คุณลักษณะของยีนดื้อยากลุ่ม Quinolones ที่อยู่บน Plasmid ในเชื้อ *Salmonella* ที่แยกได้จากผู้ป่วยและสัตว์ในประเทศไทย

นางสาว บุณฑริกา ธงรอด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHARACTERIZATION OF PLASMID-MEDIATED QUINOLONE RESISTANCE GENES IN *SALMONELLA* ISOLATES FROM PATIENTS AND ANIMALS IN THAILAND

Miss Boontarika Tongrod

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Science

Faculty of Medicine

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Thesis Title	CHARACTERIZATION OF PLASMID-MEDIATED QUINOLONE RESISTANCE GENES IN *SALMONELLA* ISOLATES FROM PATIENTS AND ANIMALS IN THAILAND
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บุณฑริกา ธงรอด : คุณลักษณะของยีนดื้อยากลุ่ม Quinolones ที่อยู่บน Plasmid ในเชื้อ *Salmonella* ที่แยกได้จากผู้ป่วยและสัตว์ในประเทศไทย (CHARACTERIZATION OF PLASMID-MEDIATED QUINOLONE RESISTANCE GENES IN *SALMONELLA* ISOLATES FROM PATIENTS AND ANIMALS IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. นพ. วันล่า กุลวิชิต, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ. ดร. ธนิษฐา ฉัตรสุวรรณ, 133 หน้า.

การติดเชื้อกลุ่ม nontyphoidal *Salmonella* พบรายงานมากขึ้นทั่วโลก ซึ่งมีสาเหตุมาจากการติดต่อจากสัตว์สู่คนผ่านทางห่วงโซ่อาหาร ยากลุ่ม fluoroquinolones ซึ่งเป็นยาหลักที่ใช้ในการรักษาโรคติดเชื้อกลุ่มนี้ พบว่ามีภาวะ reduced susceptibilityมากขึ้นและมีรายงานการรักษาที่ล้มเหลว กลไกการดื้อยากลุ่ม fluoroquinolones เกิดจากการกลายพันธุ์ของยีนที่เป็นเป้าหมายของยาที่อยู่บนโครโมโซมได้แก่ *gyrA, gyrB, parC* และ *parE* ซึ่งทำให้เกิดการดื้อยาในระดับสูง และการมียีนดื้อยาที่อยู่บนพลาสมิด ได้แก่ ยีน *qnr* และ ยีน *aac(6')-Ib-cr* ซึ่งมีความสำคัญทางคลินิคเนื่องจากสามารถถ่ายทอดยีนดื้อยาแบบ horizontal ผ่านทางพลาสมิดได้ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาคุณลักษณะและความชุกของยีน *qnr* และ *aac(6')-Ib-cr* ซึ่งอยู่บนพลาสมิดในเชื้อกลุ่ม nontyphoidal *Salmonella* จำนวน 356 สายพันธุ์โดยแบ่งเป็นเชื้อ 108 สายพันธุ์จากผู้ป่วยและเชื้อที่ได้จากสัตว์จำนวน 248 สายพันธุ์ จากการศึกษาพบว่า ความชุกของการดื้อยา nalidixic acid ในเชื้อที่แยกได้จากผู้ป่วยเป็น 81.6% และ reduced susceptibility ต่อ ciprofloxacin เป็น 72.2% โดย *S.* Choleraesuis เป็น serovar ที่มี reduced susceptibility ต่อยานี้มากที่สุด (30.56%) นอกจากนั้นยังพบว่าความชุกของการดื้อยา nalidixic acid ในเชื้อที่แยกจากสัตว์เป็น 42.6% ดื้อยา ciprofloxacin เป็น 0.4% และ reduced susceptibility ต่อ ciprofloxacin พบเป็น 41.36% โดย *S.* Enteritidis (8.06%) พบเป็น serovar ที่มี reduced susceptibility มากที่สุด การตรวจคัดกรองหายีน *qnr* และ *aac(6')-Ib-cr* ในเชื้อ nontyphoidal *Salmonella* ทั้งหมด 356 สายพันธุ์ยีน *qnrS* เป็น 8.70% (31 สายพันธุ์) และไม่พบยีน *aac(6')-Ib-cr* ในเชื้อทุกสายพันธุ์ ความชุกของยีน *qnrS* ในเชื้อที่แยกได้จากผู้ป่วยพบเป็น 3.7% (4/108) โดยพบเป็น *S.* Choleraesuis 1 สายพันธุ์ และ *S.* group D 3 สายพันธุ์ มีความชุกของยีน *qnr*S ในเชื้อที่แยกได้จากสัตว์พบเป็น 10.88% (27/248) โดย serovar พบมากคือ *S.* Anatum (9 สายพันธุ์) การศึกษานี้เป็นการรายงานครั้งแรกของความชุกของยีน *qnrS* ในเชื้อ nontyphoidal *Salmonella* ในประเทศไทย จากการศึกษาลำดับนิวคลีโอไทด์ของยีน *qnrS* ในเชื้อทั้ง 31 สายพันธุ์นี้ พบว่าลำดับนิวคลีโอไทด์และลำดับกรดอะมิโน มีความเหมือน 100% กับยีน *qnrS1* และโปรตีน QnrS1 เมื่อเทียบกับข้อมูลใน GenBank การศึกษาด้วยวิธีเหนี่ยวนำให้เกิดการดื้อยา ciprofloxacin ในหลอดทดลอง พบว่า ในกลุ่มที่มียีน *qnrS1* 5 สายพันธุ์ มี 4 สายพันธุ์ ที่มีค่า MIC ต่อยา ciprofloxacin เพิ่มสูงขึ้นถึง 32-64 µg/ml ใน 3rd generation selection ในขณะที่กลุ่มที่ไม่มียีน *qnrS1* จำนวน 5 สายพันธุ์พบว่ามีเพียง 2 สายพันธุ์ที่มีค่า MIC เพิ่มสูงขึ้นถึง 32 µg/ml ใน 3rd generation selection การกลายพันธุ์ 1 ตำแหน่งใน QRDR ของ GyrA สามารถพบได้ที่ตำแหน่ง S83F, Y หรือ D87G เมื่อเชื้อมีค่า MIC ต่อยา ciprofloxacin มากกว่าหรือเท่ากับ 1 µg/ml การเกิดการกลายพันธุ์สองตำแหน่งพร้อมกันใน GyrA ที่ตำแหน่ง S83 และ D87 พบว่าจะทำให้ค่า MIC สูงขึ้นมากกว่า อย่างไรก็ตาม พบว่ามีการเพิ่มสูงขึ้นของค่า MIC โดยไม่พบการกลายพันธุ์ใน GyrA ซึ่งแสดงถึงการมีกลไกการดื้อยาอื่นๆร่วมด้วย ได้แก่ การเกิดการกลายพันธุ์ใน ParC หรือ การเพิ่มการแสดงออกของ efflux system หรือ การลดลงของ outer membrane porins

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : SALMONELLA / QNR / AAC(6')-IB-CR / QUINOLONE RESISTANCE

BOONTARIKA TONGROD : CHARACTERIZATION OF PLASMID-MEDIATED QUINOLONE RESISTANCE GENES IN SALMONELLA ISOLATES FROM PATIENTS AND ANIMALS IN THAILAND. THESIS ADVISOR : ASSOCIATE PROFESSOR WANLA KULWICHIT, M.D., THESIS CO-ADVISOR : TANITTHA CHATSUWAN, PH.D., 133 pp.

Nontyphoidal *Salmonella* is considered to be acquired from animals to human via food chain. Fluoroquinolones, drug of choice for *Salmonella* infection has become reduced susceptible and clinical failure has been reported. The chromosomal-mediated fluoroquinolone resistance mechanisms are commonly due to the drug target alterations in *gyr*A, *gyr*B, *par*C and *par*E genes which can cause high level of resistance. Other resistance mechanism is the presence of plasmid-mediated genes, including *qnr* genes and *aac(6')-Ib-cr* genes which become more clinical concern because of the ability of horizontal gene transfer. This study characterized the plasmid-mediated quinolone resistance genes and investigated the prevalence of *qnr* genes and *aac(6')-Ib-cr* genes in 356 nontyphoidal *Salmonella* isolated from 108 patients and 248 animals. Prevalence of nalidixic acid resistance in patient isolates was 86.1% and reduced susceptibility to ciprofloxacin was found in 72.2%. High rate of reduced susceptibility was found in *S.* Choleraesuis (30.56%). Prevalence of nalidixic acid resistance was 42.6%, ciprofloxacin resistance was 0.4% and reduced susceptibility to ciprofloxacin was found in 41.36%. Reduced susceptibility was mostly found in *S.* Enteritidis (8.06%, 20/248). Screening for the presence of *qnr* and *aac(6')-Ib-cr* in 356 nontyphoidal *Salmonella* isolates showed that *qnrS* gene was found in 8.70% (31 isolates) and there was no isolate carrying *aac(6')-Ib-cr*. The prevalence of *qnrS* genes in isolates from patients was 3.7% (4/108) which were found in *S.* Choleraesuis (1 isolate) and *S.* group D (3 isolates). The prevalence of *qnrS* genes in isolates from animals was 10.88% (27/248) and the most common serovar carrying *qnr* genes was *S.* Anatum (9 isolates). This study was the first report of the prevalence of *qnrS* in nontyphoidal *Salmonella* isolated from Thailand. DNA sequencing analysis of the *qnrS* gene of all 31 *qnrS*-positive revealed 100% nucleotide and amino acid identity to *qnrS1* and QnrS1 submitted in GenBank. The *in vitro* selection of ciprofloxacin resistance demonstrated that 4 out of 5 *qnrS1*-positive parent strains raised ciprofloxacin MIC to 32-64 µg/ml in the third-generation selection whereas 2 out of 5 *qnrS1*-negative parent strains raised ciprofloxacin MIC to 32 µg/ml. Single amino acid substitutions in GyrA were found at S83F, Y and D87G when the ciprofloxacin MIC of mutants increased to $\geq$ 1 µg/ml. The double mutations at S83 and D87 in GyrA led to higher ciprofloxacin MIC. However, there was an increase in ciprofloxacin MIC without any mutation in GyrA, suggesting that there were other mechanisms involved in the development of resistance such as mutations in QRDR of ParC, overexpression of efflux system and decreased outer membrane porins.

Field of Study : Medical Science ..... Student's Signature .....

Academic Year : 2009 ..... Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

A	adenosine
AC	amoxicillin
Ala (A)	alanine
Asn (N)	asparagine
Asp (D)	aspartic acid
Arg (R)	arginine
bp	base pair
C	cytidine
CO_2	carbon dioxide
CH	clarithromycin
CLSI	Clinical and Laboratory Standards Institute
^{o}C	degree Celsius
Cys (C)	cysteine
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate

ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxytymidine 5'-triphosphate
DDW	double distilled water
ddNTPs	dideonucleotide-tri-phosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide-tri-phosphate
dTTP	deoxytymidine 5'-triphosphate
DW	distilled water
EDTA	ethylenediamine tetraacetic acid
et al.	*et alii*
g	gram
G	guanosine
Gly (G)	glycine
Glu (E)	glutamic acid
Gln (Q)	glutamine
HCl	hydrochloric acid
hr	hour
His (H)	histidine
i.e.	id test

Ile (I)	isoleucine
Lys (K)	lysine
Leu (L)	leucine
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute (s)
mL	milliliter
mM	millimolar
mmol	millimole
Met (M)	methionine
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic, anhydrous
NaOH	sodium hydroxide
Phe (F)	phenylalanine
PCR	polymerase chain reaction
pmol	picomol
Pro (P)	proline
sec	second

Ser (S)	serine
T	thymidine
TAE	tris-acetate-EDTA
Thr (T)	threonine
Tris	Tris-(hydroxymethyl)-aminoethane
Trp (W)	tryptophan
Tyr (Y)	tyrosine
U	unit
μg	microgram
μL	microliter
μM	micromolar
UV	ultraviolet
V	volt

CHAPTER I

INTRODUCTION

Salmonella spp. is one of the most important causes of gastrointestinal infection. Enteric fever was most commonly caused by *Salmonella* enterica serovars Typhi and Paratyphi A. These two serovars are exclusively human pathogens. However, due to the hygienic development, the infection by *S*. Typhi and Paratyphi A has decreased worldwide. Infection by nontyphoidal *Salmonella* was estimated to cause a larger burden of the disease and especially in developed and developing country. The infection was considered to be acquired from animals to human via food chain [1, 2]. According to the increasing use of antibiotics in animal farm, this problem seems to be more serious [3, 4]. Even though diarrhoea caused by nontyphoidal *Salmonella* is self-limiting which antimicrobial agents are not required for treatment, invasive infection with bacteremia may be occurred. The resistance to older drugs including ampicillin, chloramphenicol, trimetroprim-sulphamethoxazole has been present for many years. Nowadays, resistance to fluoroquinolones, the drug of choice for treatment *Salmonella* infection, has been increasingly reported [5].

There are two major of fluoroquinolones resistance mechanisms. The first one is chromosomal-mediated. The most important mechanis is the mutations in DNA gyrase (*gyr*A and *gyr*B) and topoisomerase IV (*par*C and *par*E) [6-8]. The alterations of amino acids on each sub unit can cause high level of drug resistance. The important position on DNA gyrase and topoisomerase IV has been described as QRDR or Quinolone-Resistance Determining Region [9].

The second fluoroquinolones resistance mechanism is plasmid-mediated. The discovery of quinolone resistance protein (Qnr) in 1998 can increase clinical concern of horizontal fluoroquinolone resistance genes transfer. Qnr protein encoding by *qnrA, qnrB* [15], *qnrS* [16], *qnrC* or *qnrD* [17], protects DNA from fluoroquinolone lethal inhibition by competitive binding to DNA gyrase and topoisomerase IV. The presence of *qnr* genes has been reported worldwide from many pathogens including *Salmonella* [18-20]. Although the pathogens carrying *qnr* genes were not resistant to fluoroquinolones by the CLSI guideline, this gene can help the pathogens to develop other resistance mechanisms or act synergistically [21] The common localization of *qnr* gene on integron, transposon or conjugative plasmid is the important factor for co-localization with other resistance genes including genes encoding for ESBL and AmpC type β-lactamase such as CTX [22], SHV, FOX and VEB. The other mechanism is the presence of *aac(6')-Ib-cr* gene. This gene is the variation of *aac(6')-Ib* gene which is the gene encoding for aminoglycoside acetyltransferase, responsible for resistance to tobramycin, amikacin and kanamycin [23, 24]. The new variation changes the effect to structurally-related resist to ciprofloxacin and norfloxacin. According to the effect of these two plasmid-mediated resistance mechanisms, the pathogens with the ciprofloxacin MIC of 0.125-1 µg/ml or show reduced susceptibility to ciprofloxacin should be determined.

Only a few cases of treatment failure due to fluoroquinolone resistance in *Salmonella* (including *Salmonella typhi*) have been reported [26, 27]. However there were many evidence of an increasing incidence of strains which were resistant to nalidixic acid and exhibit decreased susceptibility to fluoroquinolones used in human therapeutics such as ciprofloxacin [3, 28-33]. The plasmid-mediated mechanisms can cause reduced susceptibility to ciprofloxacin. However, there was no report of the prevalence of the plasmid-mediated quinolone resistance genes in nontyphoidal *Salmonella* isolates in Thailand. The purpose of this study was to investigate the prevalence of *qnr* and *aac(6')-Ib-cr* genes in nontyphoidal *Salmonella* isolates from

patients and animals and characterize the plasmid-mediated quinolone resistance genes.

CHAPTER II

OBJECTIVES

I. To investigate the prevalence of fluoroquinolone resistance genes including *qnr* and *aac(6')-Ib-cr* genes in nontyphoidal *Salmonella* isolates from patients and animals in Thailand

II. To characterize *qnr* and *aac(6')-Ib-cr* genes in nontyphoidal *Salmonella* isolates from patients and animals in Thailand

III. To study the relationship between *qnr*, *aac(6')-Ib-cr* genes and fluoroquinolone resistance in nontyphoidal *Salmonella* isolates from patients and animals

CHAPTER III

LITERATURE REVIEW

1. Bacteriology

Salmonella spp. is classified in Phylum Proteobacteria, class Gamma Proteobacteria, order Enterobateriales and family Enterobacteriaceae. It is gram-negative non-spore forming bacteria in bacilli shape. The cells are approximately 0.7 to 1.5 μM with flagella. Most species produce hydrogen sulfide which can be detected on media containing ferrous sulfate. Most of them can be found in two phase; motile and non-motile phase. It can be found worldwide in human, animal and environment.

2. *Salmonella* Taxonomy

Salmonella serotype is based on immune reactions with two surface structures. O antigen is a carbohydrate antigen and present on the outer component of lipopolysaccharide. H antigen is a protein antigen called flagellin which present in the flagella.

The genus *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into 6 subspecies that are designated by names or, more commonly, Roman numerals. These are differentiated by biochemical reactions. Subspecies I present in both warm and cold blooded animals while the other *Salmonella* subspecies are generally associated with cold-blooded animals. There are over 2,500 *Salmonella* serotypes of which approximately 60% belong to subspecies I. These accounted for about 99% of human infections. All *Salmonella*

serotypes can be designated by an antigenic formula. Subspecies I serotypes are given a name, e.g. Kentucky in the above example while other subspecies are not named. The official name for an isolate with the antigenic structure 4,5,12: i : 2 is *Salmonella enterica* subspecies *enterica* serotype Typhimurium. However, this is normally shortened to *Salmonella* Typhimurium.

3. Pathology and Diseases

Salmonella infection can cause diseases ranging from gastroenteritis, typhoid fever and bacteremia. The infectious dose is small, probably from 15 to 20 cells. The bacterial cells can penetrate into the host cells and lead to increasing of intracellular free calcium and the cytoplasm rearrangement. The invasion of bacterial cell can also make the cell membrane disorder. Salmonellosis can cause diarrhea, fever, abdominal cramps, nausea and vomiting. In mild cases diarrhea may be non-bloody, occur several per day and not to be very voluminous. On the other hand, in severe cases, it may be more frequent with bloody and mucus in high volume. Especially infected by non-typhoidal *Salmonella*, the symptoms can be resolved without treatment within 5-7 days after infection. The severity can be found in dehydrated patients or infected by antibiotic resistant strains.

Most of infection host can be recover completely except for small group can develop to Reiter's syndrome cause pain in joints, eyes irritation and painful urination. The symptoms can last long to months or year and can develop to be chronic arthritis. Bacteremia can be characterized by infection in tissue surrounding the brain and spinal cord and infection in the bloodstream or sepsis.

4. Tranmission

Salmonella infection can transmit from animal to animal, animal to human and human to human. Nowadays, there are not only many findings about the transmission to human via food chain but also found most of them were antibiotic resistant strains. The transmission usually occurred by fecal-oral route. The transmission can prevent by cooking all kind of food thoroughly and drink only pasteurized milk.

5. Treatment

Salmonella infections usually can be resolved in 5 to 7 days and most cases require no treatment by antibiotics unless the patients become more severely dehydrated or the infection spreads. The patient with severe diarrhea require only re hydration treatment.

In the cases with infection spreads out of the intestines, the antibiotic use will be required. Salmonellosis can be generally treated with ampicillin, amoxicillin, gentamicin, trimethoprim/sulfamethoxazole, ceftriaxone or fluoroquinolone (ciprofloxacin). The length of treatment varies depending on the patient's illness and can range from 14 days for enteric fever to 6 weeks for bacteremia. Some of *Salmonella* bacteria can develop antibiotic resistance mechanisms. The use of fluoroquinolones, especially ciprofloxacin, are widely use as drug of choice for salmonellosis treatment. Because of this reason, the increasing of resistance rate for ciprofloxacin in nontyphoidal *Salmonella* has become clinical concern.

6. Epidemiology

The incidence of nontyphoidal *Salmonella* has been increased since 1970s. The 2,500 serotypes of nontyphoidal *Salmonella* are able to infect humans but the most successful serotypes that were found geographically worldwide were included *Salmonella* Enteritidis and *Salmonella* Typhimurium. The infection caused by *Salmonella* Typhi decreased worldwide due to the better hygienic policy whereas nontyphoidal *Salmonella* infection in humans has been increasing by promoting of antibiotics into food animals and transmitted to humans via food chain.

Salmonella infections cause an estimated 1.4 million human illnesses and 400 deaths annually in the United States or the outbreaks of nontyphoidal *Salmonella* infections and sporadic illness have been associated with causes food and animal origin [34]. The first multistate outbreak of multidrug resistant *Salmonella* Typhimurium DT104 associated with consumption of ground beef in northern United States [35, 36]. The finding in this report also used PFGE for identification of clusters of illness, for *Salmonella* Typhimurium, Use of PulseNet to study on subtype data, laboratory Division of eight *Salmonella* Typhimurium isolates with indistinguishable PFGE pattern. These PFGE results were also sent to the National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet), the database revealed 31 patients with same PFGE pattern *Salmonella* Typhimurium infections from nine states of the United States and the District of Columbia [37]. The further study showed the source of the infection from ground beef purchased at branches of national supermarkets. [37] In 2005, the study of epidemiology of nontyphoidal *Salmonella* in Israel and internationally revealed the decrease of *Salmonella* Typhi infections but the infection caused by nontyphoidal *Salmonella* was increased the outbreak stains, *S.* Agona, in 1994. This study also reported the high rate of resistance in *Salmonella* Virchow (16% of nontyphoidal *Salmonella* illnesses) and highly invasive in children also [38]. The study based on the FoodNet data showed 8,699 *Salmonella* isolates submitted during 1996 to 1999. In the United States, the population survey based on 9,003 interviews helped to estimate

1,397,000 cases of *Salmonella* illnesses per year (1996-1999). From these data, the hospitalization and death rates were calculated, according to FoodNet data, confirmed cases were 20% (15,000) and 0.6% (400), respectively [39]. In 2000, the study in Wales and England showed the hospitalization rate caused by nontyphoidal *Salmonella* was 10.1% and the death rate was 0.8%. Interestingly, the studies from both region showed same results that *Salmonella* infection was the leading cause of death roughly one third of death due to bacterial food borne illnesses [40]. The emergence and spread of highly resistant strains in Europe, was observed in the early 1990s with *Salmonella* enterica serovar Typhimurium phage type DT204, and recently re-occurs in various serovars, such as Typhimurium, Choleraesuis or Schwarzengrund [7, 9, 41-45]. In Israel, *Salmonella* Virchows, isolated from 1997 to 2004, was reported as highly prevalence in humans and farm animals. In addition, this serovar also showed high rate of resistance to multiple antibiotics and more than 90% of them were resistant to nalidixic acid (MIC $\geq$ 128 µg/mL) with reduced susceptibility to ciprofloxacin (MIC between 0.125 to 0.250 µg/mL) [2]. On February 24, 2009, the Nebraska Department of Health and Human Services identified six isolates of *Salmonella* Saintpaul which collected from February 7-14, associated with eating alfalfa sprouts. Food and Drug Administration (FDA) released guidance to help prevent seed producers and sprout growers enhance the safety of the products [46].

