คุณลักษณะของเชื้อ Nontyphoidal *Salmonella* ที่ดื้อยาต้านจุลชีพที่แยกได้จากคนและสัตว์ที่เลี้ยงเป็นอาหารในประเทศไทย

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นางสาวสิริรัตน์ ลูกอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT NONTYPHOIDAL *SALMONELLA* ISOLATED FROM HUMANS AND FOOD ANIMALS IN THAILAND

172

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Thesis Title	CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT NONTYPHOIDAL *SALMONELLA* ISOLATED FROM HUMANS AND FOOD ANIMALS IN THAILAND 172252256
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สิริรัตน์ ลูกอินทร์ : คุณลักษณะของเชื้อ Nontyphoidal *Salmonella* ที่ดื้อยาต้านจุลชีพที่แยกได้จากคนและสัตว์ที่เลี้ยงเป็นอาหารในประเทศไทย 171251255 (CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT NONTYPHOIDAL *SALMONELLA* ISOLATED FROM HUMANS AND FOOD ANIMALS IN THAILAND 172252256) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. ธนิษฐา ฉัตรสุวรรณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ. วันล่า กุลวิชิต, 196 หน้า.

การอุบัติขึ้นและการเพิ่มสูงขึ้นของการดื้อยาต้านจุลชีพโดยเฉพาะอย่างยิ่งต่อยากลุ่ม extended-spectrum cephalosporins (ESCs) และ fluoroquinolones ในเชื้อ nontyphoidal *Salmonella* เป็นปัญหาสำคัญต่อการรักษาโรคติดเชื้อนี้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษา กลไกการดื้อต่อยาต้านจุลชีพ, clonality, และ plasmid profiles ของเชื้อ nontyphoidal *Salmonella* ที่ดื้อต่อยาต้านจุลชีพซึ่งแยกได้จากคนและสัตว์ที่ใช้เป็นอาหาร ในประเทศไทย โดยทำการศึกษาในเชื้อ nontyphoidal *Salmonella* 897 สายพันธุ์ ซึ่งประกอบด้วยเชื้อที่แยกได้จากคน 617 สายพันธุ์ และ จากสัตว์ที่ใช้เป็นอาหาร 280 สายพันธุ์ ในช่วงปี 2005 ถึง 2007 และ 2012 ถึง 2016 ผลการศึกษาพบว่าอัตราการดื้อต่อยาต้านจุลชีพ ในเชื้อ *S.* Choleraesuis สูงกว่าเชื้อในserotype อื่นๆ อย่างมีนัยสำคัญ ($p < 0.0001$) ยกเว้นยา norfloxacin โดยในเชื้อ *S.* Choleraesuis พบอัตราการดื้อต่อยากลุ่ม ESCs และ ciprofloxacin ที่สูงมากคิดเป็น 56.9% และ 19.4% ตามลำดับ ผลจากศึกษาด้วยวิธี PFGE และ RFLP พบว่ามี predominant clones และ predominant plasmids ของเชื้อ nontyphoidal *Salmonella* ที่ดื้อต่อยาต้านจุลชีพที่แยกได้จากคน โดยพบการแพร่กระจายของ self-transferable plasmid ชนิด $IncFII_s$ (E06), IncFII (E20), และ IncI1 (E21) ที่มียีน $bla_{CTX-M-14}$ และ self-transferable plasmid ชนิด IncA/C (S01) ที่มียีน bla_{CMY-2} รวมถึงพบการแพร่กระจายของ clone ของเชื้อ *S.* Choleraesuis สายพันธุ์ที่มียีน bla_{CMY-2} (C35-01) ซึ่งเป็นสาเหตุของอัตราการดื้อต่อยากลุ่ม ESC ที่พบสูงขึ้นในช่วงปี 2005 ถึง 2007 การศึกษานี้พบการอุบัติขึ้นเป็นครั้งแรกของ CTX-M-55 ในเชื้อ *S.* Choleraesuis ซึ่งพบเพิ่มขึ้นอย่างรวดเร็วและ CTX-M ชนิดนี้มีความชุกมากที่สุดในเชื้อที่แยกได้ระหว่างปี 2012 ถึง 2016 การแพร่กระจายของ clone C27-01 ซึ่งเกิดจาก การแพร่กระจายของ plasmid ชนิด IncA/C ที่มียีน $bla_{CTX-M-55}$ ร่วมกับยีน *qnrS1* ในเชื้อ *S.* Choleraesuis ซึ่งพบการกลายพันธุ์ของ GyrA ที่ D87G พบว่าเป็นสาเหตุของอัตราการดื้อต่อยากลุ่ม ESCs และ ciprofloxacin ร่วมกัน (44.9%) ในช่วงปี 2012 ถึง 2016 ความสัมพันธ์ของแบบแผนทางพันธุกรรมระหว่างเชื้อที่แยกได้จากคนและสุกร พบใน clone C27-05 ของเชื้อ *S.* Choleraesuis ที่สร้าง CTX-M-55 ที่แยกได้ในปี 2014 การศึกษานี้ พบยีน *qnrVC4* ในเชื้อ *S.* Rissen สายพันธุ์ 166ANSS50 ที่แยกได้จากสุกร ซึ่งเป็นรายงานแรกของการพบยีนในกลุ่ม *qnrVC* ใน เชื้อ *Salmonella enterica* โดยพบว่าเชื้อนี้มี non-conjugative plasmid ขนาด 17 กิโลเบสซึ่งมี *qnrVC4* อยู่ในตลับยีน *qnrVC4-qacH4-aacA4-cmlA7-*bla_{OXA-10}*-aadA1-dfrA14* ที่มียีนดื้อยาหลายชนิด ซึ่งอยู่บน integron คลาส 1 กลุ่ม In4 ขนาด 8.91 กิโลเบส ซึ่งมี unusual 3' คือ *mobC*-IS*6100* ผลจากการศึกษาครั้งนี้แสดงให้เห็นถึงความสำคัญของการควบคุมการแพร่ระบาดของการดื้อต่อยาต้านจุลชีพใน nontyphoidal *Salmonella* ซึ่งอาจสามารถเป็นภัยคุกคามต่อสุขภาพของคนทั่วโลก อันเนื่องมาจากการท่องเที่ยวและการค้าขายผลิตภัณฑ์อาหารจากสัตว์

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5587815420 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS: NONTYPHOIDAL SALMONELLA / ANTIMICROBIAL RESISTANCE / ESBL / AMPC / QNRVC

SIRIRAT LUK-IN: CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT NONTYPHOIDAL *SALMONELLA* ISOLATED FROM HUMANS AND FOOD ANIMALS IN THAILAND 172252256. ADVISOR: TANITTHA CHATSUWAN, Ph.D., CO-ADVISOR: ASSOC. PROF. WANLA KULWICHIT, M.D., 196 pp.

The emergence and increase of resistance to antimicrobial agents, especially to extended-spectrum cephalosporins (ESCs) and fluoroquinolones in nontyphoidal *Salmonella* have recently become a serious therapeutic problem. The objectives of this study were to characterize antimicrobial resistance mechanisms, the clonality, and plasmid profiles among nontyphoidal *Salmonella* isolates from human and food animals in Thailand. A total of 897 nontyphoidal *Salmonella* isolates, 617 isolates from human and 280 isolates from food animal in Thailand during 2005 to 2007 and 2012 to 2016 were included in this study. The significantly higher resistance rates were found in *S.* Choleraesuis compared with other serotypes to all antimicrobial agents tested ($p < 0.0001$), except for norfloxacin. *S.* Choleraesuis showed extremely high rates of resistance to ESCs (56.9%) and ciprofloxacin (19.4%). The results from PFGE and RFLP revealed the predominant antimicrobial resistance clones and plasmids among nontyphoidal *Salmonella* human isolates. The dissemination of the self-transferable $bla_{CTX-M-14}$-carrying $IncFII_s$ (E06), IncFII (E20), and IncI1 (E21) plasmids and bla_{CMY-2}-carrying IncA/C (S01) plasmid along with the clonal spread of bla_{CMY-2}-harbouring *S.* Choleraesuis isolates (C35-01) contributed to the high rates of ESC resistance during 2005 to 2007. Moreover, this study reported the first occurrence of CTX-M-55 in *S.* Choleraesuis isolates which dramatically increased and became the most abundant CTX-M variant among ESC-resistant isolates during 2012 to 2016. The spread of clone C27-01 was due to the dissemination of IncA/C plasmids carrying $bla_{CTX-M-55}$ along with *qnrS1* among the *S.* Choleraesuis isolates harbouring D87G in GyrA which was apparently responsible for the high rates of co-resistance to ESCs and ciprofloxacin (44.9%) during 2012 to 2016. The genetically related isolates from human and swine were found in CTX-M-55-producing *S.* Choleraesuis clone C27-05 isolated in 2014. Interestingly, we found *qnrVC4* gene in *S.* Rissen 166ANSS50 from a swine isolate. This is the first known report of *qnrVC* in *Salmonella enterica*. This isolate harbored a 17-kb non-conjugative plasmid carrying *qnrVC4* within 8.91 kb of a novel In4-like class 1 integron (In805). It contained the multi-drug resistance gene cassettes of *qnrVC4-qacH4-aacA4-cmlA7-*bla_{OXA-10}*-aadA1-dfrA14* and unusual 3'-CS of *mobC*-IS*6100*. The results of this study underlines the importance of the action plan to control the dissemination of antimicrobial resistance in nontyphoidal *Salmonella* since this could be global health threats due to travel and trade in animal food products.

Field of Study: Medical Microbiology

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Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

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Ala (A)	alanine
Asn (N)	asparagine
Asp (D)	aspartic acid
Gly (G)	glycine
Phe (F)	phenylalanine
Ser (S)	serine
Tyr (Y)	tyrosine
Ile (I)	isoleucine
Val (V)	valine
bp	base pair
kb	kilo base pair
CLSI	Clinical and Laboratory Standards Institute
^{o}C	degree Celsius
dNTPs	deoxynucleotide-tri-phosphate
DDW	double distilled water
DNA	deoxynucleic acid
DW	distilled water
DDW	Double-distilled water
EDTA	ethylenediamine tetraacetic acid
et al.	*et alii*
g	gram
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour
M	molar
mg	milligram
$MgCl_2$	magnesium chloride

MIC	minimum inhibitory concentration
min	minute (s)
mL	milliliter
mM	millimolar
mmol	millimole
NaCl	sodium chloride
NaOH	sodium hydroxide
PCR	polymerase chain reaction
pmol	picomole
sec	second
TBE	tris-borate-EDTA
Tris	Tris-(hydroxymethyl)-aminoethane
U	unit
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviolet
V	volt
Inc	incompatibility group
ESBL	Extended-spectrum beta-lactamase
ESC	extended-spectrum cephalosporin

CHAPTER I

INTRODUCTION

Nontyphoidal *Salmonella*, the major pathogen of the foodborne illnesses is estimated to cause more than one million illnesses each year, with almost 20,000 hospitalizations and 400 deaths, according to a 2011 report in the United States by CDC (1). These infections are generally self-limiting, but invasive infections can be occurred especially due to certain *Salmonella* serotypes, including *S.* Enteritidis, *S.* Choleraesuis, and *S.* Dublin, for which antimicrobial treatments are required for life-saving (2). The conventional antimicrobial agents, such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, were the drugs of choice in the treatment of salmonellosis. Lately, nontyphoidal *Salmonella* with resistance to these drugs has been reported from many countries (3). Extended-spectrum cephalosporins (ESCs) (1) and fluoroquinolones are recommended as alternatives (4). However, nontyphoidal *Salmonella* isolates resistant to ESCs and fluoroquinolones have been increasingly reported worldwide (5-8).

Cephalosporins are members of ß-lactam antibiotics which act by inhibiting bacterial cell wall synthesis. Cephalosporins are classified to the first to the fourth generations based on their antibacterial activities. Extended-spectrum cephalosporins (ESCs), commonly referred to the third-generation and fourth-generation cephalosporins, which are frequently used antibiotics for the treatment of severe infections because of their broad spectrum and low toxicity (9). The third generation cephalosporins such as ceftriaxone are commonly administrated to treat salmonellosis in children and infants (9). The major cause of ESC resistance in nontyphoidal *Salmonella* is the production of extended-spectrum ß-lactamases (ESBLs) and plasmid-mediated AmpC ß-lactamases which destroy ß-lactam ring structure of the drugs (10, 11). ESBLs that were reported in nontyphoidal *Salmonella* included TEM-3 (12), TEM-52 (13), SHV-2a (14), SHV-5 (15), SHV-12 (14, 16, 17), CTX-M-1 (7, 16), CTX-M-2 (18), CTX-

M-3 (14), CTX-M-5 (17, 19), CTX-M-9 (16, 20), CTX-M-14 (16, 21), CTX-M-15 (17, 22), CTX-M-32 (23), CTX-M-53 (24), and CTX-M-55/57 (17) which were reported in many countries. Recently, the CTX-M (Cefotaxime-hydrolyzing ß-lactamases) are rapidly growing group of ESBLs (11). The CTX-M ß-lactamase family is sub-classified into five groups, including CTX-M-1, CTX-M-2, CTX-M-9, CTXM-8, and CTX-M-25, according to the similarity of their amino acid sequences (25). Some amino acid alteration of these enzymes affects substrate hydrolysis which extends their hydrolysis spectrum (25). In nontyphoidal *Salmonella*, only three groups of CTX-M including CTX-M-1, CTX-M-2, and CTX-M-9 groups have been reported (10).

Salmonella is one of many bacteria that lack an intrinsic AmpC ß-lactamases. However, the dissemination of this resistance determinant is also mediated by plasmids (26). The plasmid-mediated AmpC ß-lactamases can be divided into six families based on amino acid sequences including MOX, CIT, DHA, ACC, EBC, and FOX family (26). Recently, CMY-2, CMY-4, CMY-7, ACC-1, and DHA-1 have been found in nontyphoidal *Salmonella* (27-31). Moreover, CMY-2, belonging to CIT family was commonly identified worldwide such as England and Wales (27), France (7), United States (32), Netherlands (30), Taiwan (6, 21), South Korea (33), China (34), and Singapore (35).

Fluoroquinolones are broad-spectrum antibiotics which directly inhibit DNA synthesis by action on the two target enzymes, DNA gyrase (two subunits, GyrA and GyrB) and topoisomerase IV (two subunits, ParC and ParE) (36). They are derived from the quinolone family of antibiotics which are created by the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity (36). Two major fluoroquinolone resistance mechanisms contributing to fluoroquinolone resistance have been demonstrated in *Salmonella* (37). The chromosomal-mediated mechanism is the alteration of quinolone targets including DNA gyrase (*gyr*A and/or *gyr*B) and/or topoisomerase IV (*par*C and/or *par*E) genes in the Quinolone Resistance-Determining Region (QRDR) (38). Recently, the plasmid-mediated quinolone resistance (PMQR) mechanisms have been reported. The most common one is the production of quinolone resistance protein (Qnr) (39-41). Qnr protein

can protect DNA gyrase and topoisomerase IV from fluoroquinolone lethal inhibition (42). Currently, five main families of *qnr* genes have been reported; *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*. The other mechanisms are the presence of the modified-acylyltransferase (AAC (6')-Ib-cr) that acetylates ciprofloxacin and norfloxacin (43) and the QepA protein that acts as an efflux pump for hydrophilic fluoroquinolones (44). According to the effect of these plasmid-mediated resistance mechanisms, the pathogens show reduced susceptibility to ciprofloxacin with MIC of 0.125-1 mg/L. However, these genes are widespread among bacteria in many countries due to conjugative plasmid and various mobile genetic elements (45). Moreover, these dissemination mechanisms are the important factor for the colocalization of PMQR genes with other resistance genes including genes encoding for ESBL and AmpC type ß-lactamases such as CTX-M (46), SHV (46), TEM (46), and CMY (47).

Currently, antimicrobial–resistant nontyphoidal *Salmonella* has been reported as a threat level serious by CDC (http://www.cdc.gov/drugresistance/threat-report-2013). The National Antimicrobial Resistance Monitoring System (NARMS) reported that resistance to ceftriaxone was about 3% of nontyphoidal *Salmonella* tested, and ciprofloxacin MIC of $\geq$ 0.125 mg/L was about 3%. About 5% of nontyphoidal *Salmonella* were resistant to five or more types of antimicrobials in the United States during 2009 to 2011. Antimicrobial resistance in nontyphoidal *Salmonella* continues to increase from <1% in 1996 to ~3% in 2011 for both ceftriaxone and ciprofloxacin. A study of nontyphoidal *Salmonella* from seven Asian countries, including Philippines, Hong Kong, Singapore, Sri Lanka, Korea, Thailand, and Taiwan showed that 3.0% were ceftriaxone-resistant during 2003-2005, except in Taiwan (38.0%) or in *S.* Typhimurium (25.0%) from all countries (48). This multinational study from Asia also showed that reduced susceptibility to ciprofloxacin in nontyphoid *Salmonella* isolates was commonly found in Taiwan (48.1%) and Thailand (46.2%) (48). A study from China in *S.* Typhimurium clinical isolates showed ciprofloxacin and ceftriaxone resistance with 20% and 4%, respectively during 2005 to 2011 (49). Despite ESCs and fluoroquinolones, broad-spectrum antimicrobial agents, have been classified as critically important antimicrobial-

classes for human medicine by the World Health Organization (WHO) (50). Currently, these two classes of antimicrobials, including ceftiofur and enrofloxacin are also commonly used in food animal production systems. Since the introduction of their use in food animal medicine, the prevalence of these antimicrobial-resistant *Salmonella* within livestock population has been increasing (51) and antimicrobial-resistant *Salmonella* were identified in imported foods in many countries (52-54). Recently, the results from Antimicrobial Resistance Surveillance in Canada demonstrated a strong correlation ($r = 0.9$, $p<0.0001$) between ceftiofur-resistant *S.* Heidelberg isolated from retail chicken and incidence of ceftiofur-resistant *S.* Heidelberg infections in humans across Canada (55).

In Thailand, the prevalence of ESC and fluoroquinolone resistance in nontyphoidal *Salmonella* has been increasingly reported in recently. The study reported that more than 17% of blood isolates of nontyphoidal *Salmonella* from Siriraj Hospital in 2005 were resistant to ceftriaxone (56). High rate of ceftriaxone resistance (15%) was also found in *S.* Choleraesuis isolates from bacteremic patients from King Chulalongkorn Memorial Hospital and the WHO National *Salmonella* and *Shigella* Center during 2003-2005, all of which also showed resistance to quinolones (57). Moreover, antimicrobial-resistant *Salmonella* spp. was found in imported food animal product from Thailand (52, 58).

The development and spread of ESC- and fluoroquinolone-resistant *Salmonella* has become a significant public health concerns and can potentially lead to treatment failures. High rates of ESC and fluoroquinolone resistance have been reported in Thailand. The over-usage of ESCs and fluoroquinolones in food animals may develop the potential reservoir of antimicrobial-resistant *Salmonella* strains or antimicrobial resistance plasmids that cause antimicrobial resistance in *Salmonella* in human population. However, there are very few studies about the mechanisms of ESC and fluoroquinolone resistance among nontyphoidal *Salmonella* in Thailand. In addition, no data on the predominant clones and antimicrobial resistance plasmids that are responsible for ESC and fluoroquinolone resistance in nontyphoidal *Salmonella* has been reported in this country. The purpose of this study is to characterize the

mechanisms of ESC and fluoroquinolone resistance among nontyphoidal *Salmonella* in Thailand, to identify the predominant antimicrobial-resistant clones and plasmids, to investigate the genetic relatedness between human isolates and food animal isolates, and to characterize novel antimicrobial resistance genes in *Salmonella* spp.

CHAPTER II

OBJECTIVE

1. To investigate the prevalence of ESC and fluoroquinolone resistance among nontyphoidal *Salmonella* in Thailand.
2. To characterize the mechanisms of ESC and fluoroquinolone resistance among nontyphoidal *Salmonella*.
3. To identify the predominant clones and antimicrobial resistance plasmids that are responsible for ESC and fluoroquinolone resistance in nontyphoidal *Salmonella* in Thailand.
4. To compare the genetic patterns and plasmid profiles of antimicrobial-resistant nontyphoidal *Salmonella* human isolates with food animal isolates.
5. To characterize the transmission mechanisms of ESC and fluoroquinolone resistance genes among nontyphoidal *Salmonella.*
6. To identify and characterize novel ESC or fluoroquinolone resistance genes which have not been reported in *Salmonella* spp.

CHAPTER III

LITERATURE REVIEW

Part I: Nontyphoidal *Salmonella*

Salmonella spp. is Gram-negative bacteria belonging to family Enterobacteriaceae. It can cause infection in both humans and animals. The genus *Salmonella* contains two species: *Salmonella enterica* and *Salmonella bongori* (formerly subspecies V). *Salmonella enterica* is divided into six subspecies, including *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). Over 99% of the serotypes belong to *Salmonella enterica* species consisting of the major serovars which cause human salmonelosis (59). Salmonellae are traditionally classified into serotypes based on the immune-reactivity of diverse surface antigens including polysaccharide O (somatic) antigens, H (flagellar) antigens and Vi (capsular) antigens (59). Agglutination reactions based on the O-antigen are used by most clinical laboratories to divide *Salmonella* into serogroups including A, B, C1, C2, D and E, which cause most of the nontyphoidal *Salmonella* infections in humans (59). The White-Kauffmann scheme is a practical summary of the antigenic structure of different *Salmonella* serotypes (59, 60). Nowadays, over 2500 *Salmonella* serotypes have been identified (59). An example of a correct *Salmonella* subspecies and serotype designation is *Salmonella enterica* subspecies *enterica* serotype Typhimurium. An accepted abbreviation of this full taxonomic designation is *Salmonella* ser. Typhimurium (capitalised and not italicised) at the first citation and subsequently *Salmonella* Typhimurium (59). *Salmonella enterica* subspecies *enterica* contains almost all the serotypes pathogenic for humans (59).

Salmonellosis can be broadly classified into two groups: enteric fever (typhoidal salmonellosis) and nontyphoidal salmonellosis. *Salmonella enterica* subspecies *enterica*

serovar Typhi (*S.* Typhi) or *S.* Paratyphi A, B and C cause enteric fever, a systemic illness with prolonged fever. These two serotypes are exclusively human pathogens and the incidences of infections have been decreasing in the last decade. Recently, nontyphoidal *Salmonella* infections which occur through the consumption of contaminated food of animal origin constitute a major public health problem in developed and developing countries according to the WHO (www.who.int). Nontyphoidal *Salmonella* have a wide range of hosts and reservoirs (61). Some serotypes are host specific, such as *S.* Pullorum (poultry), and *S.* Typhisuis (swine). Some serotypes are host adapted, such as *S.* Dublin (predominantly in cattle and humans), and *S.* Choleraesuis (predominantly in swine and human). Some serotypes are able to infect a broad host range, such as *S.* Typhimurium and *S.* Enteritidis (61). Nontyphoidal *Salmonella* usually causes a self-limited enterocolitis with diarrhea in humans. However, bloodstream infection can occur in approximately 6% of patients with diarrheal enterocolitis; infants, young children, the elderly, and the immunocompromised hosts are at particular risk for bacteremia (2, 9). Certain nontyphoidal *Salmonella* serovars have been associated with a higher mortality rate than others. The mortality rates of the 10 most lethal *Salmonella* serotypes are shown in Table 1 Four out of 10 most lethal serotypes belong to serogroup C and 2 to serogroup E (*S.* Muenster and *S.* Anatum), whereas there is only one serogroup D (62). *S.* Typhimurium is considered to be a typical host generalist with a broad host range and modest likelihood of causing invasive disease. *S.* Heidelberg, *S.* Dublin, and *S.* Choleraesuis are markedly more likely than *S.* Typhimurium to cause hospitalization, invasive disease, or death (2, 9). *S.* Newport are associated with a lower case fatality ratio (0.3%) that of than *S.* Typhimurium (2, 9). The reasons for differences in host specificity among *Salmonella* serotypes are complex and incompletely understood (2, 9). One of the leading serotypes in Asia, *S.* Choleraesuis (group C1) has been found to be invasive in up to 56% of cases in Thailand (2). *S.* Choleraesuis also has a much higher odds ratio (44:1) of being recovered from blood rather than stool, compared to other serotypes in Taiwan (62). *S.* Dublin (serogroup D) is also one of the most invasive nontyphoidal *Salmonella*

serotypes, with 64% of strains isolated from sterile sites (62). The invasiveness of a serotype and the hospitalization rate due to infection by the serotypes also shows a positive correlation with Spearman coefficient of 0.42; $P<0.002$ (62).

Table 1. Serogroup and associated mortality rates of the 10 deadliest *Salmonella* serotypes isolated in the United States between 1996 and 2006 (62)

Rank	Serotype	Serogroup	Mortality rate (%)
1	Dublin	D	3
2	Muenster	E	2
3	Choleraesuis	C	1.8
	Cerro	K	1.8
5	Johannesburg	R	1.5
6	Tennessee	C	1.3
7	Manhattan	C	1
	Anatum	E	1
9	Bovismorbificans	C	0.9
	Adelaide	O	0.9

CDC has estimated that 1 in 6 Americans gets sick and 3,000 die of foodborne diseases each year. Nontyphoidal *Salmonella*, the major cause of these foodborne illnesses is estimated to cause more than one million illnesses each year, with 23,000 hospitalizations and 450 deaths (http://www.cdc.gov/salmonella/index.html). The distribution of serotype varies worldwide, leading to a difference in the most common serotypes. In Europe, in 2012, 42% of cases were serogroup D (almost exclusively attributed to *S.* Enteritidis), followed by serogroups B (32.7%) and C (8.4%) (62). In the United States in 2012, 25.7% of all reported cases of salmonellosis were caused by serogroup C isolates, followed by serogroup B (20.5% of all cases). Serogroup D accounted for only 16.5% of all reportedcases (62). However, the increase of

prevalence of *Salmonella* serogroup C has been observed in both the United States and Europe with 22.5 to 34.7% and 5 to 8.6%, respectively, suggesting that this serogroup may become more important in the future (62). The surveillance data demonstrated that the persistence of nontyphoidal *Salmonella* in animals can introduce into the food chains via food products. The food products from food animal are mainly associated with the outbreaks including milk, poultry and eggs, as well as food products such as chocolate and peanut butter (Table 2.) (https://www.cdc.gov/salmonella/outbreaks.html)

Table 2. Major outbreaks of *Salmonella* spp. from 2002 to 2014 (https://www.cdc.gov/salmonella/outbreaks.html)

Year	Serovar	No. of cases reported	Food source	Country	Remarks
2014	*Salmonella* Infantis, *S.* Newport or *S.* Hadar	300	Live poultry	USA	80% of the reported ill people had contact with live poultry a week before the illness began
2012	*Salmonella* Bareilly and *S.* Nchanga	425	Raw yellowfin tuna	USA	Present in the frozen raw yellowfin tuna product known as Nakaochi Scrape
2010	*Salmonella* Montevideo	272	Red and black pepper/ Italian-style meats	USA	Found in the pepper added to the meats
2007	*Salmonella* Tennessee	628	Peanut butter	USA	Found in the environmental samples collected from the plant
2005	*Salmonella* Oranienburg	126	Alfalfa	Australia	Alfalfa at a production facility
2002	*Salmonella* Oranienburg	439	Chocolate	Germany	*S.* Oranienburg isolated from chocolate (high fat content) displayed a higher level of heat resistance

In Thailand, the observational study demonstrates the epidemiological trends and risk factors associated with the 10 most common *Salmonella* serotypes isolated from humans in Thailand during 2002 to 2007 (2). Among a total of 11,656 *Salmonella* isolates covering all 6 years, the top 10 *Salmonella* serotypes were *S.* Enteritidis, *S.* Stanley, *S.* Weltevreden, *S.* Rissen, *S.* I [1],4,[5],12:i:_, *S.* Choleraesuis, *S.* Anatum, *S.* Typhimurium, *S.* Corvallis, and *S.* Panama, which accounted for 69.6% of the isolates (2). Moreover, this study reports the increasing rate of human infections with *S.* Stanley,

S. Corvallis, and *S.* Choleraesuis which have been associated with swine and the decreasing rate of human infections with *S.* Weltevreden and *S.* Anatum. The distribution of the top 10 most common serotypes of nontyphoidal *Salmonella* from the different regions in Thailand is shown in Figure 1. The study revealed two serotypes, S. Enteritidis and S. Choleraesuis associated with a high odds ratio for blood samples. Moreover, the additional epidemiologic studies are recommended to investigate the increase in swine associated serotypes (*S.* Stanley, *S.* Corvallis, and *S.* Choleraesuis) (2)

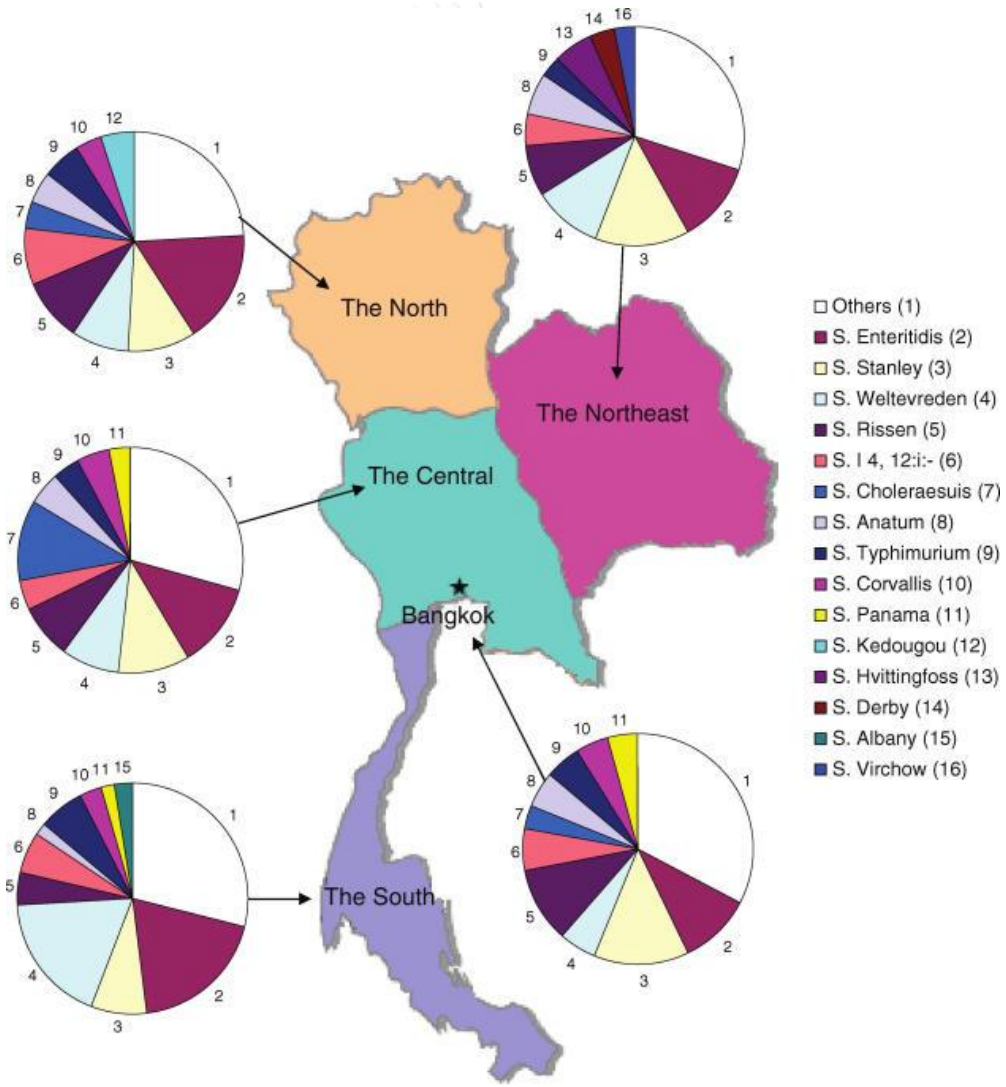


Figure 1. Distribution of the top 10 most common serovars in the different regions in Thailand from 2002 to 2007 (2)

Part II: Antimicrobial resistance in nontyphoidal *Salmonella*

Although salmonellosis is usually a self-limiting disease, antimicrobial agent is required for treatment of invasive infection. The conventional antimicrobial agents, such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (SXT), are the drugs of choice in the treatment of salmonellosis (9). Since nontyphoidal *Salmonella* with high rate of resistance to the traditional drugs has been reported from many countries (3), ciprofloxacin (fluoroquinolones) and ceftriaxone (third-generation or extended-spectrum cephalosporins, ESCs) are recommended as alternatives (4). However, nontyphoidal *Salmonella* isolates resistant to ESCs and fluoroquinolones have been increasingly reported in worldwide (5, 8).

Fluoroquinolones

Quinolones are bactericidal antibacterial agents with broad-spectrum activity. The modifications at C position 6 by adding fluorine can create the fluoroquinolones which are more potent against Enterobacteriaceae. The drug targets are DNA gyrase and topoisomerase IV. DNA gyrase or topoisomerase II consists of two subunits (GyrA and GyrB) and topoisomerase IV also consists of two subunits (ParC and ParE). Both enzymes are important for DNA replication. There are two major fluoroquinolone resistance mechanisms. The chromosomal-mediated mechanism is the modification of quinolone targets with changes of DNA gyrase and/or of topoisomerase IV genes on the quinolone-binding site of drug target enzymes which has been described as Quinolone Resistance-Determining Region (QRDR) (38). In *Salmonella*, a single mutation in *gyrA* gene can confer high-level resistance to nalidixic acid but additional mutations may be required to obtain high-level fluoroquinolone resistance (37).

The effects of individual mutations on fluoroquinolone resistance in *Salmonella* isolates were determined using *in vitro* mutants which showed that Ser83Phe amino acid substitution conferred an 8-fold increase in the ciprofloxacin MIC and 64-fold increase in

the nalidixic acid MIC (63). Amino acid substitution at Ser83 confered a higher resistance level than that at Asp87 (63). It has been reported that a single mutation in *gyrA* gene can display from low to very high level of fluoroquinolone resistance with ciprofloxacin MIC ranging from 0.5 to 8 mg/L and double mutation in *gyrA* gene can be highly resistant with ciprofloxacin MIC ranging from 4 to 16 mg/L (63). However, it is still unclear whether additional mutations leading to higher levels of quinolone resistance in *Salmonella* were similar to those previously suggested for *E. coli* (63). Although high-level resistance in clinical isolates of *Salmonella* was uncommon, these isolates were commonly found at least two mutations in the *gyrA* gene, often combined with mutations in the other topoisomerase genes or with other mechanisms (37).

The mutations occur most frequently at Ser83 and Asp87 as in *E.* coli and *Salmonella*. However, mutations at Ala67, Asp72, Gly81 and Asp82 have also been reported in *Salmonella,* as shown in Table 3 (37). Asp87Gly was found to be the most common mutation in the panel of veterinary Salmonellae in previous study(64), while Asp87Asn mutation was most commonly found in human isolates of *S.* Typhimurium DT104 and also in *S.* Hadar and *S.* Montevideo isolates from farm animals (65). It has been suggested that mutations at Ser83 and Asp87 may not be equally distributed among different serotypes, for instance Ser83 mutations were more prevalent in *S.* Newport, *S.* Virchow and *S.* Typhimurium, while Asp87 mutations were more prevalent in *S.* Hadar and *S.* Kottbus (37). Amino acid substitution at Ser83Tyr was found in all *S.* Bredeney isolates tested and Asp87Gly was found in all *S.* Senftenberg isolates tested in previous study (66). The successful distribution of particular clones after acquiring a mutation in *gyrA* may play an important part in the association between serotypes and *gyrA* mutations.

Table 3. Amino acid substitutions detected in the DNA gyrase and topoisomerase IV of *Salmonella* (37).

Gene	Codon position	Substitution
gyrA	Ala67	Pro
	Asp72	Gly
	Val73	Ile
	Gly81	Cys
		Ser
		His
		Asp
	Asp82	Gly
		Asn
	Ser83	Tyr
		Phe
		Ala
	Asp87	Asn
		Gly
		Tyr
		Lys
	Leu98	Val
	Ala119	Ser
		Glu
		Val
	Ala131	Gly
	Glu139	Ala
	Asp144	Asp (silent mutation)
gyrB	Tyr420	Cys
	Arg437	Leu
	Ser464	Tyr
		Phe
parC	Tyr57	Ser
	Thr66	Ile
	Gly78	Asp
	Ser80	Arg
		Ile
	Glu84	Lys
		Gly
parE	Glu453	Gly
	Ser458	Pro
	His461	Tyr
	Ala498	Thr
	Val512	Gly

In *Salmonella,* amino acid substitutions in ParC are not detected as frequently as in *E. coli* suggesting that they do not play an important role in quinolone resistance (37). Amino acid substitutions in ParC at codon 80 are most commonly found in *Salmonella* (37). The loss of a Ser80-Ile substitution was previously demonstrated to display a 16- to

32-fold decrease in resistance to fluoroquinolones in *S.* Typhimurium DT204 (67). To date, amino acid substitutions in ParE have still hardly been identified in *Salmonella* isolates (37).

Recently, the plasmid-mediated quinolone resistance (PMQR) mechanisms have been reported. The most common one is the production of quinolone resistance protein (Qnr) which has been reported worldwide from many pathogens including *Salmonella* (39-41). Qnr protein can protect DNA gyrase from fluoroquinolone lethal inhibition by reversibly competitive binding to DNA gyrase and topoisomerase IV before it forms DNA-DNA gyrase complex which is target binding for quinolones (42). Interestingly, ciprofloxacin MIC of transconjugant *E. coli* which obtained *qnr* gene from the *Salmonella* isolate displayed 4 mg/L, corresponding to a 64-fold increase compared with that in the wild-type recipient strain (68). However, *E. coli* carrying the recombinant DNA expression vector of *qnr* gene conferred low-level ciprofloxacin resistance, suggesting that the phenotypic expression of the *Salmonella qnr* gene is highly dependent on its genetic environment, and is likely to undergo positive selection towards higher levels, for instance by the mutational strengthening of promoters (68). Moreover, low-level fluoroquinolone resistance of *qnr* gene can facilitate the selection of chromosomal quinolone-resistance mutations by raising the level at which mutants can be selected (69, 70). Currently, five main families of *qnr* genes have been reported in Enterobacteriaceae; *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*. The levels of fluoroquinolone resistance conferred by *qnr* genes are generally lower than chromosomal mutations but the MIC can reach up to 1 mg/L (36). The other mechanisms are the presence of the modified-acylyltransferase (AAC (6')-Ib-cr) that acetylates ciprofloxacin/ norfloxacin (43) and the QepA protein that acts as an efflux pump for hydrophilic fluoroquinolones (44). According to the effect of these plasmid-mediated resistance mechanisms with the low-level fluoroquinolone MIC, suggesting that the pathogens with the ciprofloxacin MIC of 0.125-1 mg/L or reduced susceptibility to ciprofloxacin should be determined. The impact of different resistance mechanisms on susceptibility to ciprofloxacin is shown in Table 4.

Table 4. Summary of the impact of different quinolone resistance mechanisms on susceptibility to ciprofloxacin (36)

Resistance mechanism	Fold change in ciprofloxacin MIC
Gram-negative species[a]	
Topoisomerase substitutions	
gyrA	10–16
parC	0
gyrA (× 2) + *parC*	60
Permeability changes	
Efflux upregulation	4–8
Porin loss	4
PMQRs	
Carriage of *qnr* alleles	>30
Carriage of *qepA*	32
Carriage of *oxqAB*	16
Carriage of *aac(6′)Ib-cr*	4
Gram-positive species[b]	
Topoisomerase substitutions	
grlA	4–8
grlB	4–8
gyrA	0
grlA + *gyrB*	64–128
Permeability changes	
Efflux upregulation	4

[a]Based on data from *Escherichia coli*. [b]Based on data from *Staphylococcus aureus*.

PMQR genes are widespread among bacteria in many countries due to conjugative plasmid and various mobile genetic elements (45).The *qnr*-like determinants are commonly found embedded in In4 family complex class 1 integrons downstream of *orf513* in plasmid of enterobacteria (45).

Cephalosporins

Cephalosporins, bactericidal antibacterial agents, are members of ß-lactam antibiotics act on penicillin-binding proteins which catalyze the cross-linking of the peptidoglycan polymers in the bacterial cell wall, leading to the inhibition of cell wall synthesis. Cephalosporins are classified to first to fourth generation based on their antibacterial activities. Extended-spectrum cephalosporins (ESCs), commonly referred to third-generation and fourth-generation cephalosporins, are frequently used antibiotics for the treatment of severe infections, because of their broad spectrum, strong bactericidal activity, and low toxicity (9). A third generation cephalosporin, ceftriaxone is administrated to treat salmonellosis caused by fluoroquinolone-resistant *Salmonella*, or in children and infants (9).

The most common cause of resistance to ESCs in nontyphoidal *Salmonella* is the production of extended-spectrum ß-lactamases (ESBLs) and AmpC ß-lactamases which destroy ß-lactam ring of ß-lactam antibiotics, resulting in loss of antibacterial activity (10, 11). By definition, ESBLs are molecular class A or D ß-lactamases, which (i) are able to hydrolyze oxyimino, (ii) have an active-site serine, and (71) are inhibited by ß-lactamase inhibitors such as clavulanic acid (72, 73). ESBLs contain a number of mutations that allow them to include its criteria. The major of ESBLs are molecular class A ß-lactamases such as TEM (which names after the patient's name of Temoniera), SHV (which stands for SulfHydryl Variable), and CTX-M (which stands for 'active on CefoTaXime, first isolated in Munich') -type derivatives (11). Class A ß-lactamases commonly found four motifs including Ser70-X-X-Lys73 (X is any amino acid), Ser130-X-Asn, Asp233-Lys234-Thr/Ser-Gly, and Ω-loop which Ser70 was the main catalytic residue in their active site (Ambler numbering (74) (75, 76) (Figure 2.). The OXA–type ß-lactamases are molecular class D ß-lactamases and contain an active site serine. In the Bush functional classification scheme, ESBLs are placed in two subgroups of group 2 which are inhibited by clavulanate, including subgroups 2be for mainly TEM and SHV-derived ESBLs and 2d for OXA-derived ESBLs (72).

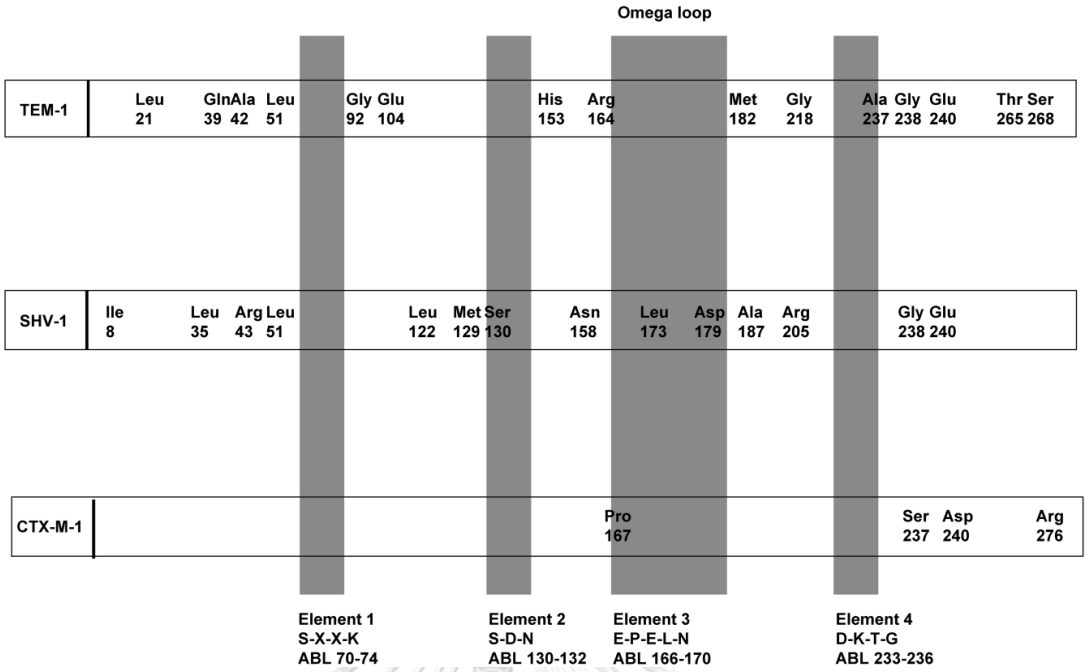


Figure 2. The key amino acid positions in molecular class A ß-lactamases TEM, SHV, and CTX-M (77). The grey shaded areas represent the evolutionary conserved structural elements that limit the active site and the numbering is according to the scheme of Ambler *et al.* (78).

