gyrase inhibition activity and antimicrobial effect are very limited, particularly in mutant *Salmonella* DNA gyrases. In addition, DNA gyrase inhibitory activity and potency of WQ-3154 have never been evaluated before. Therefore, the objectives of this chapter were to assess and compare the inhibitory effect of WQ-3034 and WQ-3154 along with WQ-3810 and ciprofloxacin on WT and mutant *S.* Typhimurium DNA gyrases, and further to understand the antimicrobial activity of these drugs against nontyphoidal *Salmonella*.

Materials & Methods

Antimicrobial compounds and reagents

WQ-3034, WQ-3154, and WQ-3810 were provided by Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan). Ciprofloxacin was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). The chemical structures of antimicrobial compounds used in this study, along with the basic structure of quinolones, are presented in Table 10 and Figure 15. Relaxed and supercoiled pBR322 DNA were procured from John Innes Enterprises, Ltd. (Norwich, UK).

Protein expression and purification

Recombinant DNA gyrase was obtained as separate subunits, GyrA and GyrB. In total, plasmids encoding nine subunits including WT GyrA and GyrB as well as seven GyrA with amino acid substitutions (Ser83Phe, Ser83Ile, Ser83Tyr, Asp87Gly, Asp87Asn, Asp87Tyr, and Ser87Phe-Asp87Asn) in the QRDR were constructed as described in our previous study (138). These plasmids were introduced into *Escherichia coli* BL21(DE3) (Merck KGaA, Darmstadt, Germany), and protein expression was induced in the bacterial cells. A transformed *E. coli* was inoculated into Luria-Bertani broth supplemented with ampicillin (50 mg/L) and incubated at 37°C until the optical density value reached 0.60. Expression of GyrA and GyrB was induced by adding 1mM isopropyl beta-D-1-thiogalactopyranoside (IPTG) (Wako Pure Chemical Industries Ltd, Osaka, Japan) followed by incubation at 16°C for 40 hr (for GyrA), or 18°C for 13 hr (for GyrB). To release the expressed protein, *E. coli* cells were harvested by centrifugation and suspended in a native binding buffer containing complete mini EDTA-free (Roche Applied Science, Mannheim, Germany), then sonicated at a 30% duty cycle with 10 cycles of 40 sec on and 40 sec off (Sonifier® 250; Branson, Danbury, CT). The recombinant DNA gyrase subunits were purified by column chromatography using Ni-NTA agarose resin (Thermo Fisher Scientific, Inc., Waltham, MA) and dialyzed against DNA gyrase dilution

buffer (50mM Tris-HCl, pH 7.5; 100mM KCl, 2mM dithiothreitol [DTT], 1mM ethylenediaminetetraacetic acid [EDTA]). The obtained protein was mixed with glycerol to prevent denaturation and stored in small aliquots at -80°C for future use. The protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ATTO, Tokyo, Japan) as shown in Figure 16.

Fluoroquinolone-inhibited DNA supercoiling assay

Fluoroquinolone-inhibited DNA supercoiling assay was performed to assess the inhibitory effects of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin. The 30 µL of reaction mixture contained DNA gyrase reaction buffer [35 mM Tris-HCl, 6 mM $MgCl_2$, 1.8 mM spermidine, 24 mM KCl, 5 mM DTT, 0.36 mg/mL of BSA, and 6.5% glycerol (w/v)], 50 mM ATP, 18 nM GyrA, 18 nM GyrB, 1.5 nM relaxed pBR322 DNA, and serially diluted fluoroquinolones. WQ-3034, WQ-3154, and WQ-3810 were used at the concentration of 0.01 – 5.12 µg/mL (for WT, Ser83Phe, Ser83Tyr, Asp87Gly, Asp87Asn, and Asp87Tyr), and 0.04 – 20.48 µg/mL (for Ser83Ile and Ser87Phe-Asp87Asn). While ciprofloxacin was used at four different concentration range; 0.01 – 5.12 µg/mL (for WT, Ser83Phe, Asp87Gly, and Asp87Tyr), 0.04 – 20.48 µg/mL (for Ser83Tyr and Asp87Asn), 0.08 – 40.96 µg/mL (for Ser83Ile) and 3.20 – 1,638.4 µg/mL (for Ser87Phe-Asp87Asn). The reaction mixture was incubated at 37°C for 60 min, then the reaction was halted by adding 8 µL of stop solution [5% SDS, 25% glycerol, and 0.25 mg/mL of bromophenol blue] (139). Next, 10 µL of each reaction mixture was subjected to gel electrophoresis for 80 min at 50 mA in 1.2% agarose gel in 1xTris-acetate-EDTA (TAE) buffer. Subsequently, the gel was stained with 0.5 µg/mL of ethidium bromide for 30 min. The presence of supercoiled DNA can be easily distinguished as a separated band under the UV light. The amount of supercoiled DNA was quantified by its intensity using ImageJ, the digital image processing software (https://imagej.nih.gov/ij/download.html).The concentration of the compounds that reduce 50% DNA gyrase activity (IC_{50}) was calculated with the AAT Bioquest IC_{50} Calculator web tool (https://www.aatbio.com/tools/ic50-calculator).