In Thailand, according to the study of *Salmonella* isolated from human and other sources in 1993 – 2002 [47], found that the most common serovars contained *S.* Weltevreden, *S.* Enteritidis, *S.* Anatum, *S.* Derby and *S.* 1,4,5,12:i. Even though the proportions of these 5 serovars were decreased between 1993 and 2002 but an increase has been reported for *S.* Rissen, *S.* Stanley, *S.* Panama and *S.* Schwarzengrund. This report also found that *S.* Weltevreden were the most common serovar isolated from human and seafood from the United States imported from Thailand and Malaysia [48]. This observation suggested the water-related source seafood. In South-East Asia, *S.* Weltevreden has been reported as a common and increasing cause of human infection and was the predominant serovar in Thailand, in contrast, this strain

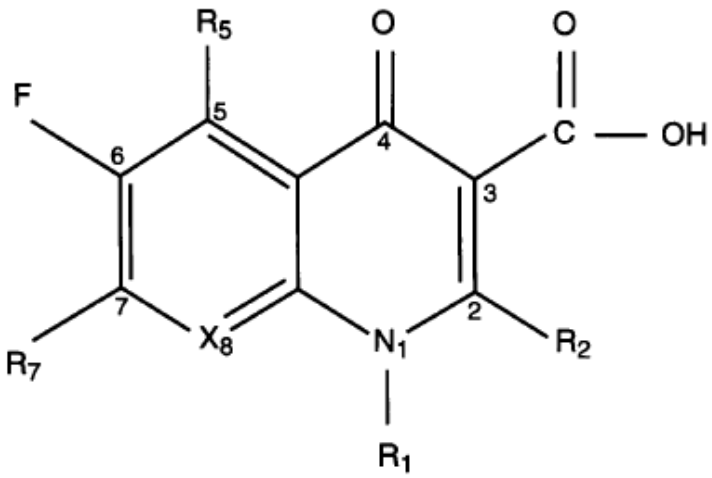
was reported as low frequency of resistance compared to other strains [49]. F. M. Aarestrup *et al.* [50] reported only 48 (9.5%), of 503 *S.* Weltevreden isolated from 10 countries in South-East Asia, were resistant to one or more of the antimicrobial agents tested. Another study of predominant serovars of *Salmonella* isolated from human and foods from Thailand found that, during 1993 and 1996, 72 and 81 serovars were isolated from humans and foods, respectively. The most common serovars from human stool were *S.* Weltevreden, *S.* Derby, *S.* Enteritidis and *S.* Typhimurium. The most common serovars from blood and other specimens were *S.* Enteritidis and the second was *S.* Choleraesuis [49]. Other study in nontyphoidal *Salmonella* isolated from bacteremic patients at King Chulalongkorn Memorial Hospital from January 2003 to October 2005 showed high frequency drug resistance. All isolates of *S.* Choleraesuis were ceftriaxone resistance and also showed high level resistance of nalidixic acid and most of them showed reduced susceptibility to ciprofloxacin [51]. In 2008, there was a study of the diversity and antimicrobial resistance of *Salmonella* Rissen isolated from various food sources (collected in 2004) in Thailand and from six patients in Denmark who had traveled to Thailand (collected from 200-2005). This study found higher level of resistance in strains originating from Thailand and pork products [1].

7. Quinolones and Fluoroquinolones

By 1962, nalidixic acid has been developed; the new chemical compound was synthesized based on the structure of quinine which had effect against malaria. Quinolones were derivatives of the 1, 8-naphthyridine molecules that possessed antibacterial activity [52]. In 1964, nalidixic acid was available in the UK for urinary tract infections but still had limited spectrum only in Enterobacteriaceae.

The 4-quinolone or known as naphthyridine carboxylic acid, were including oxolinic acid, cinoxacin, pipemidic acid. Until in 1980, there were modifications at C6 and C7 of the pharmacore by adding fluorine at C6 created “Fluoroquinolones”. The advantages of these quinolones include: much broader spectrum of antibiotic activity, good tissue distribution, improved pharmacokinetic profile and low incidence of adverse drug effect [53].

Figure 1 The quinolone pharmacore



7.1 The relationship between Structure and Activity of Quinolones

According to Figure 1 [54], the position 7 was one of the most influential part of the molecule [55]. Modifying this position can improve molecule's activity and pharmacokinetic profile. The most popular group added at position 7 was aminopyrrolidines (tosufloxacin and clinafloxacin) and piperazine (ciprofloxacin, lomefloxacin, temafloxacin and sparfloxacin). The aminopyrrolidines substituent was for increase activity against gram-positive bacteria and the piperazine for activity against gram-negative bacteria.

7.2 Quinolone Generations

The quinolones can be classified into four generations based on their chemical structure and activity against various bacteria.

First generation

The quinolones in this generation comprise of nalidixic acid, cinoxacin and oxolinic acid. This earliest quinolone was used to target gram-negative bacteria including Enterobacteriaceae family including *E.coli*, *Klebsiella* spp. and *Proteus* spp. This quinolone has little systemic distribution [56] and lack activity against *Pseudomonas aeruginosa*, gram-positive bacteria and anaerobes [57].

Second generation

The fluorine addition at the 6 position of the quinolone ring generated the fluoroquinolones.

Class I : This class contains enoxacin, norfloxacin and lomefloxacin have more activity against Enterobacteriaceae family and commonly used as urinary tract infection therapy [58]. However, the tissue penetration of enoxacin, norfloxacin and lomefloxacin still limited to cure only UTI without uncomplicated systemic infection [57].

Class II : Members of this class were ofloxacin and ciprofloxacin. The drugs possessed enhanced activity against wide gram-negative bacteria including Enterobacteriaceae and *Pseudomonas aeruginosa* [58]. And also have moderate activity against *Staphylococcus* spp. Ciprofloxacin and ofloxacin have good tissue penetration with intracellular activity so that these drugs can produce activity against *Legionella pneumophila*, *Clamydia pneumoniae and Mycoplasma pneumoniae* [56]. The drugs in this class are mostly used as drug of choice to treat most gram-negative bacteria infection including salmonellosis and other complicated urinary tract infections, nosocomial infections and sexually transmitted infections [57].

On the other hand, the drugs in this class still have low level of activity against *Streptococcus pneumoniae, Enterococcus faecalis* and *Enterococcus faecium* [58].

Third generation

Because of low activity against gram-positive bacteria in the second generation of quinolones, the third generation has been developed. This class contains sparfloxacin, levofloxacin, grepafloxacin, gatifloxacin and moxifloxacin. These newer fluoroquinolones have expanded coverage to streptococci, *Streptococcus pneumoniae* (including penicillin-resistant strains) and improved for management of community-acquired pneumonia [57, 58].

Fourth generation

This class composed of clinafloxacin and trovafloxacin. The drug activity has been extended to cover more anaerobic bacteria, atypical pathogens and methicillin-susceptible *S. aureus*. Trovafloxacin was generally has less activity against gram-negative bacteria than ciprofloxacin and have nonrenal elimination pathways so. But the longer half-life allowed for use once daily dosing [57]. The indications of this class were for nosocomial pneumonia, intra-abdominal infections [58].

8. Mechanism of Action of Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones act by inhibiting the action of DNA gyrase and topoisomerase IV. DNA gyrase is tetrameric enzyme composed of two A subunits, encoded by *gyrA* and two B subunits, encoded by *gyrB*. The major function of this enzyme is to catalyze the negative supercoiling of DNA. Topoisomerase IV is also tetrameric enzyme with A2B2 subunits, composed of *parC* and *parE* genes, respectively. The main function of topoisomerase IV is DNA catenation and decatenation during bacterial cells divisions. GyrA and GyrB are highly homologue to ParC and ParE, respectively. The actions of both enzymes were important for DNA replication, transcription, recombination and repair so inhibition of these enzymes can cause bacterial cells death.

The action of these enzymes were binding to DNA, transiently break the closed circular DNA molecule and pass another strand through the break and then reseal the DNA again. Quinolones bound to these enzymes and stabilized the drug-enzyme-cleaved DNA complex, resulted in lethally unrepaired double-stranded DNA breaks [6].

9. Mechanisms of Fluoroquinolone Resistance

9.1 Chromosomal-mediated Quinolone Resistance Mechanisms

9.1.1 Target Alterations

Alterations in DNA gyrase are the major cause of quinolones and fluoroquinolones resistance. The reconstruction by mutations of GyrA generated the holoenzyme with decreased affinity to quinolones caused quinolones resistance. The important region of targets mutations were described as QRDR or Quinolone-Resistance Determining Region, the portion of DNA-binding to the enzyme. In *E.coli*, amino acid substitution within QRDR of GyrA ranging from positions 67 to 106. Mutations at

positions 81, 83, 84 and 87 are responsible for quinolones resistance. The amino acid substitutions were included Gly81⟶ Cys or Asp, Ser83⟶Leu, Trp, Ala or Val, Ala84⟶Pro or Val and Asp87⟶Asn, Gly, Val, Tyr or His. The single mutation in QRDR of GyrA usually resulted in high level resistance to nalidixic acid but to obtained resistance to fluoroquinolones, the two or more mutations were required.

In *Salmonella* spp., the alterations of target genes were also described as major resistance mechanisms. A single mutation in the QRDR (Ala67 to Gln106) in GyrA mediated to nalidixic acid resistance and showed different level of decreased susceptibility. The most frequently found of GyrA mutations were Ser83⟶Phe [8, 29, 43, 45, 59-65], Tyr[8, 43, 59-64] or Ala[60], Asp87⟶Gly[8, 29, 43, 60-65], Asn [8, 43, 45, 59-64], Tyr [8, 43, 59-64], Ala[60] and other mutations could be found at Ala67⟶ Pro [65] Asp72 ⟶ Gly [61, 62], Val73 ⟶ Ile [62], Gly81⟶ His [59], Ser [30] or Cys [60], Asp82 ⟶ Asn [45, 60-62, 64], Leu98 ⟶ Val [62], Ala119 ⟶ Glu [10]. The combination of substitutions at positions 83 and 87 were led to high-level fluoroquinolone resistance such as MIC of ciprofloxacin: 32 μg/ml [66].

The mutations in ParC, in *Salmonella* were found between residues 57 and 84, were considered as secondary target for quinolones, including Ser80⟶Ile [45], Arg [43, 45] or Ser80 ⟶ Lys [45], Thr57⟶Ser [43, 59], Glu84 ⟶ Lys [45] were required as high level resistance [67] . The silent mutations were found at Val67 ⟶ Val [59, 64], His75 ⟶ His [59, 64], His77 ⟶ His [59, 64], Ala117 ⟶ Ala [59], Ala118 ⟶ Ala [64], Ser 123 ⟶ Ser [64], Ser124 ⟶ Ser [59] and Tyr129 ⟶ Tyr [59]. Mutations in ParC also found Gly107 ⟶ Gly, Thr66⟶Ile were less frequent than in GyrA.

The alterations in GyrB were rarely found in *Salmonella* spp. but maybe found between residues 420 and 464 including Tyr420⟶Cys [62] and Arg437⟶Leu or in other positions such as in Glu453⟶Gly, His461⟶Tyr, Ser464 ⟶ Tyr [68] or Phe, Ala498⟶Thr, Val512⟶Gly and Ser518⟶Cys. The silent mutations were also found at Leu451 ⟶ Leu, Leu462 ⟶ Leu and Ser464 ⟶ Ser [64] or Phe [7, 12, 69, 70].

The mutations in ParE, were found between residues 453 and 512 [67], most of them were silent mutations include: Ala422 ⟶ Ala, Thr447 ⟶ Thr, Glu449 ⟶ Glu, Leu456 ⟶ Leu [59], Gln459 ⟶ Gln, Thr500 ⟶ Thr [64], Glu460 ⟶ Glu, His509 ⟶ His [59, 64], Tyr520 ⟶ Tyr, Leu523 ⟶ Leu, Pro525 ⟶ Pro and Glu567 ⟶ Glu [59].

9.1.2 Efflux Pump System

In early 1980s, the efflux pump was first reported as antibiotic resistance mechanism. Until recently, the efflux-mediated resistance against several antimicrobial agents, including fluoroquinolones, has been reported in many bacterial species. Bacterial efflux pump system was classified into five groups, based on the amino acid sequence homology. Five groups contained the major facilitator superfamily (MFS), the ATP binding cassette family, the resistance nodulation cell division (RND) family [14], the small multidrug resistance (SMR) protein family and the multidrug and toxic compound extrusion (MATE) family. Antimicrobial agent efflux pumps were classified into three families including, the RND, MFS and MATE family. The RND family transporters are found in a number of gram negative bacteria and found work in coordination between periplasmic membrane protein and outer membrane protein (outer membrane efflux protein) [71].

In *Salmonella* spp., the main efflux system is AcrAB-TolC. The AcrAB-TolC uses as a wide variety of substrates transporter for various classes of antibiotic such as quinolones, β-lactams, tetracycline, chloramphenical, erythromycin and also other disinfectants, dyes, detergents, bile salts and organic solvents [13, 14, 71-80]. AcrAB is a member of RND family (resistance nodulation cell division), encoded by *acrAB* genes. There are three components in this pumps including AcrB: the transporter protein in the inner membrane, AcrA: a periplasmic lipoprotein accessory protein, most probably

trimers for interaction with AcrB and TolC [81-83], TolC: an outer membrane protein channel [13, 14, 76]. For the mechanism of this pump, the AcrB captures the substrates and transport to external via TolC. AcrB and TolC are mediated by AcrA [78, 84]. The overexpression of AcrAB-TolC is regulated by the transcriptional activators, MarA and SoxS, which are regulated by MarR and SoxR, respectively [85-87]. This mechanism was known as synergistic action with genes target alterations resistance mechanisms. It has been reported that in *S.* Typhimurium DT104 it could be of greater relevance in mediating fluoroquinolones resistance than single mutations in *gyr*A [11]. In high-level fluoroquinolone resistant *S.* Typhimurium DT204, disruption of *acrB* or *tolC* was shown to result in a 16- to 32-fold reduction in the level of resistance to fluoroquinolones [11, 12, 68, 88, 89].

9.1.3 Decrease Expression of Outer Membrane Porins

There are few studies have reported on the alterations of outer membrane protein expression in quinolone resistant *Salmonella* [3, 8, 26, 89]. However, it does not clearly report that this alterations contributed to significantly quinolone resistance. It is believed that hydrophilic fluoroquinolones such as ciprofloxacin prefer penetrating through the porin pathway [90]. In *Salmonella*, lacking of expression of OmpF has been reported in some quinolone resistant strains [3, 8, 26] but it was not clear that the lack of OmpF caused decreased accumulation of quinolones.

9.2 Plasmid-mediated Quinolone Resistance

9.2.1 Qnr (Quinolone Resistance Protein)

Quinolones were known as synthetic compound, which microorganisms were unlikely to promote of resistance mechanisms. It had been reported that the plasmid-mediated protein, MCbG, which acted as a protein protecting bacterial cell from micricin

B17. And also, McbG can cause quinolone resistance. From further study reported microcin B17 is a protein that can block DNA replication by inhibiting DNA gyrase supercoilling and stabilizing DNA-gyrase cleaved complex as same as quinolone activity do. And also, in 2001, in *Mycobacterium smegmatis*, was isolated the plasmid contained the *mfpA* gene, encoding for pentapeptide repeat family conferring reduced susceptibility to sparfloxacin and ciprofloxacin (4 to 8 folds increase of MIC) [91]. Both of these proteins had similar structure to Qnr, which was identified later in many enterobacteriaciae conferring low-level resistance to fluoroquinolones by the same mechanisms, protecting DNA gyrase from inhibiting compound like quinolones.

In 1998, the plasmid-mediated quinolone resistance had been first reported in *Klebsiella pneumoniae* isolated from a urine culture collected in 1994 in Birmingham, Alabama, USA [92]. This strain carried plasmid pMG252 which coding for *qnr* gene and has been renamed to *qnrA* and later *qnrA1* after discovery of other variants. After that there were many studies reported about the new variants of *qnr* genes. In 2005, *qnrS* genes was discovered on plasmid pAH0376 in *Shigella flexneri* 2b, which was an outbreak strain in food poisoning in 2003 in Japan [93]. This plasmid conferred low level resistance to nalidixic acid and fluoroquinolones. In 2006, *qnrB* was first identified from *Klebsiella pneumoniae* isolated from blood culture in 2002 from South India and this strain also produced CTX-M-15 β-lactamase on the same plasmid [15]. QnrC has been first identified in 2008 in China [94]. The *qnrC* genes was found in *Proteus miralbilis* which caused urinary tract infection, the susceptibility testing showed that this organism was susceptible to most antibiotic except amoxicillin, cotrimoxazole and showed reduced susceptibility to ciprofloxacin [94].

9.2.1.1 The Origin of *qnr* Genes

According to many worldwide studies of epidemiology of *qnr* genes, suggested that these genes might be circulated in clinical isolates for a long time before first

identification. This reason led to study of *qnr* genes origin by studying of the chromosome of organisms isolated from human, animals and environment. And in 2005, the screening of genome sequence of gram negative bacteria found 4 variants of *qnrA* genes (*qnrA2-A5*) in three strains of *Shewanella algae* which occurred in marine and fresh water and occasionally involved in human infection [95]. The MIC of quinolone for these strains were 4 to 8 fold higher than in other closely related strains without chromosomal *qnrA* genes. This finding suggested that *S. algae* was a reservoir of *qnrA* genes. For the *qnrS* genes, the homolog of this gene was identified in chromosomal location of *Vibrio splendidus* with high similarity of 78 % [96, 97]. And for *qnrB* genes, the homologs of this gene were found in *qnr*-like genes from *S. maltophilia* and other gram negative bacteria especially from aquatic origins [98, 99]. And the latest variants of *qnr* genes, designed as *qnrD* genes was first identified in clinical isolates of *Salmonella enterica* serovar Kentucky and Bovismorbificans in China [17].

The *qnrA* genes allele were first identified; *qnrA1-qnrA6*. The *qnrA1* was found in *K.pneumoniae* [92] , *qnrA2* in *K. oxytoca*, *qnrA3 – A5* in *S. algae* [95], *qnrA6* in *Proteus miralbilis* [100].

The *qnrB* genes were found : *qnrB1* in *K. pneumoniae* [15], *qnrB2* in *Cirtobacter koseri* [15], *qnrB3* in *E.coli* [101], *qnrB4* in *E.coli* [101], *qnrB5* in *Salmonella* Berta [19], *qnrB6* in *Pantoea agglomerans* [102], *qnrB7* in *Enterobacter cloacae* [103], *qnrB8* [103], *qnrB10* [104], *qnrB12* in *Citrobacter werkanii* [105], *qnrB13-qnrB15* [106] *qnrB18* in *Citrobacter freundii*, *qnrB12* in *Citrobacter werkmani*, *qnrB19* and *qnrB20* in *E.coli* [107].

The *qnrS* genes were found; *qnrS1* in *Shigella flexneri* 2b [93], *qnrS2* in *Salmonella* Anatum, *qnrS3* in *E.coli* and *qnrS4* in *Salmonella* Stanley [108].

9.2.1.2 Qnr Protein and *qnr* Genes Nomenclature

Since many increasingly reports of new variants of *qnr* genes, the database for avoiding confusion was created and maintained when the new allele has been submitted. It has been assigned that only the full-length sequenced and naturally occurrence alleles could be numbered, with priority for first to published numbers, then to in accepted or submitted manuscripts and last to the date of genes submission to GenBank [109]. For some *qnrB* alleles which contained two initiation codons, it was agreed that the family numbers were defined by considering the second ATG on the sequence because this initiation codon was common to all variants. Because of this, QnrB proteins have 214 amino acids, whereas QnrA and QnrS have 218 amino acids. Similar to *qnrC* genes, the study showed the functional initiation codon was TTG, 43 codons upstream of ATG ; so that QnrC have 221 amino acids [109].

The *qnr* genes found in chromosome of some gram positive or gram negative bacteria were termed as *qnr* from organism by creating short name of specific organisms such as Efs*qnr* from *Enterococcus faecalis* [110], Vp*qnr* from *Vibrio parahaemolyticus* [111] and Vc*qnr* from *Vibrio cholerae* [112].

9.2.1.3 Genetic Background of *qnr* Genes

All plasmids carrying *qnr* genes were vary in size and other associated resistance genes. The *qnrA* and occasionally *qnrB* were found as part of *sul1*-type integrons containing recombinase IS*CR1*. The resistance gene cassettes on this integron were usually associated with 59 base pair of recombinase in 3' region of integrase gene which suggested the region of mobilization and integration [113]. The *qnrB* gene was also associated with IS*CR1*, recombinase, Orf1005 and other nonresistance genes that usually found in chromosome of aquatic bacteria [15]. In the *qnrS* plasmid, *qnrS* was not part of any integron but part of conjugative plasmid. The

sequence showed the inverted repeat with insertion sequence-like region which could associate with genes mobilization [19]. For *qnrC* gene, the genes were found located in plasmid pHS10 with integrase at upstream and amidase at downstream.

The linkage between *qnr* genes and other resistance genes were reported. The *qnr* genes were described that were associated with resistance genes such as *aac(6')-Ib-cr, aadA1, aadA2, dfrA1, dfrA12, cmlA1* and *catB2* [114, 115]. There were the linkages of *qnrA* genes with the genes encoding for ESBL and AmpC type β-lactamase such as CTX-M-1 [22], CTX-M-9 [21, 116] , CTX-M-14 [117], CTX-M-15 [22], SHV-5 [118], SHV-7 [116], SHV-92 [21] , FOX-5 [92, 116, 119] and VEB-1 [118, 120, 121]. The *qnrB* genes were found located on the same plasmid carrying the genes encoding for CTX-M-15 [94], SHV-12 [94], SHV-30 [19], KPC-2, KPC-3, IMP-4 and DHA-1 [122-124]. And for *qnrS* genes were reported the co-location with the genes encoding for TEM-1 [93] and SHV-12 [121].

9.2.1.4 Structure and Mechanisms of Action of Qnr Protein

Qnr proteins were categorized to pentapeptide-repeat family. The pentapeptide-repeat protein were defined by a tandem of five amino acid repeats or called semi-conservative motif including (Ser, Thr, Ala or Val), (Asp or Asn), (Leu or Phe), (Ser, Thr or Arg) and (Gly). There were more than one thousand proteins known as pentapeptide-repeat proteins with wide range of functions in organisms. As mentioned above: MfpA and McbG were also known as quinolone resistance proteins. The MfpA homolog was found in *Mycobacterium tuberculosis*, shared 67% identical to the variants found in *M. smegmatis* [125]. Both variants acted by inhibiting DNA supercoilling and DNA relaxing by directly binding with the enzyme (DNA gyrase). The study of 3D structure of this protein revealed that MfpA had structure and molecular charge closely DNA so that this protein could competitive binding to DNA gyrase with DNA. Consequently; the DNA

gyrase that bound to MFpA, could not participate in enzyme-DNA-fluoroquinolone cleaved complex [125]. This mechanism occurred in the same way for Qnr proteins to protect DNA gyrase.

There were many studies on Qnr mechanism, most on QnrA1 variants. First of all, there was reported that Qnr protein did not take an effect on fluoroquinolones accumulation or drug inactivity. According to DNA gyrase supercoilling assay, the direct effect of Qnr was to bind directly to DNA gyrase for dose-dependent inhibition of DNA gyrase activity. For DNA gyrase supercolling assay [126], ciprofloxacin was used to inhibit gyrase-mediated supercoilling function, the inhibition could be reversed by adding purified QnrA1 protein in dose-dependent manner and also with QnrB that showed the same result [15]. The Qnr was found to bind with DNA gyrase independently without the presence of relaxed DNA, ATP or ciprofloxacin so Qnr-DNA gyrase complex could be found before exposure of ciprofloxacin and formation of cleaved complex. This suggested that the reaction between DNA gyrase and QnrA1 was recognized before interaction with DNA. As a result; the quinolones which later participate in the cleaved complex would be inhibited or reduced. Still, how QnrA1 could compete with DNA to bind with DNA gyrase is not yet known.