ESBLs differ from their parent TEM-1, TEM-2 and SHV-1 enzymes by 1 to 7 amino acid substitutions that alter the configuration and the properties of the active site (Figure 2.). The most important substitutions for extending spectrum activity are mutation at position 164 in TEMs, 179 in SHVs and 238 in both, provide enough space for the interaction of enzymes with ß-lactams that have the bulky oxyimino side-chains (77, 79).

The major of ESBLs are TEM, SHV, and CTX-M-type derivatives. Currently, over 300 variants of enzymes by accumulation of point mutations have been reported worldwide. Recently, the CTX-M-type ß-lactamases, which preferentially hydrolyze cefotaxime, has become particularly widespread in recent years (11). CTX-M-producing strains are resistant to cefotaxime, but they often appear to be susceptible to ceftazidime. The crystal structures of CTX-M enzymes have shown that the active sites of CTX-M are not large enough to recognize ceftazidime, which is larger than cefotaxime

(80). Moreover, most of CTX-M enzymes hydrolyze cefepime effectively and MIC values of cefepime for bacteria producing CTX-M tend to be higher than those producing other types of ESBLs (81). A key role in extended-spectrum activity has been attributed to Ser-237, Asp-240, and Arg-276 (82-84) (Figure 2.). It has been suggested that the serine residue at position 237, which is present in all of the CTX-M enzymes, plays an important role in the extended-spectrum activity of the CTX-M-type ß-lactamases (82). The substitutions of Asp240 and Pro167 are known to enhance hydrolytic activity against ceftazidime. Inhibition by ß-Lactamase inhibitors such as sulbactam, clavulanate, and tazobactam are commonly known as inactivators of class A ESBLs. Interestingly, CTX-M-14 is capable of hydrolyzing sulbactam, while clavulanate and tazobactam retain their ability to inactivate this enzyme (85).

In recent years, the number of CTX-M derivatives have been described from CTX-M-1 to CTX-M-172 but the amino acid sequences of CTX-M-14 and CTX-M-18 of CTX-M-55 and CTX-M-57 of CTX-M-2 and CTX-M-97, and of CTX-M-3 and CTX-M-133 are identical (86). Some amino acid alterations of these enzymes affect substrate hydrolysis which extends their hydrolysis spectrum (25). CTX-M-type enzymes are divided into five groups, namely CTX-M-1, CTX-M-2, CTX-M-9, CTXM-8, and CTX-M-25, according to the similarity of their amino acid sequences (25, 86). CTX-M derivatives were: the CTX-M-1 group, including CTX-M-1, -3, -10, -11, -12, -15, -22, -23, -29, -30, -32, -33, -28, -36, and -54; the CTX-M-2 group, including CTX-M-2, -4, -6, -7, -20, -31,and -44; the CTX-M-9 group, including CTX-M-9, -13, -14, -16, -17, -19, -24, -27, -45, -46, -47, -48, -49, and -50; the CTX-M-8 group, including CTX-M-8 and CTX-M-40; the CTX-M-25 group, including CTX-M-25, -26, -39, and -41 (25).

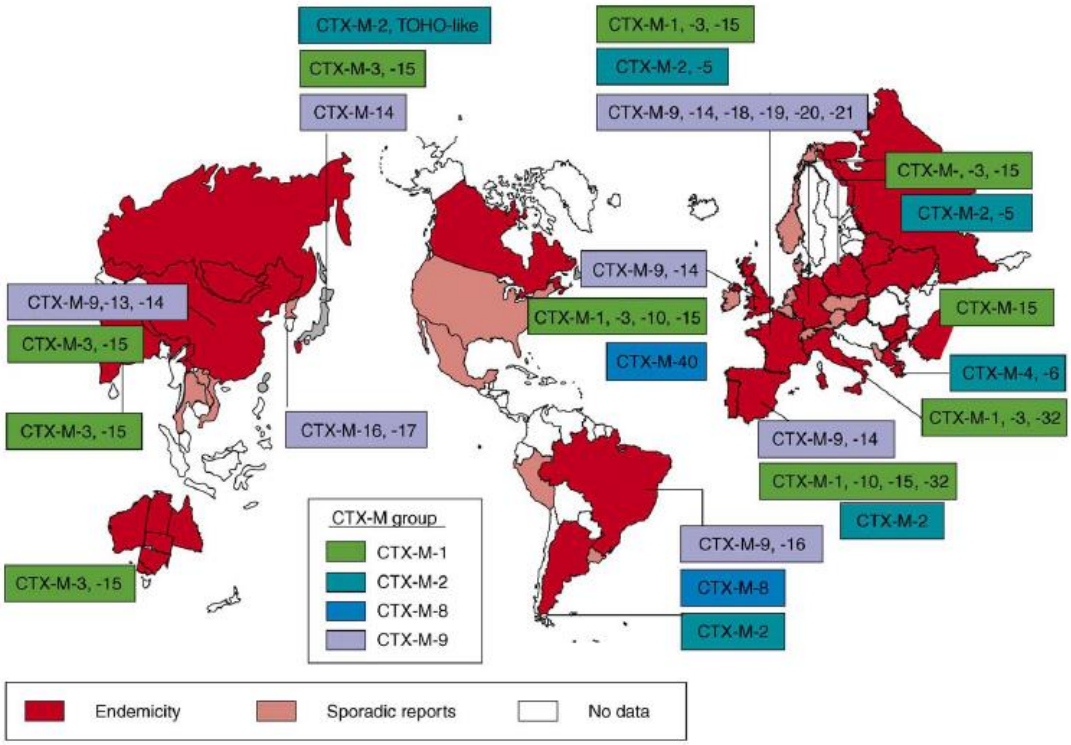


Figure 3. The current situation of CTX-M type ESBLs in different geographic areas (25).

Recently, it is interesting that an endemic situation is dominant in most countries in Europe, Asia and South America (25) (Figure 3.). The enzymes from the CTX-M-9 group are well represented in the countries surrounding the Mediterranean Sea (87, 88). The CTX-M-2 has been mainly isolated in South America and Japan (89, 90) whiles CTX-M-15 is spread nearly worldwide (91, 92). CTX-M enzymes have been reported in many outbreaks worldwide, e.g. in China (CTX-M-3, CTX-M-9, CTXM-13, and CTX-M-14) (93, 94), Vietnam (CTX-M-14 and CTX-M-17) (95), Taiwan (CTX-M-3 and CTX-M-14) (96), Korea (CTX-M-14) (97), Poland (CTX-M-3 and CTX-M-15) (98, 99). This suggests that CTX-M enzymes are widely dispersed. There is a concern that CTX-M ß-lactamases confer resistance to all cephalosporins, but are not detectable by detection tests which are based on using only ceftazidime.

ESBLs reported in nontyphoidal *Salmonella* included TEM-3 (12), TEM-52 (13), SHV-2a (14), SHV-5 (15), SHV-12 (14, 16, 17), CTX-M-1 (7, 16), CTX-M-2 (18), CTX-M-3 (14), CTX-M-5 (17, 19), CTX-M-9 (16, 20), CTX-M-14 (16, 21), CTX-M-15 (17, 22), CTX-

M-32 (23), CTX-M-53 (24), and CTX-M-55/57 (17). Recently, the CTX-M are rapidly growing group of ESBLs and extended in various types which have been reported in several countries (11). There are only three groups of CTX-M including CTX-M-1, CTX-M-2, and CTX-M-9 reported in nontyphoidal *Salmonella* (10). Interestingly, many of new variants of CTX-M enzymes have been frequently found among isolates of *S.* Typhimurium (100, 101). The outbreaks of CTX-M-producing strains of *S.* Typhimurium have also been reported in South America and Eastern Europe. It has also been found to express a variety of CTX-M type variants (18, 100, 102).

AmpC enzymes were classified into class C in the Ambler structural classification of ß-lactamases (78) and were assigned to group 1 in the functional classification scheme of Bush *et al.* (72). AmpC enzymes were consistently resistant to penicillins and provided resistance to cephalosporins in the oxyimino-cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and the cephamycins (cefoxitin, cefotetan) (26). MICs were usually higher for ceftazidime than for cefotaxime and for cefoxitin than for cefotetan (26). These enzymes were also resistant to the monobactam and aztreonam and were poorly inhibited by ß-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (26). However, the enzymes were susceptible to cefepime, cefpirome and carbapenems (imipenem, meropenem) (26). The enzymes had the motif Ser-X-X-Lys (X is any amino acid) at residues 64 to 67 which was a serine active site of the mature protein. The key catalytic residues are found in active site pocket other than Ser64 for class C enzymes including Lys67, Tyr150, Asn152, Lys315, and Ala318, with substitutions at these sites lowering enzymatic activity significantly (103).

AmpC ß-lactamases, verified to be chromosomally mediated since 1981, have been described in many genera of bacteria such as *Acinetobacter* spp., *Aeromonas* spp., *Chromobacterium violaceum*, *C. freundii*, *Enterobacter* spp., *E. coli*, *Hafnia alvei*, *Lysobacter lactamgenus*, *Morganella morganii*, *Ochrobactrum anthropi*, *Proteus rettgeri*, *Providencia stuartii*, *P. aeruginosa*, *Psychrobacter immobilis*, *Rhodobacter sphaeroides*, *S. marcescens*, and *Yersinia enterocolitica* (26, 104). However, many bacteria lack an intrinsic AmpC ß-lactamases. The dissemination of this resistance

determinant is also mediated by plasmids (26). It has been suggested that plasmid-mediated AmpC ß-lactamases originate from the transfer of chromosomal genes onto plasmids (26, 103, 105). This transfer has resulted in plasmid-mediated AmpC enzymes in several members of the family Enterobacteriaceae (26). The plasmid-mediated AmpC ß-lactamases are classified based on amino acid sequences into six families that are closely related to chromosomal-mediated AmpC ß-lactamases as indicated in Figure 4. (26, 106). The six families of plasmid-mediated AmpC ß-lactamases included MOX, CIT, DHA, ACC, EBC, and FOX family (26). Most AmpC ß-lactamases are derivatives of CIT-type ß-lactamases, including LAT-1, CMY-2 to -7, CMY-12 to -18 and CMY-20 to -50 (86, 103).

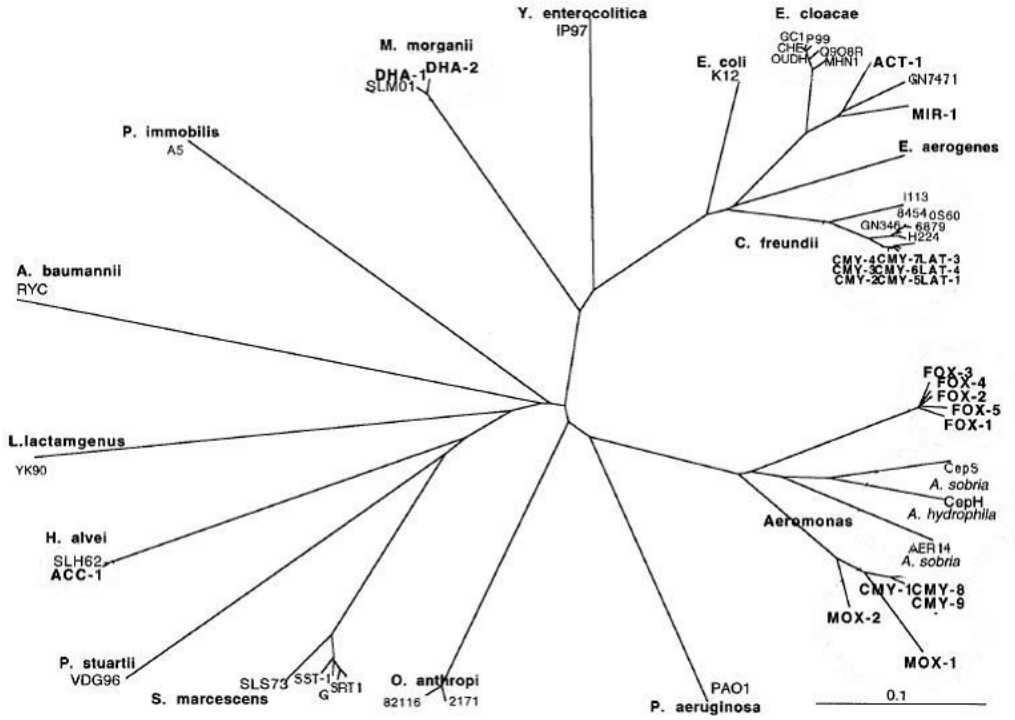


Figure 4. Dendrogram for chromosomal and plasmid-mediated AmpC ß-lactamases: Plasmid AmpC are shown in boldfaces and branch lengths are proportional to the number of amino acid exchanges (26).

Salmonella is one of many bacteria that lacks an intrinsic AmpC ß-lactamases. However, the dissemination of this resistance determinants is also mediated by

plasmids (26). Recently, CMY-2, CMY-4, CMY-7, ACC-1, and DHA-1 have been found in nontyphoidal *Salmonella* (27-31). CMY-2 was commonly identified worldwide such as England and Wales (27), France (7), United States (32), Netherlands (30), Taiwan (6, 21), South Korea (33), China (34), and Singapore (35). CMY producers were found in several serovars of *Salmonella enterica*, with *S.* Typhimurium and Newport being the most common (10, 107, 108).

Part III: Epidemiology of ESC- and fluoroquinolone-resistant nontyphoidal *Salmonella*

Currently, antimicrobial–resistant nontyphoidal *Salmonella* has been reported as a threat level serious by CDC (http://www.cdc.gov/drugresistance/threat-report-2013). The National Antimicrobial Resistance Monitoring System (NARMS) reported that resistance to ceftriaxone was about 3% of nontyphoidal *Salmonella* tested, and ciprofloxacin MIC of $\geq$ 0.125 mg/L was about 3%. About 5% of nontyphoidal *Salmonella* were resistant to five or more types of antimicrobials in the United States for 3-year average (2009–2011). Antimicrobial resistance in nontyphoidal *Salmonella* increased from <1% in 1996 to ~3% in 2011 for both ceftriaxone and ciprofloxacin. A European surveillance study in 27,000 *Salmonella* isolates reported reduced susceptibility to fluoroquinolone in 13% of *S.* Typhimurium, 8% of *S.* Enteritidis, 53% of *S.* Virchow, and 57% of *S.* Hadar isolates in 2000 (109). A study of nontyphoidal *Salmonella* from seven Asian countries, including Philippines, Hong Kong, Singapore, Sri Lanka, Korea, Thailand, and Taiwan showed that 3.0% were ceftriaxone resistance during 2003-2005, except in Taiwan (38.0%) and in *S.* Typhimurium (25.0%) from all countries (48). This multinational study from Asia also showed that reduced susceptibility to ciprofloxacin in nontyphoid *Salmonella* isolates was commonly found in Taiwan (48.1%) and Thailand (46.2%) (48). A study from China in *S.* Typhimurium clinical isolates showed ciprofloxacin and ceftriaxone resistance with 20% and 4%, respectively during 2005 to 2011 (49).

This problem seems to be more serious according to the increasing use of antimicrobials in animal farms. Almost 80% of the amounts of antimicrobials sold for both people and food animals were reserved for food animal production reported by the Food and Drug Administration (FDA) (http://www.fda.gov/AnimalVeterinary/NewsEvents/CVMUpdates.htm). ESCs and fluoroquinolones, broad-spectrum antimicrobials, have been classified as critically important antimicrobials for human medicine by WHO (50). Currently, these two classes of antimicrobials are commonly used in food animal production systems. Ceftiofur and enrofloxacin were commonly used for the treatment or prevention of infection in animals. They are closely related with ceftriaxone and ciprofloxacin used for the treatment of human for systemic salmonellosis in children and in adult, respectively (50). Since the introduction of their use in food animal medicine, the prevalence of these antimicrobial-resistant organisms within livestock population has been increasing (51) and antimicrobial-resistant *Salmonella* isolates were identified in imported foods in many countries (52-54). In US, the prevalence of ESC-resistant *Salmonella* isolates from cattle ranged from 2.4% to 17.6% from the NARMS study during 1999 to 2004 (51, 110). The prevalence of ESC-resistant *Salmonella* was dramatically increased to 37.9% of isolates in the US (111). Recently, the results from Antimicrobial Resistance Surveillance in Canada showed the high rate of ESC resistance (ceftiofur and ceftriaxone) in *S.* Heidelberg isolated from both humans and chicken. The study described a strong correlation ($r = 0.9$, $p<0.0001$) between ceftiofur-resistant *S.* Heidelberg isolated from retail chicken and incidence of ceftiofur-resistant *S.* Heidelberg infections in humans across Canada (55). In Québec, >60% of *S.* Heidelberg chicken isolates and 30% to 40% of *S.* Heidelberg human isolates were resistant to ceftiofur. Moreover, the changes of ceftiofur resistance in *S.* Heidelberg isolated from chicken are related to changing levels of ceftiofur use in hatcheries during 2003–2008, from highest to lowest levels before and after a voluntary withdrawal and to increasing levels after reintroduction of use from 62% to 7% to 20%. These events provide evidence that ceftiofur use in chickens may be result in ESC resistance in bacteria isolated from chicken and humans.

The results suggested that the extensively use of ESCs and fluoroquinolones in food animals can be a potential problem for antimicrobial resistance of *Salmonella* in human population. Moreover, some antimicrobial resistance genes can be widespread among inter-serovar or inter-species of bacteria by conjugative plasmid and various mobile genetic elements (52, 112).

ESC-resistant *Salmonella* isolates reported worldwide were attributed to the different types of ß-lactamases (10). The geographical distribution of the different ß-lactamase groups and their alleles conferring to ESC resistance in *Salmonella* isolates from human is summarized in Table 5. CMY-2 is reported in many countries widely distributed in all four regions (Table 5.). However, some enzymes are limited to specific continents, such as CTX-M-14 in Asia and TEM- 52 in Europe. For the ESC-resistant *Salmonella* isolates from food animal, the following *bla* genes have been detected from different countries including Belgium (poultry: CTX-M-2, TEM-52), Brazil (poultry: CTX-M-2), Canada (cattle: CMY-2), France (poultry: CTX-M-1/-9; cattle: CTX-M-1), Germany (poultry: CTX-M-1, TEM-20/-52, CMY-2; pigs and cattle: CTX-M-1), Ireland (poultry: SHV-12, CMY-2), Italy (poultry: SHV-12), Japan (poultry: TEM-52), the Netherlands (poultry: CTX-M-1/-2, TEM-20/-52, ACC-1), Spain (poultry: CTX-M-9; pigs: SHV-12), UK (poultry:CMY-2), USA (cattle: CMY-2, CTX-M-1; pigs: CTX-M-1) (113). ESC resistance in different serotypes of *Salmonella* was attributed to the different type of ß-lactamases (10). The distribution by serotype of *Salmonella* of the different ß-lactamase groups and their alleles conferring to ESC resistance in *Salmonella* isolates is summarized in Table 6. *S.* Typhimurium and *S.* Enteritidis, the most prevalent serotypes in many countries have been reported the most diversity type of ß-lactamases witht at least 18 and 11 different enzymes in S. Typhimurium and S. Enteritidis, respectively (Table 6). Moreover, ESBL and AmpC-encoding genes including CTX-M (46), SHV (46), TEM (46), and CMY (47) have been also reported the colocalization with plasmid-mediated quinolone resistance (PMQR) genes.

Table 5. Distribution by country of the different ß-lactamase groups and their alleles conferring to ESC resistance in *Salmonella* from human source (10)

	TEM group	SHV group	CTX-M-1 group	CTX-M-2 group	CTX-M-9 group	PER group	CMY group	DHA group	ACC g
Europe									
Austria		5							
Belarus				5					
France	3, 4, 52		15		9		2		
Greece	52	5	32	6,7			2		
Hungary	52								
Italy		12					2		
Latvia				5					
The Netherlands	20, 52, 63	2, 12	3, 15, 28	2					ACC-1
Poland		2a	3						
Romania		5					2		
Russia				4, 5					
Slovakia									
Spain	27				9, 14		2		
Turkey						PER-1			
UK	52	12	15		9, 17		2, 4	DHA-1	
Americas									
Argentina				2		PER-2			
Brazil									
Canada		2					2		
Honduras		5	15				2		
Martinique	3								
Mexico							2		
USA							2		
Africa									
Algeria	25	2	3				2		
Gambia							2		
Libya		2							
Mali		12							
Morocco	3								
Senegal		12	15						
South Africa	63, 131	5, 12					2		
Tanzania		12							
Tunisia	4	2, 2a	3		27		4		ACC-1
Asia									
Hong-Kong					14				
India		5							
Iraq							2		
Japan					14				
Korea	52				14			DHA-1	
Lebanon			15						
Pakistan			15						
Saudi Arabia								DHA-1	
Taiwan		2a, 12	3		14		2		
Thailand					17				

Table 6. Distribution by serotype of *Salmonella* of the different ß-lactamase groups and their alleles conferring to ESC resistance in *Salmonella* isolates (10)

Serotype	TEM	SHV	CTX-M-1	CTX-M-2	CTX-M-9	PER	CMY	DHA	ACC
Agona	52			2		2	2		
Ajiobo							2		
Albany			3				2		
Anatum			3, 15				2		
Babelsberg		12							
Bareilly									ACC-1
Blockley	52								
Braenderup									ACC-1
Brandenburg		5							
Bredeney							2		
Cairo							2		
Chester							2		
Choleraesuis			3				2		
Concord		12							
Cremieu							2		
Cubana									
Derby							2		
Duesseldorf							2		
Enteritidis	52	2a, 5, 12	3, 15	2	14, 17		2	DHA-1	
Give							2		
Gloucester							2		
Hadar	52								
Heidelberg							2		
Infantis		5	3, 15	2			2		ACC-1
Isangi	63, 131	5	28						
Kaduna							2		
Kedougou	3								
Kentucki			15						
Kimuenza							2		
Livingstone					27				ACC-1
London	52				14				
Mbandaka	4, 25	2a	3						ACC-1
Mikawasima							2		
Mons			3				2		
Montevideo								DHA-1	
Muenchen	63								
Newport		12					2		
Oranienburg			3	2					
Othmarschen	27								
Panama	3, 52				14				
ParaB	20, 52		15						
Redba							2		
Saint-Paul	52		3				2		
Schleissheim							2		
Schwarzengrund							2		
Senftenberg		5	3				2, 4	DHA-1	
Stanley	52				17		2		
Thompson	52						2		
Typhimurium	3, 52, 131	2, 2a, 5, 9, 12	3, 15	2, 4, 5, 6, 7		1, 2	2, 7		

In Thailand, ESCs and fluoroquinolones are also extensively used in food animal production systems for treatment and prevention of diseases and for growth promotion. Enrofloxacin is used in poultry, swine, and seafood industries and ceftiofur is used in swine industry. Recently, the prevalence of ESC and fluoroquinolone resistance in nontyphoidal *Salmonella* has been increasingly reported in Thailand. The multinational study from Asia also showed that reduced susceptibility to ciprofloxacin in nontyphoid *Salmonella* isolates was commonly found in Thailand (46.2%) from all countries (48). The study at Siriraj Hospital in 2005 showed that more than 17% of blood isolates of nontyphoidal *Salmonella* were resistant to ceftriaxone (56). Likewise, nontyphoidal *Salmonella* isolates from bacteremic patients at King Chulalongkorn Memorial Hospital and from the WHO National *Salmonella* and *Shigella* Center during 2003-2005 showed high rate of ceftriaxone resistance (15%) in *S.* Choleraesuis isolates and all of isolates with ceftriaxone resistance also showed resistance to quinolones (57). Currently, the fully ciprofloxacin-resistant *Salmonella* isolates were also found, accounted for 31% of isolates from human in 2011 (114). To date, only one study has described that the CMY-2 and CTX-M-14 ß-lactamases contribute to ESC resistance in *Salmonella* from clinical isolates in Thailand during 2003, 2007, and 2008 (115). Moreover, antimicrobial-resistant *Salmonella* as also found in imported seafood and chicken meat from Thailand (52, 58).

Part IV: Spread of antimicrobial resistance in nontyphoidal *Salmonella*

Antimicrobial resistance can be disseminated among bacterial population through the two basic ways including clonal spread of resistant strains and horizontal gene transfer of genetic elements coding for resistance determinants (116).

Clonal spread of resistant strains

The population of *Salmonella enterica* can be changed through the introduction of strains that spread and displace existing populations. Clonal spread of certain bacterial clones are referred to as “successful clones” due to favorable phenotypic traits (e.g. virulence or antibiotic resistance), which enable them to disseminate and persist in different environments, and cause human infections and outbreaks. Successful clones carrying antibiotic resistance determinants play a major role in the spread of resistance due to ability to survive in the antimicrobial selective pressure.

In the past few decades, the relationship of *Salmonella* strains is usually investigated by phenotyping technique using phage typing (117). Phage typing can classify *Salmonella* according to theirs susceptibility to a lysis by a panel of bacteriophages due to the molecular characteristics of the phage and phage receptor present on the surface of the bacterium (117). However, a stock of typing phages needs to be maintained and the technically demanding procedure is only available in major reference laboratories.

Recently, the molecular typing techniques, characterization of organism according to its genetic were widely used to investigate the relationship of *Salmonella* strains. Pulsed-field gel electrophoresis (PFGE) is currently the method used by a national food-borne disease surveillance system (118) to track the spread of foodborne pathogens and assist to identify sources of *Salmonella* outbreaks (119). PFGE is considered as the gold standard typing method for bacteria which provides a highly reproducible and discriminatory. This typing technique is based on a comparison of

DNA fragment patterns which are generated by digestion of bacterial genome using restriction endonuclease enzyme. PFGE is often performed using PulseNet protocol according to the Center for Disease Control and Prevention.

S. Typhimurim DT104 clone, one of the most important multidrug-resistant *Salmonella* clone with global dissemination, is identified by phage typing technique due to the association with a specific phage type called definitive type 104 (DT104) (120). The multidrug-resistant *S.* Typhimurium DT104 began to spread in the early 1980s in cattle in the United Kingdom. These strains displayed resistance to five antimicrobial agents including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline that commonly referred to as R-type ACSSuT (120). In 1996, *S.* typhimurium DT 104 were recognized in cattle and humans in the USA and this strain became particularly common in cattle, poultry and pigs in in many European countries and also in Israel, Canada, Turkey and Japan (121, 122). The outbreaks of multidrug-resistant *S.* Typhimurium DT104 related to unpasteurized cheese have been reported in the USA. Moreover, the strain DT 104 is apparent predilection to cause serious disease which over 15% of human isolations of multidrug-resistant *S.* Typhimurium DT104 in the USA have been reported to be associated with septicaemia (121). However, *S.* Typhimurium DT104 with R-type ACSSuT has been declining from 32% in1998 to 22% in 2005 and to 20% in 2011, all of which were collected from humans in the USA (9).

Later, the development of resistance to quinolones and fluoroquinolones has been another important resistance trend among nontyphoidal *Salmonella* isolates since 1992 (121). The multidrug-resistant *S.* Typhimurium DT104 has been developed the additional resistance to trimethoprim (R-type ACSSuTTm) and decreased susceptibility to ciprofloxacin (R-type ACSSuTCpL), leading to 15% trimethoprim resistance and 13% decreased susceptibility to ciprofloxacin in England and Wales in 1997 (123). It has been suggested that the emergence and spread of decreased susceptibility to ciprofloxacin may result from the use of enrofloxacin by the licensing for veterinary in the UK in 1993 (121).The rapid development of resistance to nalidixic acid in strains of S. typhimurium DT 104 has also been reported in food-producing animals in the UK,

particularly turkeys, but also in other livestock (124). The quinolone resistance mechanism of *S.* typhimurium DT 104 of R-type ACSSuTCp was due to the amino acid substitutions at Asp-87 and Ser-83 (121). The predominant patterns of antimicrobial resistance in *S.* typhimurium DT 104 isolated from human in England and Wales during 1990 to 2000 are shown in Table 7.

Table 7. The predominant patterns of antimicrobial resistance in *S.* typhimurium DT 104 from human in England and Wales during 1990 to 2000 (121)

Year	Total	Antibiogram (%)			
		ACSSuT	ACSSuTTm	ACSSuTCp$_L$	ACSSuTTmCp$_L$
1990	259	39	0	0	0
1991	544	44	0	0	0
1992	808	66	1	0.1	0
1993	1526	79	1	0	0
1994	2873	74	12	1	0
1995	2837	54	27	6	0
1996	4006	59	21	13	1
1997	2956	63	17	12	2
1998	2090	61	13	16	2
1999	1030	69	11	11	1
2000	1168	73	10	9	1

Drug resistance symbols: A, ampicillin; C, chloramphenicol; G, gentamicin; K, kanamycin; S, streptomycin; Su, sulfonamides; T, tetracyclines; Tm, trimethoprim; CpL ciprofloxacin (MIC of 0.125 to 1 mg/L).

In the United States, the emergence of multidrug-resistant *S.* Newport with an AmpC phenotype (*S.* Newport MDR-AmpC) was the most dramatic change in multidrug-resistant *Salmonella* during the past decade (116). These strains displayed resistance to five antimicrobial agents including ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline that commonly referred to as R-type ACSSuT(107). These S. Newport strains also carried plasmid encoding CMY-2 which mediated resistance to extended-spectrum cephalosporins (107). This is particular clinical concern for treating complicated salmonellosis in children with ceftriaxone. The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) reported the increase of *S.* Newport MDR-AmpC from human isolates in the U.S. from 1% in 1997 to

17% in 1999, 22% in 2000, and 25% in 2001 (www.cdc.gov/narms). The studies of the epidemiology of S. Newport MDR-AmpC infections in humans showed a strong link between the exposure to dairy cattle and several outbreaks. These infection were attributed to consumption of beef products (107) (116).

PFGE was used to investigate the relationship between human and animal isolates of *S.* Newport (125). The results showed that S. Newport MDR-AmpC isolates from human and animal sources had more than 80% similarity with S. Newport MDR-AmpC isolates from human, cattle, swine, and chicken. All isolates fell into the same major cluster (125). Indistinguishable PFGE patterns were also found in several isolates from human, cattle, and pig, and one chicken (125).

Horizontal gene transfer (HGT)

Horizontal gene transfer (HGT) is referred to the transfer of foreign genes between organisms. HGT can happen between related organisms and also between different species, genera, or kingdoms (126). Three mechanisms of HGT included (i) transformation which is the active uptake of free DNA from the environment by competent bacterial cells, (ii) transduction which is an infection of bacteria by bacteriophages or bacterial viruses, who inject foreign DNA into a bacterial cell, and (¡¡¡) conjugation which is the mechanism where a donor and a recipient cell physically connect to each other and share DNA carried by conjugative elements, such as plasmids and transposons. Moreover, the foreign DNA captured by transformation, transduction or conjugation can be integrated into the bacterial chromosome (126).

Plasmids are self-replicating extra-chromosomal, double-stranded DNA elements. They are harbored by most bacterial cells with various size, replication system, and host range. Broad-host range plasmids may spread among and be maintained by a wide range of bacterial hosts. Narrow-host range plasmids are only maintained by closely related bacterial hosts (126).

The plasmid backbone contains conserved core genes essential for replication, transfer and maintenance (126). Many plasmids also contain addiction systems including antibiotic resistance genes, heavy metal resistance genes, and virulence genes, which involve in environmental adaptability and persistence of bacterial host. The minimal portion of a plasmid that replicates with the characteristic copy number of the parent plasmid is called basic replicon. The replicon is a highly conserved region, which encodes genes needed for plasmid replication initiation, including an origin for initiation of replication (ori) and also the genes encoding specific replication initiator proteins. The backbone structure of plasmid is shown in Figure 5.

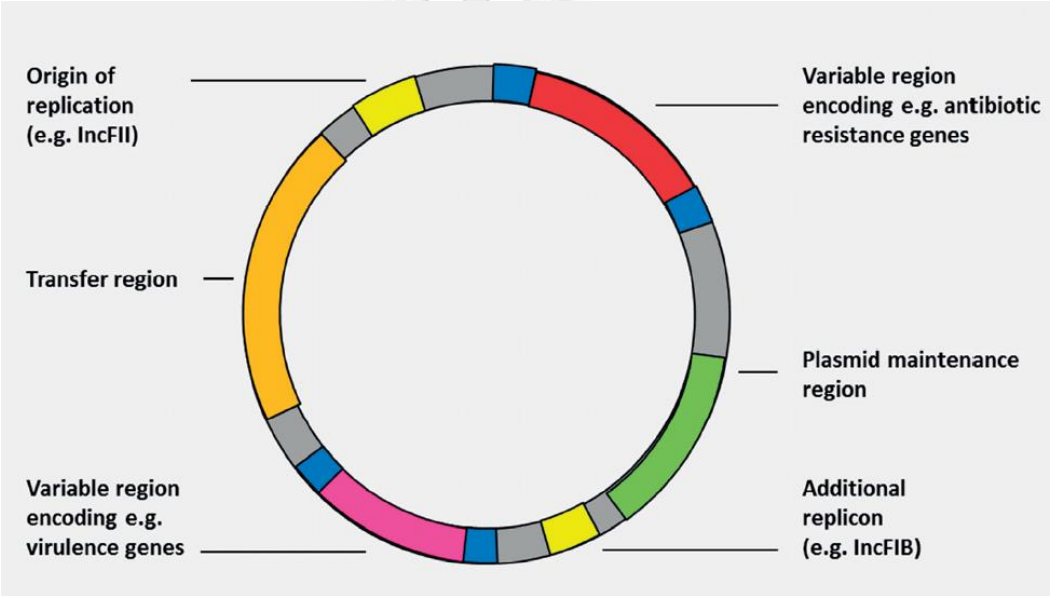


Figure 5. The backbone structure of plasmid including replicons (yellow), transfer region (orange) and maintenance region (green)

Plasmids can promote their horizontal transfer among bacterial population through the conjugation mechanism (126). Conjugation is referred when plasmids are transferred from a donor to a recipient cell, via a contact dependent transmission. Plasmid can be classified by conjugation ability including conjugative and non-conjugative plasmid. The conjugative plasmid, self-transmissible plasmid contains *tra*

genes which are necessary for non-sexual transfer of genetic material to perform the process of conjugation. However, plasmids that are not self-transmissible by conjugation can be mobilized at high frequency in the presence of a helper plasmid. These features can help the successful spread of certain specific plasmid types among bacterial population from different source and different geographical origin.

Plasmids can be classified based on plasmid incompatibility (Inc) groups using the phenomenon of the inability of two plasmids belonging to the same Inc group to be propagated stably in the same cell (127). Plasmid typing was traditionally performed by competition assays, where a new plasmid was introduced to an isolate carrying a plasmid of known incompatibility type (Inc type). Recently, a more convenient PCR-based replicon typing (PBRT) method has been developed and a PBRT scheme has been available by using multiplex PCRs, the replicons of the major plasmid families occurring in Enterobacteriaceae (127). Currently, 27 Inc groups are recognized in Enterobacteriaceae by the Plasmid Section of the National Collection of Type Culture (Colindale London, UK). Inc typing is frequently used together with other specific characteristics of the bacterial strain including resistance gene content, sequence type by multi-locus sequence typing (MLST), phylogroup by restriction fragment length polymorphism (RFLP) (127). These plasmid typing techniques are currently used as an additional marker for comparative analysis of unrelated and related strains during epidemiological investigations.

Plasmids are considered effective vehicles for the spread of antibiotic resistance determinants. In Enterobacteriaceae, IncFII, IncA/C, IncL/M, IncN and IncI are among the most common plasmid types carrying ESBLand AmpC-encoding genes (128). The following Inc group plasmids have been described for the most important *bla* genes in ESC-resistant *E. coli* and *Salmonella enterica* isolates from humans: $bla_{\text{CTX-M-1}}$ (IncN, IncI1, IncF, IncL/M), $bla_{\text{CTX-M-2}}$ (IncA/C, IncHI2, IncP, IncI1), $bla_{\text{CTX-M-9}}$ (IncHI2, IncP, IncF, IncI1, IncY, IncB/O, IncK), $bla_{\text{CTX-M 14}}$ (IncK, IncF, IncI1, IncHI2, IncB, IncA/C), $bla_{\text{CTX-M-15}}$ (IncF, IncI1, IncA/C, IncL/M, IncN), $bla_{\text{SHV-12}}$ (IncI1, IncK, and IncF, IncA/C, IncHI2), $bla_{\text{TEM-52}}$ (IncI1), $bla_{\text{CMY-2}}$ (IncI1, IncA/C, IncF), $bla_{\text{VIM-1}}$ (IncN, IncHI2, IncI1, IncW), and

bla$_{NDM-1}$ (IncN, IncL/M) (128). Moreover, the plasmids belonging to IncF, N, I1, I2, A/C, HI2, and K groups are present in both food animal and human hosts (113, 128). IncF plasmids also involved the other resistance genes including *aac(6')-Ib-cr*, *qnr*, and *armA* genes (128). ESBL and AmpC-encoding genes including CTX-M (46), SHV (46), TEM (46), and CMY (47) have also been reported to be colocalized with plasmid-mediated quinolone resistance (PMQR) genes. Major Inc group of plasmid and associated resistance genes in antimicrobial-resistant *Enterobacteriaceae* isolates from human and animal sources in worldwide are shown in Table 8.

Table 8. Major Inc group of plasmid and associated resistance genes in antimicrobial-resistant *Enterobacteriaceae* isolates from human and animal sources in worldwide (128)

Replicon	No. of plasmids	Resistance genes	Species
F	331	*aac(6')-Ib-cr*, *bla*$_{CMY-2}$, *bla*$_{CTX-M-1-2-3-9-14-15-24-27}$, *bla*$_{DHA-1}$, *bla*$_{SHV-2-5-12}$, *bla*$_{TEM-1}$, *armA*, *rmtB*, *qepA*, *qepA2*, *qnrA1*, *qnrB2*, *qnrB4*, *qnrB6*, *qnrB19*, *qnrS1*	*E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *S. enterica*, *S. marcescens*, *S. sonnei*
A/C	317	*bla*$_{CMY-2-4}$, *bla*$_{CTX-M-2-3-14-15-56}$, *bla*$_{SHV-2-5-12}$, *bla*$_{TEM-3-21-24}$, *bla*$_{IMP-4-8-13}$, *bla*$_{VIM-4}$, *bla*$_{VEB-1}$, *armA*, *rmtB*, *qnrA1*	*C. freundii*, *C. koseri*, *E. cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, *P. stuartii*, *S. enterica*, *S. marcescens*
L/M	270	*aac(6')-Ib-cr*, *bla*$_{CTX-M-1-3-15-42}$, *bla*$_{TEM-3-10}$, *bla*$_{SHV-5}$, *bla*$_{IMP-4-8}$, *armA*, *qnrA1*, *qnrB1*, *qnrB2*, *qnrB4*, *qnrS1*	*C. amalonaticus*, *C. freundii*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *M. morganii*, *P. mirabilis*, *S. enterica*, *S. flexneri*, *S. marcescens*
I1	146	*bla*$_{CMY-2-7-21}$, *bla*$_{CTX-M-1-2-3-9-14-15-24}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1-3-52}$, *bla*$_{VIM-1}$, *armA*, *rmtB*, *mphA*, *qnrA1*	*E. coli*, *K. pneumoniae*, *S. enterica*, *S. sonnei*
HI2	90	*bla*$_{CTX-M-2-3-9-14}$, *bla*$_{SHV-12}$, *bla*$_{IMP-4}$, *bla*$_{VIM-1}$, *armA*, *qnrA1*, *qnrS1*	*C. youngae*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *S. enterica*
N	70	*bla*$_{KPC-2}$, *bla*$_{CTX-M-1-3-15-32-40}$, *bla*$_{VIM-1}$, *qnrA3*, *qnrB2*, *qnrB19*, *qnrS1*, *armA*	*E. coli*, *K. ascorbata*, *K. pneumoniae*, *S. enterica*

From the current epidemiology of ESBL worldwide, the *bla*$_{CTX-M-15}$, one of the most important ESC resistance mechanisms has been located mainly on plasmids belonging to the IncF group (128). The IncF family has low copy number of plasmids and a narrow-host range which is limited to the Enterobacteriaceae family. IncF group can be sub-classified into IncFII, IncFIA, IncFIB and IncFIC (127). The IncFII replicon has been further subdivided into IncFIIS, IncFIIY and IncFIIK due to sequence variations and

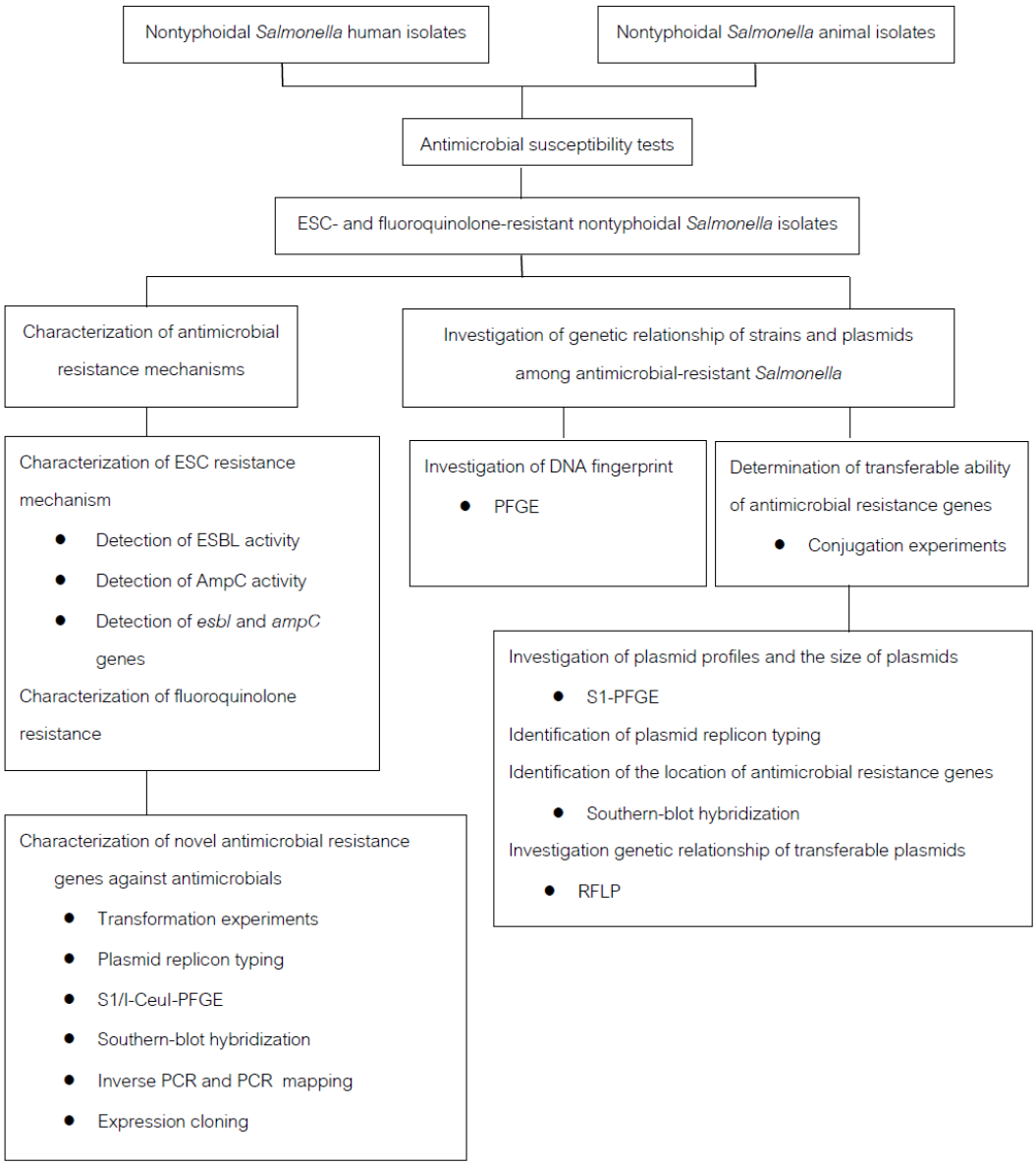
preferred host, *Salmonella spp*., *Yersinia spp. and Klebsiella spp*., respectively (127). The IncF plasmids are associated with the spread of other resistance determinants, including bla_{KPC}, bla_{CMY}, bla_{DHA}, *aac (6')-Ib-cr*, *qnr*, *qepA*, and *armA* genes (128). The $bla_{CTX-M-9}$ gene was found spreading in clinical isolates of *E. coli* and *Salmonella enterica* among European countries due to the dissemination of IncHI2 plasmids (129). The IncHI2 plasmids were also associated with the dissemination of the $bla_{CTX-M-2}$ gene in animal isolates (130).