Fluoroquinolone-mediated DNA cleavage assay

DNA cleavage assays against WT and Ser83Ile were conducted as previously described (140, 141). Instead of relaxed form, supercoiled pBR322 DNA was utilized as the substrate for the cleavage assays. The 30 µL reaction mixture consisted of DNA gyrase assay buffer, purified DNA gyrase subunits, supercoiled pBR322 DNA (0.3 µg), and increasing concentrations of

WQ-3034 and ciprofloxacin. WQ-3034 was used at the concentration of 0.01 – 5.12 μg/mL for WT and at 0.01 – 100 μg/mL for Ser83Ile. Ciprofloxacin was used at the concentration of 0.01 – 33.33 μg/mL for both of DNA gyrases. Following an incubation period of 1 hr at 37°C, 3 μL of 2% SDS and 3 μL of proteinase K (1 mg/mL) were added to the reaction mixture. After an additional 30-min incubation at 37°C, the reactions were stopped by adding 8 μL of stop solution. Subsequently, 10 μL of each reaction mixture was subjected to gel electrophoresis for 96 min at 50 mA in 1% agarose gel in 1xTAE buffer and stained with 0.5 μg/mL of GelRed (Biotium, Hayward, CA, USA) for 30 min. Plasmid pBR322 linearized by BamHI digestion was used as cleaved DNA marker. The gel was photographed under the UV illumination and the extent of DNA cleavage was quantified by ImageJ (https://imagej.nih.gov/ij/download.html). The quinolone concentrations that require to induce 25% of maximum DNA cleavage (CC_{25}s) were determined for WQ-3034 and ciprofloxacin.

Antimicrobial susceptibility testing for nontyphoidal Salmonella

The antimicrobial activity of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin were tested against *S.* Typhimurium NBRC 13245 and *S.* Enteritidis NBRC 3313 using micro-broth dilution method as a recommended protocol of the Clinical and Laboratory Standards Institute (CLSI) (142). A suspension containing approximately 5 x 10^5 CFU/mL of each *Salmonella* strain was transferred into 96-well plate containing serially diluted fluoroquinolones and incubated at 37°C for 16 hr. The minimum inhibitory concentration (MIC) was defined as the lowest FQs concentration required to entirely inhibit the visible bacterial growth in the well.

Results

Inhibitory effect of FQs on S. Typhimurium recombinant DNA gyrase

IC_{50}s estimated from fluoroquinolone-inhibited DNA gyrase supercoiling assay are summarized in Table 11, and gel electrophoresis patterns are presented in Figure 17. IC_{50}s of WQ-3034 against WT and all mutant *S*. Typhimurium DNA gyrase were found to be lowest, conversely IC_{50}s of ciprofloxacin were found to be highest. WQ-3810 had lower IC_{50}s than WQ-3145 against DNA gyrase with amino acid substitution at codon 87, on the other hand, WQ-3154 had lower IC_{50}s than WQ-3810 at DNA gyrase with amino acid substitution at codon 83 and double mutation. Among single mutant DNA gyrases, IC_{50}s of all four FQs against Ser83Ile were the highest. IC_{50}s of every fluoroquinolone against double mutant DNA gyrase were significantly higher than WT and single mutant DNA gyrase. For example, IC_{50}s of ciprofloxacin against double mutant *S*. Typhimurium DNA gyrase were 7,264-fold and 95-fold

to 1,144-fold greater than that of WT and single mutant DNA gyrase, respectively. Compared to ciprofloxacin, IC_{50}s of WQ-3034, WQ-3154, and WQ-3810 against double mutant DNA gyrases were comparatively lower. IC_{50}s of WQ-3034, WQ-3154, and WQ-3810 against double mutant DNA gyrase were 59-fold, 70-fold, and 110-fold greater than WT, respectively. However, these were considerably less than ($1/50^{th}$) that of ciprofloxacin.

As indicated in Figure 17, all four fluoroquinolones exhibited the DNA gyrase inhibitory effect in a dose-dependent manner. The inhibitory activity trends of each fluoroquinolone against WT and seven mutant DNA gyrases are summarized in Figure 18. The slope showed that when the fluoroquinolone concentration increased, the DNA gyrase enzymatic activity decreased. Generally, inhibitory activity trends were similar among WQ compounds against WT and mutant *S*. Typhimurium DNA gyrase except in double mutation, where WQ-3810 presented a different shape of the slope. In addition, ciprofloxacin also showed a distinct slope shape compared to WQ compounds.

Fluoroquinolone-mediated DNA cleavage complex by WT and GryA-Ser83Ile DNA gyrases

Table 12 provides a summary of the CC_{25}s obtained from the fluoroquinolone-mediated DNA cleavage assays. The gel electrophoresis patterns depicting these results are presented in Figure 19, and the levels of DNA cleavage are summarized in Figure 20. CC_{25}s of WQ-3034 against the WT were found to be comparable to those of ciprofloxacin. However, when estimating the CC_{25} against Ser83Ile mutant DNA gyrase of WQ-3034, it was not possible to calculate the CC_{25} of ciprofloxacin for the same mutant. This was due to ciprofloxacin failing to induce maximum (100%) DNA cleavage at the equivalent concentration of WQ-3034. Thus, this could be inferred that WQ-3034 has a better DNA cleavage effect against Ser83Ile mutant DNA gyrase than ciprofloxacin.

MICs of FQs against nontyphoidal Salmonella

MICs of four FQs are summarized in Table 13. WQ-3034 had slightly different MICs between against *S*. Typhimurium and *S*. Enteritidis at 0.08 μg/mL and 0.16 μg/mL, while WQ-3154 had similar MICs between two *Salmonella* species at 0.08 μg/mL. WQ-3810 had comparable MICs to ciprofloxacin at 0.04 μg/mL in both *Salmonella* species, which were lower than that of WQ-3034 and WQ-3154.

Discussion

From the supercoiling assay, WQ-3034 demonstrated the highest, whereas ciprofloxacin exhibited the lowest inhibitory effect against wild-type and mutant *S.* Typhimurium DNA gyrases with amino acid substitution at codon 83 and/or 87. Interestingly, WQ-3034, WQ-3154, and WQ-3810 exhibited a significantly greater inhibitory effect than ciprofloxacin against double mutation of DNA gyrase. These WQ-fluoroquinolones have identical substitutions at R_1 position with a heteroaromatic ring called 6-amino-3,5-difluoropyridine-2-yl. This difluoropyridine moiety is structurally larger than cyclopropyl moiety, a functional group of ciprofloxacin. Since the 6-amino-3,5-difluoropyridine-2-yl as a substituent at R_1 position of WQ-3810 was proposed to be closer to the amino acid at position 83 and likely to assist WQ-3810 for robust interaction with DNA gyrases (136, 137), WQ-3034 and WQ-3154 may have similarly obtained superior inhibitory activity.