9.2.1.5 Qnr Protein and Quinolone Resistance

Qnr activity could be supplementary resistance mechanisms for drug target alterations, active efflux and/or decreased outer membrane porin. The study by introducing plasmid pMG252 carrying *qnrA1* into *E.coli* containing drug target alterations in *gyrA* and *gyrB* and *parC* subunits and mutations in regulatory genes or porin loss showed increase of the MICs of several quinolones for 4 – 128 times [127]. Another study showed increase of the MICs of fluoroquinolones for 32 – 64 folds in both *acrAB* mutations and overexpression in *E.coli* [128]. This finding was similar to original discovery in *K.pneumoniae* with *qnrA1*. There were many studies of Qnr activity on clinical isolates demonstrated the difference in MICs of quinolones against the strains

carrying *qnr*. The *K. pneumoniae* clinical isolates were described the difference of *qnrA1* genes copy number but it was difficult to correlate the copy number of genes and the MIC because of additional mechanisms [129]. But in transconjugants *E.coli*, which the copy number of genes was excluded, the expression of *qnrA1* correlated with the MICs of ciprofloxacin and moxifloxacin. The effect of *qnrA1* expression was also enhanced by difference of strength of promoter [130].

The *qnr* genes also known as the genes that allowed bacteria with low-level quinolone resistance to survive long enough to grow again during quinolone exposure or treatment [131-133]. QnrA1 could help bacteria to facilitate mutant selection window by increasing their MPC or mutant prevention concentration. The Mutant Prevention Concentration (MPC) value of clinical strains *K. pneumoniae* carrying *qnrA* were ranged from 4 – 64 µg/mL, similar to the susceptible strains with transconjugant by plasmid pMG252 [132]. This study also reported that *qnr* favored the emergence of mutation in QRDR of *gyrA* (Ser83⟶ Phe and Asp87 ⟶ Tyr) and *parC* (Ser80 ⟶ Ile and Val87 ⟶ Asp) [132].

9.2.1.6 Qnr and *Salmonella* spp.

There were many reports of *qnr* variants in *Salmonella* spp. One study on *Salmonella* Enteritidis isolated in 2003 in Hong Kong, China was the first identified of *qnrA* in *Salmonella* spp. [117]. After that there were many studies worldwide. In Germany there was reported *Salmonella* Infantis isolated in 2004 was found carrying *qnrS* [134]. From the study on 335 nontyphoidal *Salmonella* isolated in 1996-2003 in the United States was found ten isolates carried *qnrB* or *qnrS* genes with ciprofloxacin $\geq$ 0.06 µg/ml [19]. In France, the study found one isolate of *Salmonella* Concord (0.2%)

carried *qnrA1* [135]. In the United Kingdom, screening of 118 isolates of *Salmonella enterica* found six isolates (5%) carried *qnrS1* [20]. According to the study of *Salmonella* Corvallis isolated from animals and patients from Thailand and Denmark found 23 isolates (100%) of the strains carried *qnrS1* [16]. In Turkey, there was the study of nine isolates of *Salmonella* Virchow isolated from chicken meat found three isolates carrying *qnrS1* [4]. From the study in the Netherlands, 39 isoaltes of *Salmonella* spp. isolated from humans and animals with MIC of ciprofloxacin of 0.25-21 µg/ml and nalidixic acid of 8-16 µg/ml found one *qnrB2*, two *qnrB5* and 31 *qnrS1*, all isolates were from human origin except *qnrB2* and the predominant strain was *Salmonella* Corvallis [136]. The study in 27 isolates of nontyphoidal *Salmonella* isolated in 1996-2004 in the United States, which showed decreased susceptibility to quinolones, found *qnrB2* in *Salmonella* Mbandaka isolate [137]. And another study in 45 nontyphoidal *Salmonella* isolated in 2006-2007, which showed ciprofloxacin MIC of 0.125 to 1.0 µg/ml and nalidixic acid MIC of $\leq$ 16 µg/ml, found 37 isolates carried *qnrS1* and one isolate with *qnrB2* and another one isolate with *qnrB5* [138]. The screening of all variants of *qnr* genes in six isolates of *Salmonella enterica* isolated from Finnish patients traveled to Malaysia and Thailand found one strain carried *qnrA*, two strains with *qnrS* and three strains with both *qnrB* and *qnrS* [139]. The screening of 160 strains of *Salmonella* isolted in 2004-2006 in Italy found one isolate of *Salmonella* Typhimurium carried *qnrB* [140]. And the other screening in 688 isolates of *Salmonella* spp. isolated from chickens in Belgium and turkeys in Germany in 2003-2005 found four isolates (0.6%) with *qnrS* (*Salmonella* Give, *Salmonella* Infantis, *Salmonella* Saintpaul) and *qnrB* (*Salmonella* Derby) [5]. And another one study in *Salmonella enterica* isolated from meat of different animals in 2007-2008 in Denmark reported 3 strains of *Salmonella* Newport and 4 strains of *Salmonella* Hadar carried *qnrB5* and 4 strains of *Salmonella* Saintpaul carried *qnrS1* [18].

9.2.2 Plasmid-Mediated Quinolone Modifying Enzyme: *aac(6')-Ib-cr* genes

According to the study on *E.coli* J53 carried plasmid containing *qnrA1* genes from Chinese clinical isolate. The study revealed two populations of *E.coli*, one with reduced susceptibility to ciprofloxacin for 4 times less than another one. By using transposon insertion analysis could identify the variation of *aac(6')-Ib* genes ; known as aminoglycoside acetyltransferase genes which responsible for resistance to tobramycin, amikacin and kanamycin [141]. This new variation was named *aac(6')-Ib-cr* (referred to ciprofloxacin resistance). This gene acted synergistically with *qnrA1* genes to cause ciprofloxacin resistance when both of them were present in the same cell. This enzyme increased the MIC of ciprofloxacin and norfloxacin for 3-4 times but take no effect on other fluoroquinolones which lacking of piperazinyl group in structure such as nalidixic acid, moxifloxacin and levofloxacin [141]. As the same result to Qnr activity, this enzyme can help bacteria increase the frequency of chromosomal mutations and facilitate quinolone treatment. And furthermore, this finding can explain the relationship between common resistance to both quinolones and aminoglygoside at the same time.

The prevalence of *aac(6')-Ib-cr* genes also has been reported. From the study in Shanghai, China, found 51% out of 71 clinical isolates carried this genes which was more common than *qnrA1* (7.7%) in the same population. The screening by PCR among 47 isolates of *E.coli* and 106 isolates of *K. pneumoniae* and 160 isolates of *Enterobacter* spp. found 15 (32%) isolates of *E.coli*, 17 (16%) isolates of *K. pneumoniae* and 12 (7.5%) isolates of *Enterobacter* spp. carried *aac(6')-Ib-cr* genes. Among these organisms also found co-occurrence with *qnr* genes for 15.9% in *aac(6')-Ib-cr* positive strains. But 24.5% of *aac(6')-Ib-cr* positive strains still negative for *qnr* genes, suggested that both genes can circulate in environment independently. In later studies, the detection of this gene can be found in *E.coli* more than other strains.

9.2.2.1 Epidemiology of *aac(6')-Ib-cr* Genes

After the first report of *aac(6')-Ib-cr* genes in *E. coli* producing CTX-M-15 isolated in the UK [142], there were many studies tried to screen this genes by using PCR base method. One study in Portugal, in *E. coli* (17) and *K. pneumoniae* (2) producing CTX-M-15, found all CTX-M-15 and OXA-1 producing isolates carried *aac(6')-Ib-cr* genes [143]. According to the screening of *aac(6')-Ib-cr* genes in 313 isolates of Enterobacteriaceae with MIC of ciprofloxacin > 0.25 µg/mL found *aac(6')-Ib-cr* genes in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae* isolates and 12 (7.5%) of 160 *Enterobacter* isolates. This study also found *qnr* genes carrying in 7 of 44 (15.9%) of *aac(6')-Ib-cr* positive strains [144]. The study of *E. coli* DH5α transformants (17) from *K. pneumoniae* producing CTX-M isolated in 2002-2003 in Nigeria, found *qnrB1* and *aac(6')-Ib-cr* on the same plasmid of *K. pneumoniae* [145]. The screening of 232 strains of gram-negative bacteria isolated from zoo animals in Japan in 2006 found one isolate of *Aeromonas* spp. carried *aac(6')-Ib-cr* genes [114]. From the study in Korea in ESBL-producing *E. coli* and *K. pneumoniae* found *aac(6')-Ib-cr* in 13 (7%) *K. pneumoniae* and one isolate also carried *qnrB4* [24] . In Spain, 2006-2007, found *aac(6')-Ib-cr* genes in *qnrS2* positive isolates [146]. Another study in Korea in 2005-2006 found one isolate of *E. coli* with cephalosporin resistance carried *aac(6')-Ib-cr* genes [147]. And another one screening in bloodstream clinical isolates found 21 isolates carried *aac(6')-Ib-cr* genes [148]. In France, screening in 538 Enterobacteriaceae isolated in 2004-2006 found *aac(6')-Ib-cr* genes 15 (34%) in *qnr* positive strains [149]. The study in Bulgaria, in 163 Enterobacteriaceae bloodstream isolates, found multiple co-carried between *qnrB2* in two *Citrobacter freundii* with *aac(6')-Ib-cr* genes [150]. From the study in *K. pneumoniae* bloodstream isolates in 2006 has been first reported *aac(6')-Ib-cr* genes in an IS*CR1* contaning class 1 integron and also found *qnrB6* genes between IS*CR1* and 3' conserved region [151]. And in Uruguay in 2006, found two isolates of *E. coli* with ciprofloxacin resistance carried *aac(6')-Ib-cr* genes [23]. From the study in *E. coli* and *K. pneumoniae* found *aac(6')-Ib-cr* genes with class 1 integrons, CTX-M-15 and *ISEcp1* on the same plasmid [152]. The

study of *E. cloacae* with third-generation cephalosporins resistance found *qnrB4* and *aac(6')-Ib-cr* [153]. According to study in China in 197 isolates of *E. coli*, *K. pneumoniae*, *C. freundii* and *E. cloacae* found 18 (9%) with co-existent of *qnr* and *aac(6')-Ib-cr* genes [25]. From the study in Italy in ESBL- producing strains found 61 *E. coli* and 1 *K. pneumoniae* isolates carried *aac(6')-Ib-cr* [154]. The screening of 248 isolates of *E. coli* and *K. pneumoniae* reported *qnrB1* and *aac(6')-Ib-cr* on different plasmids of one *K. pneumoniae* and also found 78% in ESBL-producing strains [155]. The screening of *aac(6')-Ib-cr* genes in *E. cloacae* (179), *C. freundii* (134) and *S. marcescens* (166) found *aac(6')-Ib-cr* genes 10.8%, 20% and 18.2%, respectively. This study also reported 11 isolates with *qnr* genes in the same strains (six *qnrA*, four *qnrB* and one *qnrS*) [156]. The study in Ireland in Enterobacteriaceae found *aac(6')-Ib-cr* genes in *K. pneumoniae*, *Enterobacter* spp., *E. coli* and *E. aerogenas* [157]. The study in China in 48 *E. coli* isolated in 2005-2006 found co-concurrent of *qnrS2* and *aac(6')-Ib-cr* genes in 4 isolates [158]. And the study in 16 *E. cloacae* with carbapenem resistance isolates found one isolate carried both *qnrB2* and *aac(6')-Ib-cr* genes [159].

9.2.3 Plasmid-Mediated Fluoroquinolone Efflux Pumps; QepA

The fluoroquinolone efflux pump, *qepA* gene, was first described in *E. coli* isolated caused urinary tract infection in Japan in 2002. This strain showed multiresistance to antimicrobial agents including fluoroquinolones, aminoglycosides and extended-spectrum β-lactams. The conjugative plasmid pHPA was identified from this strain with low-level resistance to norfloxacin and ciprofloxacin. The plasmid also harbored $bla_{CTX\text{-}M\text{-}12}$, *mphA* and *rmtB* genes [160]. The *qepA* gene also identified in Belgium, in *E. coli* isolates with resistance to aminoglycosides with genes on the same plasmid with *qepA* gene [161]. In 2008, *qepA2* was identified in CTX-M-15 positive *E.coli* with the same activity to *qepA1*gene.

9.2.3.1 Structure and Activity of QepA

The QepA protein composed of 511 amino acids, categorized into the major facilitator superfamily (MFS) of 14-transmembrane segment efflux pump. This efflux pump can cause increasing of the resistance in moderate level (more than 5 times). From further study showed QepA did not affect on less hydrophilic quinolones (sparfloxacin, gatifloxacin, pefloxacin, levofloxacin and moxifloxacin) or hydrophobic quinolones (nalidixic acid). QepA can cause extrusion of ciprofloxacin and norfloxacin depended on the proton motive force which generated energy to MFS proteins so this function could be interfered by using the protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP) [160, 161]. The genetic environment of *qepA* has been observed. The *qepA* gene was flanked by two copies of IS26 with *tnpA* and also contained *tnpR*, bla_{TEM-1} and *rmtB* gene [160]. And another study also reported *qepA* gene was flanked by transposase genes and *intI* gene [161]. With high G+C content (72%) compared to the flanking genes of *qepA* gene, suggested that this gene had an origin in high G+C content organisms. And further study revealed that *qepA* might be originate from actinomycetes which originally produced metabolites which had the same structure to quinolone and must be extrude out of the cells by efflux pump mechanism. For *qepA2*, was the *qepA1* with two amino acid changes at Ala99⟶Gly and Val134Ile but still confer the same phenotype as *qepA1* [162].

The expression of *qepA* gene, same to other plasmid borne quinolone resistance genes, had an effect on help facilitating of bacteria in the presence of quinolones [163].

9.2.3.2 Epidemiology of *qepA* Genes

The first report of *qepA* gene was found in two *E. coli* isolated in Japan, 2002. Two positive strains out of 751 strains were clonally unrelated and collected from different part of distant hospital in Japan [158]. Both strains carried *rmtB* gene but negative for *qnr* gene. In another study in China, *qepA* gene was found in 28 out of 48 (58.3%) *rmtB*-positive *E. coli* isolated from pig feces during 2005 and 2006. The findings of co-prevalence of *qepA* and *rmt*B genes among *E.coli* led to further study of other plasmid-mediated quinolone resistance. Of the 151 isolates of RmtB producing, *E.coli* collected during 2005 to 2006, were screened for the presence of *qepA, qnrA, qnrB, qnrS and aac(6')-ib-cr* genes by using PCR method. This study reported *qepA* (58.3%), *qnrB6* (2.1%) *qnrS1* and *qnrS2* (18.8%) and *aac(6')-ib-cr* genes (12.5%). This suggested the closely relationship between *qepA* and *rmtB* genes and showed high prevalence of plasmid-mediated quinlone resistance gene in food-producing animals. And also, this was the first identification of concurrence of three QepA, Qnr, and AAC(6_)-Ib-cr producing in the same strains [158]. Another study in 2009 among *Enterobacteriaceae* food-producing animals and pets from the hospitals found combination of *qepA* with *aac(6')-ib-cr*, CTX-M-9 group, CTX-M-1 group [164].

CHAPTER IV

MATERIALS AND METHODS

1. Bacterial Strains

There were 108 human isolates of nontyphoidal *Salmonella*. Fourty-four isolates were collected from stool, obtained from WHO National *Salmonella* and *Shigella* Center (NSSC); Department of Medical Sciences; Ministry of Public Health (Bangkok, Thailand) in 2005 and 64 isolates were collected from haemoculture of patients in the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between August 2005 and May 2006 (designed as S1-S108).

There were 248 animal isolates of nontyphoidal *Salmonella*. One hundred and fifty-one were obtained from the Department of Medical Sciences; Ministry of Agriculture and Cooperatives (Bangkok, Thailand) in 2007 (deginged as SSA109-SSA259) and 97 isolates were obtained from the Department of Livestock Development (Bangkok, Thailand) between 2003 and 2005 (designed as SA1-SA98).

1.1 Culture Preservation

All isolates were grown on trypticase soy agar (Oxiod, UK) at 37^{o}C for 18-24 hours. The colonies were suspended in cryogenic vials containing trypticase soy broth (Oxiod, UK) with 20% Glycerol and were kept at -70^{o}C until use.

2. Nalidixic Acid and Ciprofloxacin Susceptibility

Minimal inhibitory concentrations (MICs) were determined by agar-dilution technique, according to guideline of Clinical and Laboratory Standards Institute (CLSI), 2008. The antibiotics used in this study were nalidixic acid (Sigma, USA) and ciprofloxacin(Sigma, USA).

Nontyphoidal *Salmonella* isolates were grown on trypticase soy agar. MICs value were determined on Mueller-Hinton agar (BBL, Becton Dickinson and Company, Coskeysville, MD) inoculums were prepared from an overnight culture on trypticase soy agar and the turbidity was adjusted to 0.5 McFarland standard. Dilutions of antimicrobial agent for agar dilution susceptibility test were prepared in doubling dilutions at concentrations of 0.03125 and 256 µg/ml. A multipoint inoculator was used to deliver 1-2 µl of suspension to the agar plates. The final inoculums were approximately 10^4cfu/spot. The plates were incubated at 37^{o}C for 18-24 h. The MIC was determined to the lowest concentration of antimicrobial agent at which there is no visible growth.

Breakpoint criteria used in this study were those defined by the National Committee for Clinical Laboratory Standards, 2003; Clinical and Laboratory Standards Institute (CLSI), 2008. According to current CLSI breakpoint, the isolates with the MIC of $\leq$ 16 µg/ml for nalidixic acid were defined as susceptible strains, those with the MIC of $\geq$ 32 µg/ml as resistant strains. The isolates with the MIC of $\leq$ 1 µg/ml for ciprofloxacin were defined as susceptible strains and those with the MIC of $\geq$ 4 µg/ml were defined as resistant strains.

The isolates with the MIC for ciprofloxacin between 0.125 and 1 µg/ml were defined as reduced susceptibility to ciprofloxacin.

Table 1 Scheme for preparing dilutions of antimicrobial agents for agar dilution susceptibility tests

Step	Conc. (µg/ml)	Source	Volume in agar	Diluent	Intermediate concentration	Final conc. At 1:10 dilution
5,120	Stock (µg/ml)		-	-	5,120	512
1	5,120	Stock	2 ml	2 ml	2,560	256
2	5,120	Stock	1	3	1,280	128
3	5,120	Stock	1	7	640	64
4	640	Step3	2	2	320	32
5	640	Step3	1	3	160	16
6	640	Step3	1	7	80	8
7	80	Step6	2	2	40	4
8	80	Step6	1	3	20	2
9	80	Step6	1	7	10	1
10	10	Step9	2	2	5	0.5
11	10	Step9	1	3	2.5	0.25
12	10	Step9	1	7	1.25	0.125
13	1.25	Step12	2	2	0.625	0.0625
14	1.25	Step12	1	3	0.3125	0.03125

Table 2 Acceptable limits for quality control strains used in this study for minimal inhibitory concentration (MICs) (µg/ml)

Bacterial Strain	Nalidixic acid (µg/ml)	Ciprofloxacin (µg/ml)
Staphylococcus aureus ATCC® 29219	-	0.125-0.5
Enterococcus faecalis ATCC® 29212	-	0.25-2
Escherichia coli ATCC® 25922	1- 4	0.004-0.016
Pseudomonas aeruginosa ATCC® 27853	-	0.25-1

3. Quinolone resistance determinating region (QRDR) mutations detection (gyrA, gyrB, parC and parE genes) by PCR and automated DNA sequencing

Nontyphoidal *Salmonella* isolates with ciprofloxacin resistance and reduced susceptibility to ciprofloxacin were investigated for the presence of *gyrA, gyrB, parC* and *parE* genes mutations. The QRDR of these genes were amplified and then sequenced.

3.1 DNA Extraction

Nontyphoidal *Salmonella* isolates were re-suspended in 100 µl of steriled distilled water and boiled for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant was used as the DNA template in the PCR experiments.

3.2 Primers

The primers for the detection of *gyrA, gyrB, parC* and *parE* genes mutations are described in Table 3 and are based on those previously reported [62].

Table 3 Primers used for detection of QRDR mutations

Primers	Sequence (5' to 3')	Product size (bp)
gyrA forward	5' TGTCCGAGA TGGCCTGAAGC 3'	
reverse	5' TACCGTCATAGTTATCCACG 3'	347
gyrB forward	5' GCGCTGTCCGAACTGTACCT 3'	
reverse	5' TGATCAGCGTCGCCACTTCC 3'	150
parC forward	5' CTATGCGATGTCAGAGCTGG 3'	
reverse	5' TAACAGCAGCTCGGCGTATT 3'	262
parE forward	5' TCTCTTCCGATGAAGTGCTG 3'	
reverse	5' ATACGGTATAGCGGCGGTAG 3'	238

3.3 Amplification of *gyrA, gyrB, parC* and *parE* genes by PCR

The PCR was performed individually in a final volume of 25 µl containing 1X buffer, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 2 mM for *gyrA, gyrB, parC* and *parE* forward and reverse primers,0.1U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 94^{o}C for 5 min; 30 cycles of 94^{o}C for 1 min, 54^{o}C (*gyrA* and *parC*) and 57 ^{o}C (*gyrB* and *parE*) for 1 min, and 72^{o}C for 1 min; and 1 cycle at 72^{o}C for 10 min.

3.4 Analysis of Amplified DNA

The PCR products were analyzed on 1% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). Four microliters of PCR products were mixed with 2 µl of loading buffer (20% ficoll, 0.05% bromphenol blue) and 2 µl of steriled distilled water. The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light trans-illuminator.

3.5 Purification of PCR Products

The PCR products of *gyrA, gyrB, parC* and *parE* genes were purified by using QIAquick PCR purification kit as described by the manufacturers (QIAGEN , Max-Volmer-StraBe4 , Hilden, Germany). Five volume of Buffer PBI were added into the 1 volume PCR products and mixed by pulse-vortexing for 15 S. After well mixing the suspensions were placed into the 2 ml QIAquick column and centrifuged 13,000 rpm for 1 min. DNA was absorbed to the silica-membrane in the presence of high salt while contaminants pass through the column. To wash, 750 ml of Buffer PE was added into the QIAquick column and centrifuged 13,000 rpm for 1 min. Discarded flow-through and placed the QIAquick column back in the same tube. The QIAquick columns were centrifuged for 60 S and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of 10mM Tris-Cl buffer (pH 8.5). The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec tm 3000, U.S.A) and approximately adjusted to 50-100 ng/µl for preparation of sequencing reaction. The purified PCR products were stored at -20^{o}C.

3.6 Preparation of Sequencing Reaction

Automated sequencing was done at the Macrogen Inc. (Seoul, Korea). Sequencing was done by the chain termination method. DNA samples were sequenced by using three sets of primers including forward primer of each *qnrA*, *qnrB* and *qnrS*. Sequencing was conducted under BigDye™ terminator cycling conditions. The reacted products were purified by ethanol precipitation and running using automatic sequencer, Applied Biosystems DNA sequencer model 3730xl (Rochester NY, USA).

3.7 Sequence Analysis

The nucleotide sequence and the deduced protein sequence were analyzed with the software available over the Internet at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/). Multiple sequence alignment of sequences were analyzed by Multilin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

4. Screening for the Presence of *qnr* and *aac(6')-Ib-cr* Genes by Polymerase Chain Reaction (PCR)

All of 356 isolates of nontyphoidal *Salmonella* were screened for the presences of both *qnr* and *aac(6')-Ib-cr* genes by using PCR

4.1 DNA Extraction

Nontyphoidal *Salmonella* isolates were re-suspended in 100 µl of steriled distilled water and boiled for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant was used as the DNA template in the PCR experiments.

4.2 Primers

The primers for the detection of *qnr* [134] and *aac(6')-Ib-cr* [165] genes are described in Table 4 and are based on those previously reported.