Both of IncI1 and IncN have been found to involve in the transmission of the $bla_{CTX-M-1}$ gene (131). Since either IncN or IncI1 plasmid type has been demonstrated to be highly prevalent in *E. coli* of the avian fecal flora and in *Salmonella* spp. from retail meat and food-producing animals (131). Recently, genetically related IncI1 plasmids carrying the $bla_{CTX-M-1}$ gene from *E. coli* poultry isolates in the Netherlands were assigned to ST7 by plasmid-MLST. This ST type of plasmid represented 56% (75/136) of all the $bla_{CTX-M-1}$ carrying plasmids submitted in the pMLST database, suggesting that the spread of this gene is mostly due to one single plasmid circulating in different bacterial species (132). This finding suggested that these plasmids were animal reservoir for $bla_{CTX-M-1}$-carrying plasmids and the spread of this resistance determinant in animals could be sustained by the use of expanded-spectrum cephalosporins (i.e. ceftiofur) in veterinary medicine (128, 131). Moreover, IncA/C plasmid was the majority of the plasmid carrying bla_{CMY-2} gene which were identified from *E. coli* and *Salmonella* spp. isolates from beef, chicken, turkey, and pork from different regions of the United States (133). The plasmid backbone of these plasmids is also broadly disseminated among resistant zoonotic pathogens associated with agriculture in this country suggesting that plasmids are advantageous in bacterial populations that are under antimicrobial selective pressure from the use of antimicrobial agents in veterinary medicine (133).

CHAPTER IV

MATERIALS AND METHODS

Methodology Scheme



Part I: Bacterial strains and Antimicrobial susceptibility tests

1. Bacterial strains

1.1 Nontyphoidal *Salmonella* isolates

Eight hundred and ninety-seven nontyphoidal *Salmonella* isolated from humans and food animals from various provinces of Thailand during 2005 to 2007 and 2012 to 2016 were included in this study. A total of 617 nontyphoidal *Salmonella* human isolates, 523 and 94 isolates were obtained from the WHO National *Salmonella* and *Shigella* Center (NSSC), Department of Medical Science; Ministry of Public Health (Nonthaburi, Thailand) and the Department of Microbiology, King Chulalongkorn Memorial Hospital (Bangkok, Thailand), respectively. The isolates were collected from blood (67.7%), stool (20.3%), rectal swab (5.8%), pus (2.8%), urine (2.6%), tissue (0.5%), CSF (0.2%), and sputum (0.2%). The 563 isolates were collected from more than 27 provinces of Thailand during 2005 to 2007, most of which were from Bangkok (32.7%). The 54 isolates were collected during 2012 to 2016 from Bangkok, Ratchaburi, Chonburi, and Phetchabun, most of which were obtained from King Chulalongkorn Memorial Hospital (59.0%) and from Bangkok (87.0%).

A total of 280 nontyphoidal *Salmonella* food animal isolates, 231 and 49 isolates were collected during 2005 to 2007 and during 2012 to 2016, respectively from more than 5 provinces of Thailand, most of which were from Bangkok (24.6%). These isolates were isolated from swine (52.5%), chicken (33.2%), and cattle (4.6%). Of 280 food animal isolates, 134, 97, 31, and 18 isolates were obtained from the WHO National *Salmonella* and *Shigella* Center (NSSC), Department of Medical Science; Ministry of Public Health (Nonthaburi, Thailand), the Department of Livestock Development (Bangkok, Thailand), the Faculty of Veterinary Science, Chulalongkorn University (Bangkok, Thailand), and the local farm in Ratchaburi province, respectively.

For culture preservation, all isolates were grown on tryptic soy agar (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) at 35-37^{o}C for 18-24 hours.

The overnight cultures were transferred to cryogenic vials of 1 ml trypticase soy broth (BBL, Becton Dickinson and Company, Cockeysville, MD, USA) containing 10% glycerol and were kept at -70°C.

1.2 Quality control strains for MIC determination

Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains for MIC determination.

2. Antimicrobial susceptibility tests

All 897 nontyphoidal *Salmonella* isolates were determined for minimal inhibitory concentrations (MICs) of penicillin (ampicillin), cephalosporins (ceftriaxone, ceftazidime, cefotaxime, and cefoxitin), tetracycline (tetracycline), aminoglycosides (gentamicin), quinolones (nalidixic acid, norfloxacin, and ciprofloxacin), chloramphenicol (chloramphenicol), and trimethoprim-sulphamethoxazole (SXT) by agar-dilution technique and interpreted according to CLSI (Clinical and Laboratory Standards Institute, 2014) (134). The antimicrobial agents used in this study were supplied by Sigma-Aldrich (St. Louis, MO, USA).

MICs were determined on Mueller-Hinton agar (BBL, Becton Dickinson and Company, Coskeysville, MD). Inoculum was prepared from a pure overnight culture in tryptic soy broth (BBL, Becton Dickinson and Company, Coskeysville, MD) and the turbidity was adjusted to a 0.5 McFarland standard (approximately $1.5x10^8$ CFU/mL) in 0.85% NaCl. After adjusting the turbidity of inoculum, the suspension was diluted 10-fold to yield the final inoculum suspension. The suspension was inoculated on Mueller-Hinton agar plates with two-fold dilution of antimicrobial agent at concentrations of 0.015 to 256 mg/L. The final inoculum was approximately 10^4 CFU/spot. The plates were incubated at 35-37 ºC for 18-24 hours. The suspension was inoculated on Mueller-Hinton agar plates without antibiotic for the growth control. The MIC is defined as the lowest concentration of antimicrobial agent at which there is no visible growth. MICs interpretation used

breakpoint criteria recommended by CLSI (Clinical and Laboratory Standards Institute, 2014) (134) are shown in the Table 9.

Table 9. MIC interpretive standards (mg/L) for *Salmonella* spp.

Antimicrobial agents	MIC interpretive standard (mg/L)		
	Susceptible	Intermediate	Resistant
ampicillin	≤ 8	16	≥ 32
cefoxitin	≤ 8	16	≥ 32
ceftazidime	≤ 4	8	≥ 16
cefotaxime	≤ 1	2	≥ 4
ceftriaxone	≤ 1	2	≥ 4
nalidixic acid	≤ 16	-	≥ 32
ciprofloxacin	≤ 0.06	0.125-0.5	≥ 1
norfloxacin	≤ 4	8	≥ 16
tetracycline	≤ 4	8	≥ 16
gentamicin	≤ 4	8	≥ 16
chloramphenicol	≤ 8	16	≥ 32
trimethoprim-sulphamethoxazole	≤ 2/38	-	≥ 4/76

3. Statistical analysis

Fisher's exact test (two-tailed) was used to determine the significant differences in resistance using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered as a statistically significant difference (135).

The Pearson product-moment correlation was used to verify the correlation between antimicrobial-resistant *Salmonella* isolated from food animals and humans using the Statistical Package for Social Sciences SPSS version 16.0 (SPSS Inc, Chicago, USA) (55).

Part II: Characterization of ESC resistance and fluoroquinolone mechanisms among nontyphoidal *Salmonella* isolated from humans and food animals

1. Characterization of ESC resistance mechanisms

1.1 Detection of ESBL activity by combination disk test

All isolates for which the MICs of either ceftazidime, cefotaxime, or ceftriaxone ≥ 2 mg/L were considered to have a positive screening test for ESBL phenotype and subjected to clavulanate confirmatory testing using the combination disk test (134). An overnight culture suspension of isolate, which was adjusted to 0.5 McFarland in 0.85% NaCl was inoculated on Mueller-Hinton agar plate by using a sterile swab. Pairs of disks containing 30 µg ceftazidime (BBL, Becton Dickinson and Company, Coskeysville, MD), 30 µg ceftazidime with 10 µg clavulanic acid (BBL, Becton Dickinson and Company, Coskeysville, MD) and 30 µg cefotaxime (BBL, Becton Dickinson and Company, Coskeysville, MD), 30 µg cefotaxime with 10 µg clavulanic acid (BBL, Becton Dickinson and Company, Coskeysville, MD) were placed on the opposite sides of the same inoculated plate. Inhibition zones were measured following incubation at 35-37 ºC for 18-24 hours. Isolates that demonstrated the inhibition zone around the combination disk at least 5 mm larger than that of the cephalosporin alone were considered to have a positive confirmatory test for ESBL phenotype (Appendix D, Figure 15).

1.2 Detection of AmpC ß-lactamase activity by modified Hodge test with cefoxitin disk

All isolates, with the MICs of ≥ 2 mg/L for ceftazidime or cefotaxime or ceftriaxone and with cefoxitin MICs of > 8 mg/L were subjected to detect for AmpC ß-lactamase activity by modified Hodge test with cefoxitin disk, previously described by Yong *et al.* (136). A Mueller-Hinton agar plate was inoculated with an overnight culture suspension of cefoxitin-susceptible *E. coli* ATCC 25922, which was adjusted to 0.5 McFarland in 0.85% NaCl. A 30 µg cefoxitin disk (BBL, Becton Dickinson and

Company, Coskeysville, MD) was placed at the center of the plate. Two to three colonies of the overnight-cultured tested strains on tryptic soy agar were picked and heavily streaked outwards from the disk. The Mueller-Hinton agar plate was incubated at 35-37°C for 18-24 hours. After 18 hours of incubation, the decreased radius of the inhibition zone along the growth of tested strain was considered a positive of modified Hodge test (Appendix D, Figure 16). CMY-2-producing *E.coli* isolate and *E. coli* ATCC 25922 were used for positive and negative control strains, respectively.

1.3 Detection of *bla* genes encoding ESBLs

The nontyphoidal *Salmonella* isolates resistant to extended-spectrum cephalosporins with ESBL phenotype were investigated for the presence of ESBL genes, including bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB}.

1.3.1 DNA extraction

The overnight culture suspension, 4-5 colonies of pure culture nontyphoidal *Salmonella* isolate in 200 µl of steriled nuclease-free water was boiled for 10 min and centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

1.3.2 Primers

The presence of bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB} was screened by multiplex PCR using OXA-F, OXA-R, TEM-C, TEM-H, SHV-F, SHV-R, CTX-A, CTX-B, VEB-1, and VEB-2 primers. The primers are described in Table 10. and are based on those previously described by Colom *et al.* (137), Mabilat *et al.* (138), Bonnet *et al.* (139), and Udomsantisuk *et al*. (unplublished data).

Table 10. Primers of the multiplex PCR used for amplification of *bla* genes encoded for ESBLs

Specific for	Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
bla_{SHV}	SHV-F	AGGATTGACTGCCTTTTTG	392	(137)
	SHV-R	ATTTGCTGATTTCGCTCG		
bla_{TEM}	TEM-C	ATCAGCAATAAACCAGC	516	(138)
	TEM-H	CCCCGAAGAACGTTTTC		
bla_{VEB}	VEB-A	CCTTTTGCCTAAAACGTGGA	216	Udomsantisuk
	VEB-B	TGCATTTGTTCTTCGTTTGC		*et al.*
bla_{CTX-M}	CTXM-A	CGCTTTGCGATGTGCAG	550	(139)
	CTXM-B	ACCGCGATATCGTTGGT		
bla_{OXA}	OXA-F	ATATCTCTAACTGTTGCATCTCC	619	(137)
	OXA-R	AAACCCTTCAAACCATCC		

1.3.3 Amplification of bla_{OXA}, bla_{TEM}, and bla_{SHV} genes by multiplex PCR

The presence of bla_{OXA}, bla_{TEM}, and bla_{SHV} was screened using OXA-F, OXA-R, TEM-C, TEM-H, SHV-F, and SHV-R primers as described by Colom *et al.* (137) and Mabilat *et al.*(138). The PCR was performed in 25 µl PCR reaction mixture containing 1X *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.06 µM of OXA-F and OXA-R primers, 0.04 µM of TEM-C and TEM-H primers, and 0.08 µM of SHV-F and SHV-R primers, and 0.5 U *Taq* polymerase (Fermentas, USA), and 3 µL of DNA template. The amplification conditions were, initial denaturation at 94ºC for 5 minutes, 30 cycles of 94ºC for 30 seconds, 54ºC for 30 seconds and 72ºC for 1 minute, and a final elongation at 72ºC for 10 minutes.

1.3.4 Amplification of bla_{CTX-M} and bla_{VEB} genes by multiplex PCR

The presence of bla_{CTX-M} and bla_{VEB} was screened using CTX-A, CTX-B, VEB-1, and VEB-2 primers described by Bonnet *et al.* (139) and Udomsantisuk *et al.* (unplublished data). The PCR was performed in 25 µl PCR reaction mixture containing

1X *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.1 µM of CTX-A and CTX-B primers, 0.05 µM of VEB-1 and VEB-2 primers, and 0.5 U *Taq* polymerase (Fermentas, USA), and 1 µL of DNA template. Multiplex PCR conditions were performed as described previously (137). The amplification conditions were, initial denaturation at 94ºC for 5 minutes, 30 cycles of 94ºC for 30 seconds, 54ºC for 30 seconds and 72ºC for 1 minute, and a final elongation at 72ºC for 10 minutes.

1.3.5 Analysis of amplified DNA

The PCR products were analyzed on 1.5% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB} were 619 bp, 516 bp, 392 bp, 550 bp, and 216 bp, respectively. A 100 bp DNA ladder (Fermentas, USA) was used as a DNA size marker.

1.3.6 Quality control

The clinical strains of *Klebsiella pneumoniae* harbouring bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB} were used as positive control strains.

1.4 Identification of bla_{CTX-M} gene groups

The nontyphoidal *Salmonella* isolates carrying bla_{CTX-M} were investigated for group of bla_{CTX-M} genes encoding CTX-M ß-lactamase.

1.4.1 DNA extraction

The overnight culture suspension, 4-5 colonies of pure culture nontyphoidal *Salmonella* isolate in 200 µl of steriled nuclease-free water was boiled for 10 min and

centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

1.4.2 Primers

The multiplex PCR classified *bla*$_{CTX-M}$ genes into four groups, including CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-8/25 groups using CTXM7, CTXM8, CTXM17, CTXM18, CTXM19, CTXM20, CTXM11, and CTXM12 primers. The primers are described in Table 11, as those previously described by Li Xu *et al*. (140).

Table 11. Primers of the multiplex PCR used for amplification of *bla*$_{CTX-M}$ groups

Specific for	Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
bla$_{CTX-M-1}$ group	CTXM7	GCGTGATACCACTTCACCTC	260	(140)
	CTXM8	TGAAGTAAGTGACCAGAATC		
bla$_{CTX-M-2}$ group	CTXM17	TGATACCACCACGCCGCTC	341	
	CTXM18	TATTGCATCAGAAACCGTGGG		
bla$_{CTX-M-8/25}$ group	CTXM19	CAATCTGACGTTGGGCAATG	207	
	CTXM20	ATAACCGTCGGTGACAATT		
bla$_{CTX-M-9}$ group	CTXM11	ATCAAGCCTGCCGATCTGGTTA	293	
	CTXM12	GTAAGCTGACGCAACGTCTGC		

1.4.3 Amplification of *bla*$_{CTX-M}$ groups by multiplex PCR

The PCR was performed in 25 µl PCR reaction mixture containing 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.4 µM of CTXM7, CTXM8, CTXM17, CTXM18, CTXM19, CTXM20, CTXM11, and CTXM12 primers, and 1.25 U *Taq* polymerase (Fermentas, USA), and 2 µL of DNA template. The amplification conditions

were, initial denaturation at 95ºC for 2 minutes, 25 cycles of 95ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute, and a final elongation at 72ºC for 10 minutes.

1.4.4 Analysis of amplified DNA

The PCR products were analyzed on 2% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-8/25}}$ group, and $bla_{\text{CTX-M-9}}$ group were 260 bp, 341 bp, 207 bp, and 293 bp, respectively. A 100 bp DNA ladder (Fermentus, USA) was used as a DNA size marker.

1.4.5 Quality control

The clinical strains of *Klebsiella pneumoniae* harbouring $bla_{\text{CTX-M-1}}$ group and $bla_{\text{CTX-M-9}}$ group were used as positive control strains.

1.5 Detection of *bla* genes encoding AmpC ß-lactamases

The nontyphoidal *Salmonella* isolates with AmpC phenotype were investigated for the presence of plasmid-mediated *amp*C genes by using multiplex PCR. The primers and PCR conditions were modified from those previously described by Perez *et al*. (106).

1.5.1 DNA extraction

The 4-5 colonies of nontyphoidal *Salmonella* isolate were suspended in 200 µl of steriled nuclease-free water and boiled for 10 min and centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

1.5.2 Primers

The multiplex PCR used specific primers for plasmid *amp*C, encoding six groups of AmpC ß-lactamases, including MOX, CIT, DHA, ACC, EBC, and FOX. The primers are described in Table 12. and are based on those previously described by Perez *et al.* (106).

Table 12. Primers of the multiplex PCR used for amplification of plasmid *amp*C genes

Specific for	Primer	Primer sequence (5'to 3')	Product size (bp)	Reference
MOX	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	(106)
	MOXMR	CACATTGACATAGGTGTGGTGC		
CIT	CITMF	TGGCCAGAACTGACAGGCAAA	462	
	CITMR	TTTCTCCTGAACGTGGCTGGC		
DHA	DHAMF	AACTTTCACAGGTGTGCTGGGT	405	
	DHAMR	CCGTACGCATACTGGCTTTGC		
ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	
	ACCMR	TTCGCCGCAATCATCCCTAGC		
EBC	EBCMF	TCGGTAAAGCCGATGTTGCGG	302	
	EBCMR	CTTCCACTGCGGCTGCCAGTT		
FOX	FOXMF	AACATGGGGTATCAGGGAGATG	190	
	FOXMR	CAAAGCGCGTAACCGGATTGG		

1.5.3 Amplification of plasmid *amp*C genes by multiplex PCR

The presence of plasmid *amp*C, including MOX, CIT, DHA, ACC, EBC, and FOX groups was screened using MOXMF, MOXMR, CITMF, CITMR, DHAMF, DHAMR,

ACCMF, ACCMR, EBCMF, EBCMR, FOXMF, and FOXMR primers by multiplex PCR. The PCR was performed in 25 µl PCR reaction mixture containing 1X *Taq* buffer, 1.5 mM $MgCL_2$, 0.2 mM dNTPs (Fermentas, USA), 0.8 µM for MOXMF, MOXMR, FOXMF, and FOXMR primers, 0.6 µM for DHAMF and DHAMR primers, 0.5 µM for ACCMF and ACCMR primers, 0.4 µM for CITMF and CITMR primers, 0.3 µM for EBCMF and EBCMR primers, 1.25 U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. The amplification conditions were, initial denaturation at 94ºC for 3 minutes, 25 cycles of 94ºC for 30 seconds, 62ºC for 30 seconds and 72ºC for 1 minute, and a final elongation at 72ºC for 7 minutes.

1.5.4 Analysis of amplified DNA

The PCR products were analyzed on 2% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 80 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{ACC}, and bla_{FOX} were 520 bp, 462 bp, 405 bp, 302 bp, 346 bp, and 190 bp, respectively. A 100 bp DNA ladder (Fermentus, USA) was used as a DNA size marker.

1.5.5 Quality control

The *E. coli* transconjugant strains carryring bla_{CMY-2}, bla_{DHA-1}, bla_{ACC-1}, bla_{MIR-1}, and bla_{FOX-4} were used as the positive control strains for CIT, DHA, ACC, EBC, and FOX family The *E. coli* transconjugant strains were positive control strains obtained from Associate Professor Dr. Aroonwadee Chanawong, Khon Kaen University, Thailand. *Aeromonas caviae* clinical isolated harbouring bla_{MOX-8} was used as the positive control strain for MOX family.

1.6 Analysis of entire *bla* genes

The nontyphoidal *Salmonella* isolates carrying *bla* genes were characterized by PCR of entire *bla* genes and automated DNA sequencing.

1.6.1 Plasmid DNA extraction

The nontyphoidal *Salmonella* was extracted plasmid DNA for amplifying entire *bla* genes those performed by Plasmid Mini Kit (GmbH & Co. KG, Germany) according to the manufacturers. Nontyphoidal *Salmonella* isolate was cultured in Luria-Bertani broth (Pronadisa, Spain) and measured the density of bacterial cells up to 12 OD/mL (OD600). Bacterial cells were transferred to a microcentrifuge tube and centrifuged at 11,000 g for 30 seconds. The supernatant was removed. The 250 µL of resuspension solution were added and mixed by vortexing. The 250 µL of lysis solution were added to the sample and mixed by inverting 6-8 times. The 350 µL of neutralizing solution were added to the sample and mixed by inverting 6-8 times. After that, the sample was centrifuged for 5-10 min. The supernatant contained the plasmid DNA was transferred into a plasmid mini column and was centrifuged for 1 min. The filtrate was removed from the tube and was replaced into the same wash tube. The 750 µL of wash solution were added to the column and centrifuged for 1 min. The wash solution was discarded. The column was replaced into the same wash tube and centrifuged for 1 additional minute to remove residual wash solution. Finally, the plasmid mini column was transferred to a 1.5 mL microcentrifuge tube and the 50 µL of elution solution was added onto the base of the column and allowed for 1 min. After that, the column was centrifuged for 1 min. to elute the plasmid DNA. The eluted plasmid DNA samples were stored at -20^{o}C.

1.6.2 Primers for PCR and DNA sequencing

The primers for PCR and sequencing of entire *bla* genes were designed by Primer 3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) based on the sequence data in GenBank under accession no. AY458016, GQ385324, and FJ621588. The primers used for PCR and sequencing are shown in Table 13.

Table 13. Sequence of the oligonucleotides used as primers for PCR and DNA sequencing entire *bla* genes

Specific for	Primer name	Primer sequence (5'- 3')	Product size (bp)	Reference
PCR primers				
Entire bla_{CTX-M} group 1	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT	1398	(141)
	ORF477-R	CCCTCACACCTTCGAGCTAC		This study (AY458016)
Entire bla_{CTX-M} group 9	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT	1281	(141)
	IS903-R	TCGTGATGGCAAGGTCAG		This study (GQ385324)
Entire bla_{CMY}	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT	1596	(141)
	CMY-R	CAGGTTCCCAGATAGCGTTT		This study (FJ621588)
Entire bla_{TEM}	TEM-F	CAGGAAGCAAAGCTGAAAGG	1349	This study (AY458016)
	TEM-R	CGCTCAGTGGAACGAAAACT		This study (AY458016)
Sequencing primers				
Entire bla_{CTX-M} group 1	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT		(141)
	ORF477-R	CCCTCACACCTTCGAGCTAC		This study (AY458016)
Entire bla_{CTX-M} group 9	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT		(141)
	IS903-R	TCGTGATGGCAAGGTCAG		This study (GQ385324)
Entire bla_{CMY}	IS*Ecp1*-CMY-F	AAAAATGATTGAAAGGTGGT		(141)
	CMY-R	CAGGTTCCCAGATAGCGTTT		This study (FJ621588)
	AmpC-1	ATGATGAAAAAATCGTTATGC		(142)

Entire bla_{TEM}	TEM-F	CAGGAAGCAAAGCTGAAAGG	This study (AY458016)
	TEM-R	CGCTCAGTGGAACGAAAACT	This study (AY458016)

1.6.3 Amplification of the entire *bla* genes by PCR

The entire $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CIT}, and bla_{TEM} genes were amplified by PCR. The PCR was performed in 50 µl PCR reaction mixture containing 1X *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.4 µM of each forward and reverse primer, and 1.25 U *Taq* polymerase (Fermentas, USA), and 2 µL of DNA template. The amplification conditions were, initial denaturation at 95ºC for 2 minutes, 30 cycles of 95ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute, and a final elongation at 72ºC for 10 minutes.

1.6.4 Analysis of amplified DNA

The PCR products were analyzed on 1.0% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of entire $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CIT}, and bla_{TEM} genes were 1398 bp, 1281 bp, 1596 bp, and 1349 bp, respectively. A 100 bp plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

1.6.5 Purification of PCR products

The PCR products of entire $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CIT}, and bla_{TEM} genes were purifired 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. After that, 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. The filtrate was removed from the tube and 750 ml of PE buffer were added into the QIAquick column and centrifuged for 1 min. Flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick columns were centrifuged for 60 sec and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of EB buffer (Elution buffer, 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°C.

1.6.6 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 using four primer sets, entire $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, bla_{CIT}, and bla_{TEM} genes (Table 9.). 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). The sequencing primers are shown in Table 13.

1.6.7 Sequence analysis

The nucleotide and protein sequences were analyzed with the free 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 was analyzed by Multilin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

2. Characterization of fluoroquinolone resistance mechanisms

2.1 Detection of Quinolone resistance-determinatining region (QRDR) mutations in gyrA and parC genes by PCR and automated DNA sequencing

2.1.1 DNA extraction

The 4-5 colonies of nontyphoidal *Salmonella* isolate were suspended in 200 µl of steriled nuclease-free water and boiled for 10 min and centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

2.1.2 Primers

The PCR for *gyrA* and *parC* were amplified by using the primers as previously described (143, 144). The primers used for PCR and sequencing are shown in Table 14.

Table 14. Sequence of the oligonucleotides used as primers for PCR and DNA sequencing QRDR of *gyrA* and *parC* genes

Specific for	Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
gyrA	gyrA-forward	TGTCCGAGATGGCCTGAAGC	347	(143)
	gyrA-reverse	TACCGTCATAGTTATCCACG		
parC	parC-forward	CTATGCGATGTCAGAGCTGG	262	(144)
	parC-reverse	TAACAGCAGCTCGGCGTATT		

2.1.3 Amplification of QRDR of *gyrA* and *parC* genes by PCR

The PCR was performed individually in a final volume of 50 µl containing 1Xbuffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.1 µM for *gyrA* or *parC* forward and reverse primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 94°C for 5 min; 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

2.1.4 Analysis of amplified DNA

The PCR products were analyzed on 1.0% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of QRDR amplicons of *gyrA* and *parC* genes were 347 bp and 262 bp, respectively. A 100 bp plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

2.1.5 Purification of PCR products

The PCR products of QRDR amplicons of *gyrA* and *parC* genes were purifired 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. After that, 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. The filtrate was removed from the tube and 750 ml of PE buffer were added into the QIAquick column and centrifuged for 1 min. Flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick columns were centrifuged for 60 sec and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of EB buffer (Elution buffer, 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.

2.1.6 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

using the primer sets (Table 14.). 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). The sequencing primers are shown in Table 14.

2.1.7 Sequence analysis

The nucleotide and protein sequences of QRDR of *gyrA* and *parC* genes were analyzed with the free 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 was analyzed by Multilin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) compared with those of *S.* Typhimurium LT2.

2.2 Screening for the Presence of plasmid-mediated quinolone resistance (PMQR) genes by PCR

2.2.1 DNA extraction

The 4-5 colonies of nontyphoidal *Salmonella* isolate were suspended in 200 µl of steriled nuclease-free water and boiled for 10 min and centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

2.2.2 Primers

Salmonella isolates were performed multiplex PCR screening for common PMQR genes, including *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* using the primers as previously described (145-147). The *qnrVC* gene was also identified by primers qnrVC-F and qnrVC-R (Table 15.).

Table 15. Pimers used for detection of PMQR genes

Specific for	Primer names	Primer sequence (5'-3')	Product size (bp)	Reference
qnrA	qnrA-fw	TCAGCAAGAGGATTTCTCA	627	(148)
	qnrA-rv	GGCAGCACTATGACTCCCA		
qnrB	qnrB-fw	TCGGCTGTCAGTTCTATGATCG	496	(148)
	qnrB-rv	TCCATGAGCAACGATGCCT		
qnrS	qnrS-fw	TGATCTCACCTTCACCGCTTG	566	(148)
	qnrS-rv	GAATCAGTTCTTGCTGCCAGG		
qnrC	qnrC-fw	TTCCAAGGGGCAAACTGT	277	This study
	qnrC-rv	GCTCCCAAAAGTCATCAGAAA		
qnrD	qnrD-fw	TGTGATTTTTCAGGGGTTGA	350	This study
	qnrD-rv	GTGCCATTCCAGCGATTT		
qnrVC	qnrVC-F	GAACCTCCGCGATACACAA	333	This study
	qnrVC-R	GCGCCAATCCATCTATTCTC		
aac(6')-Ib	AAC-F	GATCTCATATCGTCGAGTGGTGG	435	(146)
	AAC-R	GAACCATGTACACGGCTGGAC		
qepA	Qep-F	AACTGCTTGAGCCCGTAGAT	198	(147)
	Qep-R	CGT GTTGCTGGAGTTCTTCC		

2.2.3 Amplification of *qnrA, qnrB, qnrS, qnrC* and *qnrD* genes by multiplex PCR

The PCR was performed in a final volume of 25 µl containing 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.1 µM of each primers, 0.5U of *Taq* polymerase (Fermentas, USA), and 2 µl of DNA template. The PCR conditions were 1 cycle of 94ºC for 3 min; 30 cycles of 94ºC for 1 min, 56ºC for 1 min and 72ºC for 1 min; and 1 cycle at 72ºC for 10 min.

2.2.4 Amplification of *qepA* and *aac(6')-Ib* genes by multiplex PCR

The PCR was performed in a final volume of 25 µl containing 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.1 µM of each primers,

0.5U of *Taq* polymerase (Fermentas, USA), and 2 µl of DNA template. The PCR conditions were 1 cycle of 94ºC for 3 min; 30 cycles of 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1 min; and 1 cycle at 72ºC for 10 min.

2.2.5 Amplification of *qnrVC* gene by PCR

The PCR was performed in a final volume of 50 µl containing 1X buffer, 2 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM of each primers, 1.25U of *Taq* polymerase (Fermentas, USA), and 2 µl of DNA template. The PCR conditions were 1 cycle of 94ºC for 5 min; 30 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1 min; and 1 cycle at 72ºC for 10 min.

2.2.6 Analysis of amplified DNA

The PCR products were analyzed on 1.5% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of QRDR amplicons of *qnrA, qnrB, qnrS, qnrC, qnrD, qnrVC, aac(6')-Ib,* and *qepA* genes were 627 bp, 496 bp, 566 bp, 277 bp, 350 bp, 333 bp, 435, and 198 bp, respectively. A 100 bp plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

2.2.7 Analysis of *aac(6')-Ib-cr* variant

The PCR products of *aac-6'-Ib* were were digested with BstCI (New England Biolabs) to identify *aac(6')-Ib-cr* (149). The reaction mixture was performed in a final volume of 25 µl containing 1X buffer, 20 U of BstCI and 2 µl of PCR products. The mixture was incubated at 55ºC for 1 hr and analyzed on 1.5% agarose gel electrophoresis. PCR products encoding *aac(6')-Ib-cr* were not digested with enzyme which lacks the BtsCI restriction site. PCR products encoding *aac(6')-Ib* were digested by BstCI, producing two fragments of 254 and 181 bp.

2.3 Analysis of entire *qnr* genes

2.3.1 DNA extraction

The 4-5 colonies of nontyphoidal *Salmonella* isolate were suspended in 200 µl of steriled nuclease-free water and boiled for 10 min and centrifuged at 12,000 rpm at room temperature for 5 min. The supernatant was used as the DNA template in the PCR experiments and stored at -20°C.

2.3.2 Primers for PCR and DNA sequencing

The nontyphoidal *Salmonella* isolates carrying *qnrS* and *qnrVC* genes were characterized by PCR of entire *qnr* genes and automated DNA sequencing. The entire genes are amplified by PCR using specific primers for DNA sequences of upstream and downstream regions of each gene. Entire *qnrS* gene was performed by PCR using designed primers entire-qnrS-forward (5'-CGCCAATTTGACCACTTAAAAC-3') and entire-qnrS-reverse primer (5'-GTGAAGACGCCTGAGGGTAA-3'). Entire *qnrVC* gene was performed by PCR using designed primers entire-qnrVC-forward (5'-ATGGATAAAACAGACCAGT-3') and entire-qnrVC-reverse primer (5'-TTAGTCAGGAACTACTATTAAACC-3').

2.3.3 Amplification of entire *qnr* genes by PCR

The PCR was performed individually in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for each entire-qnrS or entire-qnrVC forward and reverse primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

2.3.4 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). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The PCR product sizes of entire *qnrS* and entire *qnrVC* genes were 899 bp and 657 bp, respectively. A 100 bp plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

2.3.5 Purification of PCR products

The PCR products of entire *qnr* genes were purifired 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. After that, 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. The filtrate was removed from the tube and 750 ml of PE buffer were added into the QIAquick column and centrifuged for 1 min. Flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick columns were centrifuged for 60 sec and placed the QIAquick column in a clean 1.5 ml microcentrifuge tube. The pure DNA was eluted with 30 µl of EB buffer (Elution buffer, 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.

2.3.6 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 using the primer sets (Table 15.). 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). The sequencing primers are shown in Table 15.

2.3.7 Sequence analysis

The nucleotide and protein sequences of entire *qnr* genes were analyzed with the free 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 was analyzed by Multilin (http://bioinfo.genopoletoulouse.prd.fr/multalin/multalin.html).

Part III: Investigation of the genetic relationship among antimicrobial-resistant *Salmonella* isolated from humans and food animals

1. Investigate the genetic relationship among antimicrobial-resistant *Salmonella* by Pulse Field Gel Electrophoresis (PFGE)

Genetic relationships among antimicrobial-resistant *Salmonella* isolated from humans and food animals were determined by PFGE using the PulseNet International protocol 2009 (http://www.pulsenetinternational.org/protocols/Pages/default.aspx).

1.1 Preparation of PFGE plugs

Salmonella cultures were suspended and adjusted with cell suspension buffer to 0.8-1.0 of OD610. The 400 µl of adjusted Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0) was mixed with 20 µl of 20 mg/ml proteinase K (Amresco, Solon, OH) and 400 µl 1% melted Megabase agarose (Bio-Rad, USA) and transferred into plug mold. The plugs were allowed to solidify at 4 $^{\circ}$C for 5 min.

1.2 Lysis of bacterial cell in agarose plugs

Salmonella cell in plugs were lysed in Cell Lysis Buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl) with 20 µl of 20 mg/ml proteinase K (Amresco, Solon, OH)

at 54-55° C for 1.5-2 hr. Total bacterial DNA in plugs were washed twice with pre-heated (54-55° C) sterile ultrapure water and four times with pre-heated (54-55° C) sterile TE buffer at 54-55° C for 10-15 min.

1.3 Restriction digestion of DNA in the plugs with *Xba*I

A 2 mm-wide slice of the plug was digested with 50 U of *Xba*I (Fermentas, USA) in 1X Buffer Tango at 37° C for 4 hr. After that, the enzyme/buffer mixture was removed and replaced by 200 µl of 0.5X TBE.

1.4 PFGE condition

Total bacterial DNA in plugs which were digested with *Xba*I (Fermentas, USA) and separated by 1% agarose in 0.5X TBE using a CHEF-Mapper XA pulsed-field gel electrophoresis (PFGE) system (Bio-Rad, USA). PFGE conditions were 19 h at 6 V/cm and 14°C, with a pulse angle of 120° and initial and final pulses conducted for 2.2 and 63.8 s, respectively. *S.* Braenderup H9812 which was digested with *Xba*I was used as molecular size markers.

1.5 Staining and analysis of PFGE

The agarose gel was stained in 1 µg/ml of ethidium bromide for 30 min and de-stained twice for 20 min with Double Distilled Water. The bands of DNA were visualized and photographed under UV light transilluminator. The gel images were analyzed with InfoQuest FP software version 4.5 and a dendrogram was generated by Dice coefficient and the UPGMA clustering method to determine the clonal relationship among antimicrobial-resistant *Salmonella*.

Part IV: Characterization of the transmission mechanism of antimicrobial resistance genes among nontyphoidal *Salmonella*

1. Determine transferable ability of antimicrobial resistance genes by conjugation experiments

Conjugation ability was determined by broth-mating and filter-mating technique, as previously described technique (150). Azide-resistant *E. coli* strain UB1637 (*recA*, *his*, *lys*, *trp*, Strep^{R}) was used as a recipient strain (151). Transconjugants are confirmed by PCR amplification. MICs of antimicrobials for the donor, recipient, and transconjugant strains are compared by agar-dilution technique.

1.1 Broth-mating technique

The 4-5 colonies from each donor and recipient was grown in 5 ml of Tryptic soy broth for 4 hr. at 35-37^{o}C with shaking at 120-150 rpm. Cultures of donor and recipient cells grown in mid exponential growth phase (0.3-0.5 of OD600) was mixed at a ratio of 1:10. This mixed culture was co-incubated at 37°C for 4-6 hours. Transconjugant was selected on MacConkey agar plate containing 150 µg/ml of sodium azide combined with 2 µg/ml of ceftriaxone or 0.03 µg/ml of ciprofloxacin. Transconjugants were confirmed by PCR amplification.

1.2 Filter-mating technique

The 4-5 colonies from each donor and recipient was grown in 5 ml of Tryptic soy broth for 4 hr. at 35-37^{o}C with shaking at 120-150 rpm. Cultures of donor and recipient cells in mid exponential growth phase (0.3-0.5 of OD600) was mixed at a ratio of 1:10. This mixed culture was filtered through Millipore 0.45-mm-pore-size filters (Gelman sciences Inc., USA) and co-incubated on Mueller-Hinton agar plates at 37°C for 4-6 hours. Transconjugant was selected on MacConkey agar plate containing 150 µg/ml of

sodium azide combined with 2 µg/ml of ceftriaxone or 0.03 µg/ml of ciprofloxacin. Transconjugants were confirmed by PCR amplification.

2. Investigation of plasmid profiles and determination of the size of plasmids

Plasmid profiles and the size of plasmids were investigated by PFGE using S1 nuclease. A 2 mm-wide slice of the plug was digested with 10 U of S1 nuclease (Fermentas, UK) in 1X S1 Buffer at 37° C for 4 hr. After that, the enzyme/buffer mixture was removed and replaced by 200 µl of 0.5X TBE. Total bacterial DNA prepared in plugs and digested with S1 nuclease was separated using a CHEF-Mapper XA pulsed-field gel electrophoresis (PFGE) system (Bio-Rad, USA). PFGE conditions were 17 h at 6 V/cm and 14°C, with a pulse angle of 120° and initial and final pulses conducted for 5 and 45 s, respectively. Low-range PFG marker (New England BioLabs, Inc. U.S) was used as molecular size markers. The agarose gel was stained in 1 µg/ml of ethidium bromide for 30 min and de-stained twice for 20 min with Double Distilled Water. The bands of DNA were visualized and photographed under UV light transilluminator. The gel images were analyzed with InfoQuest FP software version 4.5.

3. Identification of plasmid incompatibility groups

Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing (PBRT) using primers and PCR conditions previously described by Carattoli *et al.* (127).

3.1 Plasmid DNA extraction

Plasmid DNA of nontyphoidal *Salmonella* was extracted by Plasmid Mini Kit (GmbH & Co. KG, Germany) according to the manufacturers which were previously described above.

3.2 Primers

PBRT were performed by 5 multiplex- and 3 simplex-PCRs, recognizing FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicon types using the specific primers in Table 16.

Table 16. Sequence of the oligonucleotides used as primers for PCR-based replicon typing

Specific for	Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
parA-	HI1-FW	GGAGCGATGGATTACTTCAGTAC	471	(127)
parB	HI1-RV	TGCCGTTTCACCTCGTGAGTA		
iterons	HI2-FW	TTTCTCCTGAGTCACCTGTTAACAC	644	
	HI2-RV	GGCTCACTACCGTTGTCATCCT		
RNAI	I1-FW	CGAAAGCCGGACGGCAGAA	139	
	I1-RV	TCGTCGTTCCGCCAAGTTCGT		
ori γ	X-FW	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	
	X-RV	TGAGAGTCAATTTTTATCTCATGTTTTAGC		
repA,B,C	L/M-FW	GGATGAAAACTATCAGCATCTGAAG	785	
	L/M-RV	CTGCAGGGGCGATTCTTTAGG		
repA	N-FW	GTCTAACGAGCTTACCGAAG	559	
	N-RV	GTTTCAACTCTGCCAAGTTC		
iterons	FIA-FW	CCATGCTGGTTCTAGAGAAGGTG	462	
	FIA-RV	GTATATCCTTACTGGCTTCCGCAG		
repA	FIB-FW	GGAGTTCTGACACACGATTTTCTG	702	
	FIB-RV	CTCCCGTCGCTTCAGGGCATT		
repA	W-FW	CCTAAGAACAACAAAGCCCCCG	242	
	W-RV	GGTGCGCGGCATAGAACCGT		
repA	Y-FW	AATTCAAACAACACTGTGCAGCCTG	765	
	Y-RV	GCGAGAATGGACGATTACAAAACTTT		
iterons	P-FW	CTATGGCCCTGCAAACGCGCCAGAAA	534	
	P-RV	TCACGCGCCAGGGCGCAGCC		

repA2	FIC-FW	GTGAACTGGCAGATGAGGAAGG	262	
	FIC-RV	TTCTCCTCGTCGCCAAACTAGAT		
repA	A/C-FW	GAGAACCAAAGACAAAGACCTGGA	465	
	A/C-RV	ACGACAAACCTGAATTGCCTCCTT		
repA	T-FW	TTGGCCTGTTTGTGCCTAAACCAT	750	
	T-RV	CGTTGATTACACTTAGCTTTGGAC		
repA	FII_S-FW	CTGTCGTAAGCTGATGGC	270	(127)
	FII_S-RV	CTCTGCCACAAACTTCAGC		
RNAI/	F_{repB}-FW	TGATCGTTTAAGGAATTTTG	270	
repA	F_{repB}-RV	GAAGATCAGTCACACCATCC		
RNAI	K/B-FW	GCGGTCCGGAAAGCCAGAAAAC	160	
	K-RV	TCTTTCACGAGCCCGCCAAA		
RNAI	K/B-FW	GCGGTCCGGAAAGCCAGAAAAC	159	
	B/O-RV	TCTGCGTTCCGCCAAGTTCGA		

3.3 Detection of HI1-, HI2-, and I1-carrying plasmids by multiplex PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for HI1-FW, HI1-RV, HI2-FW, HI2-RV, I1-FW, and I1-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.4 Detection of X-, L/M-, and N -carrying plasmids by multiplex PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for X-FW, X-RV, L/M-FW, L/M-RV, N-FW, and N-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.5 Detection of FIA-, FIB-, and W-carrying plasmids by multiplex PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for FIA-FW, FIA-RV, FIB-FW, FIB-RV, W-FW, and W-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.6 Detection of Y-, P-, and FIC-carrying plasmids by multiplex PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for Y-FW, Y-RV, P-FW, P-RV, FIC-FW, and FIC-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.7 Detection of A/C-, T-, and FII_S-carrying plasmids by multiplex PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for A/C-FW, A/C-RV, T-FW, T-RV, FII_S-FW, and FII_S-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.8 Detection of F_{repB}-carrying plasmids by PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for F_{repB}-FW, and F_{repB}-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.9 Detection of K-carrying plasmids by PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for K/B-FW, and K-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.10 Detection of B/O-carrying plasmids by PCR

The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for K/B-FW, and B/O-RV primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of plasmid DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min.

3.11 Analysis of amplified DNA

The PCR products were analyzed on 1.0% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 60 minutes. The amplified products were visualized and photographed under UV light transilluminator. The multiplex PCR product sizes of amplicons for HI1-, HI2-, and I1-carrying plasmids were 471 bp, 644 bp and 139 bp, respectively. The multiplex PCR product sizes of amplicons for X-, L/M-, and N -carrying plasmids were 376 bp, 785 bp, and 559 bp, respectively. The multiplex PCR product sizes of amplicons for FIA-, FIB- and W-carrying plasmids were 462 bp, 702 bp, and 242 bp, respectively. The multiplex PCR product sizes of amplicons for Y-, P- and FIC-carrying plasmids were 765 bp, 534 bp, and 262 bp, respectively. The multiplex PCR product sizes of amplicons for A/C-, T- and FII_S-carrying plasmids were 465 bp, 750 bp, and 270 bp, respectively. The PCR product sizes of each amplicons for F_{repB}-, K-, and B/O-carrying plasmids were 270 bp, 160 bp,

and 159 bp, respectively. A 100 bp plus DNA ladder (Fermentus, USA) was used as a DNA size marker.