The alteration at R_7 position of the quinolone ring has been proposed to increase antibacterial activity (143, 144). A previous study of WQ-3810 revealed that the addition of 3-isopropylaminoazetizine-1-yl at R_7 position was inferred to lower IC_{50s} compared to ciprofloxacin and nalidixic acid (136). In the present study, 3-hydroxyazetidinyl as a substituent at R_7 position of WQ-3034 and WQ-3154 also resulted in lower IC_{50}s compared to ciprofloxacin.

The substitution at R_8 position in the quinolone ring has been proposed to increase the drug activity against anaerobes and gram-positive bacteria (48, 55). Specifically, adding Cl atom and methyl substituents to R_8 position was verified to enhance antimicrobial potency against both gram-positive and gram-negative bacteria (50). Here, R_8 position of the quinolone ring has the addition of Cl atom in WQ-3034 and methyl group in WQ-3154 and WQ-3810. The IC_{50} against double mutation was 59-fold, 69-fold, 110-fold, and over 7,000-fold higher than WT in WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin, respectively. These enormous differences could be the result of the interaction of molecule at the R_8 position with mutant gyrases. Similar results were observed in the previous study where the correlation between R_8 substitution and the lower IC_{50} as well as the interaction of R_8 substitution of quinolone ring with double-mutant gyrase was proposed (145). Moreover, the addition of the Cl atom at the R_8 position exerts a robust electron-withdrawing effect on the heteroaromatic ring at R_1 and stabilizes the molecule (131). By considering the modification of the quinolone ring of all antibacterial compounds in this study, the combination of substituted 6-amino-3,5-difluoropyridine-2-yl at R_1 position, 3-hydroxyazetidinyl at R_7 position, and addition of Cl atom at R_8 position of WQ-3034 may have contributed to the most potent inhibitory effect in

WT and mutant *S.* Typhimurium DNA gyrases with amino acid substitution at codon 83 and/or 87.

Among the WQ compounds, WQ-3810 had the lowest MIC, suggesting the influence of the alteration at R_7 position. The addition of substituents at R_7 position was suggested to facilitate the enhancement of half-life and bacterial tissue penetration (146). Nevertheless, when compared to ciprofloxacin, the MICs of WQ-3034 and WQ-3154 were higher, which could be due to less permeability and accumulation. For the bactericidal effect, the quinolones must be taken up by the bacteria and accumulated in the bacterial cell until reaching the concentration that can inhibit DNA gyrase activity. In this case, WQ-3034 and WQ-3154 may have less permeability and are easier to excrete from the bacterial cell than ciprofloxacin. In addition, the cyclopropyl group at R_1 position of ciprofloxacin increase the volume of distribution in the bacterial cell, and manipulation at R_7 position extend the half-life of the agent by increasing the lipophilicity (47). Since previous studies have shown the correlation between IC_{50} and MIC in quinolones (136, 138), the MICs of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin against mutant DNA gyrases were estimated from IC50s. Assuming the WQ compounds and ciprofloxacin have similar drug efflux transporting system and comparable permeability, when focusing on double amino acid substitution, the MICs of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin were 4.732, 5.578, 6.635, and 145.278 μg/ml, respectively. Taking into consideration, WQ-3034, WQ-3154, and WQ-3810 will likely be effective quinolone antibacterial drugs due to a higher inhibitory activity than ciprofloxacin and a higher antimicrobial activity against double amino acid substitution strain.

As mentioned previously, MICs against *S.* Typhimurium and *S.* Enteritidis of WQ compounds were higher than ciprofloxacin. This may indicate that WQ compounds were poorly accumulated or easier to excrete from the bacterial cell, which could be the result of low permeability or efflux pumps. In Gram-negative bacteria, the permeability barrier consists of inner and outer membranes (147). The permeability of the compounds across the outer membrane is a crucial factor in drug accumulation in a bacterial cell. Generally, quinolones use both a lipid-mediated and a porin-mediated pathway (148). A previous study demonstrated that membrane permeabilizers such as Tris/EDTA, polymyxin B, polymyxin B nonapeptide, and guanidinylated polymyxins have the ability to increase the sensitivity of *E. coli* and *S.* Typhimurium to several hydrophobic antibiotics (149, 150). Efflux pumps are proteins on the bacterial cell membrane that the bacteria use for substrate excretion. Since the efflux pump may take part in the WQ compounds extrusion, it could be worthwhile to consider using efflux pump inhibitors in combination with the antimicrobial compounds. The efflux pump inhibitors

are the chemical entities that inhibit efflux pumps of the bacteria by one or more mechanisms such as direct binding to the efflux pump to prevent the substrate binding to the active site, subsiding the energy mechanism accountable for the pumps, chelating iron required for the pumps (151, 152). In light of this, the membrane permeabilizer and efflux pump inhibitors could potentially increase the concentration of WQ compounds inside the bacterial cells. While ciprofloxacin is well studied, the physiochemical properties of WQ-3034 and WQ-3154 are truly limited. Further study is needed to substantially evaluate the efficacy of utilizing the membrane permeabilizer and efflux pump inhibitors in conjunction with WQ compounds to enhance antimicrobial activity and drug effectiveness against pathogenic bacteria.