Table 4 Primers used for detection of *qnrA, qnrB, qnrS* and *aac(6')-ib-cr* genes

Primers	Sequence (5' to 3')	Product size (bp)
qnrA forward	5'TCAGCAAGAGGATTTCTCA3'	
reverse	5'GGCAGCACTATGACTCCCA3'	627
qnrB forward	5'TCGGCTGTCAGTTCTATGATCG3'	
reverse	5'TCCATGAGCAACGATGCCT3'	496
qnrS forward	5'TGATCTCACCTTCACCGCTTG3'	
reverse	5'GAATCAGTTCTTGCTGCCAGG3'	566
aac forward	5'GATCTCATATCGTCGAGTGGTGG3'	
reverse	5'GAACCATGTACACGGCTGGAC3'	435

4.3 Amplification of *qnr* and *aac(6')-Ib-cr* Genes by PCR

For *qnr* genes detection including *qnrA, qnrB* and *qnrS*, the PCR was performed in a final volume of 25 µl containing 1X buffer, 2 mM of MgCl2, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 2 mM for *qnr* forward and *qnr* reverse primers, 0.1U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 94°C for 1 min ; 35 cycles of 94°C for 1 min , 50°C (*qnr*A), 54 °C (*qnr*B) and 58 °C (*qnr*S) for 1 min , and 72°C for 1 min ; and 1 cycle at 72°C for 10 min.

For *aac(6')-Ib-cr* genes detection, the PCR was performed in a final volume of 25 µl containing 1X buffer, 2 mM of MgCl2, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 2 mM for *aac(6')-Ib-cr* forward and *aac(6')-Ib-cr* reverse primers, 0.1 U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions

were 1 cycle 94°C for 1 min; 35 cycles of 94°C for 1 min, 58 °C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

4.4 Analysis of Amplified DNA

The PCR products were analyzed on 1% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide(Sigma, USA). Four microliters of PCR products were mixed with 2 µl of loadingbuffer (20% ficoll, 0.05% bromphenol blue) and 2 µl of steriled distilled water. The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light trans-illuminator.

For *qnr* genes, the expected sizes of PCR product were 627, 496 and 566 bp for *qnrA, qnrB* and *qnrS*, respectively. And for *aac(6')-Ib-cr* genes, the expected size of PCR product was 435 bp. A 100 bp DNA ladder (Fermentus, USA) was used as a DNA size marker.

5. Differentiation of *qnr* genes Variants by Automated DNA Sequencing Analysis

5.1 Purification of PCR Products

The PCR products of *qnr* genes positive strains were purified using QIAquick PCR purification kit as described by the manufacturers (QIAGEN , Max-Volmer-StraBe4 , Hilden, Germany). Five volume of Buffer PBI were added into the 1 volume PCR products and mixed by pulse-vortexing for 15 S. After well mixing the suspensions- were placed into the 2 ml QIAquick column and centrifuged 13,000 rpm for 1 min. DNA was absorbed to the siliga-membrane in the presence of high salt while contaminants pass

through the column. To wash, 750 ml of Buffer PE was added into the QIAquick column and centrifuged at 13,000 rpm for 1 min. Discarded flow-through and placed the QIAquick column back into the same tube. The QIAquick columns were centrifuged for 60 S and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of 10mM Tris-Cl buffer (pH 8.5). The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec tm 3000, U.S.A) and was approximately adjusted to 50-100 ng/µl for preparation of sequencing reaction. The purified PCR products were stored at -20^{o}C.

5.2 Preparation of Sequencing Reaction

Automated sequencing was done at the Macrogen Inc. (Seoul, Korea). Sequencing was done by the chain termination method. DNA samples were sequenced by using three sets of primers including forward primer of each *qnrA*, *qnrB* and *qnrS.* Sequencing was conducted under BigDye^{TM} terminator cycling conditions. The reacted products were purified by ethanol precipitation and running using automatic sequencer, Applied Biosystems DNA sequencer model 3730xl (Rochester NY, USA).

5.3 Sequence Analysis

The nucleotide sequence and the deduced protein sequence were analyzed with the software available over the Internet at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/). Multiple sequence alignment of sequences were analyzed by Multilin

6. *In vitro* Selection of Fluoroquinolone Resistance

Nontyphoidal *Salmonella* strains which were susceptible to nalidixic acid and ciprofloxacin were separated into four groups including; with *qnr* genes alone, with both *qnr* and *aac(6')-Ib-cr* genes, with *aac(6')-Ib-cr* genes alone and without both of them. Each group contained 5 selected strains. All of selected strains were confirmed the MICs for nalidixic acid and ciprofloxacin.

6.1 Selection of Ciprofloxacin Resistance Strains

The bacterial cells were grown overnight on trypticase soy agar (BBL, Becton Dickinson and Company, Coskeysville, MD) at 37 °C. The selection was determined on Mueller-Hinton agar. Inoculums were prepared from an overnight culture by adjusting the turbidity to 1.0 McFarland standard. One hundred ml of turbidity adjusted suspensions were transfered to Mueller-Hinton agar contains 2 times of concentration of ciprofloxacin than the primary MIC for ciprofloxacin of each strain. The colonies which could grow on the agar were collected and preserved for further selections at higher concentration of ciprofloxacin.

CHAPTER V

RESULTS

1. Bacterial strains

A total of 108 patient isolates of nontyphoidal *Salmonella* (S1-S108) were included in this study. Fourty-four isolates were collected from stool, obtained from WHO National *Salmonella* and *Shigella* Center in 2005. These isolates included *S.* Choleraesuis (54.55%, 24/44), *S.* Enteritidis (43.18%, 19/44) and *S.* Typhimurium (2.27%,1/44). The other 64 patient isolates including *S.* Choleraesuis (25.00%, 16/64), *S.* group E (1.56%, 1/64), *S.* group D (57.81%, 37/64) and *S.* group B (15.63%, 10/64) were collected from haemoculture of patients in the King Chulalongkorn Memorial Hospital between August 2005 and May 2006,. The results are shown in Table 5. *S.* Choleraesuis was the major serovar of isolates from stool whereas *S.* group D was dominant in isolates from blood.

Table 5 Serovar variation of 108 nontyphoidal *Salmonella* isolates from patients

Source/Origin	Serovar	No. of isolates (%)
Stool	*S.* Choleraesuis	24 (54.4)
(N=44)	*S.* Enteritidis	19 (43.1)
	S. Typhimurium	1 (2.2)
Blood	*S.* group D	37 (57.8)
(N=64)	*S.* Choleraesuis	16 (25)
	S. group B	10 (15.6)
	S. group E	1 (1.6)

There were 248 animal isolates of nontyphoidal *Salmonella*. One hundred and fifty-one isolates, including *S.* Rissen (36.43%, 55/151), *S.* Stanley (11.26%, 17/151), *S.* Anatum (15.23%, 23/151), *S.* Schwarzengrend (9.27%, 14/151), *S.* Weltevreden (4.64%, 7/151), *S.* Altona (3.31%, 5/151), *S.* Agona (2.64%, 4/151), *S.* Amsterdam (2.64%, 4/151), *S.* Bovismorbificans (1.99%, 3/151) and *S.* Tennessee (1.99%, 3/151), *S.* Corvallis (1.32%, 2/151), *S.* Lexington (1.32%, 2/151) and *S.* Panama (1.32%, 2/151), and 1 isolate (0.66%) of *S.* Albany, *S.* Derby, *S.* Eastbourne, *S.* Javiana, *S.* Kendougou, *S.* Kentucky, *S.* Rabislaw, *S.* Senftenberg *S.* Thompson and *S.* I 4,5,12:i:- (SSA109 – SSA259) were obtained from WHO National *Salmonella* and *Shigella* Center in 2007. There were 23 serovars among these 151 isolates. *S.* Rissen was the major serovar among animal isolates, followed by *S.* Anatum. The majority of these isolates were obtained from swine, followed by chicken. The serovar distribution is shown in Figure 2 and Table 6.

Figure 2 Serovar distribution of 151 nontyphoidal *Salmonella* isolates from animals obtained from WHO National *Salmonella* and *Shigella* Center in 2007

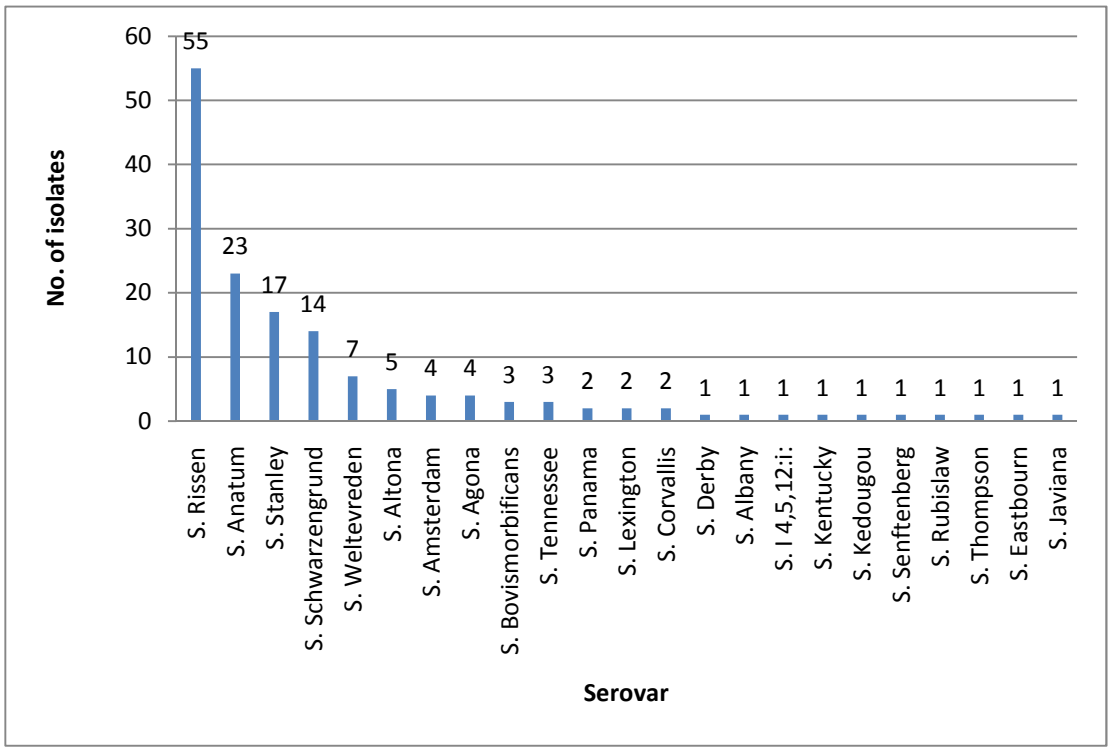
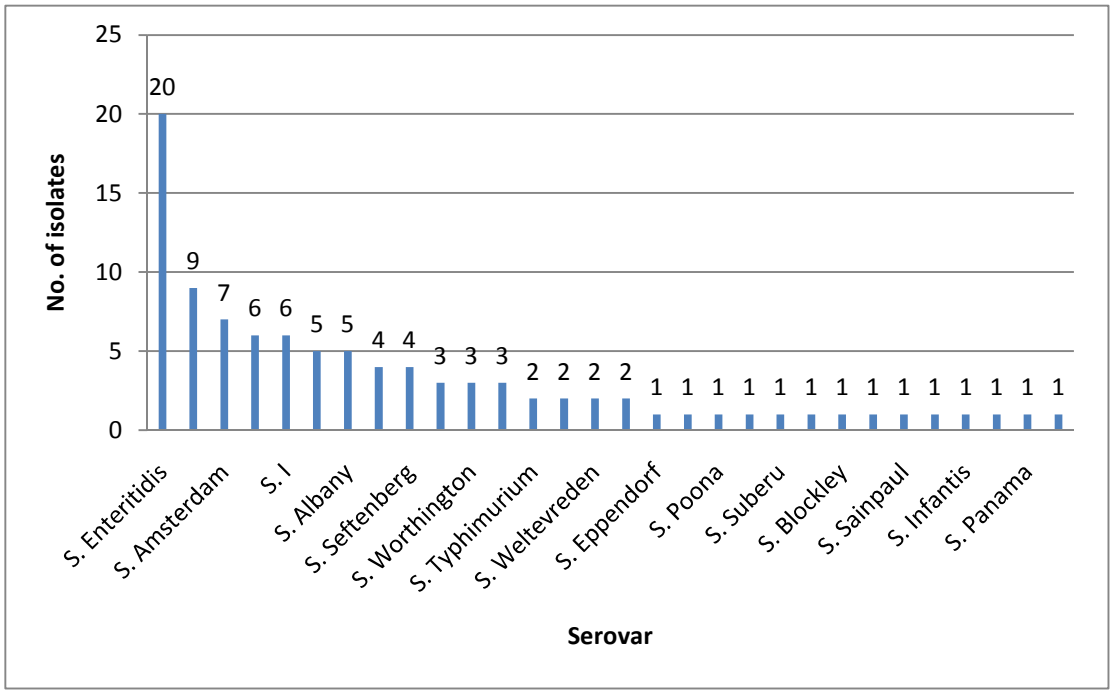


Table 6 The number of isolates and serovar distribution among 151 nontyphoidal *Salmonella* isolates from various animal species obtained from WHO National *Salmonella* and *Shigella* Center in 2007

Serovar	No. of isolates						Total
	Swine	Chicken	Cockroach	Bovine	Cricket	Shell	
S. Rissen	41	9	5				55
S. Anatum	18		5				23
S. Stanley	13			4			17
S. Schwarzengrend		14					14
S. Weltevreden	1			6			7
S. Altona	5						5
S. Amsterdam		4					4
S. Agona					4		4
S. Bovismorbificans	3						3
S. Tennessee			3				3
S. Panama	2						2
S. Lexington		1		1			2
S. Corvallis			2				2
S. Derby	1						1
S. Albany	1						1
S. I 4,5,12:i:	1						1
S. Kentucky		1					1
S. Kedougou		1					1
S. Senftenberg			1				1
S. Rubislaw						1	1
S. Thompson				1			1
S. Eastbourn				1			1
S. Javiana					1		1
Total	86	30	16	13	5	1	151

The other 97 animal isolates which were obtained from the Department of Livestock Development between 2003 and 2005 included *S.* Enteritidis (20.62%, 20/97), *S.* Bovismorbificans (9.28%, 9/97), *S.* Amsterdam (7.23%, 7/97), *S.* Corvallis (6.19%, 6/97) and *S.* I (6.19%, 6/97), *S.* Give (5.15%, 5/97), *S.* Anatum (4.12%, 4/97) and *S.* Senftenberg (4.12%, 4/97), *S.* Stanley (3.09%, 3/97), *S.* Emek (3.09%, 3/97) and *S.* Worthington (3.09%, 3/97), *S.* Typhimurium (2.06%, 2/97), *S.* Welterreden (2.06%, 2/97) and *S.* Kentucky (2.06%, 2/97), *S.* Eppendorf (1.03%, 1/97), *S.* Virchow (1.03%, 1/97), *S.* Infantis (1.03%, 1/97), *S.* Madjorio (1.03%, 1/97), *S.* Poona (1.03%, 1/97), *S.* Hvittingfoss (1.03%, 1/97), *S.* Soberu (1.03%, 1/97), *S.* Orion (1.03%, 1/97), *S.* Blockley (1.03%, 1/97), *S.* Panama (1.03%, 1/97), *S.* Muenster (1.03%, 1/97), *S.* Rissen (1.03%, 1/97), *S.* Saintpaul (1.03%, 1/97) and *S.* Virginia (1.03%, 1/97). The serovar distribution is shown in Figure 3.

Figure 3 Serovar distribution of 97 nontyphoidal *Salmonella* isolates from animals obtained from the Department of Livestock Development between 2003 and 2005



2. Determination of antibiotic susceptibility of nontyphoidal *Salmonella*

Susceptibility of nontyphoidal *Salmonella* to nalidixic acid and ciprofloxacin was determined by agar dilution method, according to guideline of Clinical and Laboratory Standards Institute (CLSI), 2008. Minimal inhibitory concentration (MIC) is the lowest concentration of antimicrobial agent required to inhibit the growth of a microorganism. The MIC_{50} and MIC_{90} are the lowest concentration of antimicrobial agents required to inhibit 50% and 90% of isolates tested, respectively.

Breakpoint criteria used in this study were those defined by CLSI, 2008. According to current CLSI breakpoint, the isolates with the MIC of $\leq$ 16 µg/ml for nalidixic acid were defined as susceptible strains, those with the MIC of $\geq$ 32 µg/ml as resistant strains. The isolates with the MIC of $\leq$ 1 µg/ml for ciprofloxacin were defined as susceptible strains, those with the MIC of 2 µg/ml were defined as intermediate resistant strains and those with the MIC of $\geq$ 4 µg/ml were defined as resistant strains. The isolates with the MIC for ciprofloxacin between 0.125 and 1 µg/ml were defined as reduced susceptibility to ciprofloxacin.

Prevalence of nalidixic acid resistance in nontyphoidal *Salmonella* isolates from patients (MIC of $\geq$ 32 µg/mL) was 86.1% (93/108). There was no isolate resistant to ciprofloxacin but 4.62% (5/108) were intermediate-resistant. However, the susceptibility testing to ciprofloxacin showed high rate of reduced susceptibility (72.2%, 78/108) in isolates from patients. Of the 248 *Salmonella* isolates from animals, 42.6% (106/248) were resistant to nalidixic acid. Ciprofloxacin susceptibility showed that 0.4% (1/248) was resistant to ciprofloxacin and 2.4% (6/248) were intermediate resistant. However, 41.36% (103/248) showed reduced susceptibility to ciprofloxacin, as shown in Table 7.

Table 7 The susceptibility of nalidixic acid and ciprofloxacin against 356 nontyphoidal *Salmonella* isolates

Isolates	No. of isolates (%)					
	Nalidixic acid			Ciprofloxacin		Reduced susceptibility to ciprofloxacin
	R	I	S	R	I	
From Patients (n=108)	93 (86.1)	0	15 (13.88)	0	5 (4.62)	78 (72.2)
From Animals (n=248)	106 (42.7)	0	143 (57.66)	1 (0.4)	6 (2.41)	102 (41.12)

R; resistant, I; intermediate resistant, S; susceptible

Source, serovar and ciprofloxacin susceptibility is summarized in Table 8. The results showed the high prevalence of reduced susceptibility to ciprofloxacin in *S.* Choleraesuis, *S.* Enteritidis and *S.* group D. However, there were four isolates of *S.* Choleraesuis which were intermediate resistant to ciprofloxacin. The most common serovar of isolates from stool with reduced susceptibility to ciprofloxacin was *S.* Choleraesuis (50%, 22/44) followed by *S.* Enteritidis (40.9%, 18/44). The most common serovar with reduced susceptibility to ciprofloxacin was *S.* group D (34.38%, 22/64) followed by *S.* Choleraesuis (20.31%, 13/64) in isolates from blood.

Table 8 Source, serovar and ciprofloxacin susceptibility of 108 nontyphoidal *Salmonella* isolates from patients

Source	Serovar	No. of isolates (%)		
		Reduced susceptibility to ciprofloxacin	Ciprofloxacin susceptibility	
			R	I
Stool (N=44)	*S.* Choleraesuis (N=24)	22 (50)	0	2 (4.5)
	S. Enteritidis (N=19)	18 (40.9)	0	0
	S. Typhimurium (N=1)	1 (2.7)	0	0
Blood (N=64)	*S.* group D (N=37)	22 (34.38)	0	1 (1.56)
	S. Choleraesuis (N=16)	11 (17.18)	0	2 (3.12)
	S. group B (N=10)	4 (6.25)	0	0
	S. group E (1)	0	0	0

R; resistant, I; intermediate resistant, S; susceptible

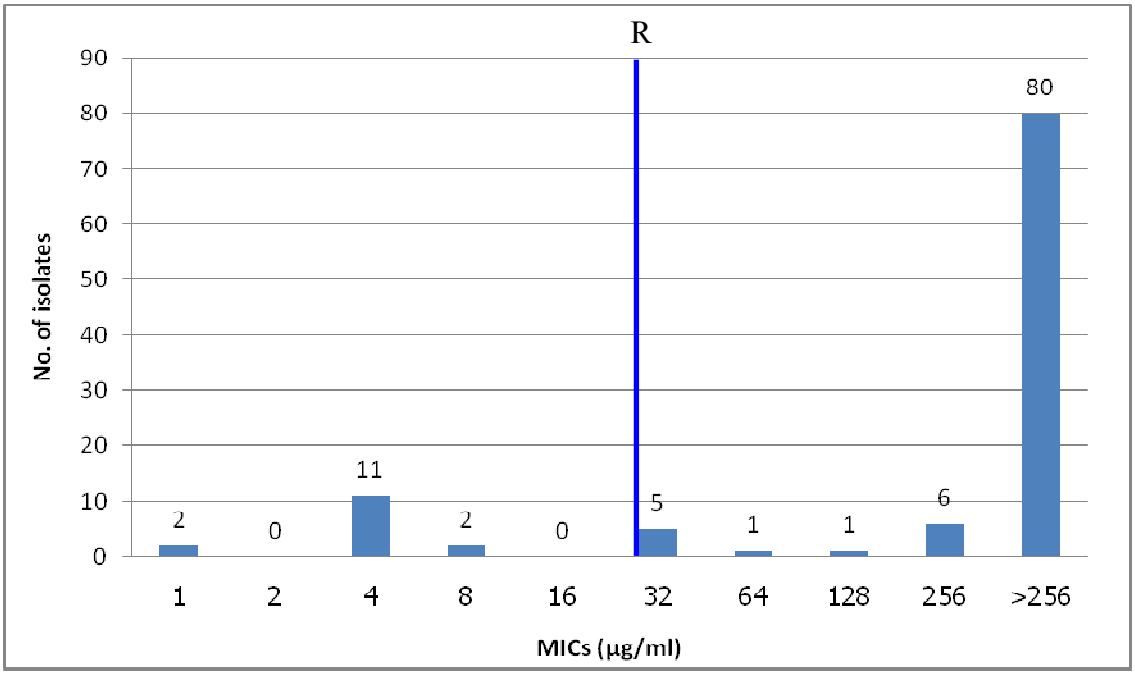
The prevalence of nalidixic acid and ciprofloxacin resistance in isolates from patients is shown in Table 9. Prevalence of nalidixic acid resistance was 86.1% (93/108). The MIC of nalidixic acid ranged from 1 to >256 µg/ml. The MIC_{50} and MIC_{90} were both >256 µg/ml. The MIC distribution of nalidixic acid is shown in Figure 4. It was demonstrated that MICs of nalidixic acid-susceptible nontyphoidal *Salmonella* isolates ranged from 1 to 16µg/ml. Most nalidixic acid-susceptible isolates (73.3%, 11/15) had nalidixic acid MIC of 4 µg/ml. Nalidixic acid-resistant isolates had the MIC range from 32 to >256 µg/ml and most of these isolates (86%, 80/93) had nalidixic acid MIC of >256 µg/ml. The MIC range for ciprofloxacin was 0.015 to 2 µg/ml and the MIC_{50} and MIC_{90} were 0.125 and 0.5 µg/ml, respectively. There was no isolate from patients resistant to

ciprofloxacin but 4.62% (5/108) were intermediate-resistant. The MIC distribution of ciprofloxacin against 108 patient isolates is shown in Figure 5. It was demonstrated that MICs of ciprofloxacin-resistant nontyphoidal *Salmonella* isolates ranged from 0.015 to 1 µg/ml. Most of the ciprofloxacin-susceptible isolates (69.4%, 75/108) had ciprofloxacin MIC range of 0.125 to 0.5 µg/ml.