4. Identification of the location of antimicrobial resistance genes

The location of antimicrobial resistance genes were determined by Southern blot hybridization by using specific probes of antimicrobial resistance gene replicons.

4.1 Transfer of DNA from agarose gel to a nylon membrane

DNA treated with S1 nuclease was blotted onto Hybond N^+ nylon membranes (Amersham, England) by using DNA capillary transfer method. The gel was incubated in depurination buffer 2 times for 30 min and in denaturing buffer 2 times for 30 min. The gel was soaked in neutralizing buffer for 15 min. The transfer apparatus for the capillary transfer were arranged from the bottom to the top (Figure 6.): the container containing 10X SSC solution; a glass plate over the support sitting; the long 2 pieces of 3MM Whatman papers soaked with 10X SSC solution; upside down the gel; the marked nylon membrane; the 2 pieces of dry 3MM Whatman papers; the 5-cm thick of paper towels; a glass plate; a weight. The transfer was continued for 12-14 hours. The membrane was placed on the 2 pieces of 3MM Whatman papers to dry at Room temperature (RT) and fixed at 80 °C for 2 hours.

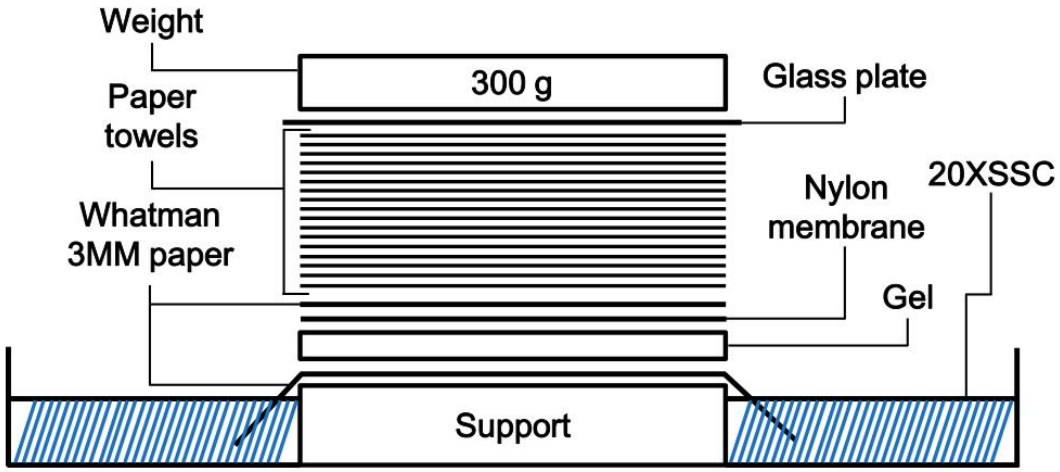


Figure 6. Scheme of capillary transfer method for Southern blotting (http://www.bio-protocol.org/e1448)

4.2 Labeling probe preparation

The probes were obtained by PCR amplifications for the screening of each antibiotic resistance gene as previously described above. The PCR products were purifired using QIAquick PCR purification kit as described by the manufacturers (QIAGEN, Max-Volmer-StraBe4 , Hilden, Germany).Probe labeling was performed with the DIG DNA labeling and detection kit (Roche Diagnostic, Indianapolis, IN, USA) according to the manufacturer's protocols. The purified DNA template (10 ng to 3 µg) was denatured by heating in a boiling water bath for 10 min and rapidly chilling on ice. DNA labeling master mix was prepared in 16 µl final volume containing 4 µl DIG-High Prime (vial 1), 1 µg DNA template, and double-distilled water to 16 µl. The reaction was incubated at 37° C for 1 h or overnight. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0) and/or by heating to 65° C for 10 min.

4.3 Hybridization and detection

Hybridization and detection were performed with the DIG DNA labeling and detection kit (Roche Diagnostic, Indianapolis, IN, USA) according to the manufacturer's protocols. The membrane was incubated in 20 ml of pre-heat DIG Easy Hyb (10 ml/100

cm^2) at 42° C for 30 min. DIG-labeled DNA probe (25 ng/ml) was denatured by boiling for 5 min and rapidly chilling on ice. Pre-heat DIG Easy was poured off. The membrane was incubated in the mixture of denatured 4 µl of DIG-labeled DNA probe and 7ml of pre-heated DIG Easy Hyb (3.5 ml/100 cm^2) for 4 hours to overnight under constant agitation avoid foaming. Probe/hybridization mixture was poured off. The membrane was washed 2 times for 5 min in 2x SSC, 0.1% SDS at 15-25° C under constant agitation. The membrane was washed 2 times for 15 min pre-heat 0.5x SSC, 0.1% SDS at 65-68° C under constant agitation. The membrane was rinsed briefly for 5 min in washing buffer at RT. The wash buffer was replaced with 100 ml 1X Blocking solution and the membrane was incubated shaking at RT for 30 min. The blocking solution was replaced with 20 ml of Antibody solution (4 µl Anti-Digoxigenin-AP (vial 4) and 20 ml 1X Blocking solution) and the membrane was incubated shaking at RT for 30 min. The membrane was washed in 100 ml wash buffer for 15 min twice shaking at RT. The membrane was incubated in 20ml Detection buffer for 5 min. The membrane was incubated in freshly prepared color substrate solution (10ml Detection buffer and 200 µl of NBT/BCIP stock solution (vial 5)) in the dark for more than 16 hours. Do not shake during purple color development. The reaction was stopped by washing the membrane in sterile TE-buffer for 5 min. The result was taken on GelDoc system (BioRad, USA).

Part V: Investigation of the genetic relationship among antimicrobial-resistant plasmid of *Salmonella* isolates from humans and food animals

The plasmid DNA from the confirmed transconjugants was extracted by the alkaline lysis method as described by Sambrook *et al.* (1989). The plasmid DNA was determined genetic relationship of transferable plasmids from antimicrobial-resistant *Salmonella* isolated from humans and food animals by Restriction Fragment Length Polymorphism (RFLP) using *Eco*RI, *Sca*I, *Hind*III, and *Hinc*II (112).

1. Plasmid extraction by the alkaline lysis method

The plasmid DNA from the confirmed transconjugants was isolated by the alkaline lysis method as described by Sambrook *et al.* (1989). The bacterial colonies were grown in 5 ml of LB broth at 37°C for overnight with shaking. The culture was centrifuged at 13,000 rpm for 30 seconds. The supernatant was discarded and the bacterial pellet was resuspended in 200 µl of Solution I. The 200 µl of Solution II and 200 µl of Solution III were added in the mixture mixing by inverting the tube gently on ice for 5 min. The mixture was centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a fresh tube and added equal volume of phenol: chloroform (1:1, v/v). The mixture was centrifuged at 13,000 rpm for 2 min and the aqueous (upper) phase was transferred to a new tube. The 2 volume of cold 100% ethanol were added in the supernatant mixing and standing at RT for 2 min. The mixtures were centrifuged with max speed for 5 min and removed the supernatant. The sediment was air dried at RT. The 1 ml of 70% ethanol was added in the mixtures and centrifuged with max speed for 15 min and then the supernatant was removed. The sediment was air dried at RT and resuspended in TE buffer. The DNA concentration was measured by NanoDrop spectrophotometer.

2. Restriction Fragment Length Polymorphism (RFLP)

The genetic relationship of transferable plasmids from antimicrobial-resistant *Salmonella* isolated from humans and food animals were determined by Restriction Fragment Length Polymorphism (RFLP) using *EcoR*I (Fermentas, USA), *Sca*I (Fermentas, USA), *Hind*III (Fermentas, USA), and *Hinc*II (Fermentas, USA) (112). The digestion reaction was individually performed at 37 °C for 4 hours in a total volume of 30 µl, containing, plasmid DNA, 1X buffer, 2 µl nuclease-free water, and 20 U of restriction enzyme. The digested plasmid DNA fragments were separated with 1.0% agarose gel electrophoresis (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 µg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 3 hours. The bands of DNA were visualized and photographed under UV light transilluminator. The gel images were analyzed with InfoQuest FP software version 4.5 and a dendrogram was generated by Dice coefficient and the UPGMA clustering method to determine the genetic relationship among antimicrobial-resistant plasmids

Part VI: Characterization of a novel quinolone resistance gene in *Salmonella* isolate

1. Bacterial strains

All nontyphoidal *Salmonella* isolates was screened for the presence of *qnrVC* genes. The *qnrVC* genes was identified in one isolate of *Salmonella* Rissen isolate number A180, isolated from rectal swab of a swine from a farm in Khon Kaen province in 2007. This is the first time of the occurrence of *qnrVC* family in *Salmonella enterica*. Therefore, the identification of the isolate was confirmed by biochemical characteristics and DNA sequencing using specific primers for 16S rRNA gene, 16SRNA-F (5'-GGAGGGTGCAAGCGTTAAT-3') and 16SRNA-R (5'-GCCCCCGTCAATTCATTT-3'). Later, we named this strain as *Salmonella* Rissen strain 166ANSS50. The potential of *qnrVC4* gene against antimicrobial agents, the dissemination of *qnrVC4* gene, and the genetic background of this resistance determinant were further characterized.

2. Antimicrobial susceptibility testing

The antimicrobials were obtained from Sigma-Aldrich (St. Louis, MO, USA), were ampicillin, ceftazidime, streptomycin, amikacin, gentamicin, kanamycin, chloramphenicol, nalidixic acid, ciprofloxacin, and norfloxacin. Minimal inhibitory concentrations (MICs) were determined by the agar-dilution technique and interpreted using the Clinical and Laboratory Standards Institute criteria (134).

3. Transfer of quinolone resistance

Transfer of *qnrVC* was determined by transconjugation and transformation experiments. The transconjugation was determined by filter-mating technique, as previously described (150). Azide-resistant *E. coli* UB1637 was used as a recipient

strain. Transconjugant was selected on MacConkey agar plate containing 150 mg/L of sodium azide and 0.03 mg/L of ciprofloxacin and confirmed by PCR.

We repeatedly failed to transfer the natural plasmid-mediated *qnrVC4* determinant of *S.* Rissen 166ANSS50 by conjugation experiments. Therefore, transformation was performed using electroporation techniques. Electrocompetent *E. coli* DH10ß (Invitrogen, Cergy Pontoise, France) was prepared according to the manufacturers for Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, USA). The 5 ml of a fresh overnight *E. coli* culture in Luria Bertani (LB) broth (BD Diagnostic System, Sparks, MD, USA) was inoculated into 500 ml of LB broth in flask. *E. coli* cells were grown at 37°C shaking at 300 rpm to an OD600 of approximately 0.5–0.7 (4–5 x10^7 cells/ml). The cells were chilled on ice for 20 minutes and centrifuged at 4000 x g for 15 minutes at 4°C. The supernatant was carefully poured off. The pellet was gently resuspended in 500 ml of ice-cold 10% glycerol centrifuged at 4000 x g for 15 minutes at 4°C and the supernatant was carefully poured off and discarded. The pellet was gently resuspended in 250 ml of ice-cold 10% glycerol centrifuged at 4000 x g for 15 minutes at 4°C; carefully pour off and discard the supernatant. The pellet was gently resuspended in 20 ml of ice-cold 10% glycerol centrifuged at 4000 x g for 15 minutes at 4°C. The supernatant was carefully poured off and discarded the supernatant. The pellet was gently resuspended in 1-2 ml of ice-cold 10% glycerol (1–3 x 10^{10} cells/ml) and frozen in aliquots at -70°C.

Plasmid DNA was isolated by the alkaline lysis method as described by Sambrook *et al.* (1989). The 2 µl of plasmid DNA was added into the 40µl of electrocompetent *E. coli* DH10ß. The mixture was transferred into an ice-cold 2mm-gap electroporation cuvette (Bio-Rad, Hercules, USA) and subjected to Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, USA) using an exponentially decaying pulse of 2.5kV, 25µF and 200 ohm. The cells were immediately added to 1 ml of LB broth with 20 mM glucose to the cuvette and gently resuspend with pipette. The cell suspension was incubated at 37°C for 1 hour with shaking at 225 rpm.

Transformant was selected on Mueller-Hinton agar plate containing 0.03 mg/L of ciprofloxacin and confirmed by PCR using specific primers for *qnrVC* gene. MICs of antimicrobials for the donor, recipient, and transformant strains were compared by the agar-dilution technique. PCR-based replicon typing was used to detect major plasmid types found in Enterobacteriaceae (127). We repeatedly failed to identify the plasmid types by PCR-based replicon typing. PCR and DNA sequencing were also performed to identify genes coding for replication protein (*repA*) and relaxase protein (*mobA*) using primers repA-F (5'-TCAGCCCTTGTATGCGATGG-3') and repA-R (5'-ATGGCCGCCAACGATCAA-3'), mobA-F (5'-GGCGCGAAACTCGATCA-3') and mobA-R (5'-GTGGCAATCGGGCGATT-3').

4. PFGE and Southern blot hybridization

Total bacterial DNA was prepared in low-melt agarose plugs which were digested with S1 nuclease (Fermentas, USA) or I-CeuI nuclease (New England BioLabs, Inc. U.S) and separated using a CHEF-Mapper XA pulsed-field gel electrophoresis (PFGE) system (Bio-Rad, Hercules, USA). The sizes of plasmids were estimated by S1 nuclease PFGE. The S1 nuclease was used to transform supercoiled plasmids into linear plasmids (152). A low-range PFG marker (New England BioLabs, Inc. U.S) was used as molecular-size markers. The chromosomal DNA was estimated by I-CeuI nuclease PFGE. The I-CeuI was used to digest bacterial genome at a 26-bp site in the *rrl* gene (23S rRNA) in the ribosomal RNA operons. The I-CeuI digestion generated an analyzable number of DNA fragments of the bacterial genome all of which contained *rrl* gene (23S rRNA) and *rrs* gene (16S rRNA) (153). *E. coli* K12 was used as a reference strain for chromosomal DNA study. DNA treated with I-CeuI or S1 nuclease was blotted onto Hybond N^+ nylon membranes (Amersham, England) by using DNA capillary transfer method. The location of the *qnrVC4* gene on plasmids or in the chromosomal DNA was determined by Southern blot hybridization by using specific probes of *qnrVC4* or 16S rRNA replicons. Probe labelling, hybridization, and detection were performed

with the DIG DNA labelling and detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocols.

5. Cloning of *qnrVC4*

The expression cloning in *E. coli* system was performed to determine the potential of antimicrobial resistance genes against antimicrobials using pBK-CMV expression vector (Stratagene, La Jolla, CA) which was transformed into an *E. coli* DH10B strain. Transformants were confirmed by PCR using specific primers for *qnrVC* gene and DNA sequencing. MICs of antimicrobials for the recipient and transformant strains are compared by agar-dilution technique.

. The entire *qnrVC4* gene was amplified from *S.* Rissen 166ANSS50 by PCR using primers, qnrVC-CF (5'-ATGGATAAAACAGACCAGT-3') and qnrVC-CR (5'-TTAGTCAGGAACTACTATTAAACC-3') as previously described above. The entire gene was cloned into a TA vector, pTZ57R/T (Fermentas, USA) using InsTAclone PCR Cloning Kit (Fermentas, USA) according to the manufacturer's protocols. Ligation reaction was performed at 4°C for overnight in a total volume of 30 µl, containing 3 µl pTZ57R/T (0.17 pmol ends), 6 µl 5X Ligation Buffer, 6 µl purified PCR product (0.52 pmol ends), 14 µl nuclease-free water, and 5U of T4 DNA Ligase. The 2.5 µl of the ligation mixture was transformed into an *E. coli* DH10B by electroporation as previously described above.

This entire gene was subcloned into pBK-CMV expression vector (Stratagene, La Jolla, CA) with *EcoR*I (Fermentas, USA) and *Apa*I (Fermentas, USA) digestion. The scheme of pBK-CMV map and multiple cloning site region of pBK-CMV vector were shown in the Figure 7.

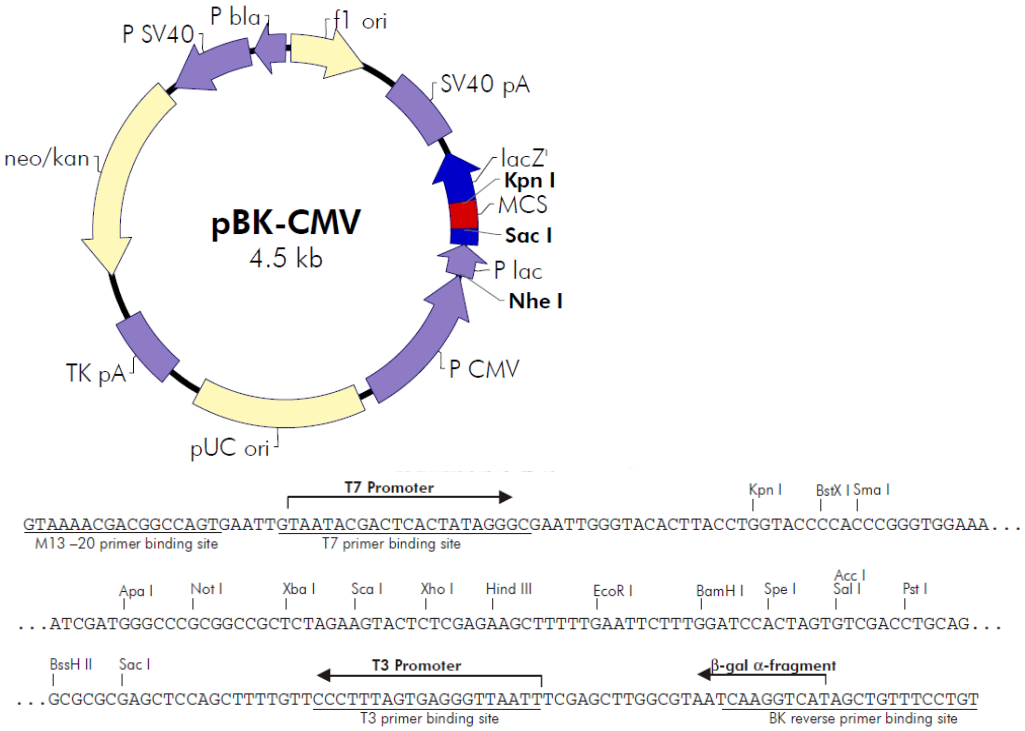


Figure 7. The scheme of pBK-CMV map (a) and multiple cloning site region of pBK-CMV vector (b) (sequence shown 952–1196)

Boths of the recombinant plasmid pTZ57R/T with *qnrVC4* and pBK-CMV expression vector were double digested with *EcoR*I and *Apa*I. The reaction mixture was individually performed at 37 °C for 4 hours in a total volume of 50 µl, containing 5 µL 10X buffer B, 20 U of *EcoR*I, 20 U of *Apa*I, 20 µl plasmid, and 11 µl nuclease-free water. The digestion reactions of *EcoR*I and *Apa*I were stopped at 80 °C for 5 min and 65 °C for 20 min, respectively. These digestion mixtures were precipitated by ethanol precipitation. The 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volume of cold 100% ethanol were added in digestion mixtures on ice for 1 hour. The mixtures were centrifuged with maximum speed for 15 min and removed the supernatant. The 1 ml of 70% ethanol was added in digestion mixtures and centrifuged with max speed for 15 min and then the supernatant was removed. The sediment was air dried at RT and resuspended in TE buffer. The DNA concentration was measured by NanoDrop spectrophotometer.

Ligation reaction was performed by 1:3 ratio of pBK-CMV expression vector and insertion DNA calculating used the formula as under:

insert (ng) = molar ratio of insert/ vector x insert lenght (bp)
/vector lenght (bp) x vector (ng)

Ligation reaction was performed at 22°C for 1 hour in a total volume of 20 µl, containing pBK-CMV expression vector, insertion DNA, 1XT4 Ligation buffer and nuclease free water. After the reaction, ligation enzyme was inactivated at 65°C for 10 min. The 2 µl of the ligation mixture was transformed into an *E. coli* DH10B by electroporation as previously described above. Transformant was selected on LB agar plates containing 50 µg/ml of kanamycin and spreading with 40 µl 100mM IPTG and 120 µl X-Gal (20 mg/ml). The colonies were selected by BlueWhite colony screening and confirmed by PCR and DNA sequencing.

6. Genetic environment of *qnrVC4* gene

The genetic environment surrounding *qnrVC4* gene was identified by PCR. The presence of *qnrVC4* located in integron was determined by PCR using specific primers for int1 and *qnrVC4* genes (154). Integron gene cassettes were detected by PCR using specific primers for the 5'conserved segment (5'-CS) and 3' conserved segment (3'-CS) regions (155). Since, this isolate does not yield an amplicon of the CS region, inverted PCR was performed to characterize the gene cassettes flanking *qnrVC4* as previously described (156). The plasmid DNA of *S.* Rissen 166ANSS50 was digested with *Pst*I. 50 ng of *Pst*I digested DNA fragments were ligated in a final volume of 50 µl and incubated at 16 °C for overnight. The T4 ligase was heat inactivate at 65°C for 10 min. The re-ligated DNA fragments were used as DNA templates for PCR using specific primers INV1 (5'-GAACCTCCGCGATACACAA-3') and INV2 (5'-CGGAGGTTCGCTCGTTTA-3'), INV3 (5'-GTTGTGATTTGAGCCACTCG-3') and INV4 (5'-GCGCCAATCCATCTATTCTC-

3') for amplification of upstream and downstream regions that flanked the known sequence, respectively. The inverse PCR amplification was performed using an initial denaturation step at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 5 min, and a final extension step at 72°C for 10 min. The inverse PCR products were purified and sequenced. PCR mapping and DNA sequencing were used to determine complete cassette of class 1 integron by primer-walking-strategy using primers in Table 17.

7. Primer designation and PCR amplification

The specific primers used in this part of the study were designed by Primer 3 program (http://www. genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). The PCR was performed in a final volume of 50 µl containing 1Xbuffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 0.4 µM for each forward and reverse primers, 1.25U of *Taq* polymerase (Fermentas, USA) and 2 µl of bacterial DNA template. Cycling conditions were 1 cycle 95°C for 2 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 10 min. The PCR products were analyzed on 1 % agarose gel electrophoresis at 100 volts for 60 minutes. The PCR products were purified and sequenced. DNA sequence comparison and annotation were performed using BLASTN and BLASTP (http://blast.ncbi.nlm.nih.gov).

8. Nucleotide sequence accession numbers

The nucleotide sequences reported here are provided on GenBank under accession number JX173955 and KU886277.

Table 17. Sequence of the oligonucleotides used as primers for PCR mapping and DNA sequencing

Target genes	Primer	Primer sequence (5'- 3')
repA	MAP1	GTCGGATTGTGTCAGGATG
intI1	MAP2	GGATCCATCAGGCAACG
intI1	MAP3	CAGTGGACATAAGCCTGTTC
qnrVC4 gene cassette	MAP4	GACTAACATTTACCAACCAGTTCTC
qnrVC4	MAP5	GAACCTCCGCGATACACAA
qnrVC4	MAP6	GCGCCAATCCATCTATTCTC
aacA4	MAP7	GAACCATGTACACGGCTGGAC
cmlA7	MAP8	TGGGTAGCTTCTTCGTCTTT
bla_{OXA-10}	MAP9	GTCTTTCGAGTACGGCATTA
bla_{OXA-10}	MAP10	TTGACTCAGTTCCCACACCA
aadA1	MAP11	TACAAATGTACGGCCAGCAA
aadA1	MAP12	ACATCATTCCGTGGCGTTAT
dfrA14	MAP13	GGATGTTTTCTTCCCGAGTAT
IS*6100*	MAP14	CCAAAGCGAGGTGAGCAT
downstream regions of *orf*	MAP15	CAAACAGCCACAAGAGGACT

CHAPTER V

RESULTS

Part I: Antimicrobial susceptibility in nontyphoidal *Salmonella* isolated from humans and food animals

1. Bacterial strains

Eight hundred and ninety-seven nontyphoidal *Salmonella* isolates were isolated from humans and food animals from various provinces of Thailand during 2005 to 2007 and 2012 to 2016. A total of 617 nontyphoidal *Salmonella* human isolates were collected from various hospitals in more than 26 different provinces, most of which were from Bangkok (37.4%), Ratchaburi (9.9%), Nonthaburi (6.8%), Chon buri (6.0%), and Sa kaeo (5.0%). These isolates were collected from blood (67.7%), stool (20.3%), rectal swab (5.8%), pus (2.8%), urine (2.6%), tissue (0.5%), sputum (0.2%), and CSF (0.2%). Four hundred and nineteen (67.9%) isolates were collected from sterile sites, most of which were from blood. One hundred and ninety-eight (32.1%) isolates were collected from non-sterile sites, most of which were from stool and rectal swab. The two most common serotypes were *S.* Enteritidis (46.4%) and *S.* Choleraesuis (40.2%). In this study, *S.* Choleraesuis (49.0%) and *S.* Enteritidis (43.1%) were the most common serotypes isolated from blood. The most common serotypes and types of clinical specimen of the 617 nontyphoidal *Salmonella* human isolates are shown in Table 18.

Table 18. The most common serotypes and types of clinical specimen of the 617 nontyphoidal *Salmonella* human isolates

Serotypes	Sterile sites (n=419)		Non-sterile sites (n=198)					Totals (n=617)(%)
	Blood (n=418)(%)	CSF (n=1)(%)	Stool (n=125)(%)	Rectal swab (n=36)(%)	Pus (n=17)(%)	Urine (n=16)(%)	Others (n=4)(%)	
S. Enteritidis	180(43.1%)	0(0%)	66(52.8%)	16(44.4%)	9(52.9%)	11(68.8%)	4(100%)	286(46.4%)
S. Choleraesuis	205(49.0%)	1(100%)	30(24.0%)	0(0%)	8(47.1%)	4(25.0%)	0(0%)	248(40.2%)
S. Typhimurium	11(2.6%)	0(0%)	27(21.6%)	18(50.0%)	0(0%)	1(6.3%)	0(0%)	57(9.2%)
S. Stanley	4(1.0%)	0(0%)	1(0.8%)	0(0%)	0(0%)	0(0%)	0(0%)	5(0.8%)
S. Dublin	2(0.5%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.3%)
S. 4,5,12:i:-	2(0.5%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.3%)
S. 4,12:i:-	2(0.5%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	2(0.3%)
S. Weltevreden	1(0.2%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.2%)
S. Rissen	0(0%)	0(0%)	1(0.8%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.2%)
S. Virchow	1(0.2%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(0.2%)
Other	10(2.4%)	0(0%)	0(0%)	2(5.6%)	0(0%)	0(0%)	0(0%)	12(1.9%)

Of the 280 nontyphoidal *Salmonella* food animal isolates, 174(62.1%), 93(33.2%), and 13(4.6%) were collected from swine, chicken, and cattle, respectively. These isolates were collected from various provinces most of which were from Bangkok (24.6%), Khon Kaen (22.9%), Ratchaburi (6.4%), and Nonthaburi (1.8%). More than 39 different serotypes were identified. The most common serotype was *S.* Rissen accounted for 19.6% of all isolates. *S.* Rissen, *S.* Enteritidis, and *S.* Welterreden were the most common serotypes found in swine, chicken, and cattle, respectively. The most common serotypes and sources of the 280 nontyphoidal *Salmonella* food animal isolates in this study are shown in Table 19.

Table 19. The most common serotypes and sources of the 280 nontyphoidal *Salmonella* food animal isolates

Serotypes	Sources (%)			Total (n=280)(%)
	Swine (n=147)(%)	Chicken (n=93)(%)	Cattle (n=13)(%)	
S. Rissen	46(31.3%)	9(9.7%)	0(0%)	55(19.6%)
S. Enteritidis	0(0%)	20(21.5%)	0(0%)	20(7.1%)
S. Stanley	16(10.9%)	0(0%)	4(30.8%)	20(7.1%)
S. Anatum	19(12.9%)	0(0%)	0(0%)	19(6.8%)
S. Schwarzengrend	0(0%0	14(15.1%)	0(0%)	14(5.0%)
S. Borismorbificans	11(7.5%)	1(1.1%)	0(0%)	12(4.2%)
S. Amsterdam	0(0%)	11(11.8%)	0(0%)	11(3.9%)
S. Choleraesuis	9(6.1%)	0(0%)	0(0%)	9(3.2%)
S. Welterreden	1(0.7%)	2(2.2%)	6(46.2%)	9(3.2%)
S. Altona	7(4.8%)	0(0%)	0(0%)	7(2.5%)
S. Corvallis	5(3.4%)	2(2.2%)	0(0%)	7(2.5%)
Other	60(40.8%)	35(37.6%)	3(23.1%)	98(34.6%)

2. Antimicrobial susceptibility tests

The results of susceptibility testing and antimicrobial resistance rates of the nontyphoidal *Salmonella* isolates from human and food animal are summarized in Table 20. Of the 617 nontyphoidal *Salmonella* human isolates, 313 (50.7%) were resistant to at least three antimicrobial subclasses whereas only 19 (3.1%) were susceptible to all antimicrobial agents tested. The most common resistance was found in nalidixic acid (90.0%), followed by ampicillin (77.3%), tetracycline (54.0%), chloramphenicol (36.0%), gentamicin (31.0%), ceftriaxone (25.8%), cefotaxime (25.8%), trimethoprim-sulfamethoxazole (22.7%), ceftazidime (20.4%), cefoxitin (12.0%), ciprofloxacin (11.8%), and norfloxacin (0.8%). Nalidixic acid, ampicillin, tetracycline, and chloramphenicol showed high level of MIC_{50}/MIC_{90} with >256/>256, >256/>256, 128/256, and 8/256 mg/L, respectively. Moreover, these isolates also had high level of MICs of ceftriaxone,

ceftazidime, and cefotaxime, ranging from 0.015 to >256, 0.125 to >256, and 0.015 to >256 mg/L, respectively.

Among 280 nontyphoidal *Salmonella* food animal isolates, the most common antimicrobial resistance was found in tetracycline (55.7%) followed by ampicillin (53.2%), nalidixic acid (41.1%), trimethoprim-sulfamethoxazole (33.6%), chloramphenicol (24.6%), gentamicin (12.1%), ciprofloxacin (7.9%), ceftriaxone (3.9%), cefotaxime (3.9%), ceftazidime (3.6%), and norfloxacin (0.7%). These isolates had MICs of ceftriaxone, ceftazidime, and cefotaxime, ranging from 0.015 to 0.015 to >256, 0.25 to 256, and 0.125 to >256 mg/L, respectively. The ciprofloxacin and norfloxacin MICs ranged from 0.015 to 32 mg/L and from 0.015 to 128 mg/L, respectively. The significantly higher resistance rates of ampicillin, cefoxitin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, and gentamicin were found among human isolates compared with food animal isolates ($p \leq 0.0001$), except for ciprofloxacin and norfloxacin (Table 20). However, nontyphoidal *Salmonella* food animal isolates showed significantly higher resistance rates of trimethoprim-sulfamethoxazole ($p = 0.0008$) than those in human isolates.

Table 20. The results of susceptibility testing of antimicrobial agents against the 617 nontyphoidal *Salmonella* human isolates and 280 nontyphoidal *Salmonella* food animal isolates

Antimicrobial agents	Human isolates (n=617) (%)				Food animal isolates (n=280) (%)				*P-values*
	MIC_{50}	MIC_{90}	MIC ranges	R (%)	MIC_{50}	MIC_{90}	MIC ranges	R (%)	
ampicillin	>256	>256	0.06->256	477(77.3%)	256	>256	0.125->256	149(53.2%)	<0.0001
cefoxitin	4	32	0.5->256	74(12.0%)	4	8	0.5-16	0(0%)	<0.0001
ceftriaxone	0.125	256	0.015->256	159(25.8%)	0.125	0.125	0.015->256	11(3.9%)	<0.0001
ceftazidime	0.5	128	0.125->256	126(20.4%)	1	2	0.25-256	10(3.6%)	<0.0001
cefotaxime	0.125	128	0.015->256	159(25.8%)	0.5	1	0.125->256	11(3.9%)	<0.0001
nalidixic acid	>256	>256	0.06->256	555(90.0%)	8	>256	0.03->256	115(41.1%)	<0.0001
ciprofloxacin	0.125	1	0.015-8	73(11.8%)	0.06	0.5	0.015-32	22(7.9%)	0.0794
norfloxacin	1	2	0.03-32	5(0.8%)	0.25	2	0.015-128	2(0.7%)	1.0000
gentamicin	0.5	128	0.06->256	191(31.0%)	0.5	16	0.125->256	34(12.1%)	<0.0001
chloramphenicol	8	256	0.125->256	222(36.0%)	8	128	2->256	69(24.6%)	0.0007
tetracycline	128	256	0.125->256	333(54.0%)	64	256	0.015-256	156(55.7%)	0.6643
trimethoprim-sulfamethoxazole	0.125	>256	0.015->256	140(22.7%)	0.125	256	0.015->256	94(33.6%)	0.0008

Among the 617 nontyphoidal *Salmonella* isolated from patients, 419(67.9%) and 198(32.1%) isolates were collected from sterile and non- sterile sites, respectively. The results of susceptibility testing of antimicrobial agent against nontyphoidal *Salmonella* human isolated from sterile and non- sterile sites are shown in Table 21. Of 419 nontyphoidal *Salmonella* human isolates from sterile sites, the most common antimicrobial resistance was found in nalidixic acid (93.8%), followed by ampicillin (80.9%), tetracycline (57.5%), chloramphenicol (41.3%), gentamicin (33.2%), ceftriaxone (29.6%), cefotaxime (29.6%), ceftazidime (23.9%), trimethoprim-sulfamethoxazole (23.4%), cefoxitin (15.0%), ciprofloxacin (13.4%), and norfloxacin (1.2%). High rates of ESC and ciprofloxacin resistance were found among the nontyphoidal *Salmonella* isolated from sterile sites, mostly from blood isolates. These isolates showed high level of MICs of ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin and norfloxacin, with the $MICs_{50}/MICs_{90}$ of 0.125/256, 0.5/128, 0.125/128, 0.125/1, and 0.5/2 mg/L respectively.

Of the 198 nontyphoidal *Salmonella* human isolates from non-sterile sites, the most common resistance was found in nalidixic acid (81.8%), followed by ampicillin (69.7%), tetracycline (46.5%), gentamicin (26.3%), chloramphenicol (24.7%), ceftriaxone (17.7%), cefotaxime (17.7%), ceftazidime (13.1%), trimethoprim-sulfamethoxazole (23.4%), ciprofloxacin (8.6%), and cefoxitin (5.6%). All of isolates were susceptible to norfloxacin. The resistance rates to ampicillin (p=0.0027), ceftriaxone (p=0.0016), ceftazidime (p=0.0019), cefotaxime (p=0.0016), cefoxitin (p=0.0005), nalidixic acid (p<0.0001), and chloramphenicol (p<0.0001) were significantly higher in isolates from sterile sites than those from non- sterile sites (Table 21).

Table 21. The results of susceptibility testing of antimicrobial agent against nontyphoidal *Salmonella* human isolated from sterile and non- sterile sites

Antimicrobial agents	Sterile site samples (n=419) (%)				Non-sterile site samples (n=198) (%)				*P*-values
	MIC_{50}	MIC_{90}	MIC ranges	R (%)	MIC_{50}	MIC_{90}	MIC ranges	R (%)	
ampicillin	>256	>256	0.06->256	339(80.9%)	>256	>256	1->256	138(69.7%)	0.0027
cefoxitin	2	32	0.5->256	63(15.0%)	4	8	0.5->256	11(5.6%)	0.0005
ceftriaxone	0.125	256	0.015->256	124(29.6%)	0.125	128	0.03->256	35(17.7%)	0.0016
ceftazidime	0.5	128	0.25->256	100(23.9%)	0.5	64	0.125->256	26(13.1%)	0.0019
cefotaxime	0.125	128	0.015->256	124(29.6%)	0.125	128	0.06->256	35(17.7%)	0.0016
nalidixic acid	>256	>256	0.125->256	393(93.8%)	>256	>256	0.06->256	162(81.8%)	<0.0001
ciprofloxacin	0.125	1	0.015-8	56(13.4%)	0.25	0.5	0.015-2	17(8.6%)	0.1085
norfloxacin	0.5	2	0.03-32	5(1.2%)	1	2	0.06-8	0(0%)	0.1821
gentamicin	0.5	128	0.125->256	139(33.2%)	0.5	128	0.06->256	52(26.3%)	0.0932
chloramphenicol	8	256	1->256	173(41.3%)	4	256	0.125->256	49(24.7%)	<0.0001
tetracycline	128	256	0.125->256	241(57.5%)	4	256	0.5->256	92(46.5%)	0.0120
trimethoprim-sulfamethoxazole	0.25	256	0.015->256	98(23.4%)	0.06	>256	0.015->256	42(21.2%)	0.6070

Remarkably, high rate of antimicrobial resistance was found among *S.* Choleraesuis isolates, especially to ESCs and ciprofloxacin. The results of susceptibility testing of antimicrobial agent against Choleraesuis isolates compared with non-Choleraesuis isolates are shown in Table 22. Among the 248 *S.* Choleraesuis human isolates, all were resistant to at least one antimicrobial agent tested and 75 (30.2%) displayed multidrug-resistance phenotype. The most common resistance was found in

nalidixic acid (98.8%), followed by tetracycline (95.2%), ampicillin (92.7%), chloramphenicol (77.0%), gentamicin (57.3%), ceftriaxone (56.9%), cefotaxime (56.9%), ceftazidime (44.0%), trimethoprim-sulfamethoxazole (35.1%), cefoxitin (29.4%), ciprofloxacin (19.4%), and norfloxacin (1.2%). These isolates showed high level of MICs of ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, and norfloxacin, with the $MICs_{50}/MICs_{90}$ of 16/256, 8/128, 8/>256, 0.25/8, and 1/4 mg/L respectively. A total of 141 ESC-resistant *S.* Choleraesuis isolates also showed high level of quinolone resistance with MICs of nalidixic acid $\geq$ 256 mg/L (99.3%) and ciprofloxacin $\geq$ 0.125 mg/L (95.0%).

Among the 369 non-Choleraesuis human isolates, 286 (77.5%) were *S.* Enteritidis. The 81(21.9%) isolates were resistant to at least three antimicrobial subclasses, whereas 19 (5.1%) were susceptible to all antimicrobial agents. The most common resistance was found in nalidixic acid (84.0%), followed by ampicillin (66.9%), tetracycline (26.3%), trimethoprim-sulfamethoxazole (14.4%), gentamicin (13.3%), chloramphenicol (8.4%), ciprofloxacin (6.8%), ceftriaxone (4.9%), cefotaxime (4.9%), ceftazidime (4.6%), norfloxacin (0.5%), and cefoxitin (0.3%). The significantly higher resistance rates were found among the Choleraesuis isolates compared with the non-Choleraesuis isolates to all antimicrobial agents tested ($p \leq 0.0001$), except for norfloxacin resistance (Table 22).

Table 22. The results of susceptibility testing of antimicrobial agents against the Choleraesuis isolates compared with the non-Choleraesuis isolates

Antimicrobial agents	Choleraesuis isolates (n=248) (%)				Non-Choleraesuis isolates (n=369) (%)				*P*-values
	MIC_{50}	MIC_{90}	MIC ranges	R (%)	MIC_{50}	MIC_{90}	MIC ranges	R (%)	
ampicillin	>256	>256	1->256	230(92.7%)	>256	>256	0.06->256	247(66.9%)	<0.0001
cefoxitin	4	64	0.5->256	73(29.4%)	2	4	0.5-64	1(0.3%)	<0.0001
ceftriaxone	16	256	0.03->256	141(56.9%)	0.125	0.25	0.015->256	18(4.9%)	<0.0001
ceftazidime	8	128	0.25->256	109(44.0%)	0.5	1	0.125->256	17(4.6%)	<0.0001
cefotaxime	8	>256	0.03->256	141(56.9%)	0.125	0.25	0.015->256	18(4.9%)	<0.0001
nalidixic acid	>256	>256	0.06->256	245(98.8%)	>256	>256	0.25->256	310(84.0%)	<0.0001
ciprofloxacin	0.25	8	0.015-8	48(19.4%)	0.125	0.5	0.015-4	25(6.8%)	<0.0001
norfloxacin	1	4	0.125-32	3(1.2%)	0.5	2	0.03-16	2(0.5%)	0.3959
gentamicin	32	256	0.125->256	142(57.3%)	0.5	32	0.06->256	49(13.3%)	<0.0001
chloramphenicol	128	256	1->256	191(77.0%)	4	8	0.125->256	31(8.4%)	<0.0001
tetracycline	256	256	0.5->256	236(95.2%)	2	256	0.125->256	97(26.3%)	<0.0001
trimethoprim-sulfamethoxazole	1	256	0.06->256	87(35.1%)	0.06	>256	0.015->256	53(14.4%)	<0.0001

Interestingly, the antimicrobial resistance rates in *S.* Choleraesuis isolates dramatically increased from those in during 2005 to 2007 to those in during 2012 to 2016 as follows: resistance to ceftriaxone (52.3 to 75.5%; p=0.0036), ceftazidime (37.7 to 69.4%; p<0.0001), cefotaxime (52.3 to 75.5%; p=0.0036), ciprofloxacin (12.6 to 46.9%; p<0.0001), norfloxacin (0.0 to 6.1%; p=0.0073), and trimethoprim-sulfamethoxazole (26.6 to 69.4%; p<0.0001). The results of susceptibility testing and antimicrobial resistance rates against *S.* Choleraesuis isolated during 2005 to 2007 and 2012 to 2016 are summarized in Table 23. Moreover, the extremely high rates of ESC and ciprofloxacin resistance were found among *S.* Choleraesuis isolates, most of which were isolated from blood. Of 205 *S.* Choleraesuis isolated from blood, 121(59.0%) and 40(19.5%) were resistant to ESCs and ciprofloxacin, respectively. The isolates showed high level of MICs of ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, and norfloxacin, with the $MICs_{50}$/$MICs_{90}$ of 32/256, 8/256, 8/>256, 0.25/2, and 1/4 mg/L, respectively.

Table 23. The results of susceptibility testing and antimicrobial resistance rates against *S.* Choleraesuis isolated during 2005 to 2007 and 2012 to 2016

Antimicrobial agents	*S.* Choleraesuis isolates: 2005-2007 (n=199)(%)				*S.* Choleraesuis isolates: 2012-2016 (n=49)(%)				*P*-values
	MIC_{50}	MIC_{90}	MIC ranges	R(%)	MIC_{50}	MIC_{90}	MIC ranges	R(%)	
ampicillin	>256	>256	1->256	185(93.0%)	>256	>256	4->256	45(91.8%)	0.7615
cefoxitin	4	64	1-128	67(33.7%)	4	32	0.5->256	6(12.2%)	0.0027
ceftriaxone	16	128	0.03->256	104(52.3%)	256	>256	0.125->256	37(75.5%)	0.0036
ceftazidime	4	128	0.25->256	75(37.7%)	128	>256	0.5->256	34(69.4%)	<0.0001
cefotaxime	8	128	0.03-256	104(52.3%)	>256	>256	0.125->256	37(75.5%)	0.0036
nalidixic acid	>256	>256	4->256	198(99.5%)	>256	>256	0.06->256	47(95.9%)	0.1005
ciprofloxacin	0.25	1	0.015-2	25(12.6%)	0.5	2	0.015-8	23(46.9%)	<0.0001
norfloxacin	0.5	2	0.125-8	0(0%)	2	8	0.25-32	3(6.1%)	0.0073
gentamicin	16	128	0.125->256	108(54.3%)	64	256	0.25->256	34(69.4%)	0.0755
chloramphenicol	128	256	1->256	148(74.4%)	128	256	4->256	43(87.8%)	0.0572
tetracycline	256	256	0.5->256	188(94.5%)	256	256	8->256	48(98.0%)	0.4693
trimethoprim-sulfamethoxazole	1	>256	0.06->256	53(26.6%)	4	16	0.125-128	34(69.4%)	<0.0001

Similarly, among nontyphoidal *Salmonella* food animal isolates, the higher rate of antimicrobial resistance was found in Choleraesuis isolates compared with those in non-Choleraesuis isolates. The significantly higher resistance rates were found among Choleraesuis isolates compared with non-Choleraesuis isolates to ampicillin (88.9 vs. 53.0%; p=0.0418), ceftriaxone (77.8 vs. 1.7%; p<0.0001), ceftazidime (66.7 vs. 1.7%; p<0.0001), cefotaxime (77.8 vs. 1.7%; p<0.0001), ciprofloxacin (44.4 vs. 7.6%; p=0.0046), gentamicin (77.8 vs. 10.2%; p<0.0001), trimethoprim-sulfamethoxazole (77.8 vs. 33.9%; p=0.0109). *S.* Choleraesuis also showed extremely high rates of ESC (77.8%) and ciprofloxacin (44.4%) resistance among food animal isolates. The isolates showed high level of MICs of ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, and norfloxacin, with the $MICs_{50}$/$MICs_{90}$ of 128/256, 16/128, 128/>256, 0.5/2, and 2/4 mg/L, respectively.