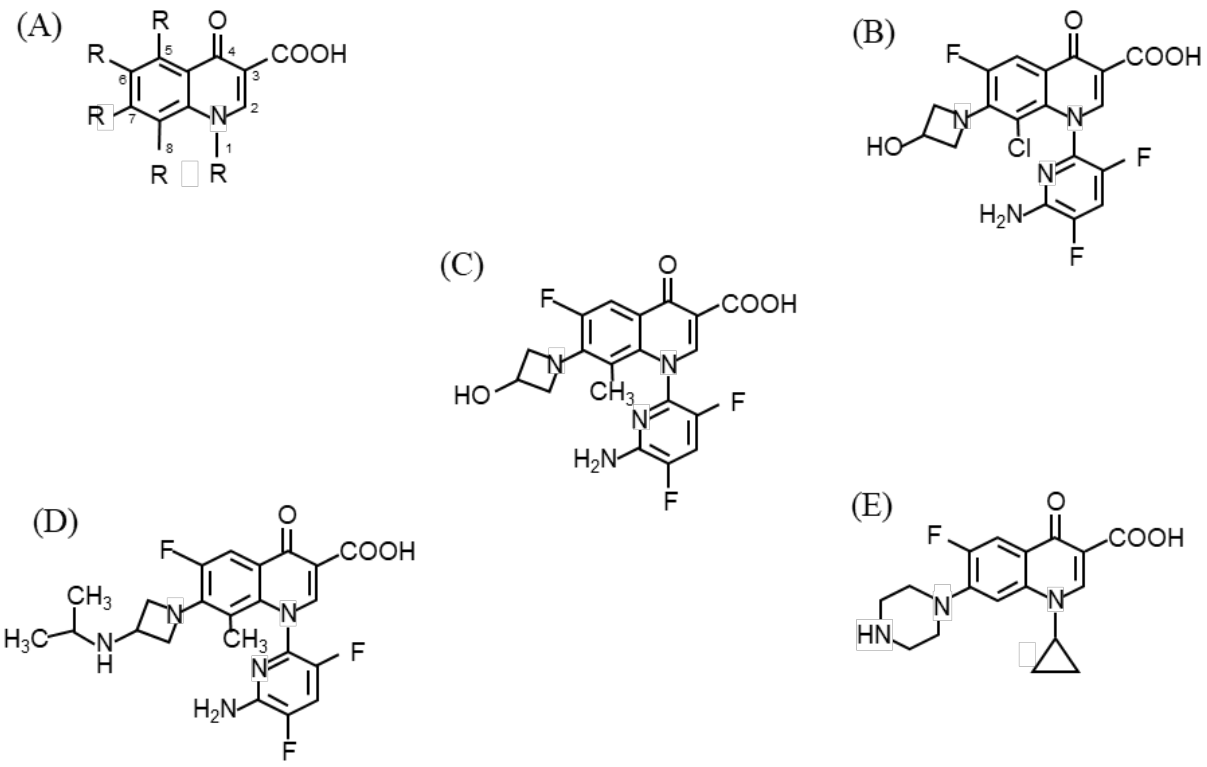


Figure 15 Chemical structures of the quinolones tested in this study. (A) Basic structure, (B) WQ-3034, (C) WQ-3154, (D) WQ-3810, and (E) ciprofloxacin.

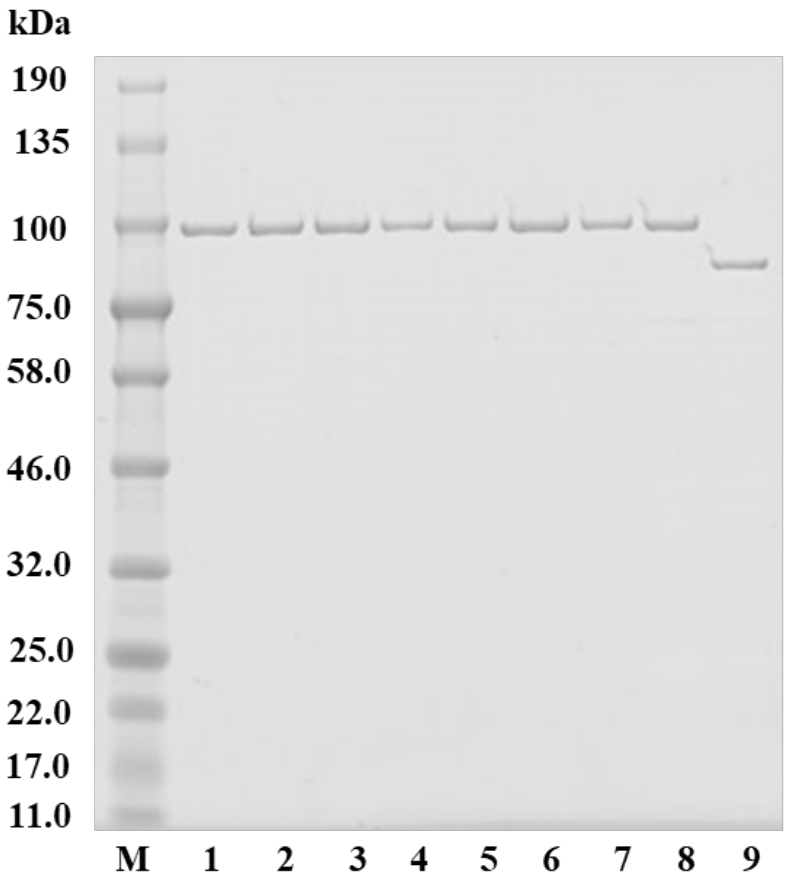


Figure 16 SDS-PAGE (5-20) (ATTO, Tokyo, Japan), of WT and mutant *S*. Typhimurium DNA gyrase subunits. Approximately, 300 ng of each protein subunit was loaded into each well. Lane M: protein size markers (kDa, Bio-Rad Lab. Inc., Japan); lane 1: WT *S*. Typhimurium GyrA subunit; lane 2 to 8: Ser83Ile, Ser83Phe, Ser83Tyr, Asp87Gly, Asp87Asn, Asp87Tyr, and Ser87Phe-Asp87Asn *S*. Typhimurium GyrA mutants, respectively; lane 9: WT *S*. Typhimurium GyrB subunit.