Table 9 Nalidixic acid and ciprofloxacin MICs and resistance rates among 108 nontyphoidal *Salmonella* isolates from patients

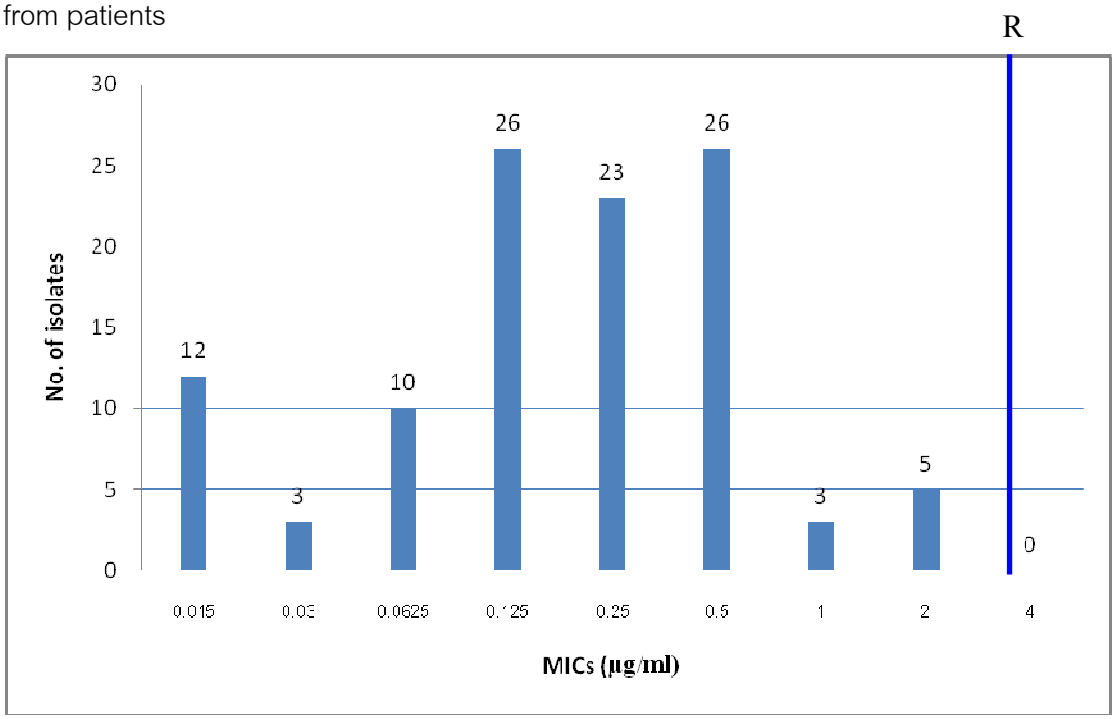
Antimicrobial agents	MICs (µg/ml)		
	Range	MIC_{50}	MIC_{90}
Nalidixic acid	1 - >256	>256	>256
Ciprofloxacin	0.015 - 2	0.125	0.5

Figure 4 MIC distribution of nalidixic acid among 108 nontyphoidal *Salmonella* isolates from patients



R; CLSI resistant break point for nalidixic acid; MIC ≥ 32 µg/ml

Figure 5 MIC distribution of ciprofloxacin among 108 nontyphoidal *Salmonella* isolates from patients



R; CLSI resistant break point for ciprofloxacin; MIC ≥ 4 µg/ml

The serovar variation and susceptibility to ciprofloxacin is summarized in Table 10. The results showed the high prevalence of reduced susceptibility to ciprofloxacin in all 20 isolates of *S.* Enteritidis, in 14 isolates of *S.* Schwarzengrend and in 11 isolates of *S.* Amsterdam. There was one isolate of *S.* Senftenberg resistant to ciprofloxacin.

The prevalence of nalidixic acid and ciprofloxacin resistance in isolates from animals is summarized in Table 11. The prevalence of nalidixic acid resistance was 42.6% (106/248) and 57.42% (143/248) were susceptible. The MIC of nalidixic acid ranged from 1 to >256 µg/ml. The MIC_{50} and MIC_{90} were 16 and >256 µg/ml, respectively. Distribution of MICs for nalidixic acid in isolates from animals is shown in Figure 6. It was demonstrated that MICs of nalidixic acid-susceptible nontyphoidal *Salmonella* isolates ranged from 1 to 16µg/ml. Most of nalidixic acid-susceptible isolates (42.16%, 105/248) had the nalidixic acid MIC of 4µg/ml. Most of nalidixic acid-resistant isolates 34.13% (85/248) had nalidixic acid MIC of >256µg/ml.

Table 10 Serovar variation and ciprofloxacin susceptibility in nontyphoidal *Salmonella* isolates from animals

Serovar	No. of isolates		
	Ciprofloxacin susceptibility		
	R	I	Reduced susceptibility to ciprofloxacin
S. Enteritidis (N=22)	0	2	20
S. Schwarzengrend (N=14)	0	0	12
S. Amsterdam (N=11)	0	0	11
S. Corvallis (N=8)	0	0	7
S. Anatum (N=27)	0	0	7
S. Albany (N=6)	0	0	6
S. Give (N=5)	0	0	5
S. I (N=6)	0	0	5
S. Rissen (N=56)	0	0	4
S. Altona (N=7)	0	0	4
S. Senftenberg (N=5)	1	2	2
S. Worthington (N=3)	0	0	2
S. Kentucky (N=3)	0	0	2
S. Agona (N=4)	0	0	1
S. Infantis (N=1)	0	0	1
S. Madjorio (N=1)	0	0	1
S. Virchow (N=1)	0	0	1
S. Weltevreden (N=9)	0	0	1
S. Poona (N=1)	0	0	1
S. Panama (N=3)	0	0	1
S. Stanley (N=20)	0	0	1
S. Tennessee (N=1)	0	0	1
S. Emek (N=3)	0	3	0
S. Orion (N=1)	0	1	0

R; resistant, I; intermediate resistant

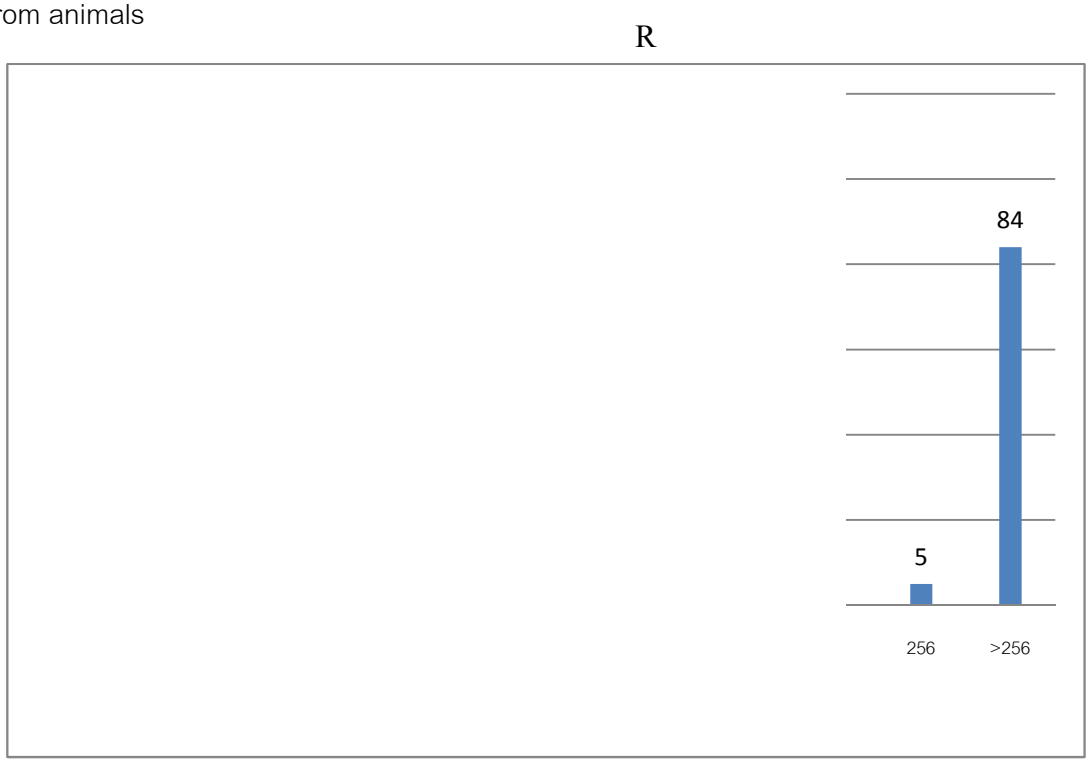
The prevalence of ciprofloxacin resistance was 0.4% (1/248) and 2.41% (6/248) were intermediate resistant. The MIC of ciprofloxacin ranged from 0.015 to 4 µg/ml. The MIC_{50} and MIC_{90} were 0.03 and 0.5 µg/ml, respectively. Distribution of MICs for ciprofloxacin in isolates from animals is shown in Figure 7. It was demonstrated that MICs of ciprofloxacin-susceptible isolates ranged from 0.015 to 2 µg/ml. Most of the ciprofloxacin-susceptible isolates had the ciprofloxacin MIC of 0.015 µg/ml. However, there was one isolate was resistant to ciprofloxacin with the MIC of 4 µg/ml.

Table 11 Nalidixic acid and ciprofloxacin MICs and resistance rate in 248 nontyphoidal *Salmonella* isolates from animals

Antimicrobial agents	MICs (µg/ml)			Susceptibility (%)		
	Range	MIC_{50}	MIC_{90}	R	I	S
Nalidixic acid	1 - >256	16	>256	106 (42.6)	0	143 (57.42)
Ciprofloxacin	0.015 - 4	0.03	0.5	1 (0.4)	6 (2.41)	242 (97.18)

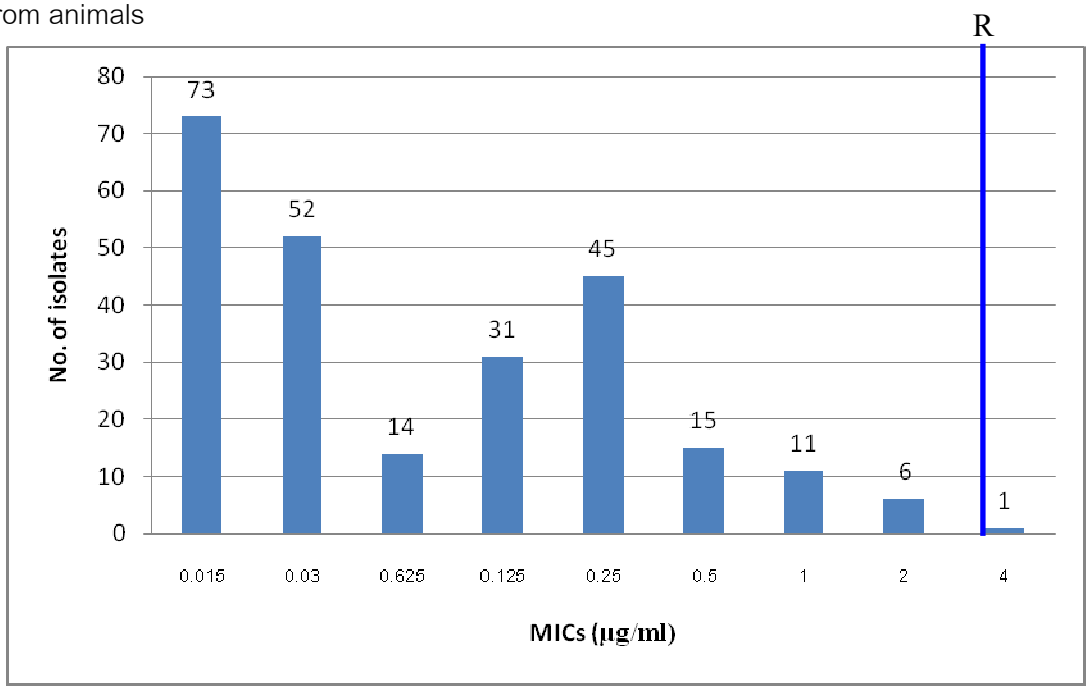
R; resistant, I; intermediate resistant, S; susceptible

Figure 6 MIC distribution of nalidixic acid among 248 nontyphoidal *Salmonella* isolates from animals



R; CLSI resistant break point for nalidixic acid MIC ≥ 32 µg/ml

Figure 7 MIC distribution of ciprofloxacin among 248 nontyphoidal *Salmonella* isolates from animals



R; CLSI resistant break point for ciprofloxacin; MIC ≥ 4 µg/ml

3. Screening for the presence of *qnr* genes by polymerase chain reaction (PCR)

A total of 356 isolates of nontyphoidal *Salmonella* were screened for the presence of *qnr* genes by using PCR. The PCR products were 627 bp for *qnrA* gene, 496 bp for *qnrB* gene and 566 bp for *qnrS* gene.

The *qnrS* gene was found in 31 isolates (8.7%). Four out of 108 isolates (0.9%) from patients had *qnrS* genes. The 4 *qnrS1*-positive isolates included one *S*. Choleraesuis isolated from stool and 3 *S*. group D isolated from blood. Other 27 (10.88%, 27/248) *qnrS1*-positive isolates from animal included *S*. Anatum (3.62%, n=9), *S*. Corvallis (2%, n=5), *S*. Emek (1.2%, n=3) and *S*. Senftenberg (1.2%, n=3), *S*. group D (0.8%, n=2), *S*. Worthington (0.8%, n=2) and, *S*. Kentucky (0.8%, n=2), *S*. Virginia (0.4%, n=1), *S*. Madjorio (0.4%, n=1), *S*. group O (0.4%, n=1) and *S*. Panama (0.4%, n=1).

4. Screening for the presence of *aac(6')-Ib-cr* genes by PCR

All 356 nontyphoidal *Salmonella* isolates were screened for the presence of *aac (6')-Ib-cr* gene by using PCR. The PCR product was 435 bp. There was no isolate from both patients and animals carried *aac (6')-Ib-cr* gene.

5. Analysis of *qnrS* genes by PCR and DNA sequencing

All 31 *qnrs*-positive nontyphoidal *Salmonella* (S2, S87, S89, S90, SSA225, SSA226, SSA227, SSA228, SSA230, SSA231, SSA233,SSA253, SA25, SA26, SA27, SA40, SA41, SA42, SA43, SA45, SA46, SA47, SA50, SA51, SA57,SA78, SA79, SA81, SA84, SA93 and SA100) with different MICs of nalidixic acid and ciprofloxacin were sequenced for *qnrS* allele. DNA sequences were analyzed by the software available over the internet at the National Center for Biotechnology Information (http;//www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/), Multiple sequence alignment was analyzed by Multalin (http://bioinfo.genopoletoulouse.prd.fr/multalin/multalin.html)

The multiple nucleotide alignment of *qnrS* allele with the submitted sequences of *qnrS1* gene from *S*. Bovismorbifican (accession no. DQ485529.1) is shown in Figure 8. DNA sequence analysis revealed the *qnrS1* allele in all of 31 isolates. Although the 566-bp fragments of *qnrS1*, not the entire gene (654 bp), were sequenced, DNA sequence analysis demonstrated that all 31 *qnrS1*-positive isolates had 100% nucleotide and amino acid identity to the *qnrS1* gene and QnrS1 submitted in GenBank, respectively. The multiple nucleotide alignments of *qnrS* alleles including *qnrS1*, *qnrS2* and *qnrS3* are shown in Figure 9. It was demonstrated that QnrS1 shared 90% and 99% of amino acid identity to QnrS2 and QnrS3, respectively.

Figure 8 Multiple nucleotide sequence alignment of *qnrS1* gene from *qnrS1* -positive isolates with those from *Salmonella* Bovismorbifican (GenBank accession no. DQ485529.1)

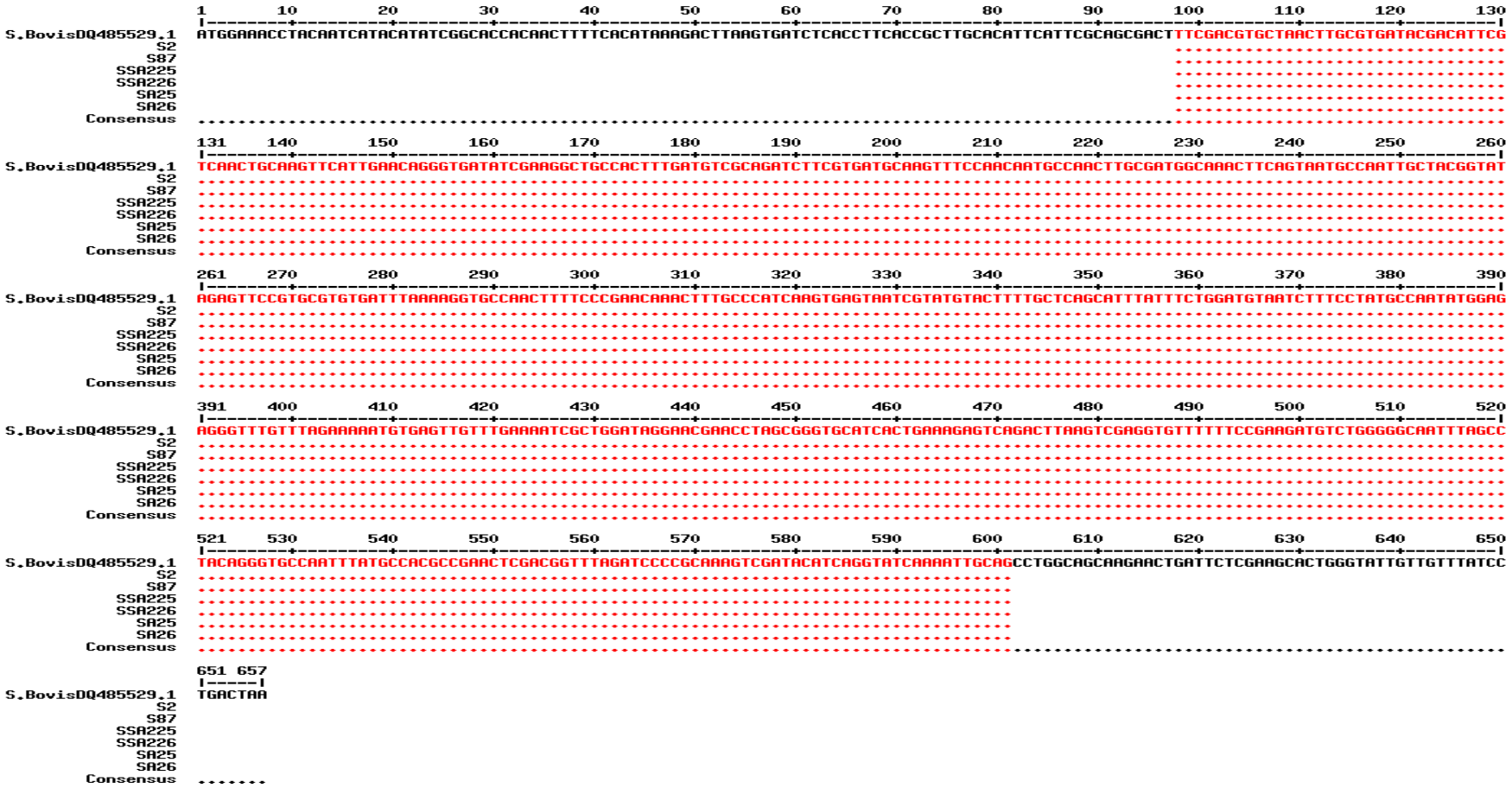


Figure 9 Multiple nucleotide sequence alignment of *qnrS1*, *qnrS2* and *qnrS3* genes with those from *S.* Bovismorbifican (GenBank accession no. DQ485529.1), *E. coli* (GenBank accession no. EU077611.1) and *S.* Anatum (GenBank accession no. DQ485530.1)

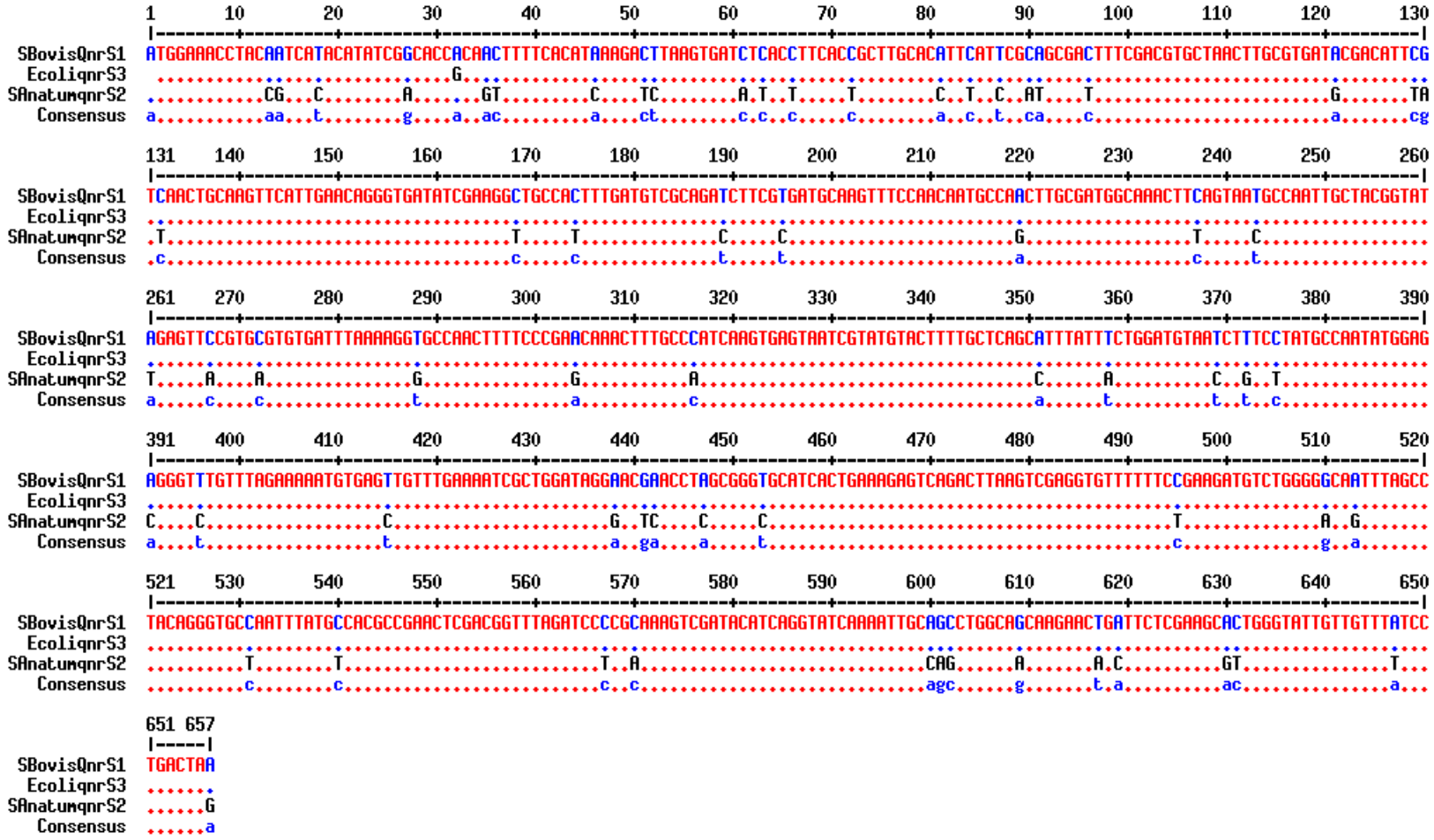


Figure 10 Multiple amino acid sequence alignment from *qnrS1*-positive isolates with those from *Salmonella* Bovismorbifican (GenBank accession no. ABF47469.1)