Part II: Characterization of ESC and fluoroquinolone resistance mechanisms among nontyphoidal *Salmonella* isolated from humans and food animals

1. Characterization of fluoroquinolone resistance mechanism

Among the 617 nontyphoidal *Salmonella* human isolates, 73 (11.8%) were resistant to ciprofloxacin. These isolates included 48 (65.7%) isolates of *S.* Choleraesuis (serogroup C), 14 (19.2%) isolates of *S.* Enteritidis (serogroup D), 8 (10.9%) isolates of *S.* Typhimurium (serogroup B), 1 (1.4%) isolate of S. Senftenberg (serogroup E), 1 (1.4%) isolate of *S. enterica* serogroup B, and 1 (1.4%) isolate of *S. enterica* serogroup C. A total of 73 ciprofloxacin-resistant isolates were resistant to ciprofloxacin which 69(94.5%) and 5(6.8%) were resistant to nalidixic acid and norfloxacin, respectively. The MICs of nallidixic acid, ciprofloxacin, and norfloxacin ranged from 4 to >256, 1 to 8 and 0.5 to 32 mg/L, respectively. The $MICs_{50}$/$MICs_{90}$ of nallidixic acid, ciprofloxacin, and norfloxacin were >256/>256, 1/2 and 4/8 mg/L, respectively. These isolates also showed high rates of resistance to ampicillin (97.3%), tetracycline (78.1%), chloramphenicol (65.8%), gentamicin (56.2%), trimethoprim-sulfamethoxazole (54.8%), ceftriaxone (52.1%), cefotaxime (52.1%), and ceftazidime (45.2%).

Of 73 ciprofloxacin-resistant isolates, 25(34.2%), 14(19.2%), and 26(35.6%) isolates had amino acid substitution in GyrA, plasmid-mediated quinolone resistance (PMQR), and both amino acid substitution in GyrA and *qnrS1*, respectively. Eight isolates were not found to have any fluoroquinolone resistance mechanisms tested in this study. All 51 ciprofloxacin-resistant isolates contained amino acid substitutions at D87G (78.4%), D87Y (7.8%), S83F (7.8%), S83Y (3.9%), and both at S83F and D87Y (1.9%) in GyrA. None of isolates had amino acid substitution in ParC. PMQR genes were detected in 40 ciprofloxacin-resistant isolates, all of which carried *qnrS1* gene. The most common ciprofloxacin resistance mechanisms were co-existence of D87G substitution in GyrA and QnrS1 production (26/73, 35.6%), followed by D87G substitution in GyrA

(14/73, 19.2%), QnrS1 producing (13/73, 17.8%), D87Y substitution in GyrA (4/73, 5.5%), S83F substitution in GyrA (4/73, 5.5%), S83Y substitution in GyrA (2/73, 2.7%), and both of S83F and D87Y substitutions in GyrA (1/73, 1.4%). Fluoroquinolone resistance mechanism and the result of antimicrobial susceptibility testing among ciprofloxacin-resistant nontyphoidal *Salmonella* human isolates are summarized in Table 24. The 26 ciprofloxacin-resistant *S.* Choleraesuis isolates, with D87G substitution in GyrA together with *qnrS1* gene showed slightly higher level of MICs of ciprofloxacin (1 to 8 vs. 1 to 2 mg/L) and norfloxacin (1 to 32 vs. 0.5 to 8 mg/L) compared with those with an amino acid substitution in GyrA alone. These isolates showed higher level of MICs of nallidixic acid, ciprofloxacin, and norfloxacin, with the $MICs_{50}/MICs_{90}$ of >256/>256, 2/4 and 4/8 mg/L, respectively. One *S.* Enteritidis isolate with S83F and D87Y substitutions in GyrA was resistant to all quinolones tested and displayed high level of MICs of nallidixic acid, ciprofloxacin, and norfloxacin with >256, 4, and 16 mg/L, respectively.

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Table 24. Fuoroquinolone resistance mechanism and the result of antimicrobial susceptibility testing among ciprofloxacin-resistant nontyphoidal *Salmonella* human isolates

GyrA/ParC substitution	PMQR	Nalidixic acid			Ciprofloxacin			Norfloxacin			Total of isolates (n=65)*		
		MIC_{50}/MIC_{90}	MIC ranges	R(%)	MIC_{50}/MIC_{90}	MIC ranges	R(%)	$MIC_{50}MIC_{90}$	MIC ranges	R(%)	Choleraesuis (n=48)	Enteritidis (n=14)	Typhimurium (n=8)
D87G/-	-	>256/>256	>256	14 (100.0%)	1/2	1-2	14 (100.0%)	4/8	0.5-8	0 (0%)	14	0	0
D87G/-	*qnrS1*	>256/>256	64->256	26 (100.0%)	2/4	1-8	26 (100.0%)	4/8	1-32	3 (11.5%)	26	0	0
D87Y/-	-	>256/>256	>256	4 (100.0%)	2/2	1-4	4 (100.0%)	2/4	0.5-8	0 (0%)	2	2	0
S83F/-	-	>256/>256	>256	4 (100.0%)	1/1	1	4 (100.0%)	2/4	1-8	0 (0%)	4	0	0
S83Y/-	-	>256/>256	>256	2 (100.0%)	1/2	1-2	2 (100.0%)	0.5/2	0.5-2	0 (0%)	2	0	0
S83F, D87Y/-	-	>256/>256	>256	1 (100.0%)	4/4	4	1 (100.0%)	16/16	16	1 (100.0%)	0	1	0
-/-	*qnrS1*	32/256	16->256	13 (92.9%)	1/1	1	14 (100.0%)	2/4	2-8	0 (0%)	0	6	7

* The isolates included one of *S. enterica* serogroup C isolate which carried *qnrS1* gene.

PMQR, plasmid-mediated quinolone resistance; -, not found; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine

Among 280 nontyphoidal *Salmonella* food animal isolates, 22 (7.9%) including 11 isolates from swine and 11 isolates from chicken were resistant to ciprofloxacin. Eleven ciprofloxacin-resistant isolates from swine included *S.* Choleraesuis (4 isolates), *S.* Virginia (3 isolates), *S.* Anatum (2 isolates), *S.* Give (one isolate) and, *S.* Senftenberg (one isolate), Eleven ciprofloxacin-resistant isolates from chicken included *S.* Senftenberg (5 isolates), *S.* Emek (3 isolates), *S.* Infantis (one isolate), *S.* Madjorio (one isolate), and *S.* Orion (one isolate).

Of 22 ciprofloxacin-resistant isolates, 5(22.7%), 7(31.8%), and 9(40.9%) isolates had amino acid substitution in GyrA, *qnrS1*, and both amino acid substitution in GyrA and *qnrS1*, respectively. The mechanism of fluoroquinolone resistance could not be identified in one isolate. Fifteen ciprofloxacin-resistant isolates were found to have amino acid substitutions at S83Y (40.0%), S83F (33.3%), D87G (6.7%) and both at S83F and D87N (13.3%) in GyrA. A total of 7 ciprofloxacin-resistant isolates harbored *qnrS1* gene. The ciprofloxacin resistance rate of nontyphoidal *Salmonella* from chicken isolates was higher than those from swine isotates (11.8% vs. 7.5%). However, two swine isolates had two amino acid substitutions at S83F and D87N in GyrA also had amino acid substitution at S80I in ParC. These isolates were resistant to all quinolones tested and displayed high level of MICs of nallidixic acid (>256 and >256 mg/L), ciprofloxacin (8 and 32 mg/L), and norfloxacin (64 and 128 mg/L). Fluoroquinolone resistance mechanism and the result of antimicrobial susceptibility testing among 22 ciprofloxacin-resistant nontyphoidal *Salmonella* food animal isolates are summarized in Table 25.

Table 25. Fluoroquinolone resistance mechanism and the result of antimicrobial susceptibility testing among 22 ciprofloxacin-resistant nontyphoidal *Salmonella* food animal isolates

Isolate	Serotype	Year	Source	GyrA/ParC mutation	PMQR gene
A84	*S.* Senftenberg	2005	chicken	-/-	*qnrS1*
A90	*S.* Senftenberg	2005	chicken	S83F/-	*qnrS1*
A245	*S.* Senftenberg	2007	chicken	S83F/-	*qnrS1*
A251	*S.* Senftenberg	2014	chicken	S83F/-	-/-
A276	*S.* Senftenberg	2014	chicken	S83F/-	*qnrS1*
A277	*S.* Senftenberg	2014	swine	-/-	*qnrS1*
A35	*S.* Choleraesuis	2005	swine	-/-	*qnrS1*
A36	*S.* Choleraesuis	2005	swine	D87G/-	*qnrS1*
A37	*S.* Choleraesuis	2005	swine	S83Y/-	*qnrS1*
A43	*S.* Choleraesuis	2005	swine	-/-	*qnrS1*
A51	*S.* Emek	2005	chicken	S83Y/-	*qnrS1*
A54	*S.* Emek	2005	chicken	S83Y/-	*qnrS1*
A56	*S.* Emek	2005	chicken	S83Y/-	*qnrS1*
A279	*S.* Virginia	2014	swine	-/-	*qnrS1*
A280	*S.* Virginia	2014	swine	S83F, D87N/S80I	-/-
A281	*S.* Virginia	2014	swine	S83F, D87N/S80I	-/-
A125	*S.* Anatum	2007	swine	-/-	*qnrS1*
A126	*S.* Anatum	2007	swine	-/-	-/-
A171	*S.* Give	2007	swine	S83Y/-	-/-
A81	*S.* Infantis	2005	chicken	S83F/-	-/-
A82	*S.* Madjorio	2005	chicken	-/-	*qnrS1*
A83	*S.* Orion	2005	chicken	S83Y/-	*qnrS1*

PMQR, plasmid-mediated quinolone resistance; -, not found; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine

2. Characterization of ESC resistance mechanism

Among the 617 nontyphoidal *Salmonella* human isolates, 159 (25.8%) were resistant to extended-spectrum cephalosporins (ceftriaxone, ceftazidime, or cefotaxime). These isolates included 141 (88.7%) isolates of *S.* Choleraesuis (serogroup C) and 18 (11.3%) isolates of *S.* Typhimurium (serogroup B). Among 167 nontyphoidal *Salmonella* isolates with MICs of $\geq$ 2 mg/L for ceftazidime or cefotaxime or ceftriaxone, 86 were also displayed cefoxitin MIC of $\geq$ 8 mg/L. Eighty-six nontyphoidal *Salmonella* isolates were subjected to detect AmpC ß-lactamase activity by modified Hodge test with cefoxitin disk. A total of 71 isolates were positive for the test and considered as AmpC producers. All 167 isolates with MICs of eithers ceftazidime, cefotaxime, or ceftriaxone $\geq$ 2 mg/L were positive for screening test of ESBL phenotype by CLSI and subjected to clavulanate confirmatory testing using the combination disk test. Eighty-nine of nontyphoidal *Salmonella* isolates, positive for the combination disk test were considered as ESBL producers.

Of the 159 ESC-resistant nontyphoidal *Salmonella* isolates, 88 (55.3%), 70 (44.0%), and 1 (0.6%) were ESBL producers, AmpC producers, and ESBL and AmpC co-producer, respectively. These isolates were screened for the presence of *bla* genes and identified for the types of *bla* genes by DNA sequencing. All 70 AmpC producers, *S.* Choleraesuis isolates carried $bla_{\text{CMY-2}}$. A total of 88 ESBL producers carried $bla_{\text{CTX-M-14}}$ (47.7%), $bla_{\text{CTX-M-55}}$ (51.1%), and $bla_{\text{CTX-M-15}}$ (1.1%). One ESBL and AmpC co-producer, *S.* Typhimurium isolate carried $bla_{\text{CMY-2}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{TEM-1}}$. Type of *bla* genes in the 159 ESC-resistant nontyphoidal *Salmonella* human isolates is summarized in Table 26.

Table 26. Type of *bla* genes in the 159 ESC-resistant nontyphoidal *Salmonella* human isolates

Type of *bla* genes	No. of isolates (%)		
	S. Choleraesuis (n=141)	*S.* Typhimurium (n=18)	Total (n=159)
AmpC producers (n=70)			
CMY-2	63 (44.7%)	0(0%)	63 (39.6%)
CMY-2 TEM-1	7 (5.0%)	0(0%)	7 (4.4%)
ESBL producers (n=88)			
CTX-M14	34 (24.1%)	0(0%)	34 (21.4%)
CTX-M-14 TEM-1	7 (5.0%)	1 (5.6%)	8 (5.0%)
CTX-M-15 TEM-1	0(0%)	1 (5.6%)	1 (0.6%)
CTX-M-55	22 (15.6%)	2 (11.1%)	24 (15.1%)
CTX-M-55 TEM-1	8 (5.7%)	13 (72.2%)	21 (13.2%)
ESBL and AmpC co-producer (n=1)			
CTX-M-14 CMY-2 TEM-1	0(0%)	1 (5.6%)	1 (0.6%)

The most common serotype among ESC-resistant human isolates was *S.* Choleraesuis (88.7%), followed by *S.* Typhimurium (11.3%). Of 141 ESC-resistant *S.* Choleraesuis isolates, 70(49.6%), 41(29.1%), and 30(21.3%) were CMY-2, CTX-M-14, and CTX-M-55 producers, respectively. The results of susceptibility testing and antimicrobial resistance rates against 141 ß-lactamase producing *S.* Choleraesuis isolates are shown in Table 27. A total 70 CMY-2 producers were resistant to all cephalosporins with MICs of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime, ranging from 32 to >256, 16 to 256, 16 to >256, and 4 to 256 mg/L, respectively. $MICs_{50}/MICs_{90}$ of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime were 32/128, 32/128, 64/128, and 16/32 mg/L, respectively. Of 41 CTX-M-14 producers, all were resistant to ceftriaxone

and cefotaxime, 9 (22.0%) were resistant to ceftazidime, and none of these were resistant to cefoxitin. These isolates showed MICs of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime, ranging from 0.5 to 8, 32 to >256, 0.5 to 32, and 4 to 256 mg/L, respectively. $MICs_{50}/MICs_{90}$ of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime were 2/4, 128/256, 8/16, and 128/256 mg/L, respectively. A total 30 CTX-M-55 producers were resistant to all extended-spectrum cephalosporin tested. These isolates showed high level of MICs of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime, ranging from 0.5 to 128, 128 to >256, 64 to >256, and 128 to >256 mg/L, respectively. $MICs_{50}/MICs_{90}$ of cefoxitin, ceftriaxone, ceftazidime, and cefotaxime were 4/16, >256/>256, 256/>256, and >256/>256 mg/L, respectively.

Noticeably, CMY-2 (67/104, 64.4%) and CTX-M-14 (37/104, 35.6%) were the two most common mechanisms of ESC resistance among the isolates during 2005 to 2007, while CTX-M-55 (30/37, 81.1%) was the most common ESC resistance mechanism followed by CTX-M-14 (4/37, 10.8%) and CMY-2 (3/37, 8.1%) among the isolates during 2012 to 2016. Moreover, the ceftazidime resistance rate was significantly higher in CTX-M-55-producing isolates than that in CTX-M-14-producing isolates (100.0% vs. 22.0%; $p<0.0001$). There was no significant difference in CMY-2-producing isolates. However, CTX-M-55-producing isolates displayed higher levels of resistance to the third-generation cephalosporins compared with CMY-2-producing isolates as follows: MIC_{50}/MIC_{90} of ceftriaxone (>256/>256 vs. 32/128), ceftazidime (256/>256 vs. 64/128), and cefotaxime (>256/>256 vs. 16/32). Moreover, CTX-M-55 producers also showed significantly higher resistance rates of ciprofloxacin (73.3%) and trimethoprim-sulfamethoxazole (83.3%) than those in CMY-2 and CTX-M-14 producers with $p<0.0001$. It should be noted that, 28(93.3%) CTX-M-55-producing isolates had MIC of ciprofloxacin $\geq$ 0.0125 mg/L. The MICs of nalidixic acid, ciprofloxacin, and norfloxacin ranged from 0.125 to >256, 0.015 to 8, and 0.25 to 32 mg/L, respectively. The $MICs_{50}/MICs_{90}$ of nalidixic acid, ciprofloxacin and norfloxacin were >256/>256, 2/4 and 4/8 mg/L, respectively. There were 22(73.3%) isolates co-resistant to ciprofloxacin carrying D87G amino acid substitution in GyrA. Of these 22 isolates, 17 also carried

qnrS1 gene. The *qnrS1* gene was found in 17 of 30 $bla_{CTX\text{-}M\text{-}55}$ carrying isolates (56.7%), while the coexistence of *qnrS1* with $bla_{CTX\text{-}M\text{-}14}$ was found in only one isolate.

Among 18 ESC-resistant *S.* Typhimurium isolates, 83.3% carried $bla_{CTX\text{-}M\text{-}55}$ gene followed by $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M15}$, and $bla_{CMY\text{-}2}$ genes. These isolates showed high level of MICs of ceftriaxone, ceftazidime, and cefotaxime, ranging from 256 to >256, 4 to >256, and 128 to >256 mg/L, respectively. $MICs_{50}/MICs_{90}$ of ceftriaxone, ceftazidime, and cefotaxime were >256/>256, 128/>256, and 256/>256 mg/L, respectively. The isolates also showed high rates of resistance to gentamicin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, and ciprofloxacin with 94.4%, 94.4%, 72.2%, 44.4%, 44.4%, and 33.3%, respectively. The MICs of ciprofloxacin, and norfloxacin ranged from 0.015 to 1 mg/L and from 0.06 to 8 mg/L, respectively. The $MICs_{50}/MICs_{90}$ of ciprofloxacin and norfloxacin were 0.5/1 and 2/2 mg/L, respectively. Six *S.* Typhimurium isolates were resistant to both ESC and ciprofloxacin, five of which carried *qnrS1* gene.

Table 27. Antimicrobial resistance of 141 ß-lactamase-producing *S.* Choleraesuis isolated from human.

Antimicrobial agent	CMY-2 producers (n=70)			CTX-M-14 producers (n=41)			CTX-M-55 producers (n=30)		
	MIC_{50}/MIC_{90}	MIC ranges	R(%)	MIC_{50}/MIC_{90}	MIC ranges	R(%)	MIC_{50}/MIC_{90}	MIC ranges	R(%)
ampicillin	>256/>256	128->256	70(100.0%)	>256/>256	>256	41(100.0%)	>256/>256	64->256	30(100.0%)
cefoxitin	32/128	32->256	70(100.0%)	2/4	0.5-8	0(0%)	4/16	0.5-128	2(6.7%)
ceftriaxone	32/128	16-256	70(100.0%)	128/256	32->256	41(100.0%)	>256/>256	128->256	30(100.0%)
ceftazidime	64/128	16->256	70(100.0%)	8/16	0.5-32	9(22.0%)	256/>256	64->256	30(100.0%)
cefotaxime	16/32	4-256	70(100.0%)	128/256	4-256	41(100.0%)	>256/>256	128->256	30(100.0%)
nalidixic acid	>256/>256	0.06->256	69(98.6%)	>256/>256	>256	41(100.0%)	>256/>256	0.125->256	29(96.7%)
ciprofloxacin	0.25/0.5	0.03-2	6(8.6%)	0.25/0.5	0.06-2	4(9.8%)	2/4	0.015-8	22(73.3%)
norfloxacin	1/2	0.25-8	0(0%)	0.5/2	0.25-4	0(0%)	4/8	0.25-32	3(10.0%)
gentamicin	64/64	0.25->256	58(82.9%)	128/>256	0.125->256	28(68.3%)	128/256	0.5->256	25(83.3%)
chloramphenicol	256/256	4->256	69(98.6%)	128/256	4-256	35(85.4%)	256/>256	64->256	30(100.0%)
tetracycline	256/256	64-256	70(100.0%)	256/256	2->256	40(97.6%)	256/256	128->256	30(100.0%)
trimethoprim-sulfamethoxazole	1/4	0.125->256	17(24.3%)	0.5/128	0.125->256	6(14.6%)	4/16	0.5-128	25(83.3%)

Of the 280 food animal isolates, ESC resistance was found in 11 isolates from swine accounted for 3.9%, all of which were ESBL producers and isolated during 2014 to 2016. The most common serotype among ESC-resistant food animal isolates was *S.* Choleraesuis (63.6%), followed by *S.* Typhimurium (36.4%). Of 11 ESBL producers carried $bla_{\text{CTX-M-55}}$ in 8 (72.7%) isolates (4 *S.* Choleraesuis isolates and 4 *S.* Typhimurium isolate) and $bla_{\text{CTX-M-14}}$ in 3 (27.3%) isolates (*S.* Choleraesuis).

All 11 ESBL producers showed high level of MICs of ceftriaxone, ceftazidime, and cefotaxime, with the $MICs_{50}/MICs_{90}$ of 256/>256, 64/128, and 256/>256, respectively. MIC of ceftazidime of CTX-M-55 and CTX-M-14 producers ranged from 32 to 256 and 8 to 16 mg/L, respectively. All of isolates displayed MIC of ciprofloxacin $\geq$ 0.125 mg/L, all of which carried *qnrS1* gene. Four CTX-M-55-producing *S.* Choleraesuis isolates were resistant to both ESCs and ciprofloxacin, two of which had either D87G or S83Y amino acid substitution in GyrA.

Part III: Investigation of the genetic relationship among antimicrobial-resistant *Salmonella* isolated from humans and food animals

1. Investigation of the genetic relationship among ciprofloxacin-resistant *Salmonella* isolates

A total of 95 ciprofloxacin-resistant *Salmonella* including 73 human isolates and 22 food animal isolates were subtyped by PFGE. There were 52 *S.* Choleraesuis, 14 *S.* Enteritidis, 8 *S.* Typhimurium, 7 S. Senftenberg, 3 *S.* Virginia, 3 *S.* Emek, 2 *S.* Anatum and one isolate of *S.* Give, *S.* Infantis, *S.* Madjorio, *S.* Orion, *S. enterica* serogroup B, and *S. enterica* serogroup C. The 40 PFGE clusters designated F01 to F40 and 66 different pulsotypes were identified using a cut-off of 85% and 95% genetic similarity, respectively (Appendix D, Table 29.). The distribution of pulsotypes and fluoroquinolone resistance mechanisms among 86 ciprofloxacin-resistant *Salmonella* isolates are summarized in Figure 8.

There were 26 PFGE clusters and 65 different pulsotypes among 73 ciprofloxacin-resistant *Salmonella* human isolates (48 *S.* Choleraesuis, 14 *S.* Enteritidis, 8 *S.* Typhimurium, 1 S. Senftenberg, 1 *S. enterica* serogroup B, and 1 *S. enterica* serogroup C). The 15 PFGE clusters and 20 different pulsotypes were identified among 22 ciprofloxacin-resistant *Salmonella* food animal isolates (11 isolates from swine and 11 isolates from chicken) included *S.* Senftenberg (6 isolate), *S.* Choleraesuis (4 isolates), *S.* Virginia (3 isolates), *S.* Emek (3 isolates), *S.* Anatum (2 isolates), and one isolate of *S.* Give, *S.* Infantis, *S.* Madjorio, and *S.* Orion. The predominant clones of ciprofloxacin-resistant *Salmonella* isolates were found among human isolates. F28 was the most common PFGE cluster among ciprofloxacin-resistant *Salmonella* human isolates including 16 isolates accounted for 21.9 % of ciprofloxacin-resistant *Salmonella* human isolates. Pulsotype F28-01, the most common pulsotype contained 7 isolates of *S.* Choleraesuis from blood during 2012 to 2015 which had D87G amino acid substitution in GyrA and *qnrS1* gene. Six isolates displayed resistance to ampicillin, ceftriaxone,

ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole, but one isolate remained susceptible to gentamicin. One isolate was also resistant to norfloxacin. This pulsotype contained two groups of 3 indistinguishable isolates, both of which were collected from the same hospital (King Chulalongkorn Memorial Hospital) but difference in the years of isolation (2012, 2014, and 2015) and hospital wards. The information of strains, PFGE pulsotypes, the antimicrobial susceptibility profiles, and resistance mechanisms are summarized in the Appendix D in Table 29.

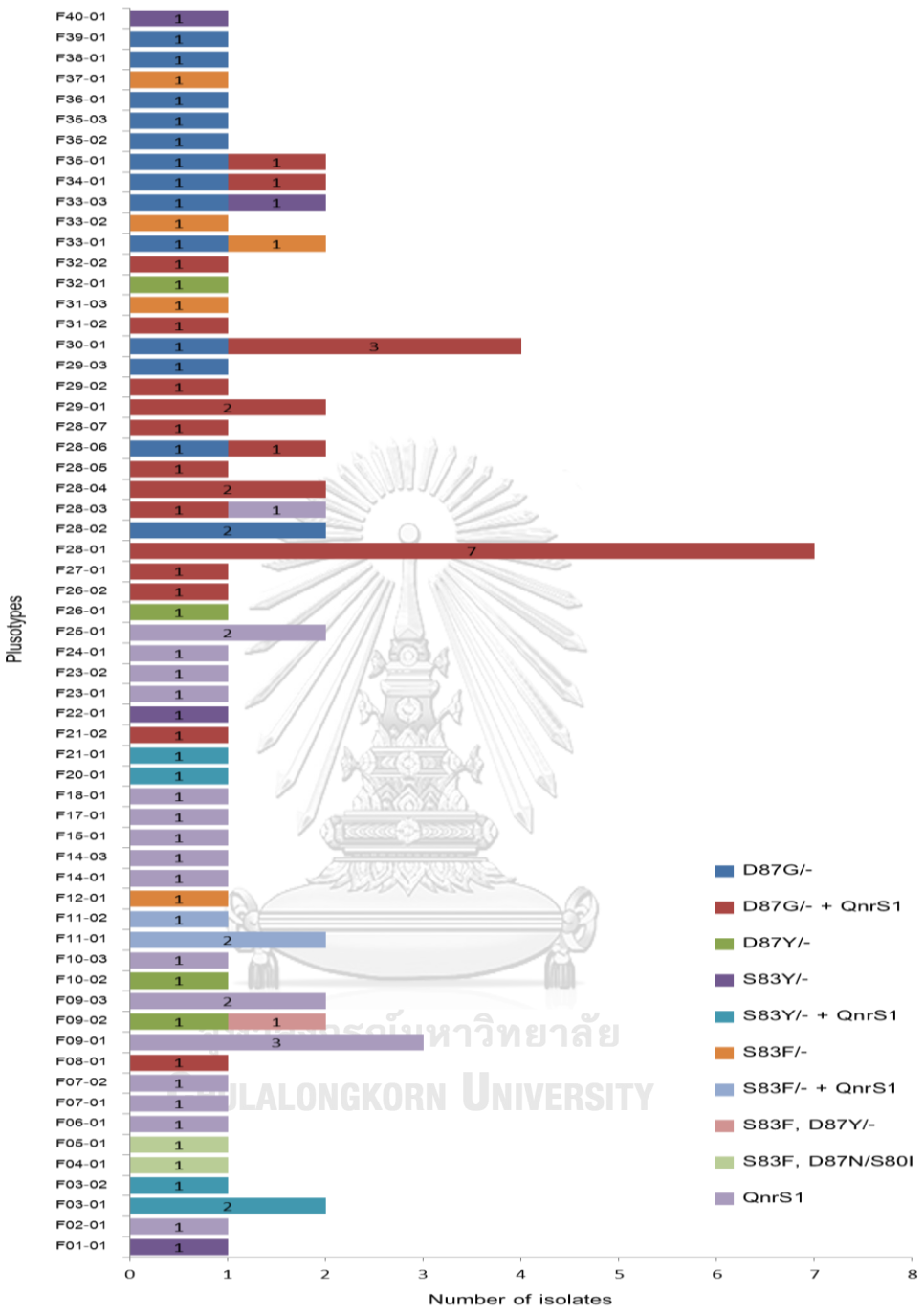

Figure 8. The distribution of pulsotype and fluoroquinolone resistance mechanisms among 86 ciprofloxacin-resistant *Salmonella* isolates: GyrA/ParC mutation and/or QnrS1 represent fluoroquinolone resistance mechanisms by mutation in GyrA and ParC and/or the production of QnrS1;D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine N, Asparagine; I, Isolucine.

2. Investigation of the genetic relationship among ESC-resistant *Salmonella* isolates

A total of 170 ESC-resistant *Salmonella* isolates including 148 *S.* Choleraesuis and 22 *S.* Typhimurium were subtyped by PFGE. These isolates were 70 CMY-2, 44 CT-M-14, 38 CTX-M-55, and 1 CTX-M-15 producers. One of these isolates was CMY-2 and CTX-M-14 co-producer. The 45 PFGE clusters designated C01 to C45 and 90 different pulsotypes were identified using a cut-off of 85% and 95% genetic similarity, respectively (Appendix D, Table 30). The distribution of pulsotypes is summarized in Figure 9.

There were 41 PFGE clusters and 83 different pulsotypes among 159 ESC-resistant *Salmonella* human isolates (141 *S.* Choleraesuis and 18 *S.* Typhimurium). A few predominant clones were found among ESC-resistant *S.* Choleraesuis human isolates. The most common pulsotype was C35-01(n=17), followed by pulsotype C19-03(n=14), C35-02(n=7), C20-02(n=7), and C27-01(n=7). The information of strains, PFGE pulsotypes, the antimicrobial susceptibility profiles, and resistance mechanisms are summarized in the Appendix D, Table 30. The two predominant pulsotypes, C35-01 and C35-02 belonged to same cluster C35. This cluster contained 26 isolates accounted for 32.9% of CMY-2-producing isolates.

The most common pulsotype, C35-01, contained 17 isolates of CMY-2-producing *S.* Choleraesuis isolates from blood and stool in 2007 which exhibited resistance to ampicillin, cefoxitin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, gentamicin, chloramphenicol, and tetracycline. It also contained 11 indistinguishable isolates (Appendix D, Figure 20.) from 4 different provinces (Ratchaburi, Bangkok, Chon buri and Nonthaburi). The second most common pulsotype, C19-03, contained 14 isolates of CMY-2-producing *S.* Choleraesuis isolates from blood in 2007 which had S83F amino acid substitution in GyrA. The isolates displayed resistance to ampicillin, cefoxitin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, gentamicin, chloramphenicol, and tetracycline except one isolate which remained susceptible to gentamicin. Five of these isolates were also resistant to trimethoprim-sulfamethoxazole. This pulsotype contained 9 indistinguishable isolates from 4 different provinces

(Ratchaburi, Bangkok, Chon buri and Phetchaburi). Pulsotype C20-02, the most common pulsotype among CTX-M-14-producing isolates, contained 7 isolates of CTX-M-14-producing *S.* Choleraesuis isolates from blood in 2007 which had GyrA mutation at D87Y. The isolates displayed resistance to ampicillin, ceftriaxone, cefotaxime, nalidixic acid, chloramphenicol, and tetracycline. This pulsotype also contained 6 indistinguishable isolates from 6 different provinces (Ratchaburi, Bangkok, Sa Kaeo, Chanthaburi, Lampang, and Nan). Pulsotype C27-01 was the most common pulsotype among CTX-M-55-producing isolates. This cluster included 19 isolates accounted for 42.2% of CTX-M-55-producing isolates. Pulsotype C27-01 contained 7 isolates of CTX-M-55-producing *S.* Choleraesuis isolates from blood in during 2012 to 2015 which had D87G amino acid substitution in GyrA and *qnrS1* gene. The isolates were resistant to ampicillin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole except one isolate which remained susceptible to gentamicin. One isolate was also resistant to norfloxacin. This pulsotype contained two groups of 3 indistinguishable isolates, both of which were collected form the same hospital (King Chulalongkorn Memorial Hospital) but difference in the years of isolation (2012, 2014, and 2015) and hospital wards. Moreover, few predominant clones were also found among CTX-M-55-producing *S.* Typhimurium isolates including pulsotype C07-01 and C09-01. Pulsotype C07-01 contained 4 indistinguishable isolates of CTX-M-55-producing *S.* Typhimurium isolates from stool and rectal swab in 2007 which showed resistance to ampicillin, ceftriaxone, ceftazidime, cefotaxime, gentamicin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. These isolates were collected from 3 different provinces including Ratchaburi, Bangkok, and Nonthaburi. Pulsotype C09-01 contained 3 indistinguishable isolates of CTX-M-55-producing *S.* Typhimurium isolates from stool and rectal swab in 2007 which displayed resistance to ampicillin, ceftriaxone, ceftazidime, cefotaxime, gentamicin, and tetracycline. These isolates were collected from 2 different provinces including Ratchaburi and Nonthaburi.

Among 11 ESC-resistant *Salmonella* food animal isolates (7 *S.* Choleraesuis and 4 *S.* Typhimurium), 7 PFGE clusters and 9 different pulsotypes were identified.The 7 PFGE clusters included C10 (n=3), C04 (n=2), C12 (n=2), C03 (n=1), C09 (n=1), C13 (n=1), and C27 (n=1). The two most common pulsotypes were pulsotype C10-02 and C12-01 which contained 2 CTX-M-55-producing *S.* Typhimurium isolates from swine in Udon Thani in 2015 and 2 CTX-M-14-producing *S.* Choleraesuis isolates from swine in provinces of the central region of Thailand in 2016, respectively. The information of strains, PFGE pulsotypes, the antimicrobial susceptibility profiles, and resistance mechanisms are summarized in the Appendix D in Table 30.

The genetically related clones between human and food animal isolates included pulsotype C10-01 and C27-05. Pulsotype C10-01, indistinguishable pattern, contained CTX-M-55-producing *S.* Typhimurium isolates (strain A252) from swine in Bangkok in 2014 and CTX-M-55-producing *S.* Typhimurium isolates (strain H612) from human in Bangkok in 2015, both of which also carried *qnrS1* gene. These isolates were resistant to ampicillin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, gentamicin, chloramphenicol, and tetracycline. Pulsotype C27-05, indistinguishable pattern, contained CTX-M-55-producing *S.* Choleraesuis isolates (strain A251) from swine in Ratchaburi in 2014 and CTX-M-55-producing *S.* Choleraesuis isolates (strain H590) from blood of bacterimic patient in Bangkok in 2014, both of which also carried *qnrS1* gene. The human isolate also had D87G mutation in GyrA. Both isolates displayed resistance to ampicillin, ceftriaxone, ceftazidime, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, chloramphenicol, and tetracycline.

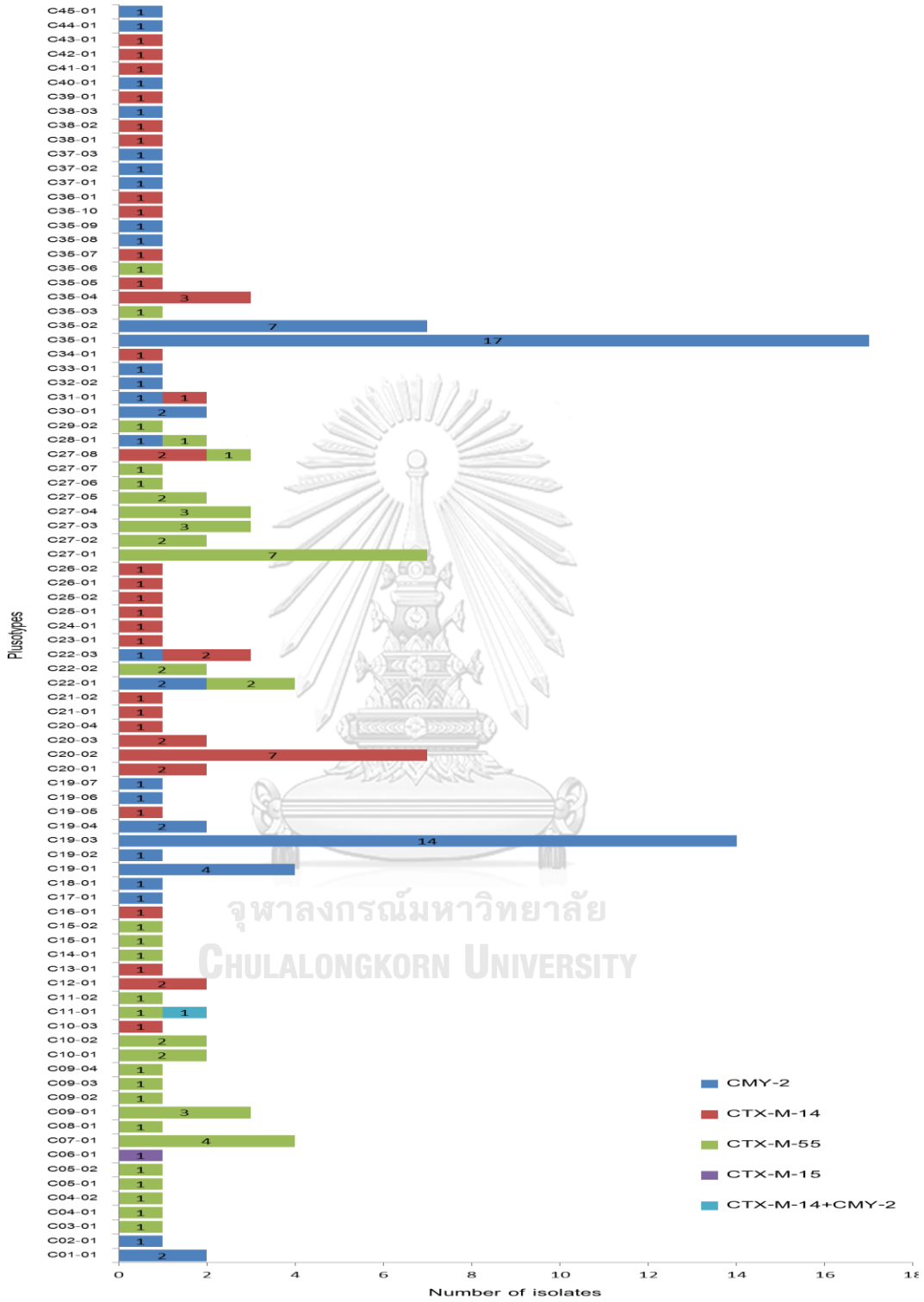

Figure 9. The distribution of pulsotype and ESC resistance mechanisms among 170 ESC-resistant *Salmonella* isolates

Part IV: Characterization of the transmission mechanism of antimicrobial resistance genes among nontyphoidal *Salmonella* isolated from humans and food animals

The transference of ESC resistance was determined in all ESC-resistant isolates by using conjugation experiment. MICs of antimicrobials for the donor, recipient, and transconjugant strains were compared by the agar-dilution technique. The size of transferable plasmids was determined by PFGE using S1 nuclease (Appendix D, Figure 21.). The estimated sizes were determined by InfoQuest FP software version 4.5. The type of transferable plasmids was determined using PCR-based replicon typing for the major plasmid types found in Enterobacteriaceae. Of 170 ESC-resistant *Salmonella* isolates, 83 (48.8%) successfully transferred *bla* genes to the recipient. The plasmid profiles, plasmid types and resistance mechanisms are summarized in Appendix D in Table 30.

Of 70 CMY-2-producing *S.* Choleraesuis isolates, 31 (44.3%) successfully transferred bla_{CMY-2} gene to recipient. The results revealed that the dissemination of bla_{CMY-2} gene was mediated by conjugative plasmid IncA/C, ranging in size from 103 to 207 kb, which were also co-transferred with tetracycline, gentamicin, and chloramphenicol resistance in most of isolates. The conjugative bla_{CMY-2}-carrying IncA/C plasmid spread among at least 9 genetically unrelated clones including cluster C01, C18, C19, C22, C28, C30, C32, C35, and C37. The distribution of plasmid types carrying *bla* gene among various clusters is shown in Figure 10.

Of 44 CTX-M-14-producing *S.* Choleraesuis isolates, 40(90.9%) successfully transferred $bla_{CTX-M-14}$ gene to the recipient. Among 31 transconjugants, the $bla_{CTX-M-14}$ was disseminated via 3 types of conjugative plasmid including IncI1 ranging from 91 to 136 kb (35.0%), FII_s ranging from 97 to 128 kb (32.5%), and FII ranging from 83 to 127 kb (30.0%). One isolate carried 38-kb of CTX-M-14-carrying plasmid which was untypable by PCR-based replicon typing for the major plasmid types found in Enterobacteriaceae. The dendrogram revealed that these plasmids spread among various genetically

unrelated clones, especially for IncI1 plasmids. IncI1 plasmids carrying $bla_{CTX-M-14}$ gene, ranging from 91 to 136 kb spread among 9 genetically unrelated clones including cluster C16, C20, C23, C24, C25, C26, C31, C36, and C41. $IncFII_s$ plasmids carrying $bla_{CTX-M-14}$ gene, ranging from 97 to 128 kb spread among 3 genetically unrelated clones including cluster C21, C27, and C43. IncFII plasmids carrying $bla_{CTX-M-14}$ gene, ranging from 83 to 127 kb spread among 3 genetically unrelated clones including cluster C34, C38 and C42. In addition, the major cluster of CTX-M-14 producers, cluster C35 and C20 had all 3 types of plasmids spreading among the isolates. Moreover, one CTX-M-15-producing *S.* Typhimurium isolate successfully transferred $bla_{CTX-M-15}$ gene to recipient via 107-kb size of IncI1 plasmid.

Of 45 CTX-M-55-producing *Salmonella* isolates (30 *S.* Choleraesuis and 15 *S.* Typhimurium), 11(24.4%) successfully transferred $bla_{CTX-M-55}$ gene to the recipient, all of which were *S.* Typhimurium. Of the 11 transconjugants, the $bla_{CTX-M-55}$ was disseminated via 3 types of conjugative plasmid including IncA/C ranging from 172 to 264 kb (45.5%), FII ranging from 57 to 255 kb (36.4%), and I1 size of 71 kb (9.1%), respectively. One isolate carried 263-kb of CTX-M-55-carrying plasmid which was untypable by PCR-based replicon typing for the major plasmid types found in Enterobacteriaceae. IncA/C plasmids carrying $bla_{CTX-M-55}$ gene, ranging from 172 to 264 kb spread among 3 genetically unrelated clones including cluster C08, C09, and C11. Two of IncA/C plasmids carrying $bla_{CTX-M-55}$ gene also co-transferred with bla_{TEM-1} gene. IncFII plasmids carrying $bla_{CTX-M-55}$ gene, ranging from 57 to 255 kb spread among 2 genetically unrelated clones including cluster C05 and C10. Moreover, Southern blot hybridization was performed on 12 $bla_{CTX-M-55}$-carrying isolates (10 *S.* Choleraesuis and 2 *S.* Typhimurium), five of which co-carried *qnrS1* gene and three of which co-carried both *qnrS1* and bla_{TEM-1} genes. (Appendix D, Figure 22. to 25). The result showed that the dissemination of $bla_{CTX-M-55}$ gene was mediated by plasmid IncA/C ranging from 61 to 202 kb in 11 of tested isolates which spread among at least 4 genetically unrelated clones including cluster C09, C27, C29 and C35 (Figure 10.). One 212-kb IncHI1 plasmid was found in a *S.* Typhimurium isolate from swine carrying $bla_{CTX-M-55}$ together

with *qnrS1*. Co-location of $bla_{CTX\text{-}M\text{-}55}$ and *qnrS1* genes on the same plasmid was found in all tested isolates, two of which also co-located with $bla_{TEM\text{-}1}$ gene.