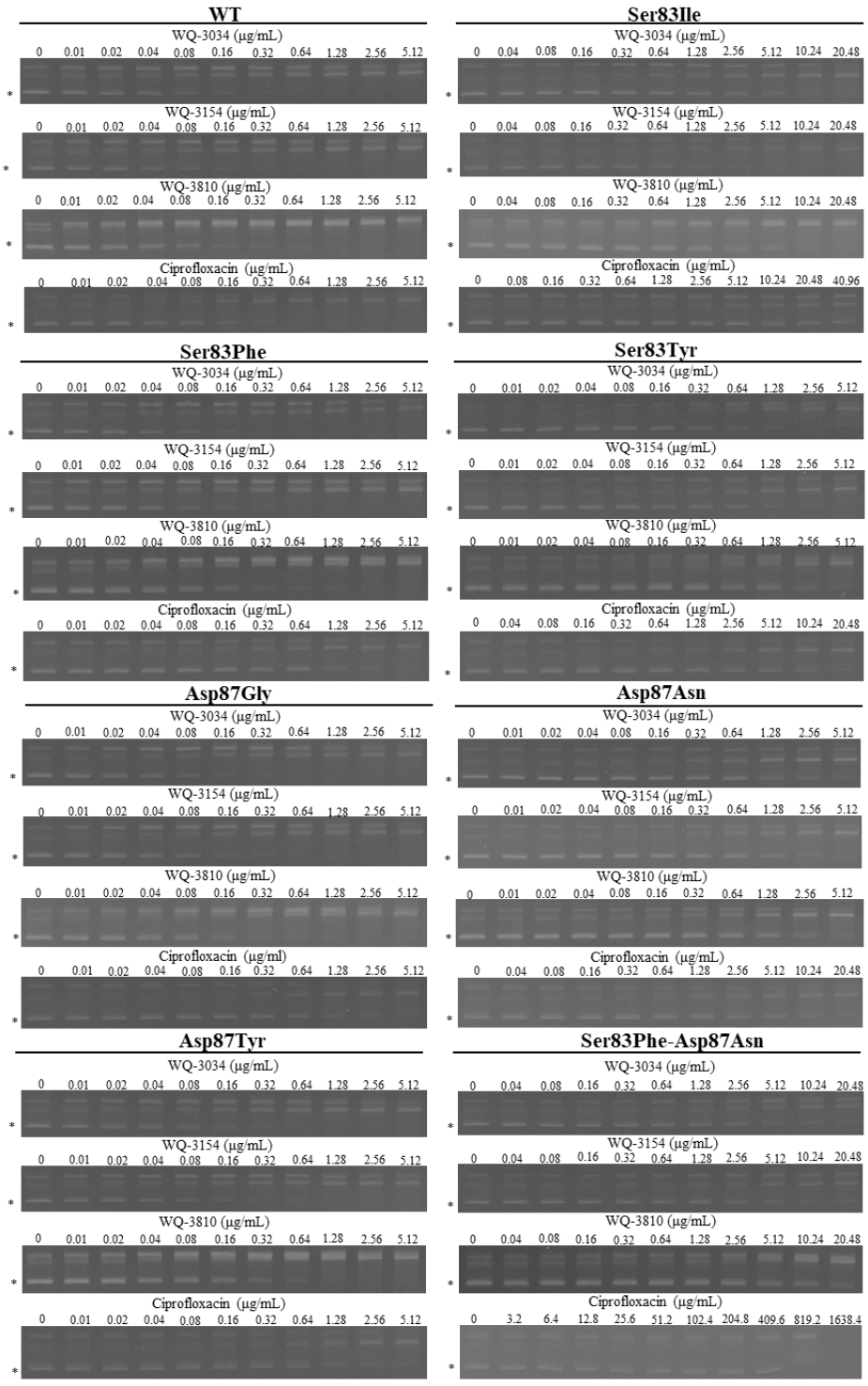


Figure 17 Inhibitory activity of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin. Asterisks (*) indicate the supercoiled DNA bands. Of note, as the concentration of quinolone increased, the intensity of supercoiled DNA band decreased.