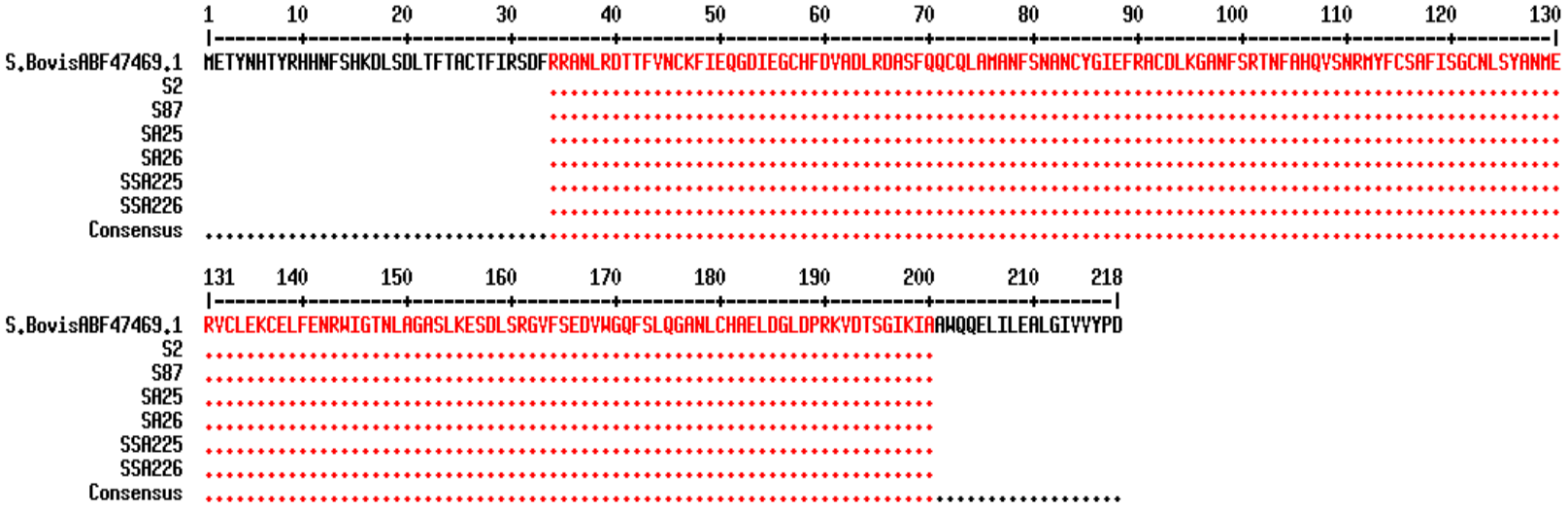
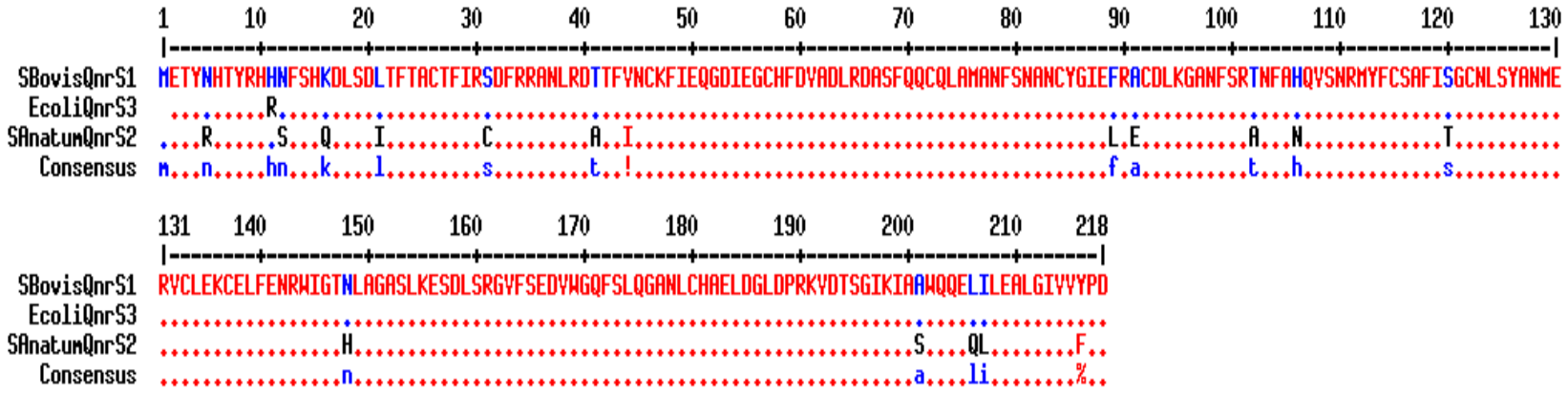


Figure 11 Multiple amino acid sequence alignment of QnrS1, QnrS2 and QnrS3 from *S.* Bovismorbificans (GenBank accession no. ABF47469.1), *E. coli* (GenBank accession no. ABU52984.1) and *S.* Anatum (GenBank accession no. ABF47470.1)



Susceptibility, serovar and source of 31 *qnrS1*-positive nontyphoidal *Salmonella* isolates obtained from WHO National *Salmonella* and *Shigella* Center are shown in Table 12. It was demonstrated that *qnrS1*-positive isolates from patients were found in both blood and stool samples. All 4 isolates from patients were resistant to nalidixic acid. Of the 4 isolates, one stool isolate had high-level nalidixic acid resistance (MIC of >256 µg/ml) and were intermediate resistant to ciprofloxacin. Other 3 blood isolates had nalidixic acid MIC of 32 µg/ml and two of these isolates had reduced susceptibility to ciprofloxacin while the other one was intermediate resistant. Other 8 *qnrS1*-positive isolates were *S.* Anatum and most of them (7/8 isolates) were isolated from swine. The result demonstrated that 2 out of 11 farms were the sources of *qnrS1* gene. The MIC of nalidixic acid for *qnrS1*-positive isolates from animals ranged from 4 to 64 µg/ml and 50% (4/8) were resistant to nalidixic acid. However, the susceptibility testing to ciprofloxacin showed wide range of ciprofloxacin MIC from 0.03 to 1 µg/ml and most of the isolates had reduced susceptibility to ciprofloxacin. One isolate carrying *qnrS1* gene had low-level ciprofloxacin MIC (MIC of 0.03 µg/ml).

Table 12 Susceptibility, serovar and source of 12 *qnrS1*-positive nontyphoidal *Salmonella* isolates obtained from WHO National *Salmonella* and *Shigella* Center

Isolates	Serovar	Origin/Source	NAL MIC (µg/ml)	CIP MIC (µg/ml)	*qnrS* gene
Patient isolates					
S2	*S.* Choleraesuis	Stool	>256	2	*qnrS1*
S87	*S.* group D	Blood	32	2	*qnrS1*
S89	*S.* group D	Blood	32	0.5	*qnrS1*
S90	*S.* group D	Blood	32	0.5	*qnrS1*
Animal isolates					
SSA227	*S.* Anatum	Swine, Farm A	64	1	*qnrS1*
SSA231	*S.* Anatum	Swine, Farm A	64	1	*qnrS1*
SSA253	*S.* Anatum	Cockroach, Farm B	64	1	*qnrS1*
SSA226	*S.* Anatum	Swine, Farm A	32	1	*qnrS1*
SSA228	*S.* Anatum	Swine, Farm A	16	0.25	*qnrS1*
SSA230	*S.* Anatum	Swine, Farm A	16	0.03	*qnrS1*
SSA233	*S.* Anatum	Swine, Farm B	8	0.5	*qnrS1*
SSA225	*S.* Anatum	Swine, Farm A	4	0.125	*qnrS1*

NAL; nalidixic acid, CIP; ciprofloxacin

Susceptibility and serovar of 19 *qnrS1*-positive nontyphoidal *Salmonella* isolates obtained from Department of Livestock Development between 2003 and 2005 is shown in Table 13. It was demonstrated that there were 10 serovars carrying *qnrS1* genes including *S.* Emek, *S.* Senftenberg, *S.* Worthington, *S.* Corvallis, *S.* Anatum, *S.* Virginia, *S.* Kentucky, *S.* Madjorio and *S.* Panama. The nalidixic acid MIC ranged from 4 to >256 µg/ml and ciprofloxacin MIC ranged from 0.03 to 2 µg/ml. There were 9 out of 12 isolates showed reduced susceptibility to ciprofloxacin and 2 isolates were intermediate resistant.

Table 13 Susceptibility and serovar of 19 *qnrS1*-positive nontyphoidal *Salmonella* isolates obtained from Department of Livestock Development between 2003 and 2005

Isolates	Serovar	NAL MIC(μg/ml)	CIP MIC (μg/ml)	*qnrS* gene
SA25	*S.* Emek	>256	2	*qnrS1*
SA26	*S.* Emek	>256	2	*qnrS1*
SA27	*S.* Emek	>256	2	*qnrS1*
SA42	*S.* Senftenberg	>256	4	*qnrS1*
SA43	*S.* Senftenberg	>256	2	*qnrS1*
SA45	*S.* Senftenberg	>256	2	*qnrS1*
SA47	*S.* Worthington	>256	0.5	*qnrS1*
SA78	*S.* Corvallis	>256	0.5	*qnrS1*
SA84	*S.* Anatum	>256	0.0625	*qnrS1*
SA100	*S.* Virginia	32	1	*qnrS1*
SA40	*S.* Kentucky	16	0.25	*qnrS1*
SA41	*S.* Kentucky	16	0.25	*qnrS1*
SA46	*S.* Worthington	16	0.5	*qnrS1*
SA50	*S.* Corvallis	16	0.25	*qnrS1*
SA51	*S.* Corvallis	16	0.5	*qnrS1*
SA57	*S.* Madjorio	16	1	*qnrS1*
SA79	*S.* Corvallis	16	0.5	*qnrS1*
SA81	*S.* Corvallis	16	0.5	*qnrS1*
SA93	*S.* Panama	16	0.5	*qnrS1*

NAL; nalidixic acid, CIP; ciprofloxacin

6. Determination of nalidixic acid and ciprofloxacin susceptibility of *qnrS1*-positive nontyphoidal *Salmonella*

The susceptibility to nalidixic acid and ciprofloxacin against 31 *qnrS1*-positive isolates is shown in Table 14. A total of 4 *qnrS1*-positive isolates from patients showed nalidixic acid resistance and reduced susceptibility to ciprofloxacin whereas none of the isolate showed resistant to ciprofloxacin. Prevalence of nalidixic acid resistance in *qnrS1*-positive isolates from animals was 53.57% (15/28) and 3.57% (1/28) showed resistance to ciprofloxacin with the MIC of 4µg/ml. Of the 31 *qnrS1*-positive isolates, 61.29% (19/31) were resistant to nalidixic acid and 70.96% (22/31) showed reduced susceptibility to ciprofloxacin and 3.22% (1/31) was resistant to ciprofloxacin.

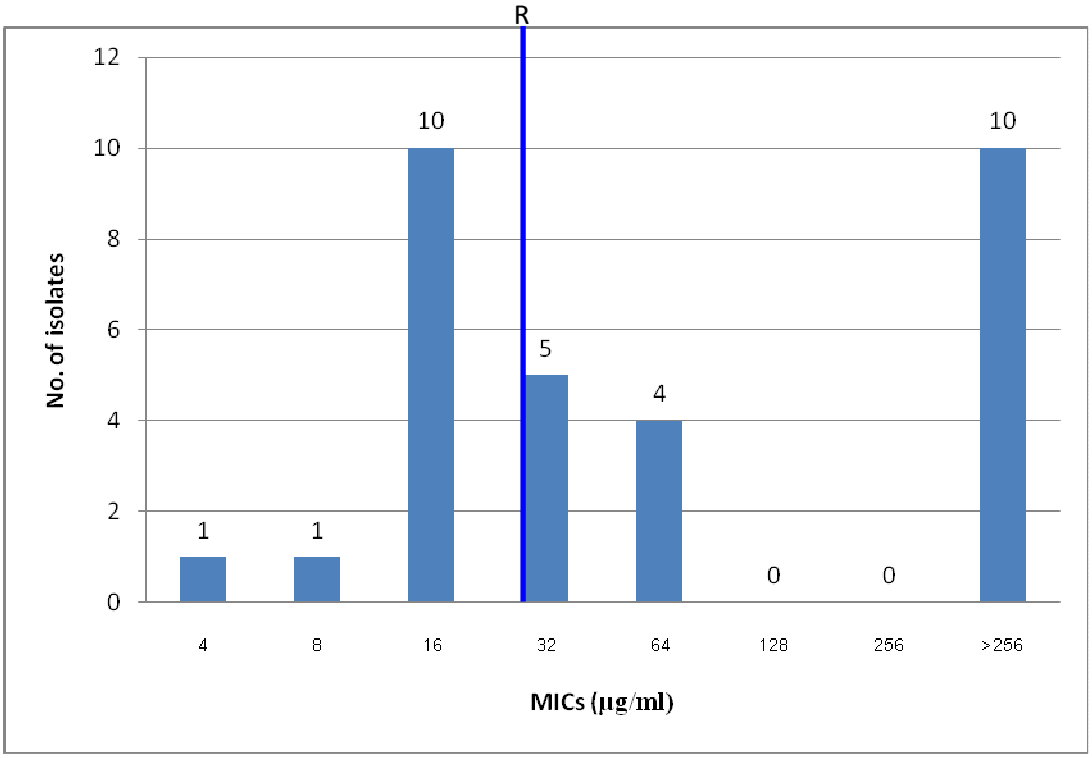
Distribution of the nalidixic acid MICs among 31 *qnrS1*-positive isolates is shown in Figure 14. It was demonstrated that MICs of nalidixic acid-susceptible isolates ranged from 4 to 16 µg/ml. Most of the nalidixic acid-susceptible isolates (31.25%, 10/31) had the MIC of 16 µg/ml and most of nalidixic acid-resistant isolates had the MIC of >256 µg/ml. Distribution of the ciprofloxacin MICs among *qnrS1*-positive isolates is shown in Figure 15. It was demonstrated that MICs of ciprofloxacin-susceptible isolates ranged from 0.03 to 1 µg/ml. Most of the ciprofloxacin-susceptible isolates (31.25%, 10/31) had the MIC of 0.5 µg/ml. There was one isolate resistant to ciprofloxacin.

Table 14 The susceptibility of nalidixic acid and ciprofloxacin against 31 *qnrS1*-positive isolates obtained from patients and animals

Bacterial Strains	No. of isolates (%)			
	Nalidixic acid resistance	Ciprofloxacin		Reduced susceptibility to ciprofloxacin
		R	I	
From Patients (n=4)	4 (100)	0	2 (50)	2 (50)
From Animals (n=27)	15 (53.57)	1 (3.5)	5 (18.51)	19 (71.42)
Total (n=31)	19 (59.37)	1 (3.1)	7 (22.58)	22 (68.75)

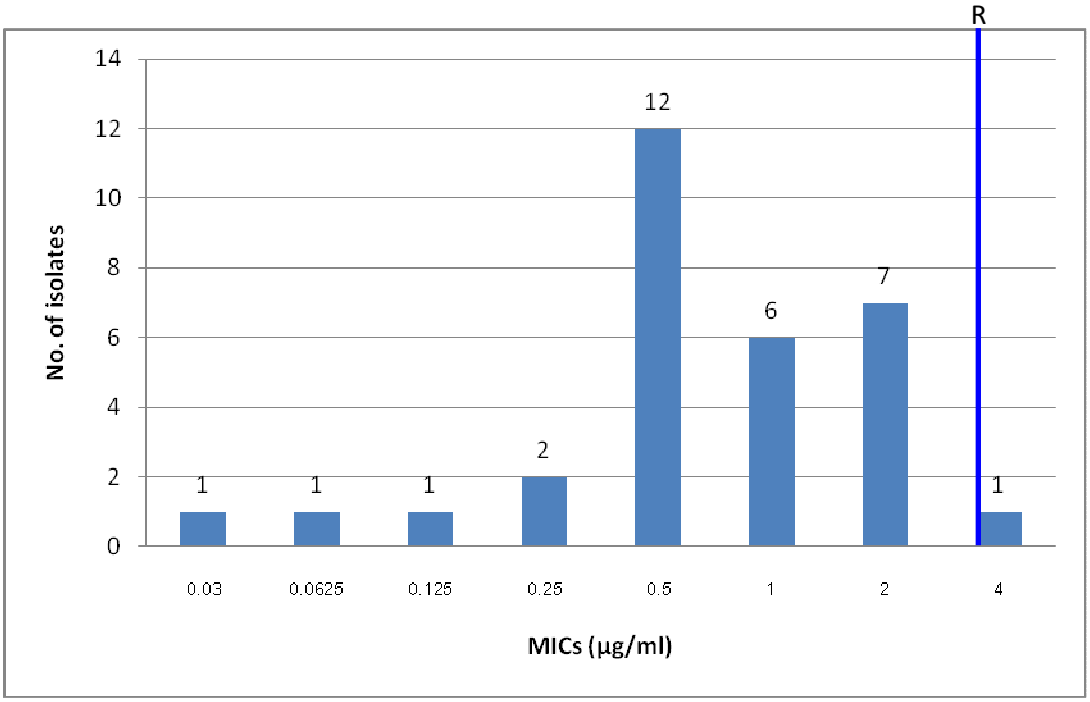
R; resistant, I; intermediate resistant

Figure 12 Distribution of nalidixic acid MIC among 31 *qnrS1*-positive isolates



R; CLSI resistant break point for nalidixic acid; MIC ≥ 32 µg/ml

Figure 13 Distribution of ciprofloxacin MIC among 31 *qnrS1*-positive isolates



R; CLSI resistant break point for ciprofloxacin; MIC ≥ 4 µg/ml

7. Analysis of mutations in QRDR of GyrA, GyrB, ParC and ParE in *qnrS1*-positive isolates

The amino acid substitutions in QRDR of DNA gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) of 31 *qnrS1*-positive isolates are shown in Table 15 and 16. Only GyrA substitutions at either S83 or D87 (*E. coli* numbering) were found in 7 *qnrS1*-positive isolates. One isolate from patient with the nalidixic acid MIC of >256 µg/ml and ciprofloxacin MIC of 2 µg/ml had D87⟶G in GyrA. Three animal isolates with the nalidixic acid MIC of >256 µg/ml and ciprofloxacin MIC of 2 µg/ml had S83⟶Y. Three animal isolates with nalidixic acid MIC of >256 µg/ml and ciprofloxacin MIC range from 2 to 4 µg/ml carried S83⟶F.

There were silent mutations in the QRDR of GyrB at G435 (ggg⟶ggc), R438 (cgc⟶cgt), K439 (aag⟶aaa), K447 (aag⟶aaa), K449 (gtc⟶gtt), F458 (ctt⟶ctc) and K460 (tcc⟶tct), in the QRDR of ParC at V67 (gtt⟶gtc), H75 (cac⟶cat), H77 (cat⟶cac) and in ParC at A117 (gcg⟶gca) and S123 (tcc⟶tct) which was not in the QRDR and in QRDR of ParE at T 500(act⟶acg) and H509(cac⟶cat). All of isolates could be found silent mutations. The results are shown in Appendices.

Table 15 Amino acid substitutions in QRDRs of GyrA, GyrB, ParC and ParE in 4 *qnrS1*-positive isolates from patients

Bacterial Strains	Amino acid substitutions					NAL MIC (µg/ml)	CIP MIC (µg/ml)
	GyrA		GyrB	ParC	ParE		
Salmonella LT2	S83	D87					
S2	-	G	-	-	-	>256	2
S87	-	-	-	-	-	32	2
S89	-	-	-	-	-	32	0.5
S90	-	-	-	-	-	32	0.5

Table 16 Amino acid substitutions in QRDRs of GyrA, GyrB, ParC and ParE in 27 *qnrS1*-positive isolates from animals

Bacterial Strains	Amino acid substitutions					NAL MIC (µg/ml)	CIP MIC (µg/ml)
	GyrA		GyrB	ParC	ParE		
Salmonella LT2	S83	D87					
SA25	Y	-	-	-	-	>256	2
SA26	Y	-	-	-	-	>256	2
SA27	Y	-	-	-	-	>256	2
SA42	F	-	-	-	-	>256	4
SA43	F	-	-	-	-	>256	2
SA45	F	-	-	-	-	>256	2
SA47	-	-	-	-	-	>256	0.5
SA78	-	-	-	-	-	>256	0.5
SA84	-	-	-	-	-	>256	0.0625
SSA227	-	-	-	-	-	64	1
SSA231	-	-	-	-	-	64	1
SSA253	-	-	-	-	-	64	1
SSA226	-	-	-	-	-	32	1
SA100	-	-	-	-	-	32	1
SSA228	-	-	-	-	-	16	0.25
SSA230	-	-	-	-	-	16	0.03125
SA40	-	-	-	-	-	16	0.5
SA41	-	-	-	-	-	16	0.5
SA46	-	-	-	-	-	16	0.5
SA50	-	-	-	-	-	16	0.25
SA51	-	-	-	-	-	16	0.5

Bacterial Strains	Amino acid substitutions					NAL MIC (μg/ml)	CIP MIC (μg/ml)
	GyrA		GyrB	ParC	ParE		
Salmonella LT2	S83	D87					
SA57	-	-	-	-	-	16	1
SA79	-	-	-	-	-	16	0.5
SA81	-	-	-	-	-	16	0.5
SA93	-	-	-	-	-	16	0.5
SSA233	-	-	-	-	-	8	0.5
SSA225	-	-	-	-	-	4	0.125

NAL; nalidixic acid, CIP; ciprofloxacin

8. *IN VITRO* SELECTION OF FLUOROQUINOLONE RESISTANCE

The *in vitro* selection of ciprofloxacin was performed by multiple exposures of 10 representative nontyphoidal *Salmonella* isolates to ciprofloxacin with the 2x higher concentration than that of parent ciprofloxacin MIC. The results are described in Table 14. The *in vitro* selection included 5 *qnrS1*-positive and 5 *qnrS1*-negative strains. The results demonstrated that in the *qnrS1*-positive isolates, S89 (parent strain) the ciprofloxacin MIC was increased from 0.5 to 64 µg/ml (128-fold increased) and the amino acid substitution in QRDR of GyrA was found at S83⟶F in the first-generation selection (8-fold increased) and additional mutation at D87⟶G was found at the second-generation selection (4-fold increased). There were three isolates including, S227, SA25 and SA84 in *qnrS1*-positive group which the ciprofloxacin MIC increased for 32-fold. There was 4-fold increased in ciprofloxacin MIC in the third-generation selection but no additional mutations in QRDR of GyrA was detected. In S227, the ciprofloxacin MIC was increased from 1 to 32 µg/ml (32-fold increased) and the amino acid substitutions in QRDR was found at S83⟶F in the first-generation selection. In the second-generation selection (2-fold increased), no additional mutation was found. In the third-generation selection (2-fold increased) additional was found at D87⟶G. In SA25, the ciprofloxacin MIC was increased from 2 to 64 µg/ml (32-fold increased) and the amino acid substitution was found at D87⟶G in the first-generation selection. In the second- and third generation selection, the MIC increased 4- and 2- fold, respectively, but no additional mutations was found. In SA84, the ciprofloxacin MIC was increased from 0.06 to 0.5 µg/ml in the second-generation mutant (8-fold increased). However, no mutation in QRDR was found in both first- and second- generation selection but in the third-generation selection (4-fold increased), the GyrA mutation was found at S83⟶Y. In SA42, the ciprofloxacin was increased from 4 to 64 µg/ml (16-fold increased) and the amino acid substitution was found at D87⟶G in the first-generation selection. In the second- and third-generation selection, the MIC increased for 2-fold for both generation selections but there was no additional mutation.