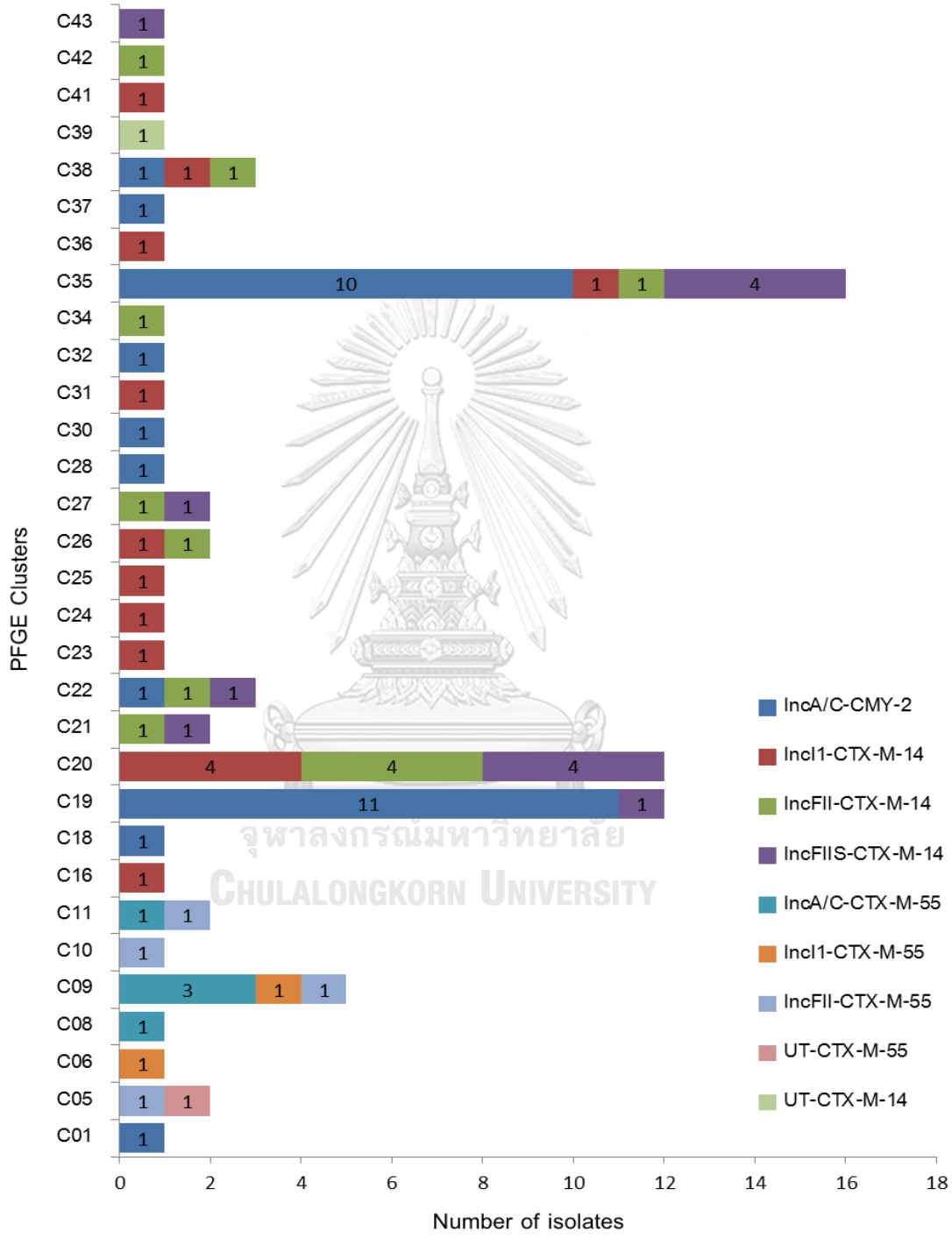

Figure 10. The distribution of plasmid type carrying *bla* gene among various clusters: Inc-(type of *bla* gene), incompatibility group of plasmid carrying *bla* gene; UT, untypable of plasmid incompatibility group.

Part V: Investigation of the genetic relationship of transferable plasmids from antimicrobial-resistant *Salmonella* isolated from humans and food animals

A total of 83 transferable plasmids carrying *bla* gene were extracted by the alkaline lysis method from 83 different transconjugants. These plasmids were determined for the genetic relatedness by restriction fragment length polymorphism (RFLP) using *Eco*RI, *Sca*I, *Hind*III, and *Hinc*II which were labeled by E, S, H, and HC, respectively. These plasmids including 31 IncA/C-carrying bla_{CMY-2}, 14 IncI1-carrying $bla_{CTX-M-14}$, 13 IncFII$_S$-carrying $bla_{CTX-M-14}$, 12 IncFII-carrying $bla_{CTX-M-14,}$ 5 IncA/C-carrying $bla_{CTX-M-55,}$ 4 IncFII-carrying $bla_{CTX-M-55,}$ 2 IncI1-carrying $bla_{CTX-M-55}$ were subtyped by RFLP. There were 2 untypable plasmids carrying $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$. The RFLP clusters and RFLP types were identified using a cut-off of 85% and 95% genetic similarity, respectively. The plasmid size, type of plasmid, the antimicrobial susceptibility profiles, resistance mechanisms, and RFLP type are summarized in the Appendix D in Table 31. to 34.

Among 31IncA/C-carrying bla_{CMY-2} plasmids, 17 RFLP clusters and 22 different RFLP types were identified. The distribution of each RFLP cluster is summarized in Figure 11a. The most common plasmid belonged to RFLP cluster S01 (n=6), followed by RFLP cluster S08 (n=3), HC01 (n=3), and HC05 (n=3). The most common RFLP types, S01-01, indistinguishable RFLP patterns, contained 6 plasmids of IncA/C-carrying bla_{CMY-2} accounted for 19.4%. These plasmids were found to co-transfer resistance to ceftriaxone with gentamicin, chloramphenicol, and tetracycline. The plasmid spread among 4 genetically unrelated clones of nontyphoidal *Salmonella* including PFGE cluster C19, C29, C32, and C35.

Among 14 of IncI1-carrying $bla_{CTX-M-14}$ plasmids, 4 RFLP clusters and 6 different RFLP types were identified. The distribution of each RFLP cluster is summarized in Figure 11b. The most common plasmid belonged to RFLP cluster E21 (n=9), followed by RFLP cluster E18 (n=3). The RFLP cluster E21 was found in 64.3% of these plasmids.

The most common RFLP type, E21-02, indistinguishable RFLP patterns (Appendix D, Figure 26.), contained 6 plasmids of IncI1-carrying $bla_{CTX-M-14}$ accounted for 42.9%. These plasmids were found to co-transfer resistance to ceftriaxone with gentamicin. The plasmids spread among 4 genetically unrelated clones of nontyphoidal *Salmonella* including PFGE cluster C20, C26, C31, and C35.

Among 13 $IncFII_S$-carrying $bla_{CTX-M-14}$ plasmids, 8 RFLP clusters and 12 different RFLP types were identified. The distribution of each RFLP cluster is summarized in Figure 11c. The most common plasmid belonged to RFLP cluster E06 (n=5), followed by RFLP cluster E14 (n=2). The RFLP cluster E06 was found in 38.5% of these plasmids. The RFLP types E06-01, indistinguishable RFLP patterns, contained 2 plasmids of $IncFII_S$-carrying $bla_{CTX-M-14}$ which spread among 2 genetically unrelated clones of nontyphoidal *Salmonella* including PFGE cluster C20 and C27.

Among 12 IncFII-carrying $bla_{CTX-M-14}$ plasmids, 7 RFLP clusters and 10 different RFLP types were identified. The distribution of each RFLP cluster is summarized in Figure 11d. The most common plasmid belonged to RFLP cluster E06 (n=3), followed by RFLP cluster E20 (n=2) and E11 (n=2). The RFLP cluster E06 and E20 was found in 50% of these plasmids. Both RFLP type E20-01 and E11-01, indistinguishable RFLP patterns, contained 2 plasmids of IncFII-carrying $bla_{CTX-M-14}$ which spread among 2 genetically unrelated clones of PFGE cluster C20 and C26 and C34 and C38, respectively.

Among 12 $bla_{CTX-M-55}$-carrying plasmids (5 InA/C-carrying $bla_{CTX-M-55}$, 4 InFII-carrying $bla_{CTX-M-55}$, and 2 IncI1-carrying $bla_{CTX-M-55}$), 6 RFLP clusters and 10 different RFLP types were identified. The RFLP type E21-03, indistinguishable RFLP patterns, contained 3 plasmids of IncA/C-carrying $bla_{CTX-M-55}$, was found. These plasmids found co-transferred of resistance to ceftriaxone with gentamicin. However, these plasmids were found within the genetically related clone of pulsotype C19-01.

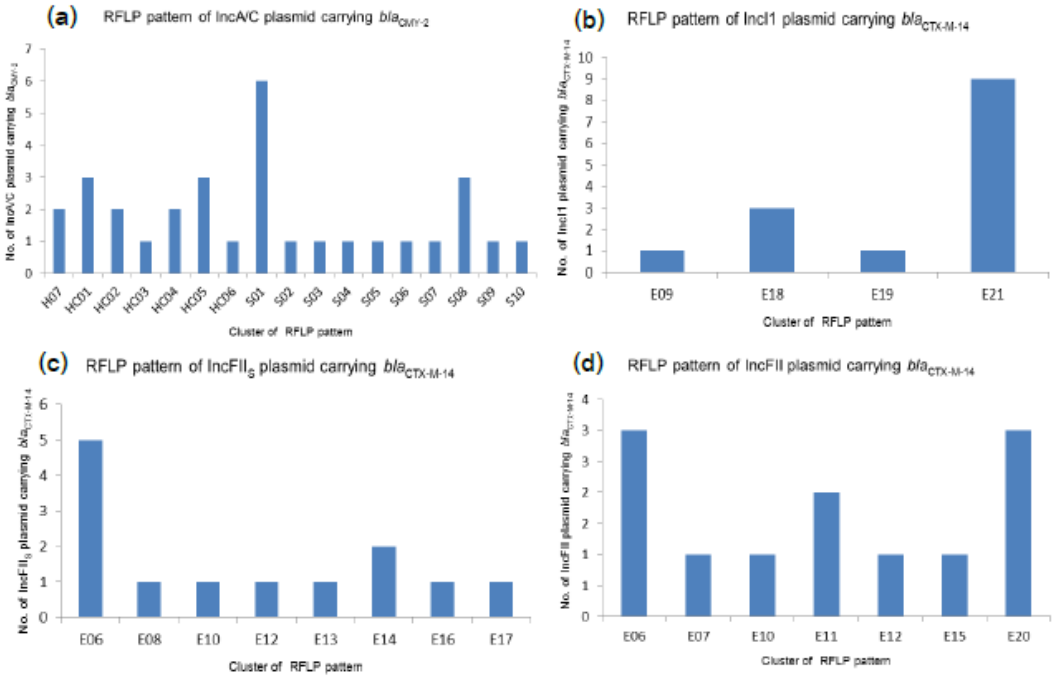

Figure 11. The distribution of each RFLP cluster among *bla*-carrying plasmids (a), 31 IncA/C-carrying bla_{CMY-2}; (b), 14 IncI1-carrying $bla_{CTX-M-14}$; (c), 13 $IncFII_S$-carrying $bla_{CTX-M-14}$; (d), 12 IncFII-carrying $bla_{CTX-M-14}$

Part VI: Characterization of a novel quinolone resistance gene in *Salmonella* isolate

As a part of our study on quinolone resistance mechanisms, *Salmonella* isolates that displayed nonsusceptible to ciprofloxacin (MIC > 0.06 mg/L) were screened for quinolone resistance mechanisms. We found that *Salmonella* Rissen strain 166ANSS50, isolated from rectal swab of a swine from a farm in Khon Kaen province in 2007, was resistant to nalidixic acid (MIC 32 mg/L) and intermediate resistant to ciprofloxacin (MIC 0.5 mg/L) and norfloxacin (MIC 1 mg/L). Mutations in *gyrA* and *parC* genes and the presence of known PMQR genes of Enterobacteriaceae were not detected. The screening of *qnrVC* gene was performed and the *qnrVC4* gene was identified in this isolate. This is the first report of the occurrence of *qnrVC* family in *Salmonella enterica*. Therefore, the antimicrobial susceptibility, the dissemination mechanism, and the genetic background of this resistance determinant were further characterized.

1. QnrVC4 Determinant from *Salmonella enterica*

The *qnrVC4* gene was detected in a swine isolate of *S.* Rissen 166ANSS50. Antimicrobial susceptibility to ampicillin, ceftazidime, streptomycin, amikacin, gentamicin, kanamycin, chloramphenicol, nalidixic acid, ciprofloxacin, and norfloxacin was determined in this isolate and its transformants. The results are shown in Table 28. *S.* Rissen 166ANSS50 showed resistance phenotype to quinolones with resistance to nalidixic acid (MIC 32 mg/L) and intermediate resistance to ciprofloxacin (MIC 0.5 mg/L) and norfloxacin (MIC 1 mg/L). This mutation in QRDR of *gyrA* or *parC* and PMQR genes were not detected in this isolate. The isolate also displayed resistance phenotype to other groups of antimicrobials including chloramphenicol, aminoglycosides (streptomycin but not amikacin, gentamicin or kanamycin). It showed resistance to narrow spectrum beta-lactam (ampicillin) but remained susceptible to broad-spectrum cephalosporin (ceftazidime).

Table 28. MICs (mg/L) of antimicrobials for *S.* Rissen 166ANSS50 and transformants

Strain	MIC (mg/L)									
	AMP	CAZ	STR	AMK	GEN	KAN	CHL	NAL	CIP	NOR
S. Rissen 166ANSS50 (pSR166)	256	1	>256	4	4	16	32	32	0.5	1
E. coli DH10B (pSR166)	128	1	256	4	2	16	16	16	0.25	0.5
E. coli DH10B (pBK-CMV*qnrVC4*)	8	1	256	4	1	>256	4	8	0.125	0.5
E. coli DH10B (pBK-CMV)	8	1	256	4	1	>256	4	2	0.015	0.03
E. coli DH10B	8	1	256	4	1	4	4	2	0.015	0.03

AMP, Ampicillin; CAZ, Ceftazidime; STR, Streptomycin; AMK, Amikacin; GEN, Gentamicin; KAN, Kanamycin; CHL, Chloramphenicol; NAL, Nalidixic acid; CIP, Ciprofloxacin; NOR, Norfloxacin.

2. Characterization of *qnrVC4* and the transference of quinolone resistance

The nucleotide sequences of *qnrVC4* from *S.* Rissen 166ANSS50 showed 657 nucleotides with 100% identity to *qnrVC4* from *A. punctate* 159 (GenBank accession number GQ891757). The translated sequence for QnrVC4 predicted a 218-amino acid protein belonging to pentapeptide repeat protein family. *E. coli* DH10B containing recombinant plasmid of pBK-CMV*qnrVC4* resulted in 4-, 8-, and 16-fold increases in the MICs of nalidixic acid (2 to 8 mg/L), ciprofloxacin (0.015 to 0.125 mg/L), and norfloxacin (0.03 to 0.5 mg/L), respectively, compared with those in *E.coli* DH10B containing pBK-CMV alone.

We repeatedly failed to transfer the natural plasmid-mediated *qnrVC4* determinant of *S.* Rissen 166ANSS50 by conjugation experiments. However, this plasmid was successfully transferred to *E. coli* DH10B by transformation using electroporation techniques. Transformant was selected on Mueller-Hinton agar plate

containing 0.03 mg/L of ciprofloxacin and confirmed by PCR using specific primers for *qnrVC* gene. MICs of antimicrobials for the donor, recipient, and transformant strains were compared by the agar-dilution technique. The transformant of pSR166 carrying *qnrVC4* conferred an 8-fold increase in the MIC of nalidixic acid and a 16-fold increase in the MIC of ciprofloxacin and norfloxacin compared with those in *E.coli* DH10B (Table 28.). This transformant also displayed 16-, 2-, and 4-fold increases in the MICs of ampicillin, gentamicin, and chloramphenicol, respectively, compared with those in *E.coli* DH10B (Table 28.). The location of *qnrVC4* in *S.* Rissen 166ANSS50 was identified by PFGE using S1 nuclease and I-CeuI digestion and Southern blot hybridization with the *qnrVC4* and the 16S rRNA gene probes. Total bacterial DNA was prepared in low-melt agarose plugs which were digested with S1 nuclease or I-CeuI nuclease and separated using a CHEF-Mapper XA pulsed-field gel electrophoresis (PFGE) system. The sizes of plasmids were estimated by S1 nuclease PFGE. The chromosomal DNA was estimated by I-CeuI nuclease PFGE. *E. coli* K12 was used as a reference strain for chromosomal DNA study. The location of the *qnrVC4* gene on plasmids or in the chromosomal DNA was determined by Southern blot hybridization by using specific probes of *qnrVC4* or 16S rRNA replicons. The I-CeuI-generated DNA fragments can be hybridized with the 16S rRNA gene probe but did not co-hybridize with the *qnrVC4* probe, excluding chromosomal locations for this gene (Figure 12.). In contrast, the hybridization signal of the *qnrVC4* probe was detected in a S1-generated DNA fragment in *S.* Rissen 166ANSS50 and its transformant. The results indicated that *qnrVC4* was located on a single 17-kb plasmid (pSR166) and it can be co-transferred with other antimicrobial resistance determinants to other bacteria through this plasmid by transformation.

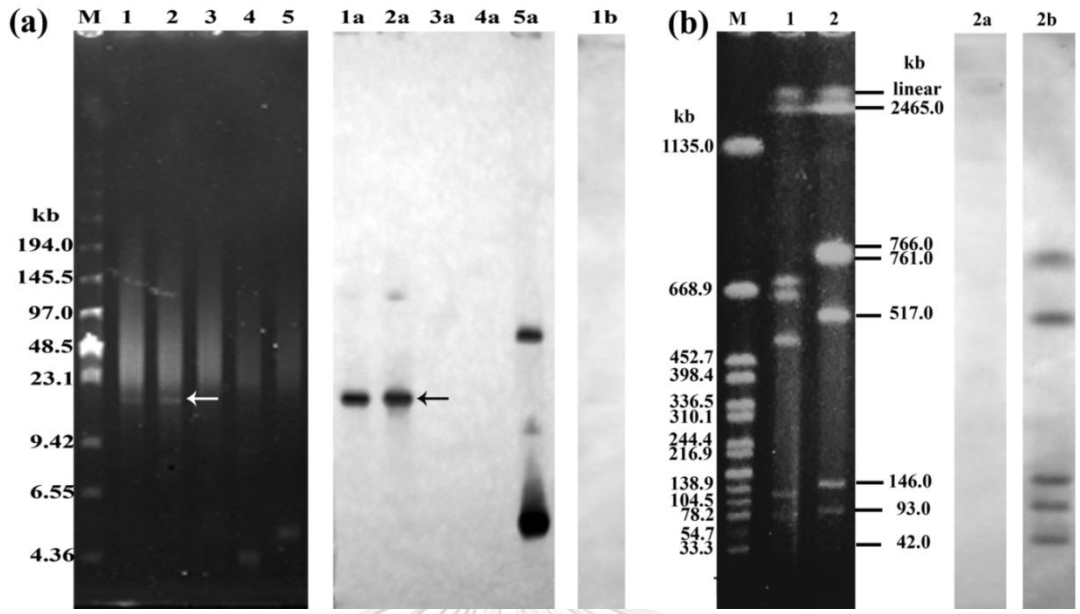

Figure 12. Identification of the *qnrVC4* location in *S.* Rissen 166ANSS50 by S1/I-CeuI PFGE and Southern blot hybridization.

PFGE profiles of total DNA digestion with S1 nuclease and the relative hybridization with *qnrVC4* probe and 16S rRNA probe (a). Lane 1, plasmid analysis of *S.* Rissen 166ANSS50 (pSR166); lane 2, *E. coli* DH10B (pSR166); land 3, *E. coli* DH10B; land 4, *E. coli* DH10B (pBK-CMV); land 5, *E. coli* DH10B (pBK-CMV*qnrVC4*); land M, Low range PFG marker (NEB #N0350S), marker labels are in kilo-bases. Lane 1a and 1b show hybridization results of *S.* Rissen 166ANSS50 plasmid with *qnrVC4* probe and 16S rRNA probe, respectively. PFGE profiles of total DNA digestion with I-CeuI and the relative hybridization with *qnrVC4* probe and 16S rRNA probe (b). Lane 1, genomic mapping of *S.* Rissen 166ANSS50; lane 2, genomic mapping of *E. coli* K12; lane M, *S.* Braenderup H9812 with X-bal digestion, marker labels are in kilo-bases. Lanes 2a and 2b show hybridization results of genomic mapping of *S.* Rissen 166ANSS50 with *qnrVC4* probe and 16S rRNA probe, respectively. Arrows indicate plasmid locations.

3. Characterization of the transferable element containing *qnrVC4*

The possibility of *qnrVC4* being located within an integron was determined by PCR using specific primers for *int1* and *qnrVC4* genes. The results showed that *qnrVC4* was located in class 1 integron. Gene cassettes within this integron were detected by PCR using specific primers for the 5'-CS and 3'-CS regions. However, it did not yield an amplicon. So, Inverted PCR and PCR mapping were performed to determine the DNA sequences flanking *qnrVC4*. The results showed that *qnrVC4* gene was located in 8.91 kb of a novel class 1 integron which was numbered as In805 according to INTEGRALL database (Figure 13a.). The nucleotide sequences reported here are provided on GenBank under accession number JX173955 and KU886277 for the nucleotide sequences of complete *qnrVC4* gene and In805, respectively. In805 is bounded by two 25-bp imperfect inverted repeats (2 mismatches), designated IRi and IRt. It contained the 5'-CS and seven integrated cassettes but unusual 3'-CS with one full copy of the insertion sequence IS*6100* flanked by 123 and 152-bp fragments of the end of the Tn*402* region in the opposite orientations which both included IRt. The 3' region displayed the *dfrA14* cassette followed by interrupted putative sequence of *mobC* gene, encoding mobilisation protein. The 5'-CS of In805 had P_c promoter, responsible for the expression of inserted gene cassettes but P_2 promoter was not detected. The P_c promoter belonged to a weak promoter variant (PcW) with a –35 box (TGGACA) and a –10 box (TAAGCT) separated by a 17-bp sequence. However, this *qnrVC4* cassette carried its own putative promoter sequences (P_{qnrVC4}) with a –35 box (TTGAGA) and a –10 box (TAGTCT) separated by a 16-bp sequence.

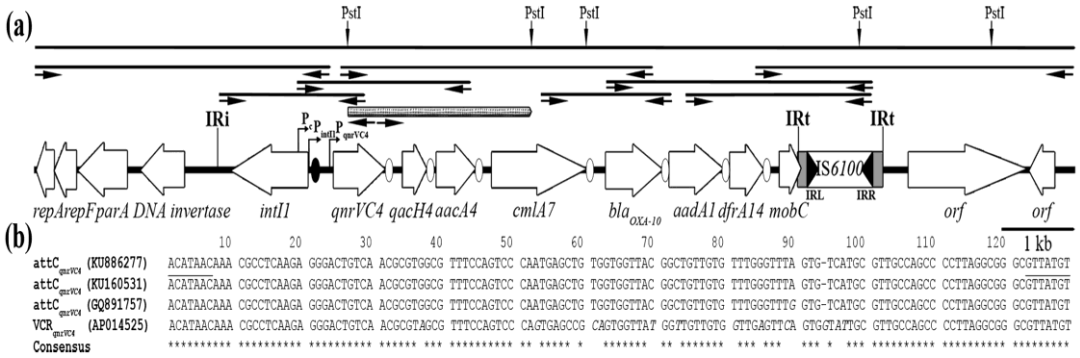

Figure 13. Schematic map of a novel class 1 integron carrying *qnrVC4* gene and alignment of attC recombination sites.

Schematic map of a novel class 1 integron carrying *qnrVC4* gene (In805) in *S.* Rissen 166ANSS50 (a). Open arrows indicate open reading frames, open ovals indicate attC sites, and a filled oval indicates the attI1 site. The initial and terminal inverted repeats of class 1 integron are shown in uppercase. The gray boxes represent nucleotide sequence 100% identical to that of the IRt end of Tn*402* (GenBank accession number U67194). The white bar represents IS*6100* and filled triangles indicate left and right inverted repeats of IS*6100*. The locations of the primers are indicated by small arrows, and PCR products are indicated by lines above the structure. The PstI recognition sites are indicated by vertical arrows. Cross-hatched bar represents PCR products from inverse PCR. Alignment of attC recombination sites of the *qnrVC4* gene cassettes (b); $attC_{qnrVC4}$ of In805 from *S.* Rissen 166ANSS50 (GenBank accession number KU886277), $attC_{qnrVC4}$ of class 1 integron from pVAS3-1 of *V. alginolyticus* VAS3-1 (GenBank accession number KU160531), $attC_{qnrVC4}$ of class 1 integron from *A. punctata* 159 (GenBank accession number GQ891757), and VCR_{qnrVC4} of superintegron (SI) in chromosome 2 from *V. cholerae* MS6 (GenBank accession number AP014525). The core site and inverse core site are underlined. The conserved residues are indicated by stars and the mismatched nucleotide residues are indicated in italics.

We repeatedly failed to identify the plasmid types by PCR-based replicon typing. The genetic environments of In805 revealed the putative backbone of this 17-kb long pSR166 including genes involved in plasmid replication (*repA*, *repF*), partitioning (*parA*), DNA invertase/recombinase, and two hypothetical protein (Figure 13a.). This RepA plasmid replication protein shares 100% identity with RepA from pG5A4 plasmids carrying bla_{GES-5} in clinical isolates of *E. coli* and *S. marcescens* which has been reported as a novel replicon type (Figure 14a). Moreover, MobA relaxase protein of pSR166 contained the three motifs of relaxase domain (225 amino acids) which showed 100% identity to MobA from pG5A4 plasmids (Figure 14b). It belonged to the MOB_{Q1} group whose prototype is the broad-host-range mobilisable IncQ1 plasmid RFS1010. In805 carried *qnrVC4* gene cassette adjacent to 5'-CS followed by the other antimicrobial resistance gene cassettes, including *qacH4* (quaternary ammonium compound resistance), *aacA4* (kanamycin and gentamicin resistance), *cmlA7* (chloramphenicol resistance), bla_{OXA-10} (beta-lactam resistance), *aadA1* (streptomycin and spectinomycin resistance), and *dfrA14* (trimethoprim resistance). The sequences revealed the fused gene cassettes of *aacA4-cmlA7* and bla_{OXA-10}-*aadA1-dfrA14*, created by the loss of the 59-base elements, which have not been reported before.

Moreover, this 1014-bp *qnrVC4* cassette showed more than 99% similarity with *qnrVC4* cassette present in class 1 integron of *V. alginolyticus, A. hydrophila, A. punctata,* and *E. coli*, all of which were isolated from aquatic environments in China and Portugal during 2008-2015 (GenBank on NCBI). This *qnrVC4* cassette contained 128-bp attC site (Figure 13b.) which showed 100% similarity with attC site of *qnrVC4* of class 1 integron in pVAS3-1 from *V. alginolyticus* VAS3-1 and 99% similarity with attC site of *qnrVC4* of complex class 1 integron from *A. punctata* 159. In addition, it also showed 90% similarity with *V. cholerae* repeats (VCR) of *qnrVC4* cassette from 144-kb long superintegron (SI) carrying 279 cassettes of ORFs and was located within chromosome 2 of *V. cholerae* O1 El Tor serotype Ogawa strain MS6 from Thailand–Myanmar border area during 2007-2010.

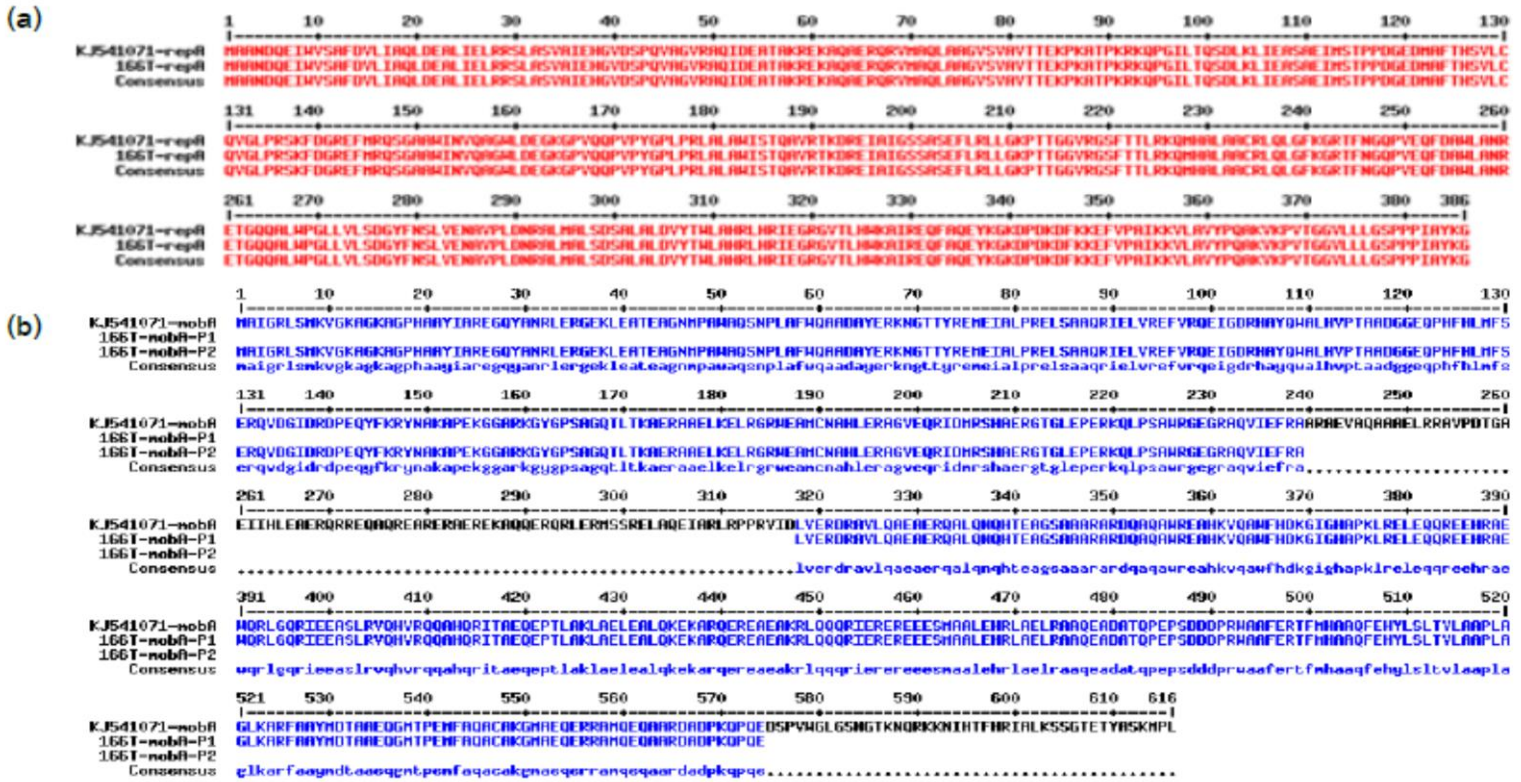

Figure 14. Alignments for amino acid sequences of RepA and MobA. Alignments for amino acid sequences of RepA from pG5A4 plasmids (GenBank accession number KJ541071) **(a).** Alignments for amino acid sequences of MobA from pG5A4 plasmids (GenBank accession number KJ541071) **(b). The 166T represents** pSR166 from transformant of *S.* Rissen 166ANSS50

CHAPTER VI

DISCUSSION

Nontyphoidal *Salmonella*, the major pathogen of the foodborne illnesses is estimated to cause more than one million illnesses each year, with almost 20,000 hospitalisations and 400 deaths, according to a 2011 report in the United States by CDC (1). These infections are generally self-limiting, but invasive infections can occurre especially due to certain *Salmonella* serotypes, for which antimicrobial treatments are required for life-saving (2). In this study, we investigated 897 nontyphoidal *Salmonella* isolates from humans and food animals from various provinces of Thailand during 2005 to 2007 and 2012 to 2016. Nontyphoidal *Salmonella* isolates from blood were the most common clinical isolates, accounted for 67.7% of all isolates. *S.* Enteritidis (46.4%) and *S.* Choleraesuis (40.2%) were the most common serotypes. According to previous reports, *S.* Choleraesuis and *S.* Enteritidis ranked the first and sixth most common *Salmonella* serotypes isolated from humans in Thailand during 2002 to 2007, respectively (2).

Our results revealed high rate of antimicrobial resistance among nontyphoidal *Salmonella* isolates from human in Thailand. The 50.7% of isolates were resistant to at least three antimicrobial subclasses, whereas only 3.1% of isolates were susceptible to all antimicrobial agents tested. The significantly higher resistance rates to cephalosporins, ampicillin, nalidixic acid, and chloramphenicol were found among the isolates from sterile sites, compared with those from non- sterile sites. High rates of ESC and fluoloquinolone resistantce in *Salmonella* isolates were found in Thailand with 25.8% and 12.0%, respectively. The ESC-resistant *Salmonella* isolates of 3.1% and fluoloquinolone-resistant *Salmonella* isolates of 2.4% were reported by using 2004–2012 data from the National Antimicrobial Resistance Monitoring System (NARMS) and the National Laboratory-based Enteric Disease Surveillance (LEDS) which included 19,410

and 369,254 *Salmonella* isolates from the United States, respectively (157), In Asia, reduced susceptibility to ciprofloxacin (MIC of 0.125 to 1 mg/L) was previously reported common in Taiwan (48.1%) and Thailand (46.2%) (48). However, the reduced susceptibility to ceftriaxone (MIC of 2 to 8 mg/L) remained uncommon, except in Taiwan (38.0%), according to the study in 400 clinical isolates of nontyphoid *Salmonella* from seven Asian countries during 2003 to 2005 (48).

Noticeably, high rate of antimicrobial resistance in nontyphoidal *Salmonella* was found in certain serotype, *S.* Choleraesuis. The significantly higher resistance rates were found among Choleraesuis isolates compared with non-Choleraesuis isolates to all antimicrobial agents tested ($p \leq 0.0001$), except for norfloxacin resistance. Similarly, antimicrobial resistance has been frequently reported in *S.* Choleraesuis in Asian countries including Taiwan (48, 158, 159), Thailand (48, 57, 115), China (160), and Philippines (48). However, antimicrobial resistance was relatively more prevalent in isolates of *S.* Newport (7, 39, 118), *S.* Typhimurium (123), *S.* Enteritidis, S. Heidelberg (55), and *S.* Hadar (39) in the US and the European countries.

Remarkably, *S.* Choleraesuis showed extremely high rates of ESC and ciprofloxacin resistance with 56.9% and 19.4%, respectively. *S.* Choleraesuis was commonly reported for reduced susceptibility to ciprofloxacin with 68.8% of isolates from seven countries in Asia during 2003 to 2005 (48). ESC resistance in *S.* Choleraesuis isolates has posed a serious threat to the population of Taiwan since it emerged in 2002 (159) and dramatically increased to be 17.8% of isolates in 2004 (6). Since most isolates are resistant to traditional drugs (48, 161) and also to fluoroquinolones with over 60% rate of resistance in recent reports (6, 158, 161), ESCs are once considered the most important antimicrobial agents with reliable activity against *S.* Choleraesuis in Taiwan.

S. Choleraesuis, a host-adapted pathogen usually causes paratyphoid in swine and also frequently causes systemic infection in human with little involvement of the gastrointestinal tract (161). In Thailand, *S.* Choleraesuis was the sixth and the second most common serotypes causing human salmonellosis and septicemia, respectively,

during 2002 to 2007(2). *S.* Choleraesuis isolates rapidly increased from 2.8% in 2002 to 9.2% in 2006 and *S.* Choleraesuis showed the highest ability to cause septicemia (OR 44.00; 95% CI 34.28-56.47) compared with the other serotypes (2). Among *S.* Choleraesuis isolates from blood in Thailand, ceftriaxone resistance increased from 15% during 2003 to 2005 (57) to 59% in the present study. These isolates also displayed reduced susceptibility (76.6%) or even resistance (19.5%) to ciprofloxacin, indicating a worrisome situation in this country. In this study, the antimicrobial resistance rates in *S.* Choleraesuis dramatically increased from those during 2005 to 2007 to those during 2012 to2016, for ESC resistance (52.3 to 75.5%; p=0.0036) and ciprofloxacin resistance (12.6 to 46.9%; p<0.0001).

Our results showed that ciprofloxacin resistance among *Salmonella* isolates in human in Thailand was attributed to mutations in Quinolone-Resistance Determining Region (QRDR) of GyrA, QnrS1 production, and co-existence of mutations in GyrA and QnrS1 production. Mutations in GyrA related to resistance included amino acid substitutions at position 83 (S83->F and Y) and 87 (D87->G and Y). The alterations of target genes were previously described as the major fluoroquinolone resistance mechanism in *Salmonella* spp. The most common GyrA mutations were S83->F (144, 162, 163), Y (144, 162, 163) or A (163), D87->G (144, 162, 163), N (144, 162, 163), Y (144, 162, 163), or A (163), D72 -> G (144), and V73 ->I (144). There were 40 ciprofloxacin-resistant *Salmonella* isolates (54.8%) harboring *qnrS1* gene. Qnr proteins, pentapeptide repeat proteins are known to confer low-level quinolone resistance, due to the protection of DNA gyrase from binding with quinolones (42). Acquisition of *qnr* genes increased fluoroquinolone MICs by 8- to 64-fold, which the final MICs remained below the susceptibility breakpoints, according to CLSI (69). However, the *qnr* gene alone is described to allow bacteria to survive long enough to grow again during quinolone exposure or treatment or consequently to develop other resistance mechanisms (69, 70). According to previous studies, the single amino acid substitution in QRDR of GyrA usually resulted in high level resistance to nalidixic acid, but two or more mutations were required to obtain resistance to fluoroquinolones (36, 63). The

result suggested that other resistance mechanisms might involve in the development of resistance to fluoroquinolones, including overexpression of efflux system or decreased outer membrane porins (68). The most common ciprofloxacin-resistant *Salmonella* isolates, *S.* Choleraesuis had a D87G substitution in GyrA together with the acquisition of *qnrS1* gene leading to high level MICs of ciprofloxacin and norfloxacin. The acquisition of *qnrS1* gene can reativate to enhance resistance attributable from GyrA mutations with 32-fold increased in MIC of ciprofloxacin (69).

We found that the two most common ESC-resistant *Salmonella* isolates from human in Thailand were *S.* Choleraesuis and *S.* Typhimurium. Noticeably, CMY-2 and CTX-M-14 were the two most common mechanisms of ESC resistance among *S.* Choleraesuis isolates during 2005 to 2007, while CTX-M-55 was the most common ESC resistance mechanism among the isolates during 2012 to 2016. There are very limited reports of ESC resistance mechanism in *S.* Choleraesuis. CMY-2, CTX-M-3, and CTX-M14 have been described to be contributed to ESC resistance in *S.* Choleraesuis in Taiwan and Thailand (14, 115), while ESC resistance in *S.* Typhimurium has been reported to be attributed to various types of ß-lactamases including SHV-2, SHV-5, SHV-9, SHV-12, CTX-M-3, CTX-M-15, CTX-M-2, CTX-M-5, CTX-M-6, CMY-2, and CMY-7 in many countries (10, 27, 101, 164).

The clonal and plasmid analysis revealed the genetically-related clones of CMY-2-producing isolates spreading among various provinces in Thailand. The most common pulsotype, C35-01, contained 11 indistinguishable isolates of CMY-2-producing *S.* Choleraesuis in 2007 which were collected from 4 different provinces. The self-transferable plasmid IncA/C played a role in the dissemination of bla_{CMY-2} gene among unrelated clones which indistinguishable RFLP patterns (S01-01) spreaded among 4 genetically unrelated clones of nontyphoidal *Salmonella*. In addition, these plasmids co-transferred of resistance to ceftriaxone together with gentamicin, chloramphenicol, and tetracycline.

Our results revealed that the dissemination of CTX-M-14-producing isolates among ESC-resistant *S.* Choleraesuis isolates might be mediated by the horizontal gene

transfer. Three types of self-transferable plasmids were found to disseminate $bla_{CTX-M-14}$ gene among various genetically unrelated clones of *S.* Choleraesuis isolates, including IncI1, $IncFII_s$, and IncFII. In addition, the major cluster of CTX-M-14 producers, cluster C20 also had all these plasmids spreading among the isolates. The self-transferable $bla_{CTX-M-14}$-carrying IncI1 plasmid with ~110-kb in size, indistinguishable RFLP patterns (E21-02), were found to spread among 4 genetically unrelated clones of nontyphoidal *Salmonella.* Interestingly, this $bla_{CTX-M-14}$-carrying IncI1 plasmid was described for the first time in *S.* Choleraesuis isolates in this present study. Similarly, it has been reported that the dissemination of bla_{CMY-2}-carrying IncA/C plasmid and the $bla_{CTX-M-14}$-carrying IncF plasmid variants was found among 23 ESC-resistant *S.* Choleraesuis clinical isolates from Bangkok and Ratchaburi provinces in Thailand during 2003, 2007, and 2008 (115). The CTX-M-14 was reported for the first time in these *S.* Choleraesuis isolates and the acquisition of CTX-M-14-producing *S.* Choleraesuis isolate by a Danish traveler during a stay in Bangkok was demonstrated (115). In contrast to the study in Taiwan, it has been demonstrated that ESC resistance among *S.* Choleraesuis isolates is mediated by a self-transferable IncI1 plasmid carrying bla_{CMY-2} gene (165).

Our results suggested that the acquisition of self-transferable bla_{CMY-2}-carrying IncA/C plasmid and these 3 types of self-transferable $bla_{CTX-M-14}$-carrying plasmids by *S.* Choleraesuis might possibly occur at or before that time and continuously spread among the isolates in Thailand. This contributed to high rate of ESC resistance in *S.* Choleraesuis isolates in this country. The bla_{CMY-2}-carrying IncA/C plasmid has been described mostly in *E. coli* and also in few other serotypes of *Salmonella enterica*, especially food animal isolates (166). The antimicrobial-resistant isolates might be selected by the selective pressure from antimicrobial usage in the production of food animals, especially ESC (ceftiofur), leading to the development and spread of resistance strains. The result of conjugation experiment revealed the co-transfer of ESC resistance with the additional antimicrobial resistance (gentamicin, tetracycline, and chloramphenicol), most of which were observed in IncA/C plasmids. These multidrug

resistance plasmids may have potential for co-selection by the usage of various antimicrobial agents other than beta-lactams.

Moreover, this study reported the first description of ceftazidime-hydrolyzing CTX-M-55 in *S.* Choleraesuis isolates. CTX-M-55 is a derivative of CTX-M-15 with A77V amino acid substitution which was first identified in *E. coli* and *K. pneumoniae* isolates in 2007 in Thailand (167). We found a significantly higher resistance rate of ceftazidime in CTX-M-55-producing isolates than that of CTX-M-14-producing isolates and CTX-M-55-producing isolates displayed higher levels of resistance to ESCs compared with CMY-2-producing isolates. Noticeably, the proportion of $bla_{\text{CTX-M-55}}$-carrying isolates dramatically increased and recently became the most abundant among ESC-resistant *S.* Choleraesuis isolates in Thailand, especially in Bangkok which previously reported the highest odds ratio for *S.* Choleraesuis infection in comparison to the other regions (2). Remarkably, CTX-M-55-producing *S.* Choleraesuis isolates showed an extremely high rate of resistance to ciprofloxacin (73.3%), all of which had D87G amino acid substitution in GyrA. We repeatedly failed to transfer $bla_{\text{CTX-M-55}}$ gene by conjugation experiments in all CTX-M-55-producing *S.* Choleraesuis isolates. The result of Southern blot hybridization revealed that the dissemination of $bla_{\text{CTX-M-55}}$ gene was all mediated by IncA/C plasmid. Co-location of $bla_{\text{CTX-M-55}}$ and *qnrS1* genes on the same plasmid was found in all tested isolates. Plasmids carrying *qnr* genes have previously been reported to cotransfer with *bla* genes encoding CTX-M (46), SHV (46), TEM (46), and CMY (47). The association with *bla* genes was relatively more prevalent in *qnrB*-carrying plasmid which was reported to be cotransferred with *bla* genes encoding SHV-12, CTX-M-9, CTX-M-14, and CTX-M-15 (168-171). However, the *qnrS1* gene was commonly associated with $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M-15}}$ which was mainly driven by the Inc A/C type plasmid from *E. coli* isolated from food animals (172, 173). Moreover, we found the most common cluster C27 among CTX-M-55-producing isolates which included 42.2% of all CTX-M-55-producing isolates which pulsotype C27-01 is a major pulsotype. Our results demonstrated the spread of clone C27-01 was due to the dissemination of IncA/C plasmids carrying $bla_{\text{CTX-M-55}}$ along with *qnrS1* among the *S.* Choleraesuis isolates

harbouring D87G in GyrA which was apparently responsible for the high rates of co-resistance to ESCs and ciprofloxacin (44.9%) during 2012 to 2016.