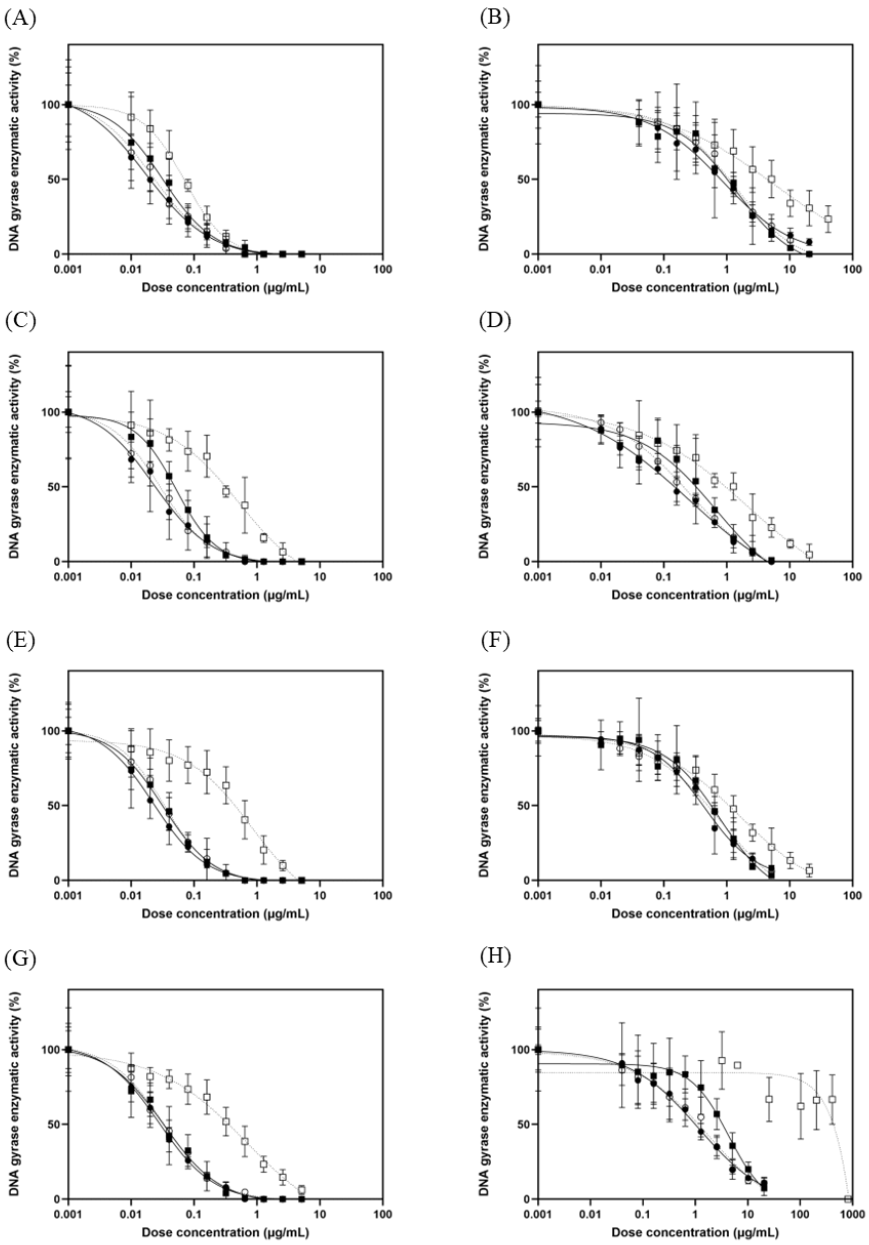


Figure 18 Sigmoidal graphs represent the inhibitory effect of fluoroquinolones against WT and mutants *S.* Typhimurium DNA gyrase in dose-dependent manner. The inhibitory activity of WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin against (A) WT, (B) Ser83Ile, (C) Ser83Phe, (D) Ser83Tyr, (E) Asp87Gly, (F) Asp87Asn, (G) Asp87Tyr, and (H) Ser83Phe-Asp87Asn. The solid circle, open circle, solid square, and open square denoted WQ-3034, WQ-3154, WQ-3810, and ciprofloxacin, respectively.

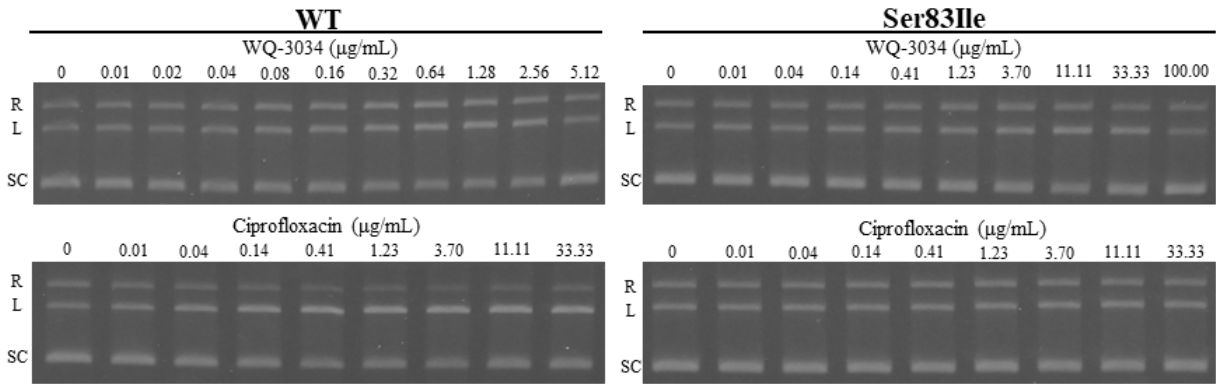


Figure 19 DNA cleavage activity of WQ-3034 and ciprofloxacin against WT and GryA-Ser83Ile *S.* Typhimurium DNA gyrases. R, L, and SC denote relaxed, linear, and supercoiled pBR322 DNA, respectively. Of note, as the concentration of quinolones increased, the intensity of linear DNA increased and then decreased after reaching the maximum DNA cleavage.