In *qnrS1*-negative group, ciprofloxacin MIC of SA48 was increased from 0.5 to 32 µg/ml (64-fold increased in MIC) and the amino acid substitution was found at S83⟶F in the first-generation selection. In the second-generation selection (2-fold increased in MIC), additional mutation was found at D87⟶G and no additional mutation was found in the third-generation selection (4-fold increased in MIC). The ciprofloxacin MIC of S18, S62 and SA8 increased for 32-fold. In S18, the ciprofloxacin MIC was increased from 0.125 to 4 µg/ml but the amino acid substitution was not found in the first-generation selection. In the second-generation selection (2-fold increased in MIC), the mutation was found at S83⟶Y and no additional mutation was found in the third-generation selection (4-fold increased in MIC). In S62, the ciprofloxacin MIC was increased from 0.5 to 16 µg/ml and the amino acid substitution was found at S83⟶F in the first-generation selection. No additional mutation was found in the second- (2-fold increased in MIC) and third-generation (4-fold increased in MIC) selection. In SA8, the ciprofloxacin MIC was increased from 0.125 to 4 µg/ml and the amino acid substitution was not found in the first- (4-fold increased in MIC) and second-generation (2-fold increased in MIC) selection but the mutation was found in the third-generation selection (4-fold increased) at S83⟶F. In S12, the ciprofloxacin MIC was increased from 2 to 32 µg/ml (16-fold increased in MIC) and the amino acid substitution was found at D87⟶G in the first-generation selection (4-fold increased in MIC) but no additional mutation was found in both of the second- (2-fold increased in MIC) and third-generation (2-fold increased in MIC) selection.

Table 17 *In vitro* selection of ciprofloxacin resistance

Bacterial strains	Ciprofloxacin MIC (μg/ml)	No. of fold increased	GyrA amino acid substitutions		*qnrS* gene
S. Typhimurium LT2			S83	D87	
S89	0.5	-	S	D	*qnrS1*
S89.1	4	8	F	D	
S89.2	16	4	F	G	
S89.3	64	4	F	G	
S227	1	-	S	D	*qnrS1*
S227.1	8	8	F	D	
S227.2	16	2	F	D	
S227.3	32	2	F	G	
SA25	2	-	Y	D	*qnrS1*
SA25.1	8	4	Y	G	
SA25.2	32	4	Y	G	
SA25.3	64	2	Y	G	
SA42	4	-	F	D	*qnrS1*
SA42.1	16	4	F	G	
SA42.2	32	2	F	G	
SA42.3	64	2	F	G	
SA84	0.06	-	S	D	*qnrS1*
SA84.1	0.25	4	S	D	
SA84.2	0.5	2	S	D	
SA84.3	2	4	Y	D	

Bacterial strains	Ciprofloxacin MIC (μg/ml)	No. of fold increased	GyrA amino acid substitutions		*qnrS* gene
S. Typhimurium LT2			S83	D87	
S12	2	-	Y	D	-
S12.1	8	4	Y	G	
S12.2	16	2	Y	G	
S12.3	32	2	Y	G	
S18	0.125	-	S	D	-
S18.1	0.5	4	S	D	
S18.2	1	2	Y	D	
S18.3	4	4	Y	D	
S62	0.5	-	S	D	-
S62.1	2	4	F	D	
S62.2	4	2	F	D	
S62.3	16	4	F	D	
SA48	0.5	-	S	D	-
SA48.1	4	6	F	D	
SA48.2	8	2	F	G	
SA48.3	32	4	F	G	
SA8	0.125	-	S	D	-
SA8.1	0.5	4	S	D	
SA8.2	1	2	S	D	
SA8.3	4	4	F	D	

.1; first-generation, .2; second-generation, .3; third-generation

S; serine, F; phenylalanine, Y; tyrosine, D; aspartic acid, G; glycine

CHAPTER VI

DISCUSSIONS

Fluoroquinolones has become the drug of choice for treatment of nontyphoidal *Salmonella* infection. Because of the increase of older drugs resistance including ampicillin, chloramphenicol, trimetroprim-sulfamethoxazole, the newer and more activity antibiotics such as ciprofloxacin are become more effective against *Salmonella* infection. However, fluoroquinolone-resistant strains of nontyphoidal *Salmonella* are increasing worldwide. This was considered to be the effect of excessive antibiotics use in environment, especially in animals. There were many evidence of transmission of nontyphoidal *Salmonella* and other gastrointestinal infection pathogens from animals to human by eating contaminated food [39]. Furthermore, on the account of the use of antibiotics such as fluoroquinolones in animal farm, this can cause the transmission of the fluoroquinolone-resistant pathogens into human. As a result, there were many reports of treatment failure in patients infected by the pathogens [166-169].

In this study, *S*. Choleraesuis (37.07%, 40/108) followed by *S*. group D (34.25%, 37/108) was the most common serovar in isolates from patients. On the other hand, other studies in Thailand and south-east Asia (1993-2002) reported that the most common serovar was *S*. Weltevreden and *S*. Enteritidis. *S*. Enteritidis was the member of *S*. group D, therefore, serotyping should be further determined in our isolates. Other studies also found the increasing number of *S*. Rissen and *S*. Stanley in patient isolates [30, 65].

The most common serovar in animal isolates was *S*. Rissen (22.17%, 55/248) followed by *S*. Enteritidis (8.06%, 20/248). This finding was different from previous studies reported by PulseNet that the most common serovar in isolates from animal food was

S. Typhimurium [36-38]. According to the reports worldwide, nontyphoidal *Salmonella* infection has been increasingly reported. In 2005, the study in Isarael found *S*. agona as an outbreak strains with the decrease of report of *Salmonella* Typhi infection. In the study in 1997 to 2004, the most common serovar was *S*. Virchows in both isolates from human and farm animals [38] but in this study we found only one *S*. Virchow in animal isolate. In animal isolates obtained from WHO *Salmonella* and *Shigella* Center, *S*. Rissen (36.42%, 55/151) was the most common serovar and the most common source of *S*. Rissen was swine (47.6%), followed by chicken (30%). Similar to the present study, *S*. Rissen was the most common serovar among the isolates from food source in 2008 in Thailand and Denmark [1]. The second most common serovar was *S*. Anatum (15.23%, 23/151) which was commonly isolated from swine. In other animal isolates obtained from the Department of Livestock, the most common serovar was *S*. Enteritidis (20.61%, 20/97) and *S*. Bovismorbificans (9.27%, 9/97). This study suggested that the presence of the same serovars in both animal and patient isolates could be referred to the relationship between animal food and human pathogen.

In this study, antimicrobial susceptibility of nalidixic acid and ciprofloxacin against 356 isolates of nontyphoidal *Salmonella* has been investigated. The susceptibility testing showed the high prevalence of nalidixic acid resistance (86.1%) in 108 nontyphoidal *Salmonella* isolates from patients. The MIC_{50} and MIC_{90} revealed that most isolates showed high-level nalidixic acid resistance.

Our results were similar to the previous study in nontyphoidal *Salmonella* isolates from bacteremic patients at King Chulalongkorn Memorial Hospital in 2003 to 2005 which showed high resistance rate to nalidixic acid and reduced susceptibility to ciprofloxacin [51]. In the present study, the prevalence of nalidixic acid resistance among 248 isolates of nontyphoidal *Salmonella* isolates from animals was 42.7%. Ciprofloxacin resistance in animal isolates was found in 0.4% (1 isolate, *S*. Senftenberg) which showed ciprofloxacin MIC of 4µg/ml. Previous study in France reported that *S*. Typhimurium isolates in three

cases of patients were resistant to ciprofloxacin [7] which is similar to another study from Europe, Middle East, USA and Thailand which showed that the high prevalence of ciprofloxacin or multidrug resistance was mostly found in *S.* Enteritidis, *S.* Typhimurium and *S.* Virchow [170].

We also found the high prevalence of reduced susceptibility to ciprofloxacin among 108 isolates from patients. Prevalence of reduced susceptibility to ciprofloxacin was 72.2% (78/108) in isolates from patients and 41.12% (102/248) in isolates from animals. The ciprofloxacin MIC_{50} and MIC_{90} demonstrated that most of the nontyphoidal *Salmonella* isolates had reduced susceptibility to ciprofloxacin (MIC of 0.125-1 µg/ml). Our results are similar to the multinational study in Asia which showed that reduced susceptibility to ciprofloxacin was commonly found in *S.* Choleraesuis and *S.* Virchow and reduced susceptible nontyphoidal *Salmonella* isolates were commonly found in Taiwan and Thailand [171].

The screening for the presence of *qnrS* and *aac(6')-ib-cr* genes was also investigated. There were 31 (8.7%) out of 356 isolates carrying *qnrS* genes. The prevalence of *qnrS* gene in nontyphoidal *Salmonella* was 0.9% (4/108) in patient isolates and 10.88% (27/248) in animal isolates. The multiple alignment of amino acid showed the conserved sequences among QnrS allele in various kinds of bacteria. Our results showed that 566-bp fragments of *qnrS* of all 31 *qnrS*-positive isolates had 100% nucleotide and amino acid identity to *qnrS1* gene and QnrS1 submitted to GenBank. The study by Covaco *et al*. showed that *qnrs1* was found in 2.29% of 175 *Salmonella* isolates and all of *qnrS1*-positive isolates were *S.* Saintpaual [18] in isolates from meat in Germany. There were many reports of *qnrS* genes in various serovars of *Salmonella* spp. In the United Kingdom, the screening for *qnr* in 118 isolates of *Salmonella enterica* showed that six isolates (5%) carried *qnrS1* [20]. In Turkey, the study of nine isolates of *Salmonella* Virchow from chicken meat found three isolates carrying *qnrS1* [4]. From the study in the Netherlands, 39 isolates of *Salmonella* spp. from humans and animals with ciprofloxacin MIC of 0.25-21 µg/ml and

nalidixic acid MIC of 8-16 µg/ml had one *qnrB2*, two *qnrB5* and 31 *qnrS1*, all of which were from isolates with human origin except *qnrB2*, and the predominant strain was *Salmonella* Corvallis [136]. In Germany, it was reported that *Salmonella* Infantis isolated in 2004 was carried *qnrS* [134].

For *qnrA* genes in *Salmonella* spp. were also reported in many countries. The qnrA was firstly identified in *Salmonella* Enteritidis isolated in 2003 in Hong Kong, China [117]. The study of 335 nontyphoidal *Salmonella* isolated in 1996-2003 in the United States was found that ten isolates carried *qnrB* or *qnrS* genes with ciprofloxacin MIC $\geq$ 0.06 µg/ml [19]. The screening of of *qnr* genes in six isolates of *Salmonella enterica* isolated from Finnish patients traveled to Malaysia and Thailand found that one strain carried *qnrA*, two strains carried *qnrS* and three strains had both *qnrB* and *qnrS*. Another study in *Salmonella enterica* isolated from meat of different animals in 2007-2008 in Denmark reported that 3 strains of *Salmonella* Newport and 4 strains of *Salmonella* Hadar carried *qnrB5* and 4 strains of *Salmonella* Saintpaul haboured *qnrS1* [18]. The screening of *qnr* in 160 strains of *Salmonella* isolted in 2004-2006 in Italy showed that one isolate of *Salmonella* Typhimurium carried *qnrB* [140]. These reports showed that there was more prevalence of *qnrB* genes among *Salmonella* spp. than the *qnrA* and *qnrS* genes and the most common serovar found to be carrying *qnrS1* gene included *S*. Saintpaual, *S*. Virchow, *S*. Corvallis and *S*. Infantis. In our study, we also found *qnrS1* gene in *S*. Choleraesuis which was previously reported carrying *qnrA3* [117, 172]. We found *qnrS1* in serovars which have not been reported before including, *S*. Emek, *S*. Senftenberg, *S*. Worthington, *S*. Kentucky, *S*. Virginia, *S*. Madjorio, *S*. group O and *S*. Panama. *S*. Anatum was previously reported to be carrying *qnrS2* gene. The presence of *qnrS4* was reported in *Salmonella* Stanley [108]. The *qnrS1* gene was also found in *Shigella flexneri* 2b [93] and *qnrS3* was found in *E.coli*. The presence of all variant of *qnr* genes could be found in many serovars of *Salmonella* spp. worldwide especially *qnrB* allele, and *qnrB* was the most common of *qnr* genes found in many pathogens.

Our results showed that isolates from animals had higher prevalence of *qnrS1* gene than isolates from patients. Eight *qnrS1*-positive animal isolates obtained from WHO *Salmonella* and *Shigella* Center were *S*. Anatum. Of the 19 *qnrS1*-positive isolates obtained from the Department of livestock between 2003 and 2005, S. Corvallis was the most common isolates. The results were in agreement with the study of *Salmonella* Corvallis isolated from animals and patients in Denmark which found that all 23 isolates (100%) carried *qnrS1* [16]. This suggested that *S*. Corvallis might be the reservoir of *qnrS1* genes in Thailand. The geographic distributions of *qnrS1* genes could be found worldwide, however there were few studies on the presence of *qnr* gene in South East Asia.

We also screened for the presence of *aac(6')-Ib-cr* genes in all of 356 nontyphoidal *Salmonella*. There was no isolate carrying this gene. However, there was no previous report of this gene in *Salmonella* spp. The *aac(6')-Ib-cr* genes was firstly reported in *E. coli* producing CTX-M-15 isolated in the UK [142] and in *K. pneumoniae* (2) producing CTX-M-15. The screening of *aac(6')-Ib-cr* genes in 313 isolates of Enterobacteriaceae with MIC of ciprofloxacin > 0.25 µg/ml showed that *aac(6')-Ib-cr* was present in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae* isolates and 12 (7.5%) of 160 *Enterobacter* isolates. This gene was also identified in *Aeromonas* spp. isolated from zoo animals in Japan in 2006 [114] and in ESBL-producing *E. coli* and *K. pneumonia* isolated in Korea.

Among the *qnrS1*-positive isolates, we found that 70.97% (22/31) showed reduced susceptibility to ciprofloxacin. The prevalence of ciprofloxacin reduced susceptibility was 50.56% (180/356), therefore; in the isolates carrying *qnrS1* gene showed in 12.22% (22/180) reduced susceptibility to ciprofloxacin. The sequencing analysis showed that 8 out of 31 isolates was highly resistant to nalidixic acid (MIC of $\geq$64 µg/ml) but had low ciprofloxacin MIC. This suggesting that the *qnrS1* gene was not the common cause of ciprofloxacin reduced susceptibility so there must be other mechanisms involved in ciprofloxacin resistance. The study of QRDR mutations in GyrA showed that the isolates with nalidixic acid MIC of >256 µg/ml and had ciprofloxacin MIC of 2 to 4 µg/ml found at least one

mutation in QRDR of GyrA. There were three isolates with nalidixic acid MIC of 32 to >256 µg/ml no mutation in QRDR of GyrA and all of them had the ciprofloxacin MIC of 0.06 to 1 µg/ml. This suggested that there were other resistance mechanisms involved or it might be due to the presence of *qnrS1* gene.

The *in vitro* selection of fluoroquinolone resistance was performed to study the effect of the *qnrS1* genes on the resistance mechanisms. Previous study demonstrated that the presence of *qnr* gene could help the pathogens facilitate the resistance to fluoroquinolones [161]. The results demonstrated that 4 out of 5 *qnrS1*-positive parent strains raised ciprofloxacin MIC to 64 µg/ml whereas 3 out of 5 *qnrS1*-negative parent strains raised ciprofloxacin MIC to $\leq$ 16 µg/ml in the third-generation selection. The amino acid substitutions at S83 included S83⟶F and S83⟶Y followed by D87⟶G in GyrA were found when the MIC increased. We found that the single S83 or D87 mutation led to the increase of ciprofloxacin MIC from 1- to 4- fold and the isolates with mutation at S83⟶F (MIC of 4 µg/ml) showed higher ciprofloxacin MIC compared to at S83⟶Y (MIC of 1 to 2 µg/ml). This phenomenon was described previously in *Salmonella* spp. that isolates with one mutation exhibited reduced susceptibility or intermediate resistant to ciprofloxacin [65].

We also found double mutations in GyrA at S83⟶F,Y and D87⟶G in the isolates with ciprofloxacin MIC of 8 to 32 µg/ml which was similar to other studies which was study on the effect of mutations acquired when the MIC of fluoroquinolone increased or the mutations found in ciprofloxacin-resistant strains [8, 27, 30, 33, 63, 65, 173, 174] [29, 30, 63, 65]. This finding was previously described in *Salmonella* spp. that additional mutations at D87 conferred higher MIC than single mutation at S83 [65]. We found the additional mutation at D87⟶G in the parent strains carrying GyrA mutation at S83⟶F, Y in the second-generation selection. This suggested the primary target of alteration was at S83.

The double mutations in GyrA (S83 and D87) led to significant increase in ciprofloxacin MIC which was similar to the previous study in both *E. coli* [175] and *Salmonella* spp. [67]. However, there was an increase in ciptofloxacin MIC without any mutationin GyrA, suggesting that there were other mechanisms involved in the development of resistance including mutations in QRDR of ParC, overexpression of efflux system or decreased outer membrane porin. The mutation frequency of *qnrS1*–positive and *qnrS1*–negative strains should be further performed to evaluate the role of *qnrS1* in the development of ciprofloxacin resistance.

CHAPTER VII

CONCLUSIONS

Infection by nontyphoidal *Salmonella* was estimated to cause a larger burden worldwide. Due to increasing of antibiotics use in animal farm, the infection by antibiotic resistance strains was considered to be acquired via food chain. Nowadays, an increase in resistance to fluoroquinolones, the drug of choice for treatment *Salmonella* infection, has been reported. The most important of resistance mechanism was the mutations in genes encoding for DNA gyrase (*gyr*A and *gyr*B) and topoisomerase IV (*par*C and *par*E). The amino acid substitutions which were related to resistance included the GyrA mutations at amino acid position 83 (Ser83⟶Phe, Tyr, Ala) and 87 (Asp87⟶Gly, Asn, Tyr and Ala). The combination of substitutions at positions 83 and 87 conferred high-level fluoroquinolone resistance. The other mechanism was the presence of *qnr* and *aac(6')-Ib-cr* genes which were plasmid-mediated resistance mechanisms. Therefore, the horizontal fluoroquinolone resistance genes transfer is of increasing clinical concern.

In this study, we investigated the prevalence of plasmid-mediated quinolone resistance genes in 356 isolates from patients and animals in Thailand.

S. Choleraesuis was the most common serovar (37.07%, 40/108) found in patient isolates. Reduced susceptibility to ciprofloxacin was commonly found in *S.* Choleraesuis (30.56%, 33/108), followed by *S.* group D (34.25%, 37/108). The prevalence of nalidixic acid resistance in patient isolates was 86.1% with MIC range of 1 to >256 µg/ml. The MIC_{50} and MIC_{90} were both >256 µg/ml. The MIC range for ciprofloxacin susceptibility was 0.015 to 2 µg/ml and the MIC_{50} and MIC_{90} were 0.125 and 0.5 µg/ml, respectively.

There was no isolate from patients resistant to ciprofloxacin but 4.62% (5/108) were intermediate-resistant. *S.* Choleraesuis was dominant serovar which showed reduced susceptibility to ciprofloxacin.

The most common serovar in animal isolates was *S.* Rissen (22.17%, 55/248), followed by *S.* Enteritidis (8.06%, 20/248). The prevalence of nalidixic acid resistance was 42.6% and the MIC ranged from 1 to >256 µg/ml. The MIC_{50} and MIC_{90} were 16 and >256 µg/ml, respectively. The prevalence of ciprofloxacin resistance was 0.4% (1/248) and 2.41% (6/248) were intermediate resistant. The MIC of ciprofloxacin ranged from 0.015 to 4 µg/ml. The MIC_{50} and MIC_{90} were 0.03 and 0.5 µg/ml, respectively.

Prevalence of the *qnrS* genes in 356 nontyphoidal *Salmonella* isolates was 8.7% (31 isolates). This included 0.9% (4/108) from patient isolates(1 *S.* Choleraesuis and 3 *S.* group D isolates) and 10.88% (27/248) were found in animal isolates, including *S.* Anatum (3.62%), *S.* Corvallis (2%), *S.* Emek (1.2%) and *S.* Senftenberg (1.2%), *S.* group D (0.8%), *S.* Worthington (0.8%) and, *S.* Kentucky (0.8%) and *S.* Virginia (0.4%), *S.* Madjorio (0.4%), *S.* group O (0.4%) and *S.* Panama (0.4%). Two out of 11 farms were the sources of *qnrS* and the majority of isolates were isolated from swine. Prevalence of nalidixic acid resistance was 53.57% (15/28) in *qnrS*-positive animal isolates and 3.57% (1/28) was resistance to ciprofloxacin with the MIC of 4 µg/ml. The result showed high rate (75%, 21/28) of reduced susceptibility to ciprofloxacin among *qnrS*-positive animal isolates. Of all *qnrS1*-positive strains, 61.29% (19/31) were resistant to nalidixic acid and 70.96% (22/31) showed reduced susceptibility to ciprofloxacin. The DNA sequence analysis revealed the *qnrS1* allele in all of 31 *qnrS*-positive isolates.

The amino acid substitutions in QRDR of GyrA of *qnrS1*-positive isolates showed that one isolate from patient had D87⟶G. Three aminal isolates had amino acid substitution at S83⟶Y and other 3 isolates carried S83⟶F . There was no amino acid substitution found in QRDR of GyrB, ParC and ParE of all 31 *qnrS1*-positive isolates. However, there were silent mutations in QRDR of GyrB (G435, R438, K439, K447, K449, F458 and K460), ParC (V67, H75, H77) and ParE at T500 and H509. There were silent mutations in ParC at A117 and S123 which were out of the QRDR.

The *in vitro* selection of ciprofloxacin resistance demonstrated that 4 out of 5 *qnrS1*-positive parent strains raised ciprofloxacin MIC to 32-64 µg/ml in the third-generation selection whereas 2 out of 5 *qnrS1*-negative parent strains raised ciprofloxacin MIC to 32 µg/ml. The results showed that there were single amino acid substitutions in GyrA at S83⟶F, Y and D87⟶G when the MIC increased to $\geq$ 1 µg/ml. The double mutations at S83 and D87 conferred higher ciprofloxacin MIC. However, there was an increase in ciptofloxacin MIC without any mutation in GyrA, suggesting that there were other mechanisms involved in the development of resistance such as mutations in QRDR of ParC, overexpression of efflux system and decreased outer membrane porins.

This study was the first report of the prevalence of *qnrS1* genes in nontyphoidal *Salmonella* in patient and animal isolates in Thailand. Our findings revealed the high rate of resistance to nalidixic acid in both patient and animal isolates and high rate of reduced susceptibility to ciprofloxacin which was the drug of choice for treatment of *Salmonella* infection. The serovars of *Salmonella* found in both patient and animal isolates showed the possibility of infection through food chain. The finding of plasmid-mediated fluoroquinolone resistance genes, *qnrS1*, showed the clinical concern of horizontal genes transfer from food animal to human pathogens.

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APPENDICES

APPENDIX A

REAGENTS, MATERIALS AND INSTRUMENTS

A. REAGENTS

Absolute ethanol	(Merck, Germany)
Agarose	(Biorad, USA)
Brain heart infusion agar	(Oxoid, England)
EDTA	(Ameresco, USA)
Ethidium bromide	(Ameresco, USA)
Tryticase soy agar	(Oxoid, England)
McConkey agar	(Oxoid, England)
NaCL	(Merck, Germany)
Tris	(Ameresco, USA)

B. METERIALS

-

C. INSTRUMENTS

Water bath	(Memmert, USA)
Perkin Elmer GeneAmp PCR system 9600	(Perkin Elmer, USA)
Camera Gel Doc TM MZL	(BIO-RAD, USA)
Incubator	(BIO-RAD, USA)
Microcentifuge	(Eppendorf,USA)
Spectrophotometer	(BIO-RAD, USA)

APPENDIX B

MEDIA, SOLUTION AND IDENTIFICATION PRODURES

1. Trypticase soy agar

Trypticase agar base	40 g/L
Distilled water	1000 ml

The medium was sterilized by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 50oC. Dispense 20 ml per Petri dish. Cool and store at 4°C until used.

2. McConkey agar

McConkey agar base	51.1 g/L
Distilled water	1000 ml

The medium was sterilized by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. The sterile medium was cooled to 50oC. Dispense 20 ml per Petri dish. Cool and store at 4°C until used.