Interestingly, pulsotype C07-01, a major pulsotype contained 4 indistinguishable isolates of CTX-M-55-producing *S.* Typhimurium isolates from stool and rectal swab in 2007 from 3 different provinces which also displayed resistance to ampicillin, streptomycin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole. This resistance phenotype has been demonstrated in the international multiresistant clone *S.* Typhimurium phage type DT104 with the additional resistance to trimethoprim (R-type ACSSuTTm) (123). This clone has been recognized in humans and cattle in the USA and it became particularly common in cattle, poultry and pigs in in many European countries and also in Israel, Canada, Turkey and Japan (121, 122). Moreover, previous studies reported that human *Salmonella* cases were attributed to contact with infected pet animals at home or in veterinary clinics (174, 175). The outbreaks of antimicrobial-resistant *S.* Typhimurium among humans in Washington in 1999 and in New York in 2003 have been demonstrated the link to dogs and cats in animal veterinary clinics (174, 175).

S. Rissen, *S.* Enteritidis, *S.* Welterreden were the most common serotypes found in swine, chicken, and cattle, respectively. The significantly higher resistance rates were also found among Choleraesuis isolates compared with non-Choleraesuis isolates and *S.* Choleraesuis showed extremely high rates of ESC (77.8%) and ciprofloxacin (44.4%) resistance among food animal isolates. ESC and fluoloquinolone resistance has been reported common in *S.* Choleraesuis isolates from swine in Taiwan (158, 176). In Japan, *S.* Choleraesuis swine isolates has been found high rates of resistance to ampicillin (100%) and streptomycin (100%), gentamicin (99.7%), tetracycline (99.7%), sulfamethoxazole/trimethoprim, and nalidixic acid (40.1%), but remained susceptible to ESCs and fluoloquinolones (177). Two isolates of *S.* Virginia from swine had double amino acid substitution at S83F and D87N in GyrA and a S80I in ParC which were displayed high level of fluoroquinolone resistance. Accordingly, the previous study also described that the combination of amino acid substitutions at positions 83 and 87 can

lead to a high-level fluoroquinolone resistance the MIC of ciprofloxacin up to 32 mg/L (178). We found that all of ESC-resistant *Salmonella* including *S.* Choleraesuis and *S.* Typhimurium were isolated from swine. ESC resistance in *Salmonella* isolates from food animal was attributed to the production of CTX-M-55 and CTX-M-14. All of these isolates displayed MIC of ciprofloxacin $\geq$ 0.125 mg/L, all of which also carried *qnrS1* gene.

Our results suggested that the rapid increase of resistance to ESCs and fluoroquinolones was apparently due to plasmid-mediated resistance mechanism. Plasmids play an important role in disseminating antimicrobial-resistant genes among bacterial populations (11, 26, 170). This study provided the information about the plasmid platform carrying the antimicrobial-resistant determinants causing antimicrobial resistance problems in Thailand. Currently, there are techniques that relied on identifying the incompatibility groups of the target plasmids and then introducing plasmids with the same incompatibility group or small molecule mimics of plasmid incompatibility to induce the curing of antimicrobial-resistant plasmid which makes resistant bacteria susceptible to antimicrobials again (179, 180). This could be a good approach to reduce the burden of antibiotic resistance among bacterial population.

In the present study, *S.* Choleraesuis was the most common *Salmonella* serotype which showed ESC and ciprofloxacin resistance in both human and food animal isolates and the significant difference of resistance rates were not found between *S.* Choleraesuis isolates from human and food animal. Moreover, the ESC resistance rates of *S.* Choleraesuis isolated from humans and swine showed a strong positive correlation ($r = 0.837$, $p = 0.077$) which held across time and within five provinces of the central region including Bangkok, Nonthaburi, Ratchaburi, Saraburi, and Suphan Buri. We analyzed this data based on the provinces of hospitals where the patiens were admitted to. Therefore, we cannot verify exactly whether the patiens live in those provinces or not. The genetically related clone between human and swine was found in CTX-M-55-producing *S.* Choleraesuis isolates (C27-05). The presence of the closely related strain of CTX-M-55-producing *S.* Choleraesuis (the primary host: swine) isolates between human and swine isolates suggested the potential spread of ESC-resistant

Salmonella isolates between animal and human population through the food chain. However, a small number of *S.* Choleraesuis isolates from food animal (n=9, 3.2%) limited a reliable comparison of genetic relatedness to the isolates from human.

S. Choleraesuis is increasingly recognized as a major cause of systemic salmonellosis in Asian countries; the emergence of *S.* Choleraesuis with resistance to traditional drugs (ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole) and now to alternative drugs (extended-spectrum cephalosporins (ESCs), and fluoroquinolones) has become public health concern (6, 48, 57). According to the previous studies, ESC and fluoroquinolone resistance in *S.* Choleraesuis isolates has been known to pose a threat to public health of Taiwan for a long time (6, 158, 159, 161). However, Taiwan government has taken actions by using the implementation of several national control programs in the whole country to control the situation since 2007. It has been demonstrated recently that the application of effective control measures on farms and in agricultural practices can lead to the successful control of *S.* Choleraesuis infection among humans (181).

This study reported for the first time the occurrence of *qnrVC* family in *Salmonella enterica*. We identified *qnrVC4 S.* Rissen 166ANSS50 isolate from swine. The nucleotide sequences of *qnrVC4* from *S.* Rissen 166ANSS50 showed 657 nucleotides 100% identity to *qnrVC4* from *Aeromonas punctate* 159 (GenBank accession number GQ891757) (182). The translated sequence for QnrVC4 predicted a 218-amino acid protein belonging to pentapeptide repeat protein family. The recombinant plasmid of pBK-CMV*qnrVC4* in *E. coli* DH10B revealed that QnrVC4 showed slight effect on nalidixic acid and the level of decreased susceptibility to fluoroquinolones was close to that previously observed with *qnrVC1* gene (183). However, *qnrVC1* have higher level of decreased susceptibility with 42- and 22-fold increases in MICs of ciprofloxacin and norfloxacin, respectively (183). The results revealed that *qnrVC4* was located on a single 17-kb plasmid (pSR166) and it can be co-transferred with other antimicrobial resistance determinants to other bacteria through this plasmid by transformation. Unlike previous

study in *A. punctate* 159, *qnrVC4* was located on a large plasmid (>100 kb) and it was not transferable by transformation or conjugation (182).

The *qnrVC4* was located within gene cassettes in 8.91 kb of a novel class 1 integron which was numbered as In805 according to INTEGRALL database (184). However, it did not yield an amplicon by PCR using specific primers for the 5'-CS and 3'-CS regions due to the loss of a common 3'-CS (*qacEdelta1-sul1*) (156). In805 is bounded by two 25-bp imperfect inverted repeats (2 mismatches), designated IRi and IRt. It contained the 5'-CS and seven integrated cassettes but unusual 3'-CS with one full copy of the insertion sequence IS*6100* flanked by 123 and 152-bp fragments of the end of the Tn*402* region in the opposite orientations which both included IRt (185). Tn*402* (also called Tn*5090*) was probably the immediate common ancestor of class 1 integrons (185). Since In805 lost all of the *tni* genes for transposition, it was designated a transposition-defective transposon derivative (186). However, its transposition has been demonstrated when the two inverted repeats are present and transposition enzymes can be supplied in trans (187). In addition, class 1 integrons containing the IR of Tn*402* can target plasmid and transposon resolution sites (*res*) by the res site-hunter characteristic of Tn*5053*-family elements, which allows the spread of these integrons and their resistance gene cassettes on Tn*21*-like transposons or other mobile elements (186-188). In805 had a backbone structure related to that of In4 which has lost most of the *tni* region due to an IS*6100*-mediated deletion (188). However, the part of 3'-CS region, including *qacEdelta1*, *orf5*, *orf6*, and *sul1* genes and the partial copy of IS*6100* were missing in In805. In In805, the 3' region displayed the *dfrA14* cassette followed by interrupted putative sequence of *mobC* gene, encoding mobilisation protein, presumably resulting from IS*6100*-mediated deletion arising at internal IRt extended into the cassette (188). Class 1 integron with this backbone structure was previously found in the IncHI1 plasmid pHCM1 from multidrug-resistant *S.* Typhi isolated in Vietnam in 1993 (189). Although we did not find a direct duplication flanking In805, the DNA adjacent to IRt-end of integron revealed that In805 is located within the *res* site of Tn*1696* (*resI* region) (188). The absence of direct repeats presumably resulted from a homologous

recombination between the two integrons, each with different direct repeat sequences (190) and the absence of the 19-bp duplication in attI1 that is seen in In4 is consistent with this conclusion (186). Additionally, the complete sequence of IncA/C plasmid pVAS3-1 carrying bla_{CMY-2} from *V. alginolyticus* VAS3-1 (GenBank accession number KU160531) showed that In805 was located in the different DNA context and flanked by 5-bp direct duplication indicating transposition event by this integron. We repeatedly failed to identify the plasmid types by PCR-based replicon typing. The genetic environments of In805 revealed the putative backbone of this 17-kb long pSR166 including genes involved in plasmid replication (*repA*, *repF*), partitioning (*parA*), DNA invertase/recombinase, and two hypothetical protein. This RepA plasmid replication protein shares 100% identity with RepA from pG5A4 plasmids carrying bla_{GES-5} in clinical isolates of *E. coli* and *S. marcescens* which has been reported as a novel replicon type (191). Moreover, MobA relaxase protein of pSR166 contained the three motifs of relaxase domain (225 amino acids) which showed 100% identity to MobA from pG5A4 plasmids. It belonged to the MOB_{Q1} group whose prototype is the broad-host-range mobilisable IncQ1 plasmid RFS1010 (191). In805 carried *qnrVC4* gene cassette adjacent to 5'-CS followed by the other antimicrobial resistance gene cassettes, including *qacH4* (quaternary ammonium compound resistance), *aacA4* (kanamycin and gentamicin resistance), *cmlA7* (chloramphenicol resistance), bla_{OXA-10} (beta-lactam resistance), *aadA1* (streptomycin and spectinomycin resistance), and *dfrA14* (trimethoprim resistance). The sequences revealed the fused gene cassettes of *aacA4-cmlA7* and bla_{OXA-10}-*aadA1-dfrA14*, created by the loss of the 59-base elements, which have not been reported before. According to the reports on the NCBI database, the *qnrVC* gene cassette frequently found to coexist with the *aacA4* gene cassette in various Gram-negative bacteria. It suggested the possibility of co-selection and persistence of these resistance genes. The 5'-CS of In805 presented P_c promoter, responsible for the expression of inserted gene cassettes but absence of P_2 promoter. This P_c promoter belonged to a weak promoter variant (PcW) with a –35 box (TGGACA) and a –10 box (TAAGCT) separated by a 17-bp sequence. The PcW was demonstrated

to be 25-fold less active than the strong promoter (PcS) (192). However, this *qnrVC4* cassette carried its own putative promoter sequences (P_{qnrVC4}) with a –35 box (TTGAGA) and a –10 box (TAGTCT) separated by a 16-bp sequence. Moreover, this 1014-bp *qnrVC4* cassette showed more than 99% similarity with *qnrVC4* cassette carried in class 1 integron of *V. alginolyticus, A. hydrophila, A. punctata,* and *E. coli*, all of which were isolated from aquatic environments in China and Portugal during 2008-2015 (GenBank on NCBI). This *qnrVC4* cassette contained 128-bp attC site which showed 100% similarity with attC site of *qnrVC4* of class 1 integron in pVAS3-1 from *V. alginolyticus* VAS3-1 and 99% similarity with attC site of *qnrVC4* of complex class 1 integron from *A. punctata* 159 (182). In addition, it also showed 90% similarity with *V. cholerae* repeats (VCR) of *qnrVC4* cassette from 144-kb long superintegron (SI) which carries 279 cassettes of ORFs and located within chromosome 2 of *V. cholerae* O1 El Tor serotype Ogawa strain MS6 from Thailand–Myanmar border area during 2007-2010 (193). The sequences of the repeats of gene cassettes within a SI appeared to be species-specific and *qnrVC* may originate from Vibrionaceae (194). Rowe-Magnus *et al.* previously demonstrated the recruitment of *catB9* cassette, encoding chloramphenicol acetyltransferases for chloramphenicol resistance from *V. cholerae* SI by the In3-carrying plasmid through class 1 integrase activity (195). This indicates that *qnrVC4* cassette may be derived from the SI of *V. cholera* into a plasmid-borne class 1 integron by class 1 integrase, leading to the spread of this resistance gene to other species via horizontal gene transfer. Considering that we found the *qnrVC4* in *Salmonella* isolated from swine in Thailand in 2007, this resistance determinant might develop and persist in this area at or before that time. *Salmonella* could acquire the *qnrVC* from its progenitor and act as a reservoir of this resistance determinant for transferring this genetic formation to Enterobacteriaceae. As *qnrVC4* was identified from *Salmonella* food animal isolate, it could play a role as a shuttle between the environment and humans, resulting in a high impact on public health.

Although a limited number of *S.* Choleraesuis isolates from food animal limited a reliable comparison genetic relatedness to the isolates from human, we cannot rule out

the possibility that antimicrobial-resistant *Salmonella* isolates might develop among food animal isolates. *S.* Choleraesuis, had swine as a primary host and main reservoir, was the most common *Salmonella* serotype found antimicrobial resistance in both human and food animal isolates. In addition, the platform carrying the antimicrobial-resistant determinant was also reported mostly from animal isolates. Moreover, the first occurrence of a novel class 1 integron harbouring quinolone resistant determinant *qnrVC4* gene in *Salmonella enterica* isolates from swine is consistent with this conclusion.

CHAPTER VII

CONCLUSION

The present study demonstrated high rate of antimicrobial resistance among 897 nontyphoidal *Salmonella* isolates, 617 isolates from human and 280 isolates from food animal in Thailand during 2005 to 2007 and 2012 to 2016. Nontyphoidal *Salmonella* isolates from blood were the most common clinical isolates, accounted for 67.7% of all isolates. *S.* Enteritidis (46.4%) and *S.* Choleraesuis (40.2%) were the most common serotypes among human isolates, whereas *S.* Rissen (19.6%) was the most common serotypes among food animal isolates.

The significantly higher resistance rates were found in *S.* Choleraesuis compared with other serotypes to all antimicrobial agents tested ($p < 0.0001$), except for norfloxacin resistance. *S.* Choleraesuis clinical isolates showed extremely high rates of ESC and ciprofloxacin resistance with 56.9% and 19.4%, respectively. Ciprofloxacin resistance mechanism were found to be related to the amino acid substitutions in GyrA at position 83 (S83->F and Y) and 87 (D87->G and Y). Ciprofloxacin-resistant *S.* Choleraesuis isolates from human and swine harbored D87G substitution in GyrA together with *qnrS1* gene. Ciprofloxacin resistance in *S.* Senftenberg isolated from chicken was commonly attributed to S83F substitution in GyrA together with the acquisition of *qnrS1* gene. Double amino acid substitutions at S83F and D87N in GyrA, and S80I in ParC were detected in 2 *S.* Virginia isolates from swine with extremely high-level fluoloquinolone resistance. ESC resistance in *S.* Choleraesuis was attributed to the productions of CMY-2 AmpC ß-lactamase (49.6%) and ESBLs including CTX-M-14 (29.1%) and of CTX-M-55 (21.3%).

The antimicrobial resistance rates in *S.* Choleraesuis showed dramatically increased from those during 2005 to 2007 to those during 2012 to 2016, for ESC resistance (52.3 to 75.5%) and ciprofloxacin resistance (12.6 to 46.9%). Noticeably,

CMY-2 (64.4%) and CTX-M-14 (35.6%) were the two most common mechanisms of ESC resistance among *S.* Choleraesuis human isolates during 2005 to 2007, while CTX-M-55 (81.1%) was the most common ESC resistance mechanism among the human isolates during 2012 to 2016. Moreover, *S.* Choleraesuis also showed extremely high frequencies of resistance to ESC (77.8%) and ciprofloxacin (44.4%) among swine isolates during 2014 to 2016, most of which were CTX-M-55 producers (57.1%).

The results from PFGE and RFLP revealed the predominant antimicrobial resistance clones and plasmids among nontyphoidal *Salmonella* human isolates in Thailand. The 41 PFGE clusters and 83 different pulsotypes were identified among 159 ESC-resistant *Salmonella* human isolates and the 7 PFGE clusters and 9 different pulsotypes were identified among 11 ESC-resistant *Salmonella* food animal isolates. The dissemination of the self-transferable $bla_{CTX\text{-}M\text{-}14}$-carrying $IncFII_s$ (E06), IncFII (E20), and IncI1 (E21) plasmids and $bla_{CMY\text{-}2}$-carrying IncA/C (S01) plasmid along with the clonal spread of $bla_{CMY\text{-}2}$-harbouring *S.* Choleraesuis isolates (C35-01) contributed to the high rates of ESC resistance during 2005 to 2007.

This study reported the first occurrence of ceftazidime-hydrolyzing CTX-M-55 in *S.* Choleraesuis isolates which dramatically increased and became the most abundant CTX-M variant among ESC-resistant isolates during 2012 to 2016. The spread of clone C27-01 was due to the dissemination of IncA/C plasmids carrying $bla_{CTX\text{-}M\text{-}55}$ along with *qnrS1* among the resistant *S.* Choleraesuis isolates harbouring D87G in GyrA which was apparently responsible for the high rates of co-resistance to ESCs and ciprofloxacin (44.9%) in these recent years. This clone also showed significantly higher resistance rates of multiple antimicrobial agents, especially ESCs and ciprofloxacin that would make the treatment for invasive *Salmonella* infections even more challenging. Moreover, we found that ESC resistance was also attributed to the productions of CTX-M-55 (72.7%) and CTX-M-14 (27.3%) in *S.* Choleraesuis isolates from food animals, all of which carried *qnrS1* gene and displayed MIC of reduced susceptibility to ciprofloxacin. The genetically related clone, indistinguishable PFGE pattern (C27-05) between human and swine isolates was found in CTX-M-55-producing *S.* Choleraesuis isolated in 2014.

These isolates were also resistant to multiple antimicrobial agents, especially ciprofloxacin.

Interestingly, this is the first known report of quinolone resistance protein from QnrVC family in *Salmonella enterica*. The *qnrVC4* gene was identified in *S.* Rissen from swine isolates. This gene was located within a novel plasmid-borne In805, containing the multi-drug resistance gene cassettes of *qnrVC4-qacH4-aacA4-cmlA7-bla*$_{OXA-10}$-*aadA1-dfrA14* and unusual 3'CS of *mobC*-IS*6100*. This element could possibly spread these resistance determinants to Enterobacteriaceae.

This study underlines the importance of actions to control and prevent the dissemination of antimicrobial resistance in nontyphoidal *Salmonella* and provide a strict policy on antimicrobial usage in the production of food animals, especially the ESC ceftiofur and the fluoroquinolone enrofloxacin. This could be global health threats due to travel and trade in animal food products.

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APPENDIX

APPENDIX A

REAGENTS AND INSTRUMENT

Reagents

Absolute ethanol	(Merck, Germany)
Agarose	(Biorad, USA)
Boric acid	(Sigma, USA)
dNTPs	(Promega, USA)
EDTA	(Amresco, USA)
Ethidium bromide	(Amresco, USA)
NaCl	(Merck, Germany)
Taq DNA Polymerase	(Fermentas, USA)
Tris	(Amresco, USA)
100 bp DNA ladder	(Fermentas, USA)
100 bp plus DNA ladder	(Fermentas, USA)
Trytic soy agar	(BBL, USA)
Trytic soy broth	(BBL, USA)
Muller-Hinton II agar	(BBL, USA)
LB broth	(Pronadisa, Spain)
NaOH	(Sigma, USA)
CTX, CTX/CLA, CAZ, CAZ/CAL, and FOX disks	(BBL, USA)
Antimicrobial agents	(Sigma, USA)
Sodium azide	(Sigma, USA)
Plasmid Mini Kit	(QIAGEN, Germany)
PCR purification kit	(QIAGEN, Germany)
Megabase agarose	(Bio-Rad, USA)
Proteinase K	(Amresco, Solon, OH)
*Xba*I, *EcoR*I, *Sca*I, *Hind*III,	(Fermentas, USA)

*Hinc*II, *Apa*I, and *Pst*I	(Fermentas, USA)
*S*1 nuclease	(Fermentas, USA)
T4 ligase	(Fermentas, USA)
*I-Ceu*I	(New England Biolabs, UK)
Millipore 0.45-mm-pore-size filters	(Gelman sciences Inc., USA)
Low-range PFG marker	New England Biolabs, UK)
CHEF DNA Size Standard-Lambda Ladder	(Bio-Rad, USA)
CHEF DNA Size Standard-8-48 kb Ladder	(Bio-Rad, USA)
Hybond N+ nylon membranes	(Amersham, UK)
DIG DNA labeling and detection kit	(Roche Diagnostic, USA)
pBK-CMV expression vector	(Stratagene, La Jolla, CA)
InsTAclone PCR Cloning Kit	(Fermentas, USA)

Instruments

Automatic pipette	(Gilson, Lyon, France)
Camera Gel Doc™ MZL	(BIO-RAD, USA)
Incubator	(Forma Scientific, USA)
Perkin Elmer GeneAmp PCR system 9600	(Perkin Elmer, USA)
Microcentrifuge	(Eppendorf, USA)
Spectrophotometer	(BIO-RAD, USA)
Water bath	(Memmert, USA)
Nanodrop Spectrophotometer	(Thermo Scientific, USA)
CHEF-Mapper XA PFGE system	(Bio-Rad, USA)
Gene Pulser Xcell electroporation system	(Bio-Rad, USA)
InfoQuest FP software version 4.5	(Bio-Rad, USA)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. Muller-Hinton II agar (BBL, USA)

Suspend 38 grams of the dehydrated medium in 1,000 ml of distilled water. Dissolve by heating with frequent agitation until complete dissolution. Adjust final volume to 1,000 ml. Sterilize at 121°C (15 lbs. sp) for 15 minutes. Once the medium is prepared, store at 4°C.

2. Tryptic soy broth (BBL, USA)

Suspend 30 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium is prepared, store at 4°C.

3. LB broth (Pronadisa, Spain)

Suspend 20 grams of the dehydrated medium in 900 ml of distilled water. Dissolve by heating with frequent agitation until complete dissolution. Adjust final volume to 1,000 ml. Sterilize at 121°C (15 lbs. sp) for 15 minutes. Once the medium is prepared, store at 4°C.

4. Trytic soy agar (BBL, USA)

Suspend 40 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium is prepared, store at 4°C.

5. MacConkey agar plate

Suspend 50 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium is prepared, store at 4°C.

5. Sterile 0.85% NaCl (Merck, Germany)

NaCl 8.5 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium is prepared, store at room temperature.

6. Antibiotic solution preparation

Cefoxitin, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Ceftazidime, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 50 µL of 0.1 N NaOH and 4.95 ml sterile distilled water

Cefotaxime, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Ceftriaxone, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water

Nalidixic acid, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 50 µL of 0.1 N NaOH and 4.95 ml sterile distilled water

Ciprofloxacin, stock concentration 5120 mg/L

- Prepare a stock solution; dissolve 0.0256 g in 50 µL of 0.1 N NaOH and 4.95 ml sterile distilled water

APPENDIX C

REAGENTS PREPARATION

1. 10x Tris-Borate buffer (TBE)

Tris base	108 g/L
Boric acid	55 g/L
0.5 M EDTA (pH 8.0)	40 ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 min.

2. 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetra-acelate $2H_2O$	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. 10x TE buffer

Tris	12.11 g/L
0.5 M EDTA	20 ml

Adjust to pH 8.0 by adding conc. HCl. Adjust volume to 1,000 ml and sterilized by autoclaving at 121°C for 15 min.

5. 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°C.

6. Reagent for plasmid DNA extraction

6.1. Solution I (Lysis buffer I): 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0. Store at 0°C

500mM Glucose	10 ml
500mM EDTA pH 8.0	2 ml
1M Tris pH 8.0	2.5 ml

Adjust volume to 100 ml with distilled water. Mix the solution. Autoclave and store at 4°C

6.2. Solution II (Lysis buffer II): Freshly prepared 0.2 N NaOH, 1% SDS. Store at room temperature (RT)

6.3. Solution III (Lysis buffer III): 3M KOAc, pH 6.0

a. 60ml 5M potassium acetate (49.07g potassium acetate in 100ml H2O)

b. 11.5ml glacial acetate

c. 28.5ml H2O

APPENDIX D

THE RESULTS OF ALL TESTS IN THIS STUDY

1. The result of the detection of AmpC and ESBL phenotypes

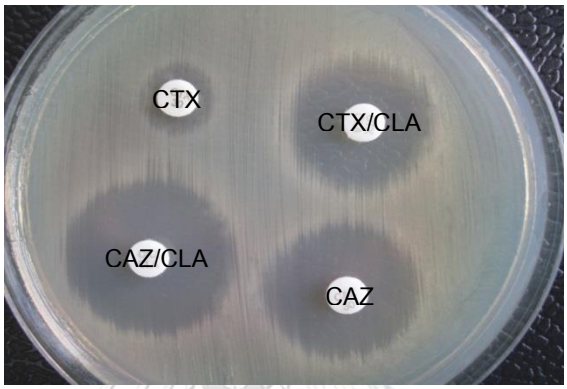


Figure 15. The positive confirmatory test for ESBL phenotype by the combination disk. clavulanate: cefotaxime (CTX), cefotaxime/clavulanic acid (CTX/CLA), ceftazidime (CAZ), ceftazidime/clavulanic acid (CAZ/CLA)

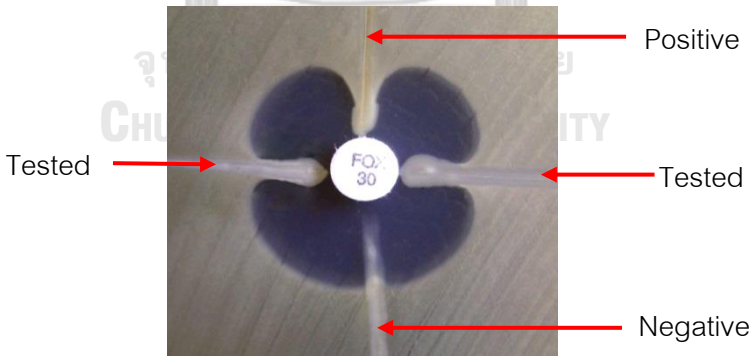


Figure 16. Detection of AmpC ß-lactamase phenotype by modified Hodge test. The decreased radius of the inhibition zone of 30 µg cefoxitin disk along the growth of tested strain showed a positive of AmpC ß-lactamase activity.

2. The result of PCR screening for the presence of *esbl* genes

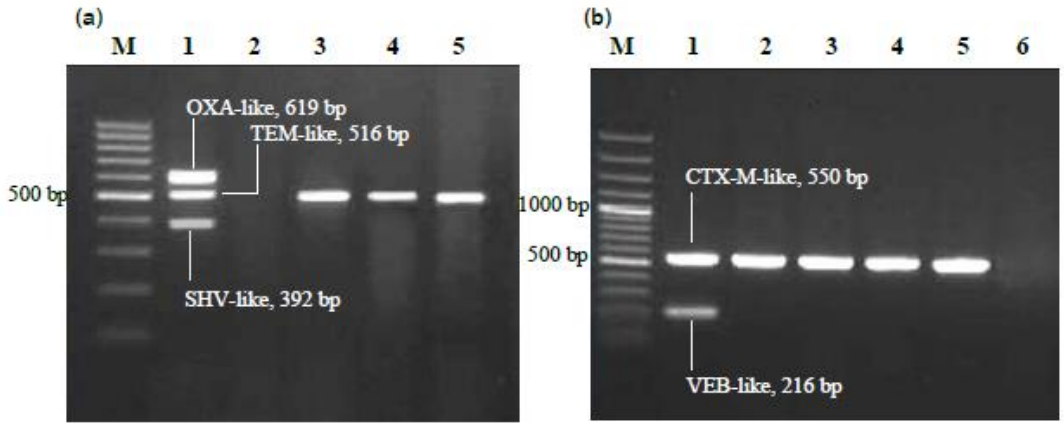


Figure 17. The result of PCR screening for the presence of *esbl* genes

(a), The multiplex PCR analysis for bla_{OXA}, bla_{TEM}, and bla_{SHV} genes: M, 100-bp DNA ladder; Lanes 1, 3 Templates, $bla_{OXA\text{-}like}$ (619 bp), $bla_{TEM\text{-}like}$ (516 bp), and $bla_{SHV\text{-}like}$ (392 bp); Lanes 2, negative control (sterile DDW); Lanes 3-5, Nontyphoidal *Salmonella* isolates harboring the $bla_{TEM\text{-}like}$ gene and (b), The multiplex PCR analysis of $bla_{CTX\text{-}M}$ and bla_{VEB} genes: M, 100-bp plus DNA ladder; Lanes 1, 2 Templates, $bla_{CTX\text{-}M\text{-}like}$ (550 bp) and $bla_{VEB\text{-}like}$ (216 bp); Lanes 2-5, Nontyphoidal *Salmonella* isolates harboring the $bla_{CTX\text{-}M\text{-}like}$ gene; Lanes 6, negative control (sterile DDW).

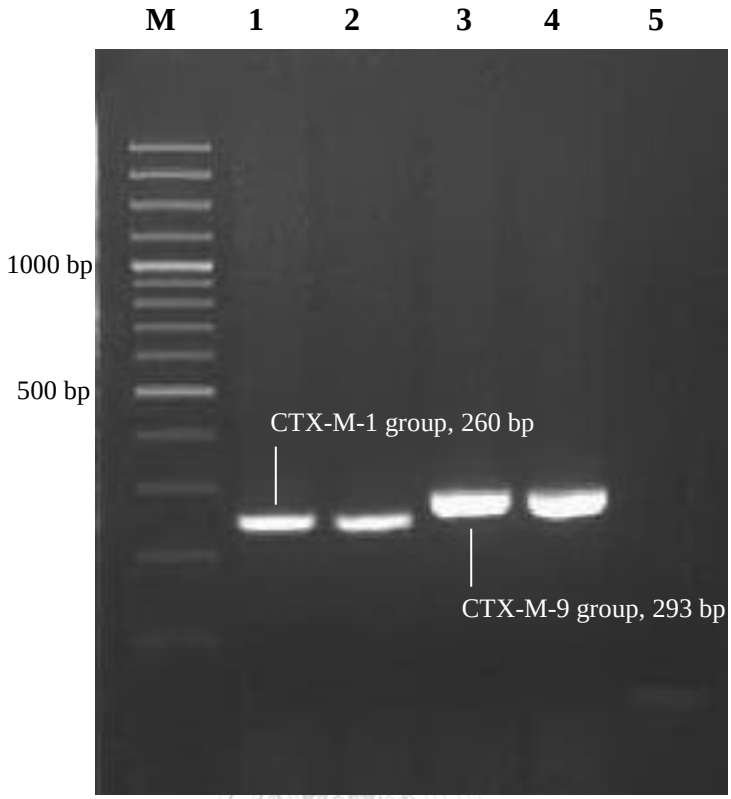


Figure 18. The multiplex PCR analysis of $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CTX-M-2}}$ group, $bla_{\text{CTX-M-8/25}}$ group, and $bla_{\text{CTX-M-9}}$ group genes

M, 100-bp plus DNA ladder; Lanes 1, Template $bla_{\text{CTX-M-1}}$ group (260 bp); Lanes 2, Nontyphoidal *Salmonella* isolates harboring the $bla_{\text{CTX-M-1}}$ group gene; Lanes 3, Template $bla_{\text{CTX-M-9}}$ group (293 bp); Lanes 4, Nontyphoidal *Salmonella* isolates harboring the $bla_{\text{CTX-M-9}}$ group gene; Lanes 5, negative control (sterile DDW)

3. The result of PCR screening for the presence of *ampC* genes

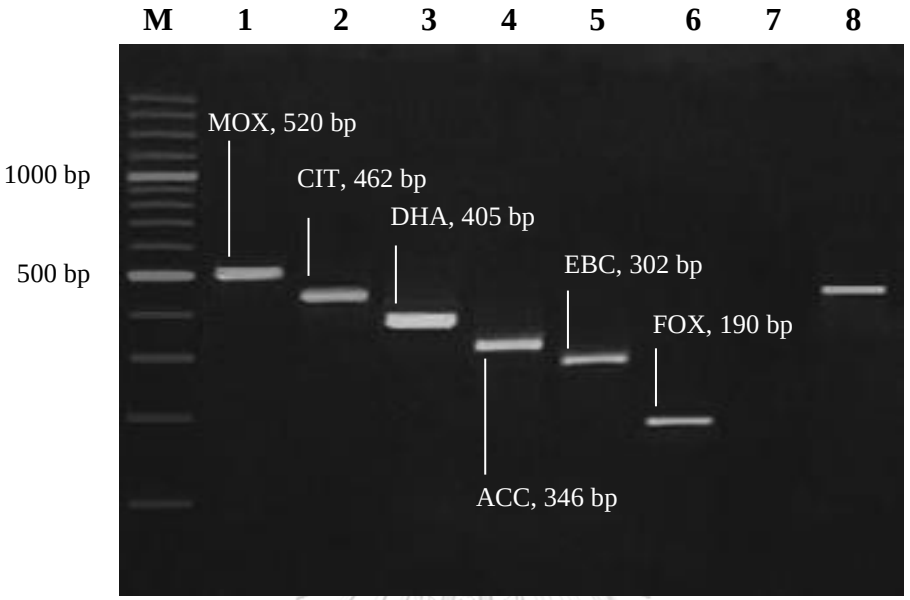


Figure 19. The multiplex PCR analysis of *ampC* genes

M, 100-bp plus DNA ladder; Lanes 1, Template bla_{MOX-8} (bla_{MOX}, 520 bp); Lanes 2, Template bla_{CMY-2} (bla_{CIT}, 462 bp); Lanes 3, Template bla_{DHA-1} (bla_{DHA}, 405 bp); Lanes 4, Template bla_{ACC-1} (bla_{ACC}, 346 bp); Lanes 5, Template bla_{MIR-1} (bla_{EBC}, 302 bp); Lanes 6, Template bla_{FOX-4} (bla_{FOX}, 190 bp); Lanes 7, negative control (sterile DDW); Lanes 8, Nontyphoidal *Salmonella* isolate harboring $bla_{CIT-like}$.

4. The result of the investigation of the genetic relationship among antimicrobial-resistant *Salmonella* isolates by PFGE using *Xba*I.

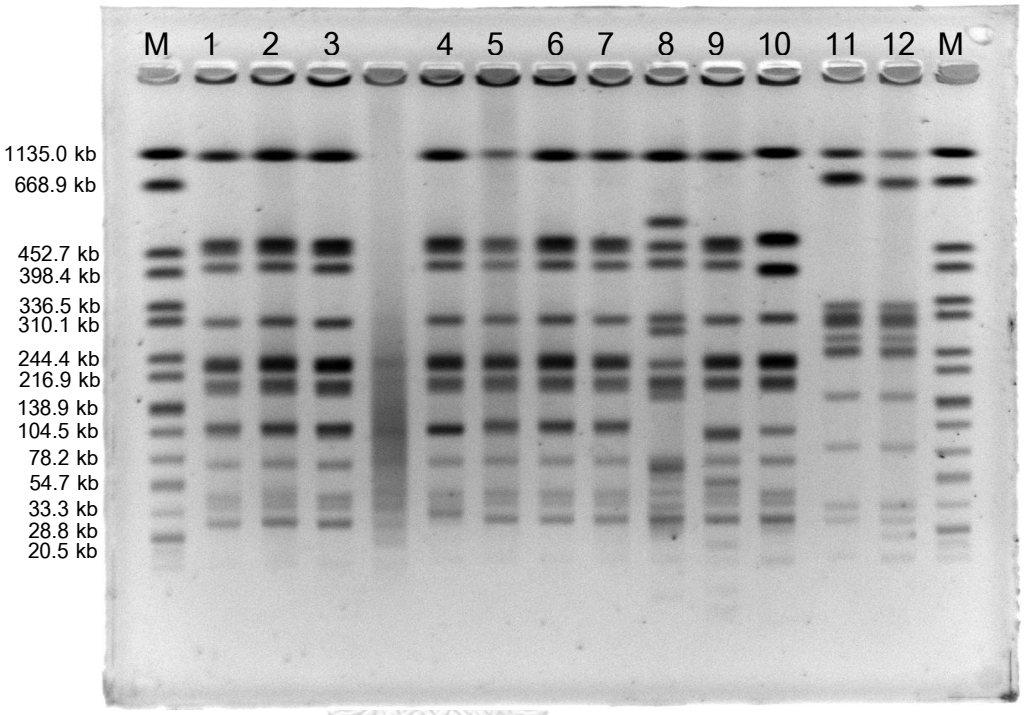


Figure 20. The result of *Xba*I-PFGE

M, *S.* Braenderup H9812; 1, H552; 2, H553; 3, H408; 4, H542; 5, H543; 6, H544; 7, H545; 8, H568; 9, H582; 10, H583; 11, A63; 12, A66; M, *S.* Braenderup H9812; 1-7, pulsotype C35-01.

5. The result of plasmid profile analysis of antimicrobial-resistant *Salmonella* isolates by PFGE using *S*1 nuclease.

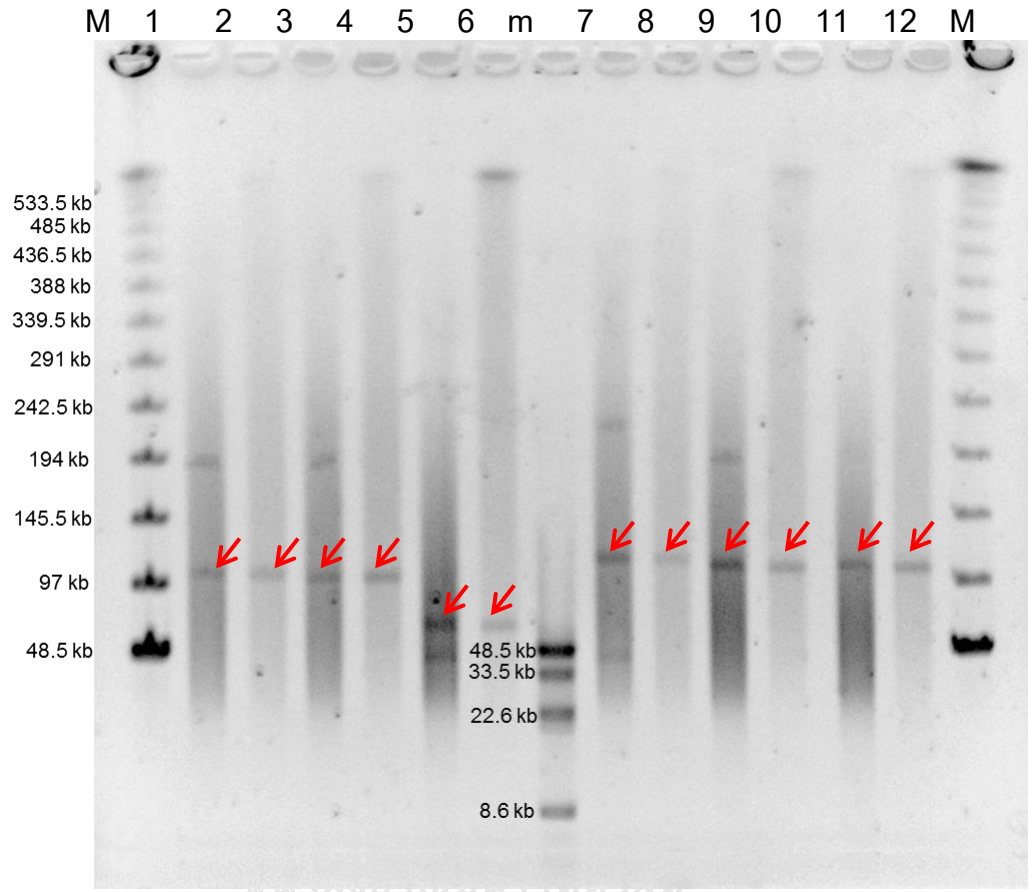


Figure 21. Plasmid profile analysis of $bla_{CTX\text{-}M\text{-}14}$-carrying *Salmonella* isolates and their transconjugants (*E. coli* UB1637 Az^R as a recipient) by *S*1-PFGE

M, CHEF DNA Size Standard-Lambda Ladder; 1, H435; 2, H435U (transconjugant); 3, H441; 4, H441U (transconjugant); 5, H407; 6, H407U (transconjugant); m, CHEF DNA Size Standard-8-48 kb Ladder; 7, H370; 8, H370U (transconjugant); 9, H386; 10, H386U (transconjugant); 11, H397; 12, H397U (transconjugant); M, CHEF DNA Size Standard-Lambda Ladder. Arrows indicate $bla_{CTX\text{-}M\text{-}14}$-carrying plasmid locations.

6. The result of plasmid profile analysis and Southern blot hybridization of *Salmonella* isolates with non-conjugative plasmid carrying $bla_{CTX\text{-}M\text{-}55}$ gene.

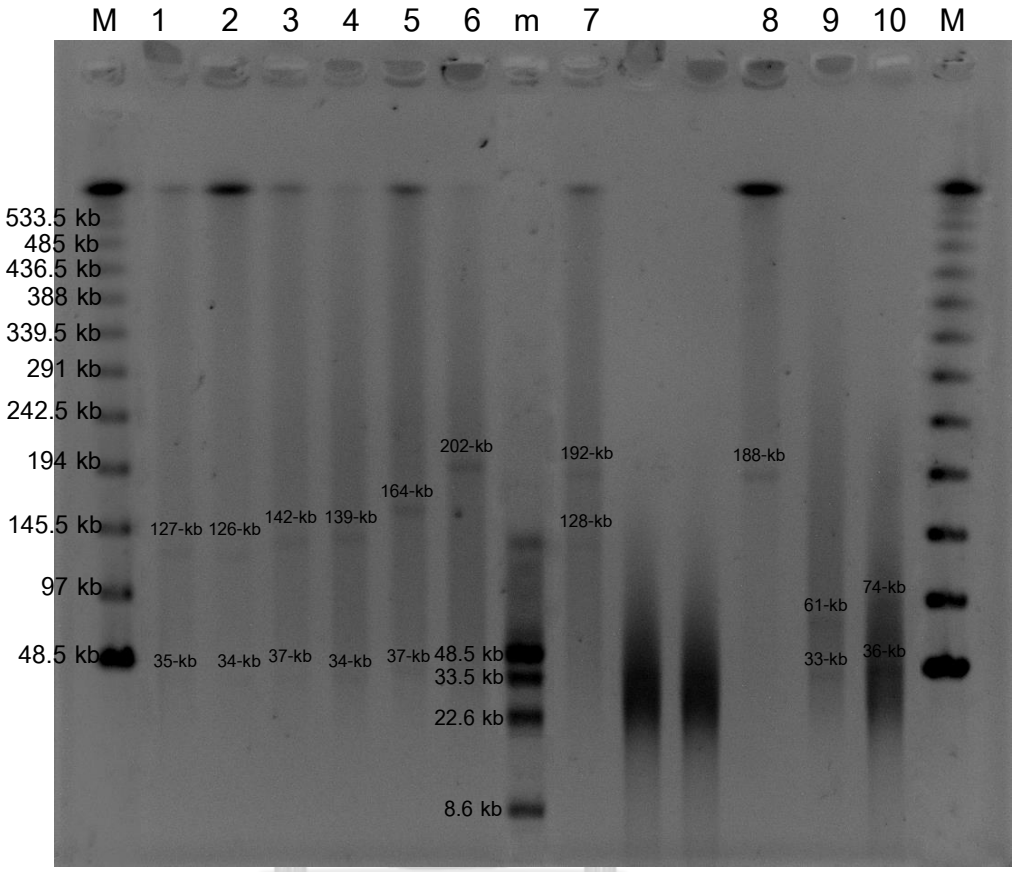


Figure 22. Plasmid profile analysis of *Salmonella* isolates with non-conjugative plasmid carrying $bla_{CTX\text{-}M\text{-}55}$ gene by S1-PFGE

M, CHEF DNA Size Standard-Lambda Ladder; 1, H591; 2, H595; 3, H589; 4, H615; 5, H607; 6, H617; m, CHEF DNA Size Standard-8-48 kb Ladder; 7, H606; 8, H611; 9, H583; 10, H603; M, CHEF DNA Size Standard-Lambda Ladder.