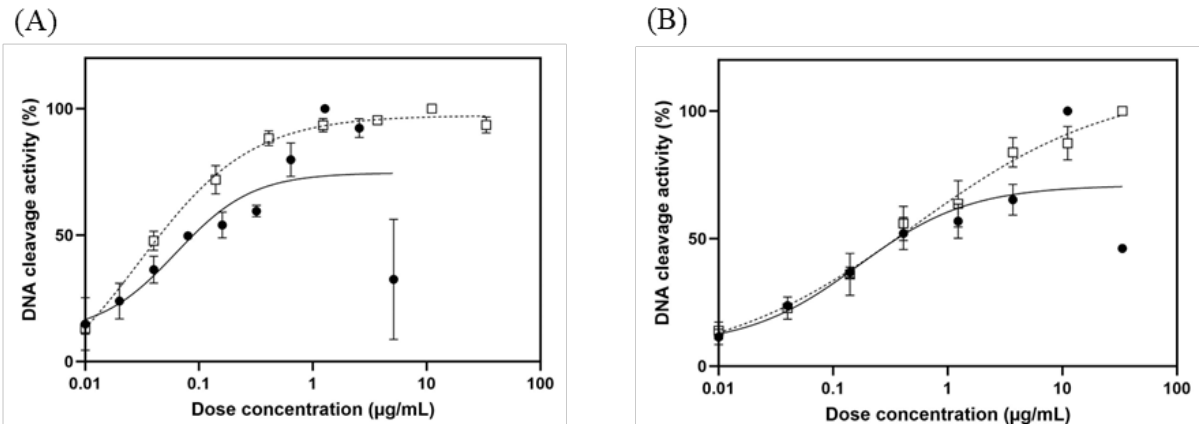


Figure 20 Graphs represent the DNA cleavage levels of fluoroquinolones against WT and mutant *S.* Typhimurium DNA gyrase. The DNA cleavage levels of WQ-3034 and ciprofloxacin against (A) WT and (B) Ser83Ile. The solid circle and open square denoted WQ-3034 and ciprofloxacin, respectively.

Table 10 Chemical structures of the quinolones tested in this study

Fluoroquinolones	**R_1**	**R_6**	**R_7**	**R_8**
WQ-3034	6-amino-3,5-difluoropyridine-2-yl	F	3-hydroxyazetidinyl	Cl
WQ-3154	6-amino-3,5-difluoropyridine-2-yl	F	3-hydroxyazetidinyl	CH_3
WQ-3810	6-amino-3,5-difluoropyridine-2-yl	F	3-isopropylaminoazetizine-1-yl	CH_3
Ciprofloxacin	Cyclopropyl	F	Piperazine	H

Table 11 IC_{50}s of the quinolones against WT and mutant *Salmonella* DNA gyrases

Quinolones	IC_{50}S ± SD (μg/mL)							
	WT	Ser83Ile	Ser83Phe	Ser83Tyr	Asp87Gly	Ap87Asn	Asp87Tyr	Ser83Phe-Asp87Asn
WQ-3034	0.020 ± 0.003	0.840 ± 0.029	0.026 ± 0.003	0.218 ± 0.023	0.024 ± 0.002	0.450 ± 0.066	0.030 ± 0.002	1.183 ± 0.132
WQ-3154	0.026 ± 0.005	1.154 ± 0.064	0.030 ± 0.002	0.249 ± 0.036	0.035 ± 0.003	0.875 ± 0.031	0.034 ± 0.002	1.813 ± 0.033
WQ-3810	0.033 ± 0.010	1.158 ± 0.223	0.052 ± 0.014	0.414 ± 0.083	0.032 ± 0.002	0.593 ± 0.121	0.033 ± 0.005	3.649 ± 0.468
Ciprofloxacin	0.072 ± 0.008	5.484 ± 0.182	0.457 ± 0.106	1.219 ± 0.012	0.631 ± 0.064	1.673 ± 0.145	0.668 ± 0.139	523.0 ± 161.1

IC_{50}: Concentration of quinolones that inhibit DNA gyrase activity by 50%

WT: Wild-type

SD: Standard Deviation

Table 12 CC_{25}s of the quinolones against WT and mutant *Salmonella* DNA gyrases

Quinolones	CC_{25}s (μg/mL)	
	WT	Ser83Ile
WQ-3034	0.16	0.58
Ciprofloxacin	0.15	1.11

CC_{25}: Concentration of quinolones that induce 25% of maximum DNA cleavage

WT: Wild-type

Table 13 MICs of the quinolones against *S.* Typhimurium and *S.* Enteritidis

Quinolones	MIC ± SD (μg/mL)	
	S. Typhimurium	*S. Enteritidis*
WQ-3034	0.08 ± 0.00	0.016 ± 0.00
WQ-3154	0.08 ± 0.00	0.08 ± 0.00
WQ-3810	0.04 ± 0.00	0.04 ± 0.00
Ciprofloxacin	0.04 ± 0.00	0.04 ± 0.00

MIC: Minimum Inhibitory Concentration

SD: Standard Deviation

Summary

Quinolone-resistant nontyphoidal *Salmonella*, one of the prominent pathogens causing acute gastroenteritis, has become a public health concern globally. The World Health Organization has ranked fluoroquinolone-resistant *Salmonella* as a high-priority pathogen for researching and developing new antibiotics. WQ-3034 and WQ-3154 are relatively new synthetic fluoroquinolones with distinctive structures. WQ-3034 has 6-amino-3,5-difluoropyridine-2-yl at R_1, 3-hydroxyazetidinyl at R_7, and the addition of Cl atom at R_8. WQ-3154 has a similar basic pharmacophore to WQ-3034 except for the modification at R_8 with methyl group.

In this study, the inhibitory effect and DNA cleavage effect against WT and mutant *S.* Typhimurium DNA gyrases of WQ-3034 and WQ-3154 were examined along with WQ-3810 and ciprofloxacin by measuring the drug concentration that inhibits half of the enzyme activity (IC_{50}) and the drug concentration that induces 25% of maximum DNA cleavage (CC_{25}). The minimum inhibitory concentration (MIC) of the compounds was assessed against *S.* Typhimurium and *S.* Enteritidis.

Among four compounds, WQ-3034 demonstrated the highest inhibitory effect against both WT and mutant *Salmonella* Typhimurium DNA gyrases with amino acid substitution at codon 83 and/or 87, while ciprofloxacin showed the lowest inhibitory effect. Remarkably, WQ-3034 and WQ-3154 exhibited a significantly higher inhibitory effect than ciprofloxacin against *S.* Typhimurium DNA gyrase with double amino acid substitution, Ser83Phe-Asp87Asn. Similarly, CC_{25} of WQ-3034 against mutant *S.* Typhimurium DNA gyrase was lower than ciprofloxacin. Notably, MICs of WQ-3034 and WQ-3154 were higher than ciprofloxacin. In conclusion, this study revealed that WQ-3034 and WQ-3154 could potentially be effective therapeutic agents against quinolone-resistant nontyphoidal *Salmonella*.