3. Sterile saline solution

Sodium chloride	8.5 g/L
Distilled water	1 L

The solution was sterilized by autoclaving at 121°C, 15 pound/inch^2 pressure, for 15 minutes. Store at room temperature.

4. Antibiotic solution preparation

Nalidixic acid

Prepare a stock solution ; dissolve 0.0128 g in 2.5 ml distilled water

Ciprofloxacin

Prepare a stock solution ; dissolve 0.0128 g in 2.5 ml distilled water

APPENDIX C

REAGENTS AND PREPARATION

1. 5X Tris-borate buffer (TBE)

Tris base	54 g/L
Boric acid	27.5 g/L
0.5 M EDTA	20 ml

Adjust volume to 1 liter with distilled water, The solution was mixed and sterilized by autoclaving at 121^{o}C for 15 min.

2. 0.5 MEDTA (pH 8.0)

Disodium ethylene diamine tetra-acetate $2H_2O$	186.1 g/L
Distilled water	1 L

Adjust pH to 8.0 and volume to 1 liter. Store at room temperature for no longer than 1 year.

3. 1.5% Agarose gel

Agarose	1.5 g
0.5X BE	100 ml

Dissolved by heating in microwave oven and occasional mix unit no granules of agarose are visible.

4. 6X Loading buffer 100 ml

Tris HCL	0.6 g
EDTA	1.68 g
SDS	0.5 g
Bromphenol Blue	0.1 g
Sucrose	40 g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtubes and store at 4^{o}C.

5. Reagent for PCR product purification

Buffer PE

Buffer PE is supplied as a concentrate. Before using for the first time, add 55 ml of ethanol (96-100%) to buffer PE concentrate as indicated on the bottle.

Buffer PB (Ready to used)

APPENDIX D

THE RESULTS OF ALL TESTS IN THIS STUDY

Results of antibiotic susceptibility of *Salmonella* spp. patient isolates obtained from WHO National *Salmonella* and *Shigella* Center (NSSC) (Bangkok, Thailand) in 2005 (S1-S44).

No. of isolates	Serovar	Nalidixic acid (R $\geq$ 32)	Ciprofloxacin (R$\geq$ 4)
S1.	*S.* Choleraesuis	>256	0.25
S2.	*S.* Choleraesuis	>256	2
S3.	*S.* Enteritidis	>256	0.5
S4.	*S.* Choleraesuis	>256	0.25
S5.	*S.* Choleraesuis	>256	0.5
S6.	*S.* Enteritidis	>256	0.5
S7.	*S.* Typhimurium	>256	0.25
S8.	*S.* Enteritidis	>256	0.25
S9.	*S.* Enteritidis	>256	0.25
S10.	*S.* Choleraesuis	>256	0.25
S11.	*S.* Enteritidis	>256	0.5
S12.	*S.* Choleraesuis	>256	2
S13.	*S.* Choleraesuis	>256	0.5
S14.	*S.* Choleraesuis	>256	0.5

No. of isolates	Serovar	Nalidixic acid (R ≥ 32)	Ciprofloxacin (R≥ 4)
S15.	*S.* Enteritidis	>256	0.25
S16.	*S.* Choleraesuis	>256	0.25
S17.	*S.* Choleraesuis	>256	0.5
S18.	*S.* Choleraesuis	>256	0.125
S19.	*S.* Choleraesuis	>256	0.5
S20.	*S.* Enteritidis	>256	0.5
S21.	*S.* Enteritidis	64	0.0625
S22.	*S.* Enteritidis	>256	0.25
S23.	*S.* Choleraesuis	>256	0.125
S24.	*S.* Enteritidis	>256	0.125
S25.	*S.* Enteritidis	>256	0.5
S26.	*S.* Choleraesuis	>256	0.25
S27.	*S.* Enteritidis	>256	0.5
S28.	*S.* Choleraesuis	>256	0.25
S29.	*S.* Choleraesuis	>256	1
S30.	*S.* Choleraesuis	>256	0.5
S31.	*S.* Choleraesuis	>256	0.5
S32.	*S.* Enteritidis	>256	0.5
No. of	Serovar	Nalidixic acid (R ≥ 32)	Ciprofloxacin (R≥ 4)

isolates			
S33.	*S.* Enteritidis	>256	0.25
S34.	*S.* Choleraesuis	>256	0.25
S35.	*S.* Choleraesuis	>256	0.5
S36.	*S.* Choleraesuis	>256	0.5
S37.	*S.* Enteritidis	>256	0.5
S38.	*S.* Enteritidis	>256	0.5
S39.	*S.* Enteritidis	>256	0.5
S40.	*S.* Enteritidis	>256	0.25
S41.	*S.* Choleraesuis	>256	1
S42.	*S.* Choleraesuis	>256	0.125
S43.	*S.* Enteritidis	>256	0.125
S44.	*S.* Choleraesuis	>256	0.125

Results of antibiotic susceptibility of *Salmonella* spp. patient isolates collected from haemoculture of patients in the King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between August 2005 and May 2006 (S45-S108).

No. of isolates	Serovar	Nalidixic acid (R≥32)	Ciprofloxacin (R≥4)
S45.	*Salmonella* gr. E	4	0.015625
S46.	*Salmonella* gr. D	>256	0.25
S47.	*Salmonella* gr.B	4	0.25
S48.	*Salmonella* gr.D	256	0.25
S49.	*Salmonella* gr.D	256	0.5
S50.	*Salmonella* gr.C	256	0.5
S51.	*Salmonella* gr.D	256	0.25
S52.	*Salmonella* gr.D	256	0.25
S53.	*Salmonella* gr.B	1	0.015625
S54.	*Salmonella* gr.B	4	0.03125
S55.	*Salmonella* gr.D	>256	0.125
S56.	*Salmonella* gr.D	8	0.015625
S57.	*Salmonella* gr.B	>256	0.125
S58.	*Salmonella* gr.D	32	0.125
S59.	*Salmonella* gr.B	1	0.015625
S60.	*Salmonella* gr.D	>256	0.25

No. of isolates	Serovar	Nalidixic acid (R≥32)	Ciprofloxacin (R≥4)
S61.	*Salmonella* gr.D	4	0.015625
S62.	*Salmonella* gr.D	>256	0.5
S63.	*Salmonella* gr.D	>256	0.25
S64.	*Salmonella* gr.D	>256	0.0625
S65.	*Salmonella* gr.B	>256	0.25
S66.	*Salmonella* gr.D	>256	0.0625
S67.	*Salmonella* gr.D	128	0.03125
S68.	*Salmonella* gr.D	>256	0.0625
S69.	*Salmonella gr.D*	4	0.015625
S70.	*Salmonella gr.D*	256	0.0625
S71.	*Salmonella gr.D*	>256	0.0625
S72.	*Salmonella gr.D*	>256	0.0625
S73.	*Salmonella gr.D*	>256	0.125
S74.	*Salmonella gr.C*	>256	0.125
S75.	*Salmonella gr.D*	>256	0.125
S76.	*Salmonella gr.D*	>256	0.0625
S77.	*S. Choleraesuis*	4	0.015625
S78.	*Salmonella gr.B*	>256	0.5

No. of isolates	Serovar	Nalidixic acid (R≥32)	Ciprofloxacin (R≥4)
S79.	*Salmonella gr.D*	>256	0.125
S80.	*Salmonella gr.D*	8	0.015625
S81.	*Salmonella gr.D*	>256	0.125
S82.	*S. Choleraesuis*	>256	0.125
S83.	*Salmonella gr.D*	32	0.5
S84.	*S. Choleraesuis*	>256	1
S85.	*S. Choleraesuis*	>256	0.125
S86.	*S. Choleraesuis*	>256	2
S87.	*Salmonella gr.D*	32	2
S88.	*S. Choleraesuis*	>256	0.125
S89.	*Salmonella gr.D*	32	0.5
S90.	*Salmonella gr.D*	32	0.5
S91.	*Salmonella gr.D*	>256	0.25
S97.	*S. Choleraesuis*	>256	0.125
S98.	*S. Choleraesuis*	>256	0.125
S99.	*Salmonella gr.D*	>256	0.0625
S100.	*Salmonella gr.D*	4	0.015625
S101.	*S. Choleraesuis*	>256	0.125

No. of isolates	Serovar	Nalidixic acid (R≥32)	Ciprofloxacin (R≥4)
S102.	*S. Choleraesuis*	>256	0.125
S103.	*Salmonella gr.D*	>256	0.125
S104.	*Salmonella gr.B*	4	0.015625
S105.	*S. Choleraesuis*	>256	0.125
S106.	*Salmonella gr.C*	4	0.03125
S107.	*Salmonella gr.D*	>256	0.125
S108.	*S. Choleraesuis*	>256	2

Results of antibiotic susceptibility of *Salmonella* spp. animal isolates obtained from WHO National *Salmonella* and *Shigella* Center (NSSC) in 2007 (SSA109-SSA259).

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA109.	*Salmonella* Rissen	4	0.03125
SSA110.	*Salmonella* Rissen	4	0.03125
SSA111.	*Salmonella* Stanley	4	0.03125
SSA112	*Salmonella* Rissen	4	0.03125
SSA113.	*Salmonella* gr.B	4	0.015625
SSA114.	*Salmonella* Stanley	4	0.015625
SSA115.	*Salmonella* Stanley	4	0.015625
SSA116.	*Salmonella* Rissen	4	0.015625
SSA117.	*Salmonella* Rissen	4	0.03125
SSA118.	*Salmonella* Rissen	4	0.03125
SSA119.	*Salmonella* Panama	4	0.015625
SSA120.	*Salmonella* Rissen	8	0.03125
SSA121.	*Salmonella* Rissen	4	0.015625
SSA122.	*Salmonella* Anatum	4	0.015625
SSA123.	*Salmonella* Anatum	4	0.015625
SSA124.	*Salmonella* Anatum	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA125.	*Salmonella* Anatum	4	0.015625
SSA126.	*Salmonella* Rissen	8	0.03125
SSA127.	*Salmonella* Rissen	4	0.015625
SSA128.	*Salmonella* Rissen	8	0.0625
SSA129.	*Salmonella* Rissen	4	0.015625
SSA130.	*Salmonella* Rissen	4	0.03125
SSA131.	*Salmonella* Rissen	4	0.03125
SSA132.	*Salmonella* Rissen	4	0.015625
SSA133.	*Salmonella Anatum*	4	0.015625
SSA134.	*Salmonella* Anatum	4	0.015625
SSA135.	*Salmonella* Rissen	4	0.03125
SSA136.	*Salmonella* Rissen	4	0.03125
SSA137.	*Salmonella* Rissen	4	0.015625
SSA138.	*Salmonella* Rissen	4	0.03125
SSA139.	*Salmonella* Stanley	4	0.015625
SSA140.	*Salmonella* Rissen	4	0.03125
SSA141.	*Salmonella* Rissen	4	0.03125
SSA142.	*Salmonella* Rissen	4	0.03125

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA143.	*Salmonella* Amsterdam	>256	0.25
SSA144.	*Salmonella* Amsterdam	>256	0.25
SSA145.	*Salmonella* Rissen	4	0.015625
SSA146.	*Salmonella* Rissen	1	0.015625
SSA147.	*Salmonella* Tennessee	4	0.015625
SSA148.	*Salmonella* Tennessee	4	0.03125
SSA149.	*Salmonella* Rissen	4	0.015625
SSA150.	*Salmonella* Corvallis	4	0.015625
SSA151.	*Salmonella* Corvallis	8	0.25
SSA152.	*Salmonella* Hexington	4	0.015625
SSA153.	*Salmonella* Rissen	8	0.015625
SSA154.	*Salmonella* Rissen	2	0.015625
SSA155.	*Salmonella* Rissen	32	1
SSA156.	*Salmonella* Rissen	4	0.015625
SSA157.	*Salmonella* Rissen	4	0.03125
SSA158.	*Salmonella* Rissen	4	0.03125
SSA159.	*Salmonella* Rissen	4	0.03125
SSA160.	*Salmonella* Rissen	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA161.	*Salmonella* Rissen	4	0.03125
SSA162.	*Salmonella* Rissen	4	0.03125
SSA163.	*Salmonella* Albany	256	0.25
SSA164.	*Salmonella* Rissen	4	0.03125
SSA165.	*Salmonella* Stanley	4	0.03125
SSA166.	*Salmonella* Rissen	8	0.5
SSA167.	*Salmonella* Altona	32	0.125
SSA168.	*Salmonella* Altona	4	0.03125
SSA169.	*Salmonella* Rissen	4	0.015625
SSA170.	*Salmonella* Altona	16	0.25
SSA171.	*Salmonella* Altona	16	0.125
SSA172.	*Salmonella* Altona	16	0.125
SSA173.	*Salmonella* Stanley	16	0.125
SSA174.	*Salmonella* Stanley	4	0.015625
SSA175.	*Salmonella* Anatum	4	0.03125
SSA176.	*Salmonella* Stanley	4	0.015625
SSA177.	*Salmonella* Rissen	4	0.015625
SSA178.	*Salmonella* Stanley	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA179.	*Salmonella* Welterreden	4	0.015625
SSA180.	*Salmonella* Stanley	4	0.03125
SSA181.	*Salmonella* Thompson	4	0.03125
SSA182.	*Salmonella* Stanley	4	0.03125
SSA183.	*Salmonella* Welterreden	4	0.015625
SSA184.	*Salmonella* Stanley	4	0.015625
SSA185.	*Salmonella* Rissen	4	0.03125
SSA186.	*Salmonella* Stanley	4	0.015625
SSA187.	*Salmonella Anatum*	4	0.03125
SSA188.	*Salmonella Rissen*	1	0.015625
SSA189.	*Salmonella Stanley*	4	0.03125
SSA190.	*Salmonella Anatum*	4	0.03125
SSA191.	*Salmonella Stanley*	4	0.015625
SSA192.	*S. Borismorbificans*	4	0.03125
SSA193.	*Salmonella Stanley*	4	0.03125
SSA194.	*Salmonella Rissen*	4	0.03125
SSA195.	*S. Borismorbificans*	4	0.015625
SSA196.	*Salmonella Rissen*	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA197.	*Salmonella Rissen*	4	0.03125
SSA198.	*Salmonella Rissen*	4	0.03125
SSA199.	*Salmonella Rissen*	4	0.015625
SSA200.	*Salmonella Rissen*	4	0.015625
SSA201.	*Salmonella Rissen*	4	0.03125
SSA202.	*Salmonella Rissen*	4	0.03125
SSA203.	*Salmonella Rissen*	4	0.015625
SSA204.	*Salmonella Amsterdam*	>256	0.25
SSA205.	*Salmonella Amsterdam*	>256	0.25
SSA206.	*S. Schwarzengrend*	>256	0.5
SSA207.	*S. Schwarzengrend*	>256	0.25
SSA208.	*S. Schwarzengrend*	>256	0.25
SSA209.	*S. Schwarzengrend*	>256	0.25
SSA210.	*S. Schwarzengrend*	>256	0.25
SSA211.	*S. Schwarzengrend*	>256	0.25
SSA212.	*S. Schwarzengrend*	>256	0.25
SSA213.	*S.* Schwarzengrend	>256	0.25
SSA214.	*S.* Schwarzengrend	>256	0.25

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA215.	*S.* Schwarzengrend	64	0.0625
SSA216.	*S.* Schwarzengrend	>256	0.25
SSA217.	*S.* Schwarzengrend	>256	0.25
SSA218.	*S.* Schwarzengrend	>256	0.25
SSA219.	*S.* Rabialaw	4	0.03125
SSA220.	*S.* Schwarzengrend	64	0.0625
SSA221.	*S.* Kentucky	8	0.015625
SSA222.	*S.* Kedougou	2	0.03125
SSA223.	*Salmonella* Rissen	4	0.03125
SSA224.	*Salmonella* Rissen	4	0.015625
SSA225.	*Salmonella* Anatum	4	0.125
SSA226.	*Salmonella* Anatum	32	1
SSA227.	*Salmonella* Anatum	64	1
SSA228.	*Salmonella* Anatum	16	0.25
SSA229.	*Salmonella* Anatum	16	0.03125
SSA230.	*Salmonella* Anatum	64	1
SSA231.	*Salmonella* Anatum	64	1
SSA232.	*Salmonella* Rissen	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA233.	*Salmonella* Anatum	8	0.5
SSA234.	*Salmonella* Anatum	16	0.03125
SSA235.	*Salmonella* Rissen	4	0.03125
SSA236.	*Salmonella* Anatum	16	0.0625
SSA237.	*Salmonella* Rissen	>256	0.25
SSA238.	*S.* Borismorbificans	>256	0.015625
SSA239.	*Salmonella* Welterreden	4	0.015625
SSA240.	*Salmonella* Welterreden	8	0.03125
SSA241.	*Salmonella* Stanley	4	0.015625
SSA242.	*Salmonella* Welterreden	4	0.015625
SSA243.	*Salmonella* Eastbourne	2	0.015625
SSA244.	*Salmonella* Hexington	4	0.015625
SSA245.	*Salmonella* Welterreden	4	0.015625
SSA246.	*Salmonella* Agona	4	0.015625
SSA247.	*Salmonella* Agona	4	0.015625

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SSA248.	*Salmonella* Javiana	4	0.015625
SSA249.	*Salmonella* Agona	4	0.015625
SSA250.	*Salmonella* Agona	4	0.015625
SSA251.	*Salmonella* Agona	16	0.03125
SSA252.	*Salmonella* Agona	4	0.0625
SSA253.	*Salmonella* Agona	64	1
SSA254.	*Salmonella* Senftenberg	>256	0.125
SSA255.	*Salmonella* Welterreden	4	0.015625
SSA256.	*Salmonella* Tennessee	>256	0.125
SSA257.	*Salmonella* Rissen	32	0.03125
SSA258.	*Salmonella* Rissen	>256	0.125
SSA259.	*Salmonella* Derby	4	0.015625

Results of antibiotic susceptibility of *Salmonella* spp. animal isolates obtained from the Department of Livestock Development (Bangkok, Thailand) between 2003 and 2005 (designed as SA1-SA98)

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA1	*Salmonella* Enteritidis	>256	0.25
SA2	*Salmonella* Enteritidis	>256	0.125
SA3	*Salmonella* Enteritidis	>256	0.25
SA4	*Salmonella* Enteritidis	>256	0.25
SA5	*Salmonella* Enteritidis	>256	0.25
SA6	*Salmonella* Enteritidis	>256	0.25
SA7	*Salmonella* Enteritidis	>256	0.25
SA8	*Salmonella* Enteritidis	>256	0.125
SA9	*Salmonella* Enteritidis	>256	0.25
SA10	*Salmonella* Enteritidis	>256	0.125
SA11	*Salmonella* Enteritidis	>256	0.25
SA12	*Salmonella* Enteritidis	>256	0.25
SA13	*Salmonella* Enteritidis	>256	0.25
SA14	*Salmonella* Enteritidis	>256	0.25
SA15	*Salmonella* Enteritidis	>256	0.125
SA16	*Salmonella* Enteritidis	>256	0.25

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA17	*Salmonella* Enteritidis	>256	0.25
SA18	*Salmonella* Enteritidis	>256	0.125
SA19	*Salmonella* Enteritidis	>256	0.25
SA20	*Salmonella* Enteritidis	>256	0.125
SA21	*Salmonella* Give	>256	0.25
SA22	*Salmonella* Give	>256	0.25
SA23	*Salmonella* Give	>256	0.25
SA24	*Salmonella* Give	>256	0.25
SA25	*Salmonella* Emek	>256	2
SA26	*Salmonella* Emek	>256	2
SA27	*Salmonella* Emek	>256	2
SA28	*Salmonella* Amsterdam	>256	0.125
SA29	*Salmonella* Amsterdam	>256	0.125
SA30	*Salmonella* Amsterdam	>256	0.125
SA31	*Salmonella* Amsterdam	>256	0.125
SA32	*S.* Amsterdam	>256	0.125
SA34	*Salmonella* Amsterdam	>256	0.125
SA35	*Salmonella* Amsterdam	>256	0.125

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA36	*Salmonella* Albany	>256	0.125
SA37	*Salmonella* Albany	>256	0.125
SA38	*Salmonella* Albany	>256	0.125
SA39	*Salmonella* Albany	>256	0.125
SA40	*Salmonella* Kentucky	16	0.25
SA41	*Salmonella* Kentucky	16	0.25
SA42	*S.* Senftenberg	>256	4
SA43	*S.* Senftenberg	>256	2
SA44	*S.* Senftenberg	>256	1
SA45	*S.* Senftenberg	>256	2
SA46	*S.* Worthington	16	0.5
SA47	*S.* Worthington	>256	0.5
SA48	*S.* Welterreden	32	0.5
SA49	*S.* Welterreden	4	0.015
SA50	*Salmonella* Corvallis	16	0.25
SA51	*Salmonella* Corvallis	16	0.5
SA53	*Salmonella* Eppendorf	>256	0.25
SA54	*S.* BoVismorbificans	>256	0.125

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA55	*Salmonella* Virchow	>256	0.06
SA56	*Salmonella* Infantis	>256	1
SA57	*Salmonella* Madjorio	16	1
SA58	*Salmonella* I	64	0.5
SA59	*Salmonella* Poona	4	0.5
SA60	*S.* Hvittingfoss	4	0.015
SA61	*Salmonella* Suberu	>256	0.06
SA62	*Salmonella* Orion	>256	2
SA64	*Salmonella* Blockley	>256	0.25
SA65	*S.* Borismorbificans	>256	0.125
SA66	*S.* Borismorbificans	32	0.03
SA67	*S.* Borismorbificans	4	0.015
SA68	*S.* Borismorbificans	4	0.015
SA69	*S.* Borismorbificans	4	0.015
SA70	*S.* Borismorbificans	4	0.03
SA71	*S.* Borismorbificans	32	0.015
SA72	*S.* Borismorbificans	32	0.03
SA73	*Salmonella* I	>256	0.25

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA74	*Salmonella* I	>256	0.25
SA75	*Salmonella* I	16	0.125
SA76	*Salmonella* I	8	0.015
SA77	*Salmonella* I	>256	0.5
SA78	*Salmonella* Corvallis	>256	0.5
SA79	*Salmonella* Corvallis	16	0.5
SA80	*Salmonella* Corvallis	16	0.5
SA81	*Salmonella* Corvallis	16	0.5
SA82	*Salmonella* Anatum	>256	0.06
SA83	*Salmonella* Anatum	>256	0.06
SA84	*Salmonella* Anatum	>256	0.06
SA85	*Salmonella* Anatum	4	0.015
SA86	*S.* Typhimurium	256	0.125
SA87	*S.* Typhimurium	256	0.125
SA88	*Salmonella* Stanley	256	0.06
SA89	*Salmonella* Stanley	>256	0.06
SA90	*Salmonella* Stanley	8	0.015
SA91	*Salmonella* Altona	4	0.015

No. of isolates	Serovar	Nalidixic acid(R≥32)	Ciprofloxacin (R≥ 4)
SA92	*Salmonella* Altona	8	0.015
SA93	Salmonella Panama	16	0.5
SA94	*Salmonella* Give	>256	1
SA95	*S.* Worthington	>256	0.06
SA96	*Salmonella* Muenster	>256	0.06
SA97	*Salmonella* Rissen	>256	0.015
SA98	*Salmonella* Sainpaul	8	0.015
SA99	*Salmonella* Albany	256	0.125
SA100	*Salmonella* Virginia	32	1

APPENDIX E

THE DNA CODONS

Amino Acid	SLC	DNA codons
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Stop codons	Stop	TAA, TAG, TGA

BIOGRAPHY

Miss Boontarika Tongrod was born on February 9, 1984 in Bangkok, Thailand. She graduated with the Bachelor of Science (Medical technology) from Faculty of Allied Health Science, Chulalongkorn University in 2005. She has studied in the MS degree in Medical Science (Molecular Biology and Genetics), Faculty of Medicine, Chulalongkorn University since 2006.