CONCLUSION

Nontyphoidal *Salmonella* is a leading cause of acute gastroenteritis globally, with Southeast Asia having the highest incidence. Fluoroquinolones are a preferred treatment for *Salmonella* infection, but their overuse leads to resistance. Moreover, their stable residues in wastewater may contribute to antimicrobial resistance in aquatic environments. This study explored the drug resistance in *Salmonella* and counter measures for it.

CHAPTER I examined *Salmonella* spp. isolated from 6 canals in Bangkok during 2016 and 2020. The results revealed a high prevalence (92.2%, n = 307/333) of *Salmonella* spp. in canal water, with the dominance of serogroup B and C. A significant proportion of isolates (35.8%, n = 143/399) carried PMQR genes with *qnrS* being the most prevalent. Notably, six isolates harbored multiple PMQR genes and *qnrD* was first detected in *Salmonella* spp. isolated from environmental water in Thailand. Moreover, 9.3% (n = 37/399) and 3.8% (n = 15/399) of the isolates were resistant to nalidixic acid and ciprofloxacin, respectively. This chapter represents the initial investigation into PMQR genes of *Salmonella* spp. from canal water in Bangkok, Thailand. It highlights the potential public health concerns posed by resistant bacteria in canal water, the introduction of resistance genes into the country, and emphasizes the urgent need to include environmental sampling in comprehensive surveillance programs.

CHAPTER II presents the first comprehensive genomic analysis of *Salmonella* strains isolated from Bangkok canal water, utilizing WGS to examine 30 *Salmonella* strains. The predominant serotype identified was *S.* Agona. The analysis revealed the presence of 35 antimicrobial resistance genes and 30 chromosomal-mediated gene mutations, with 21 strains harboring both acquired genes and mutations associated with fluoroquinolone resistance. Notably, three-quarters of the strains exhibited multidrug resistance, and all strains carried essential virulence factors for the pathogenesis of salmonellosis. The identification of various plasmid types suggests the potential for horizontal transfer of antimicrobial resistance genes among the strains. Furthermore, the phylogenetic analysis revealed the potential circulation of *S.* Agona between canal water and food sources in Thailand and the possible introduction of drug-resistant *Salmonella* to Thailand from foreign countries or vice versa via international transportation. This chapter underscores the significance of environmental water in urban settings as a reservoir of pathogens. The data obtained from this study can inform public health risk assessments and guide intervention strategies to address antimicrobial resistance challenges in Thailand.

The inappropriate usage of antimicrobials in Thailand may create selective pressure, leading to the widespread dissemination of antimicrobial resistance genes. As such, it is imperative to implement strict regulations on antimicrobial usage and conduct extensive surveillance on a broad scale that includes environmental sampling to tackle AMR issues in Thailand. Furthermore, considering that canals receive water from wastewater treatment plants, enhancing wastewater treatment systems could be instrumental in addressing the AMR problem. Presently, Bangkok's wastewater treatment plants employ activated sludge systems, which might not be sufficiently effective in eliminating ARGs. Hence, integrated units that leverage the benefits of various technologies, such as combining membrane filtration with activated sludge processes, could present a promising strategy. Additionally, local authorities may explore alternative treatment methods like constructed wetlands and tridimensional eco-biological reactors.

CHAPTER III explored the inhibitory effect, DNA cleavage effect, and antimicrobial activity of relatively new synthetic fluoroquinolones, WQ-3034 and WQ-3154. WQ-3034 has 6-amino-3,5-difluoropyridine-2-yl at R_1, 3-hydroxyazetidinyl at R_7, and the addition of Cl atom at R_8. WQ-3154 has a similar basic pharmacophore to WQ-3034 except for the modification at R_8 with methyl group. The results showed that WQ-3034 and WQ-3154 exhibited a higher inhibitory and cleavage effect against WT and mutant *S*. Typhimurium DNA gyrases than ciprofloxacin. Remarkably, WQ-3034 and WQ-3154 showed a notably greater inhibitory effect compared to ciprofloxacin against *S*. Typhimurium DNA gyrase with the double amino acid substitution Ser83Phe-Asp87Asn. This enhanced effectiveness of WQ-3034 and WQ-3154 could be attributed to their distinct structural features at positions R_1, R_7, and R_8. Notably, MICs and of WQ-3034 and WQ-3154 were higher than ciprofloxacin. WQ-3034 and WQ-3154 show potential as effective therapeutic agents against quinolone-resistant *Salmonella enterica* with amino acid substitutions. Additionally, combining them with membrane permeabilizers and efflux pump inhibitors could enhance their antimicrobial activity. This chapter lays the groundwork for antimicrobial drug development and potential *in vivo* studies to evaluate the compounds' effectiveness in real-world scenarios, facilitating the development of effective countermeasures against drug-resistant *Salmonella*.

In conclusion, all chapters in this study address the pressing global need for the comprehensive surveillance of antimicrobial resistance in *Salmonella* spp., in accordance with the One Health approach, as well as the research and development of new treatments against fluoroquinolone-resistant *Salmonella*.

APPENDIX

Supplementary data

Table S1 Metadata of recorded isolates in NCBI gene database isolates browser used in plotting the worldwide distribution of *qnr* genes.

Table S2 Detailed information of *Salmonella* spp. isolated from water samples from six canals in Bangkok during 2016 and 2020.

Table S3 *S.* Agona strains from the global collection included in phylogenetic analysis.

Accession link for Table S1 – S3:
https://drive.google.com/drive/folders/1YIyC03QqMztOR_1DE4HFAfVm-ZYIlIxN?usp=drive_link

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