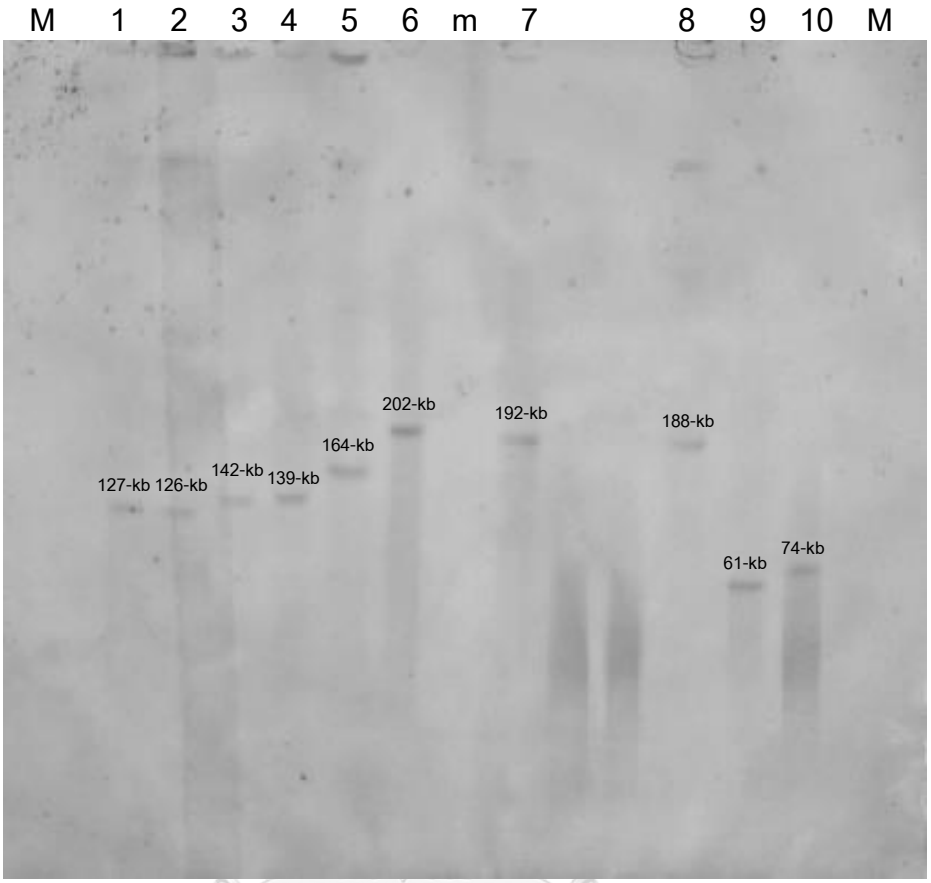


Figure 23. Southern blot hybridization using specific probe for *bla*$_{CTX-M-55}$ gene

M, CHEF DNA Size Standard-Lambda Ladder; 1, H591; 2, H595; 3, H589; 4, H615; 5, H607; 6, H617; m, CHEF DNA Size Standard-8-48 kb Ladder; 7, H606; 8, H611; 9, H583; 10, H603; M, CHEF DNA Size Standard-Lambda Ladder.

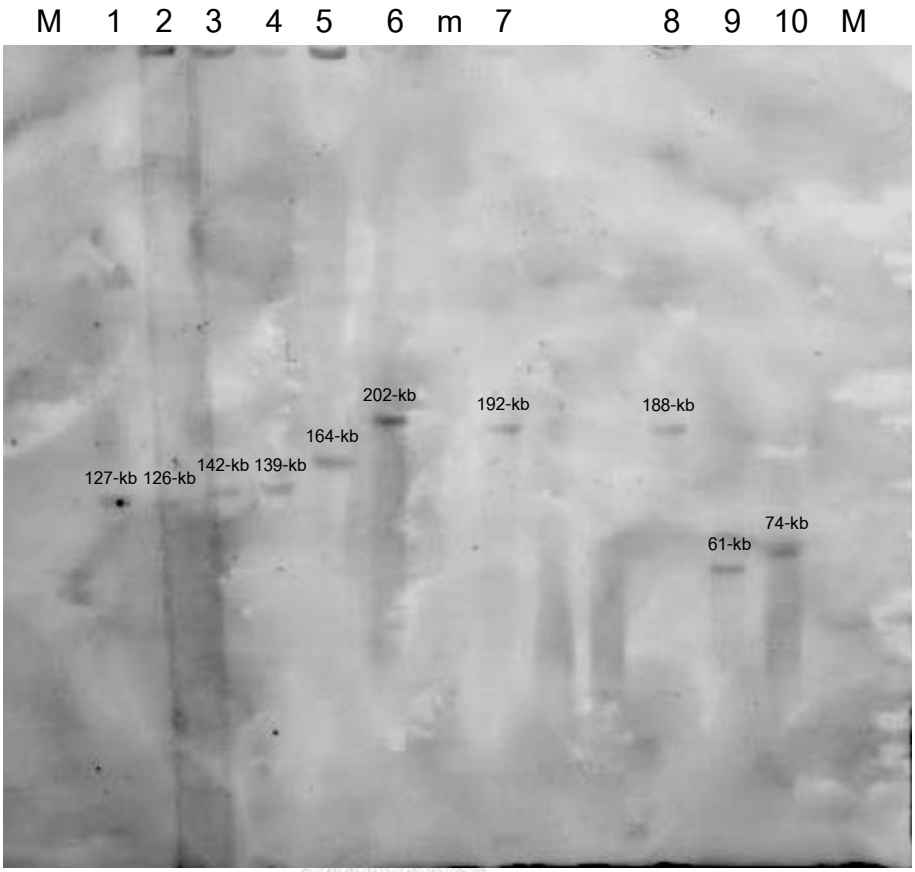


Figure 24. Southern blot hybridization using specific probe for replicon type A/C
M, CHEF DNA Size Standard-Lambda Ladder; 1, H591; 2, H595; 3, H589; 4, H615; 5, H607; 6, H617; m, CHEF DNA Size Standard-8-48 kb Ladder; 7, H606; 8, H611; 9, H583; 10, H603; M, CHEF DNA Size Standard-Lambda Ladder.

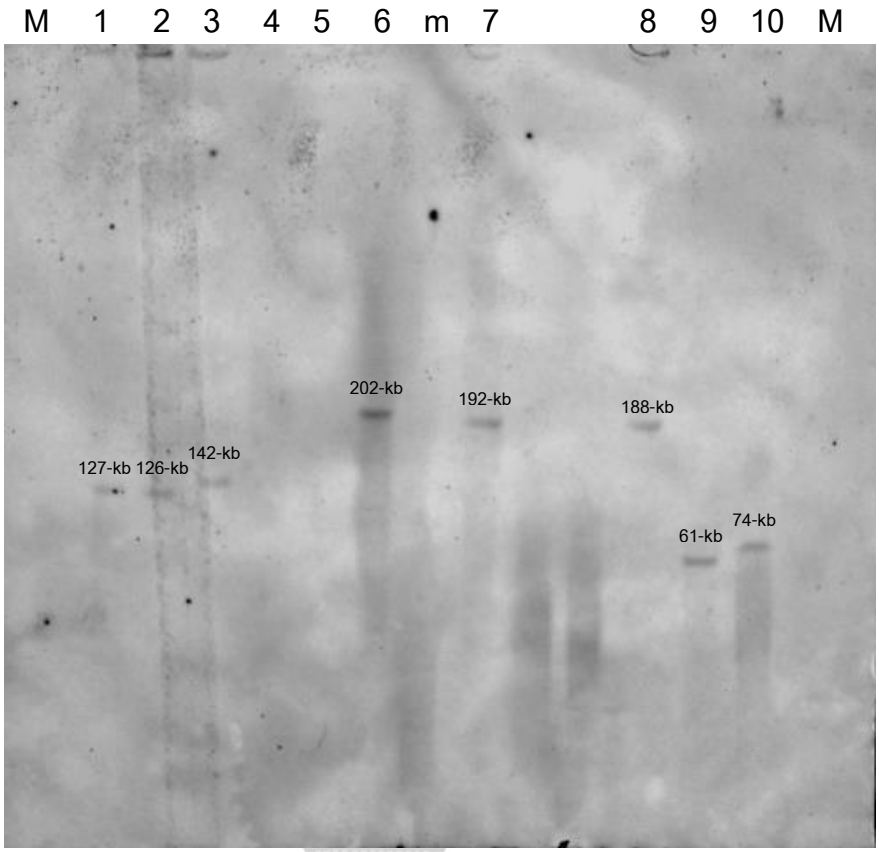
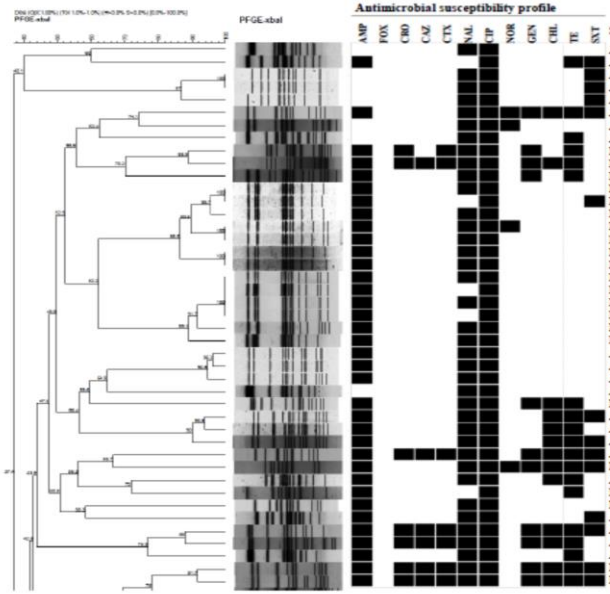


Figure 25. Southern blot hybridization using specific probe for *qnrS1* gene

M, CHEF DNA Size Standard-Lambda Ladder; 1, H591; 2, H595; 3, H589; 4, H615; 5, H607; 6, H617; m, CHEF DNA Size Standard-8-48 kb Ladder; 7, H606; 8, H611; 9, H583; 10, H603; M, CHEF DNA Size Standard-Lambda Ladder.

Table 29. The information of strains, the antimicrobial susceptibility profiles, and resistance mechanisms of each pulsotype among 95 ciprofloxacin-resistant *Salmonella* isolates

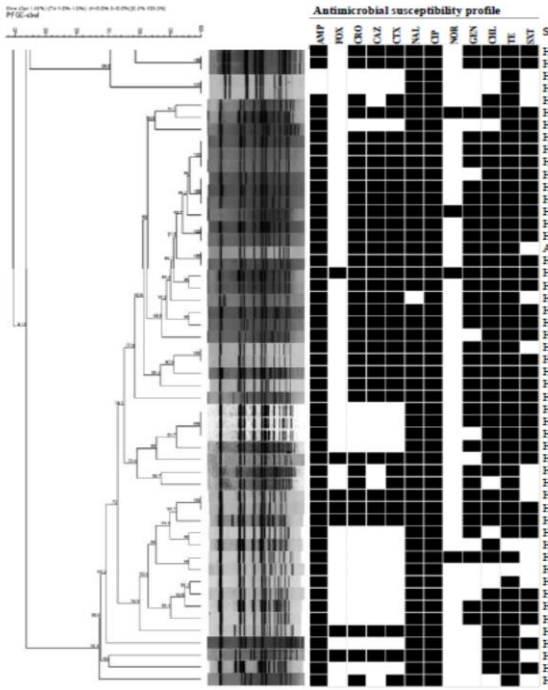


Strain	Organism	Source	Province	Year	GyrA/ParC substitution	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotyp
A51	*S.* Infantis	Chicken		2005	S83Y/-	-	-	ND	ND	F01-01
A54	*S.* Madjorio	Chicken		2005	-/-	QnrS1	-	No	ND	F02-01
A35	*S.* Emek	Chicken		2005	S83Y/-	QnrS1	-	No	ND	F03-01
A37	*S.* Emek	Chicken		2005	S83Y/-	QnrS1	-	No	ND	F03-01
A36	*S.* Emek	Chicken		2005	S83Y/-	QnrS1	-	No	ND	F03-02
A280	*S.* Virginia	Swine		2014	S83F, D87N/S80I	-	-	ND	ND	F04-01
A281	*S.* Virginia	Swine		2014	S83F, D87N/S80I	-	-	ND	ND	F05-01
A90	*S.* Virginia	Swine		2005	-/-	QnrS1	-	No	ND	F06-01
H138	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-14, TEM-1	No	ND	F07-01
H612	*S.* Typhimurium	Blood	Bangkok	2015	-/-	QnrS1	CTX-M-55	Yes	221-kb, 70-kb(FII)	F07-02
H64	*S.* Choleraesuis	Stool		2005	D87G/-	QnrS1	-	No	ND	F08-01
H165	*S.* Enteritidis	Blood	Ratchaburi	2007	-/-	QnrS1	-	No	ND	F09-01
H158	*S.* Enteritidis	Blood	Uttaradit	2007	-/-	QnrS1	-	No	ND	F09-01
H175	*S.* Enteritidis	Blood	Sa kaeo	2007	-/-	QnrS1	-	No	ND	F09-01
H239	*S.* Enteritidis	Blood	Bangkok	2007	S83F, D87Y/-	-	-	ND	ND	F09-02
H260	*S.* Enteritidis	Blood	Nan	2007	D87Y/-	-	-	ND	ND	F09-02
H164	*S.* Enteritidis	Blood	Nan	2007	-/-	QnrS1	-	No	ND	F09-03
H163	*S.* Enteritidis	Blood	Bangkok	2007	-/-	QnrS1	-	No	ND	F09-03
H162	*S.* Enteritidis	Blood	Phetchabun	2007	-/-	-	-	ND	ND	F10-01
H167	*S.* Enteritidis	Blood	Lampang	2007	-/-	-	-	ND	ND	F10-01
H189	*S.* Enteritidis	Blood	Nan	2007	-/-	-	-	ND	ND	F10-01
H190	*S.* Enteritidis	Blood	Nan	2007	-/-	-	-	ND	ND	F10-01
H296	*S.* Enteritidis	Rectal swab	Bangkok	2007	D87Y/-	-	-	ND	ND	F10-02
H446	*S.* Enteritidis	Urine	Chon buri	2007	-/-	QnrS1	-	No	ND	F10-03
A81	*S.* Senftenberg	Chicken		2005	S83F/-	QnrS1	-	No	ND	F11-01
A82	*S.* Senftenberg	Chicken		2005	S83F/-	QnrS1	-	No	ND	F11-01
A84	*S.* Senftenberg	Chicken		2005	S83F/-	QnrS1	-	No	ND	F11-02
A83	*S.* Senftenberg	Chicken		2005	S83F/-	-	-	ND	ND	F12-01
H35	*S.* Senftenberg	Blood	Bangkok	2006	-/-	-	-	ND	ND	F13-01
A171	*S.* Senftenberg	Chicken		2007	-/-	QnrS1	-	No	ND	F14-01
A126	*S.* Anatum	Swine		2007	-/-	-	-	ND	ND	F14-02
A245	*S.* Senftenberg	Swine		2007	-/-	QnrS1	-	No	ND	F14-03
A279	*S.* Choleraesuis	Swine		2014	ND	QnrS1	CTX-M-55	No	ND	F15-01
H605	*Salmonella* gr.B	Blood	Bangkok	2014	-/-	-	-	ND	ND	F16-01
A125	*S.* Anatum	Swine		2007	-/-	QnrS1	-	No	ND	F17-01
H598	*Salmonella* gr.C	Blood	Bangkok	2014	-/-	QnrS1	-	No	ND	F18-01
H36	*S.* Enteritidis	Blood	Bangkok	2006	-/-	-	-	ND	ND	F19-01
A56	*S.* Orion	Chicken		2005	S83Y/-	QnrS1	-	No	ND	F20-01
A277	*S.* Choleraesuis	Swine		2014	S83Y/-	QnrS1	CTX-M-55	No	209-kb(UT)*	F21-01
A276	*S.* Choleraesuis	Swine		2014	D87G/-	QnrS1	CTX-M-55	No	ND	F21-02
A43	*S.* Give	Swine		2005	S83Y/-	-	-	ND	ND	F22-01
H150	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	255-kb(FII)	F23-01
H123	*S.* Typhimurium	Rectal swab	Ratchaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	263-kb(UT), 204-kb	F23-02

Ampicillin, AMP; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefotaxime, CTX; nalidixic acid, NAL; ciprofloxacin, CIP; norfloxacin, NOR; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE; trimethoprim-sulfamethoxazole, SXT. PMQR, plasmid-mediated quinolone resistance; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine; ND, not determined; -, not found. *Plasmid sizes, replicon types, and antimicrobial resistance mechanisms transferred to the transconjugants following conjugation are underlin
a Plasmid sizes, replicon types, and antimicrobial resistance mechanisms confirmed the locations by Southern blot and hybridisation are underlined and superscribed with 'a'.

Ampicillin, AMP; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefotaxime, CTX; nalidixic acid, NAL; ciprofloxacin, CIP; norfloxacin, NOR; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE; trimethoprim-sulfamethoxazole, SXT. PMQR, plasmid-mediated quinolone resistance; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine; ND, not determined; -, not found.
*Plasmid sizes, replicon types, and antimicrobial resistance mechanisms transferred to the transconjugants following conjugation are underlined.
[a] Plasmid sizes, replicon types, and antimicrobial resistance mechanisms confirmed the locations by Southern blot and hybridisation are underlined and superscribed with 'a'.

Table 29. The information of strains, the antimicrobial susceptibility profiles, and resistance mechanisms of each pulsotype among 95 ciprofloxacin-resistant *Salmonella* isolates (continue)



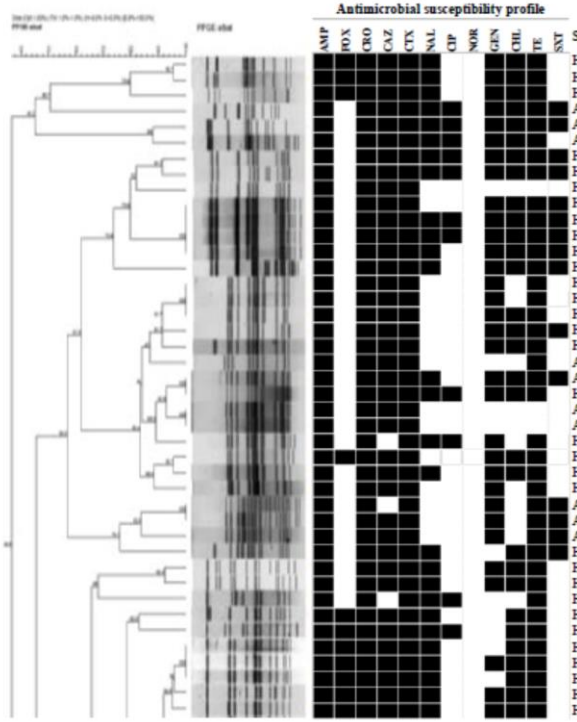
Strain	Organism	Source	Province	Year	GyrA/ParC substitution	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotype
H154	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	No	ND	F24-01
H156	*S.* Typhimurium	Stool	Bangkok	2007	-/-	-	CTX-M-55, TEM-1	No	ND	F24-01
H133	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	-	No	ND	F25-01
H135	*S.* Typhimurium	Stool	Chiangmai	2007	-/-	QnrS1	-	No	ND	F25-01
H528	*S.* Choleraesuis	Pus	Roiet	2007	D87Y/-	-	CTX-M-14	Yes	127-kb(FII)	F26-01
H583	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	91-kb(A/C)*, 33-kb	F26-02
H570	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	-	No	ND	F27-01
H611	*S.* Choleraesuis	Blood	Bangkok	2015	D87G/-	QnrS1	CTX-M-55, TEM-1	No	188-kb(A/C)*	F28-01
H608	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	F28-01
H609	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	F28-01
H602	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	F28-01
H595	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	126-kb(A/C)*, 34-kb	F28-01
H589	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	142-kb(A/C)*, 37-kb	F28-01
H603	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	74-kb(A/C)*, 36-kb	F28-01
H600	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55, TEM-1	No	ND	F28-02
H607	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	164-kb(A/C)*, 37-kb	F28-02
A251	*S.* Choleraesuis	Swine	Ratchaburi	2014	-/-	QnrS1	CTX-M-55	No	ND	F28-03
H590	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	F28-03
H588	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	F28-04
H591	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	127-kb(A/C)*, 35-kb	F28-04
H617	*S.* Choleraesuis	Blood	Bangkok	2016	D87G/-	QnrS1	CTX-M-55	No	202-kb(A/C)*	F28-05
H593	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	F28-06
H606	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	192-kb(A/C)*, 128-kb	F28-06
H592	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	F28-07
H581	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	ND	F29-01
H585	*S.* Choleraesuis	Stool	Ratchaburi	2012	D87G/-	QnrS1	CTX-M-55	No	ND	F29-01
H582	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	ND	F29-02
H576	*S.* Choleraesuis	Blood	Bangkok	2012	D87G/-	-	CTX-M-55	No	ND	F29-03
H586	*S.* Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	ND	F30-01
H418	*S.* Choleraesuis	Blood	Chon buri	2007	D87G/-	QnrS1	-	No	ND	F31-01
H510	*S.* Choleraesuis	Blood	Bangkok	2007	D87G/-	QnrS1	-	No	ND	F31-01
H394	*S.* Choleraesuis	Blood	Chang rai	2007	D87G/-	QnrS1	-	No	ND	F31-01
H537	*S.* Choleraesuis	Blood	Bangkok	2007	D87G/-	QnrS1	-	No	ND	F31-02
H521	*S.* Choleraesuis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	Yes	188-kb(A/C)	F31-03
H515	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87Y/-	-	CTX-M-14	Yes	127-kb(FII)	F32-01
H423	*S.* Choleraesuis	Blood	Bangkok	2007	D87G/-	QnrS1	CTX-M-14, TEM-1	No	ND	F32-02
H516	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	No	ND	F33-01
H522	*S.* Choleraesuis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	Yes	164-kb(A/C)	F33-01
H559	*S.* Choleraesuis	Unknown	Bangkok	2007	S83F/-	-	CMY-2	Yes	138-kb(A/C)	F33-02
H369	*S.* Choleraesuis	Blood	Sa kaeo	2007	D87G/-	-	-	ND	ND	F33-03
H74	*S.* Choleraesuis	Stool		2005	S83Y/-	-	-	ND	ND	F33-03
H53	*S.* Choleraesuis	Blood	Bangkok	2006	D87G/-	-	-	ND	ND	F34-01
H506	*S.* Choleraesuis	Blood	Sara buri	2007	D87G/-	QnrS1	-	No	ND	F34-01
H33	*S.* Choleraesuis	Blood	Bangkok	2006	D87G/-	-	-	ND	ND	F35-01
H511	*S.* Choleraesuis	Blood	Bangkok	2007	D87G/-	QnrS1	-	No	ND	F35-01
H426	*S.* Choleraesuis	Blood	Sa kaeo	2007	D87G/-	-	-	ND	ND	F35-02
H368	*S.* Choleraesuis	Blood	Sa kaeo	2007	D87G/-	-	-	ND	ND	F35-03
H412	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	No	ND	F36-01
H103	*S.* Choleraesuis	Stool		2005	S83F/-	-	-	ND	ND	F37-01
H527	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	Yes	114-kb(A/C), 35-kb	F38-01
H539	*S.* Choleraesuis	Pus	Bangkok	2007	D87G/-	-	-	ND	ND	F39-01
H91	*S.* Choleraesuis	Stool		2005	S83Y/-	-	CTX-M-14, TEM-1	Yes	108-kb(I1)	F40-01

Ampicillin, AMP; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefotaxime, CTX; nalidixic acid, NAL; ciprofloxacin, CIP; norfloxacin, NOR; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE; trimethoprim-sulfamethoxazole, SXT. PMQR, plasmid-mediated quinolone resistance; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine; ND, not determined; -, not found.

*Plasmid sizes, replicon types, and antimicrobial resistance mechanisms transferred to the transconjugants following conjugation are underlined.

[a] Plasmid sizes, replicon types, and antimicrobial resistance mechanisms confirmed the locations by Southern blot and hybridisation are underlined and superscribed with 'a'.

Table 30. The information of strains, the antimicrobial susceptibility profiles, and resistance mechanisms of each pulsotype among 170 ESC-resistant *Salmonella* isolates



Strain	Organism	Source	Province	Year	GyrA/ParC substitution	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotyp
H469	*S.* Choleraesuis	Blood	Buriram	2007	ND	-	CMY-2	No	ND	C01-01
H498	*S.* Choleraesuis	Blood	Ratchaburi	2007	ND	-	CMY-2, TEM-1	Yes	169-kb(A/C)	C01-01
H367	*S.* Choleraesuis	Blood	Nakhon phanom	2007	ND	-	CMY-2	No	ND	C02-01
A279	*S.* Choleraesuis	Swine		2014	ND	QnrS1	CTX-M-55	No	ND	C03-01
A277	*S.* Choleraesuis	Swine		2014	S83Y/-	QnrS1	CTX-M-55	No	ND	C04-01
A276	*S.* Choleraesuis	Swine		2014	D87G/-	QnrS1	CTX-M-55	No	ND	C04-02
H150	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	255-kb(FII)	C05-01
H123	*S.* Typhimurium	Rectal	Ratchaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	263-kb(UT), 204-kb	C05-02
H157	*S.* Typhimurium	Urine	Bangkok	2007	ND	-	CTX-M-15, TEM-1	Yes	107-kb(I1)	C06-01
H136	*S.* Typhimurium	Stool	Ratchaburi	2007	-/-	-	CTX-M-55, TEM-1	No	ND	C07-01
H154	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	No	ND	C07-01
H156	*S.* Typhimurium	Stool	Bangkok	2007	-/-	-	CTX-M-55, TEM-1	No	ND	C07-01
H129	*S.* Typhimurium	Rectal	Nonthaburi	2007	-/-	QnrS1	CTX-M-55, TEM-1	No	ND	C07-01
H128	*S.* Typhimurium	Rectal	Bangkok	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	264-kb(A/C), 34-kb, 6-kb	C08-01
H144	*S.* Typhimurium	Stool	Ratchaburi	2007	-/-	-	CTX-M-55, TEM-1	Yes	186-kb(A/C)	C09-01
H143	*S.* Typhimurium	Stool	Ratchaburi	2007	-/-	-	CTX-M-55, TEM-1	Yes	184-kb(A/C)	C09-01
H127	*S.* Typhimurium	Rectal	Nonthaburi	2007	-/-	-	CTX-M-55, TEM-1	Yes	183-kb(A/C)	C09-01
H125	*S.* Typhimurium	Rectal	Ratchaburi	2007	-/-	-	CTX-M-55, TEM-1	Yes	273-kb, 71-kb(I1)	C09-02
H616	*S.* Typhimurium	Blood	Bangkok	2016	ND	-	CTX-M-55	No	113-kb(A/C)*	C09-03
A291	*S.* Typhimurium	Swine	Bangkok	2016	ND	QnrS1	CTX-M-55, TEM-1	Yes	57-kb(FII)	C09-04
A252	*S.* Typhimurium	Swine	Ratchaburi	2014	ND	QnrS1	CTX-M-55	No	212-kb(HI1)* 221-kb	C10-01
H612	*S.* Typhimurium	Blood	Bangkok	2015	-/-	QnrS1	CTX-M-55	Yes	70-kb(FII)	C10-01
A283	*S.* Typhimurium	Swine	Udon Thani	2015	ND	QnrS1	CTX-M-55, TEM-1	No	ND	C10-02
A285	*S.* Typhimurium	Swine	Udon Thani	2015	ND	QnrS1	CTX-M-55, TEM-1	No	ND	C10-02
H138	*S.* Typhimurium	Stool	Nonthaburi	2007	-/-	QnrS1	CTX-M-14, TEM-1	No	ND	C10-03
H137	*S.* Typhimurium	Stool	Bangkok	2007	-/-	-	CTX-M-14, CMY-2, TEM-1	No	ND	C11-01
H126	*S.* Typhimurium	Rectal	Bangkok	2007	-/-	QnrS1	CTX-M-55, TEM-1	Yes	172-kb(A/C)	C11-01
H145	*S.* Typhimurium	Stool	Ratchaburi	2007	-/-	-	CTX-M-55, TEM-1	Yes	108-kb(FII)	C11-02
A295	*S.* Choleraesuis	Swine		2016	ND	QnrS1	CTX-M-14, TEM-1	No	ND	C12-01
A294	*S.* Choleraesuis	Swine		2016	ND	QnrS1	CTX-M-14, TEM-1	No	ND	C12-01
A292	*S.* Choleraesuis	Swine		2016	ND	QnrS1	CTX-M-14, TEM-1	No	ND	C13-01
H599	*S.* Choleraesuis	Blood	Bangkok	2014	ND	-	CTX-M-55, TEM-1	No	ND	C14-01
H566	*S.* Choleraesuis	Blood	Bangkok	2012	ND	-	CTX-M-55	No	ND	C15-01
H567	*S.* Choleraesuis	Blood	Bangkok	2012	ND	-	CTX-M-55	No	ND	C15-02
H91	*S.* Choleraesuis	Stool		2005	S83Y/-	-	CTX-M-14, TEM-1	Yes	108-kb(I1)	C16-01
H471	*S.* Choleraesuis	Blood	Phayao	2007	D87G/-	-	CMY-2	No	ND	C17-01
H527	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	Yes	114-kb(A/C), 35-kb	C18-01
H470	*S.* Choleraesuis	Blood	Phayao	2007	D87G/-	-	CMY-2	No	ND	C19-01
H439	*S.* Choleraesuis	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	No	ND	C19-01
H514	*S.* Choleraesuis	Blood	Sa Kaeo	2007	D87G/-	-	CMY-2, TEM-1	No	ND	C19-01
H524	*S.* Choleraesuis	Blood	Bangkok	2007	D87G/-	-	CMY-2	Yes	180-kb(A/C)	C19-01
H473	*S.* Choleraesuis	Blood	Nonthaburi	2007	D87G/-	-	CMY-2	No	ND	C19-02

Ampicillin, AMP; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefotaxime, CTX; nalidixic acid, NAL; ciprofloxacin, CIP; norfloxacin, NOR; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE; trimethoprim-sulfamethoxazole, SXT.

PMQR, plasmid-mediated quinolone resistance; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine; ND, not determined; -, not found.

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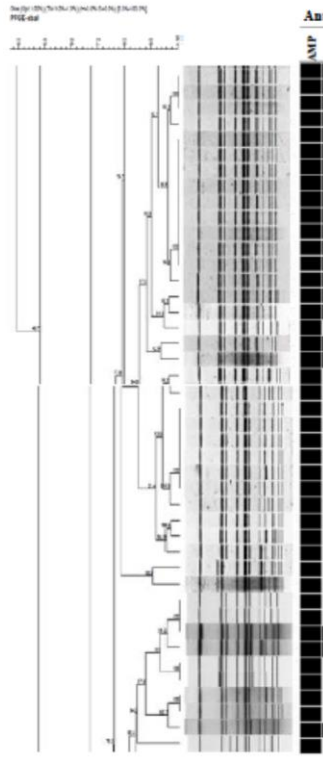

Strain	Organism	Source	Province	Year	GyrA/ParC substitution	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotype
H512	*S. Cholerae*suis	Blood	Bangkok	2007	S83F/-	-	CMY-2	Yes	146-kb(A/C), 43-kb	C19-03
H536	*S. Cholerae*suis	Blood	Nonthaburi	2007	S83F/-	-	CMY-2, TEM-1	Yes	160-kb(A/C)	C19-03
H554	*S. Cholerae*suis	Blood	Suphan Buri	2007	S83F/-	-	CMY-2	Yes	137-kb(A/C)	C19-03
H405	*S. Cholerae*suis	Blood	Sa Kaeo	2007	S83F/-	-	CMY-2	Yes	177-kb(A/C), 40-kb	C19-03
H495	*S. Cholerae*suis	Blood	Phetchaburi	2007	ND	-	CMY-2	No	ND	C19-03
H432	*S. Cholerae*suis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	No	ND	C19-03
H479	*S. Cholerae*suis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	No	ND	C19-03
H437	*S. Cholerae*suis	Blood	Chon buri	2007	S83F/-	-	CMY-2	No	ND	C19-03
H521	*S. Cholerae*suis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	Yes	188-kb(A/C)	C19-03
H431	*S. Cholerae*suis	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	Yes	152-kb(A/C), 36-kb	C19-03
H378	*S. Cholerae*suis	Blood	Bangkok	2007	S83F/-	-	CMY-2	Yes	153-kb(A/C)	C19-03
H429	*S. Cholerae*suis	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C19-03
H546	*S. Cholerae*suis	Blood	Chonburi	2007	S83F/-	-	CMY-2, TEM-1	Yes	156-kb(A/C)	C19-03
H385	*S. Cholerae*suis	Blood	Chanthaburi	2007	D87G/-	-	CMY-2, TEM-1	No	ND	C19-03
H494	*S. Cholerae*suis	Blood	Chon buri	2007	D87G/-	-	CMY-2	Yes	154-kb(A/C)	C19-04
H395	*S. Cholerae*suis	Blood	Chang rai	2007	S83F/-	-	CMY-2	Yes	137-kb(A/C)	C19-04
H531	*S. Cholerae*suis	Blood	Chanthaburi	2007	D87Y/-	-	CTX-M-14, TEM-1	Yes	102-kb(FII$_{s}$)	C19-05
H549	*S. Cholerae*suis	Blood	Chon buri	2007	S83F/-	-	CMY-2	No	ND	C19-06
H371	*S. Cholerae*suis	Blood	Sa Kaeo	2007	D87G/-	-	CMY-2, TEM-1	No	ND	C19-07
H507	*S. Cholerae*suis	Blood	Saraburi	2007	ND	-	CTX-M-14	Yes	92-kb(I1)	C20-01
H430	*S. Cholerae*suis	Blood	Phayao	2007	D87Y/-	-	CTX-M-14	Yes	83-kb(FII)	C20-01
H384	*S. Cholerae*suis	Blood	Bangkok	2007	D87Y/-	-	CTX-M-14	Yes	186-kb, 102-kb(I1)	C20-02
H526	*S. Cholerae*suis	Blood	Ratchaburi	2007	ND	-	CTX-M-14	Yes	183-kb, 103-kb(I1)	C20-02
H441	*S. Cholerae*suis	Blood	Sa Kaeo	2007	D87Y/-	-	CTX-M-14	Yes	190-kb, 99-kb(FII$_{s}$)	C20-02
H532	*S. Cholerae*suis	Blood	Chanthaburi	2007	D87Y/-	-	CTX-M-14	Yes	194-kb, 97-kb(FII$_{s}$)	C20-02
H508	*S. Cholerae*suis	Blood	Lampang	2007	D87Y/-	-	CTX-M-14	Yes	183-kb, 97-kb(FII)	C20-02
H435	*S. Cholerae*suis	Blood	Nan	2007	D87Y/-	-	CTX-M-14	Yes	190-kb, 102-kb(FII$_{s}$)	C20-02
H409	*S. Cholerae*suis	Blood	Nonthaburi	2007	D87Y/-	-	CTX-M-14	Yes	118-kb(FII)	C20-02
H401	*S. Cholerae*suis	Blood	Bangkok	2007	D87Y/-	-	CTX-M-14	Yes	119-kb(FII)	C20-03
H481	*S. Cholerae*suis	Blood	Bangkok	2007	D87Y/-	-	CTX-M-14	Yes	178-kb, 91-kb(I1)	C20-03
H393	*S. Cholerae*suis	Blood	Sa Kaeo	2007	D87Y/-	-	CTX-M-14	Yes	220-kb, 120-kb(FII$_{s}$)	C20-04
H497	*S. Cholerae*suis	Blood	Ratchaburi	2007	D87Y/-	-	CTX-M-14	Yes	119-kb(FII$_{s}$)	C21-01
H515	*S. Cholerae*suis	Blood	Ratchaburi	2007	D87Y/-	-	CTX-M-14	Yes	127-kb(FII)	C21-02
H581	*S. Cholerae*suis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	ND	C22-01
H585	*S. Cholerae*suis	Stool	Ratchaburi	2012	D87G/-	QnrS1	CTX-M-55	No	ND	C22-01
H85	*S. Cholerae*suis	Stool		2005	ND	-	CMY-2	Yes	129-kb(A/C)	C22-01
H34	*S. Cholerae*suis	Blood		2006	-/-	-	CMY-2	No	ND	C22-01
H575	*S. Cholerae*suis	Blood	Bangkok	2012	ND	-	CTX-M-55	No	ND	C22-02
H576	*S. Cholerae*suis	Blood	Bangkok	2012	D87G/-	-	CTX-M-55	No	ND	C22-02
H528	*S. Cholerae*suis	Pus	Roiet	2007	D87Y/-	-	CTX-M-14	Yes	127-kb(FII)	C22-03
H476	*S. Cholerae*suis	Urine	Bangkok	2007	ND	-	CTX-M-14	Yes	221-kb, 118-kb(FII$_{s}$)	C22-03
H565	*S. Cholerae*suis	Pus	Bangkok	2012	S83F/-	-	CMY-2	No	ND	C22-03
H577	*S. Cholerae*suis	Pus	Ratchaburi	2012	ND	-	CTX-M-14	Yes	136-kb(I1)	C23-01

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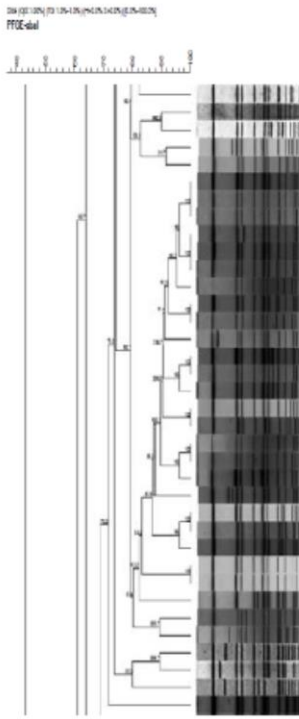

Strain	Organism	Source	Province	Year	GyrA/ParC substitution	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotype
H534	S. Choleraesuis	Blood	Chanthaburi	2007	D87G/-	-	CTX-M-14, TEM-1	Yes	113-kb(I1)	C24-01
H423	S. Choleraesuis	Blood	Bangkok	2007	D87G/-	QnrS1	CTX-M-14, TEM-1	No	ND	C25-01
H370	S. Choleraesuis	Blood	Sa Kaeo	2007	D87G/-	-	CTX-M-14	Yes	219-kb, 113-kb(I1), 41-kb	C25-02
H584	S. Choleraesuis	Pus	Ratchaburi	2012	ND	-	CTX-M-14	Yes	134-kb(I1)	C26-01
H381	S. Choleraesuis	Blood	Nonthaburi	2007	D87Y/-	-	CTX-M-14	Yes	243-kb, 108-kb(11)	C26-02
H611	S. Choleraesuis	Blood	Bangkok	2015	D87G/-	QnrS1	CTX-M-55, TEM-1	No	188-kb(A/C)$_a$	C27-01
H608	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	C27-01
H609	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	C27-01
H602	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	C27-01
H595	S. Choleraesuis	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	126-kb(A/C)$_a$, 34-kb	C27-01
H589	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	142-kb(A/C)$_a$, 37-kb	C27-01
H603	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	74-kb(A/C)$_a$, 36-kb	C27-01
H615	S. Choleraesuis	Blood	Bangkok	2016	ND	-	CTX-M-55	No	139-kb(A/C)$_a$, 34-kb	C27-02
H592	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	C27-02
H617	S. Choleraesuis	Blood	Bangkok	2016	D87G/-	QnrS1	CTX-M-55	No	202-kb(A/C)$_a$	C27-06
H600	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55, TEM-1	No	ND	C27-03
H607	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	164-kb(A/C)$_2$, 37-kb	C27-03
H596	S. Choleraesuis	Blood	Bangkok	2014	ND	-	CTX-M-55	No	ND	C27-03
A251	S. Choleraesuis	Swine	Ratchaburi	2014	-/-	QnrS1	CTX-M-55	No	ND	C27-05
H590	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	ND	C27-05
H587	S. Choleraesuis	Blood	Bangkok	2014	ND	-	CTX-M-55	No	ND	C27-04
H591	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55	No	127-kb(A/C)$_a$, 35-kb	C27-04
H588	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	C27-04
H604	S. Choleraesuis	Blood	Bangkok	2014	ND	-	CTX-M-55	No	ND	C27-07
H572	S. Choleraesuis	Blood	Bangkok	2012	ND	-	CTX-M-14	Yes	128-kb(FII$_{48}$)	C27-08
H410	S. Choleraesuis	Blood	Ratchaburi	2007	ND	-	CTX-M-14	Yes	99-kb(FII)	C27-08
H593	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	QnrS1	CTX-M-55, TEM-1	No	ND	C27-08
H579	S. Choleraesuis	Blood	Bangkok	2012	ND	-	CMY-2	Yes	160-kb(A/C)	C30-01
H580	S. Choleraesuis	Pus	Bangkok	2012	ND	-	CMY-2	Yes	103-kb(A/C), 40-kb	C30-01
H93	S. Choleraesuis	Stool		2005	ND	-	CMY-2	Yes	141-kb(A/C)	C28-01
H606	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	192-kb(A/C)$_a$, 128-kb	C29-01
H586	S. Choleraesuis	Blood	Bangkok	2014	D87G/-	-	CTX-M-55	No	ND	C29-02
H503	S. Choleraesuis	Blood	Nan	2007	D87G/-	-	CMY-2	Yes	133-kb(A/C)	C32-01
H406	S. Choleraesuis	Blood	Sa Kaeo	2007	D87G/-	-	CMY-2	Yes	124-kb(A/C), 50-kb	C32-02
H366	S. Choleraesuis	Blood	Nakhon phanom	2007	D87G/-	-	CMY-2	No	ND	C33-01
H402	S. Choleraesuis	Blood	Bangkok	2007	ND	-	CTX-M-14	Yes	97-kb(FII)	C34-01

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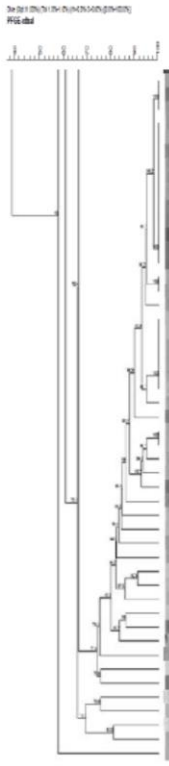


Strain	Organism	Source	Province	Year	GyrA/ParC substitutio	PMQR	Beta-lactamase	Conjugation	Plasmid profile* (replicon type)	Pulsotype
H519	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-01
H542	*S. Cholerasuis*	Urine	Bangkok	2007	ND	-	CMY-2	Yes	148-kb(A/C)	C35-01
H538	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-01
H440	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C35-01
H400	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	Yes	170-kb(A/C), 66-kb	C35-01
H518	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-01
H547	*S. Cholerasuis*	Blood	Chon buri	2007	ND	-	CMY-2	No	ND	C35-01
H548	*S. Cholerasuis*	Blood	Chon buri	2007	ND	-	CMY-2	No	ND	C35-01
H552	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	Yes	168-kb(A/C), 40-kb	C35-01
H553	*S. Cholerasuis*	Blood	Chon buri	2007	ND	-	CMY-2	No	ND	C35-01
H408	*S. Cholerasuis*	Stool	Nonthaburi	2007	ND	-	CMY-2	No	ND	C35-01
H543	*S. Cholerasuis*	Stool	Chon buri	2007	ND	-	CMY-2	Yes	132-kb(A/C)	C35-01
H544	*S. Cholerasuis*	Stool	Chon buri	2007	ND	-	CMY-2	Yes	133-kb(A/C)	C35-01
H545	*S. Cholerasuis*	Stool	Chon buri	2007	ND	-	CMY-2	No	ND	C35-01
H442	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C35-01
H513	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	Yes	137-kb(A/C)	C35-01
H520	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-01
H516	*S. Cholerasuis*	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	No	ND	C35-02
H522	*S. Cholerasuis*	Blood	Ratchaburi	2007	S83F/-	-	CMY-2	Yes	164-kb(A/C)	C35-02
H422	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-02
H428	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C35-02
H438	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C35-02
H482	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C35-02
H417	*S. Cholerasuis*	Blood	Chiang Mai	2007	ND	-	CMY-2	Yes	144-kb(A/C)	C35-02
H583	*S. Cholerasuis*	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	61-kb(A/C)[a], 33-kb	C35-03
H474	*S. Cholerasuis*	Blood	Ratchaburi	2007	D87Y/-	-	CTX-M-14	Yes	296-kb, 203-kb, 107-kkb(FII$_s$)	C35-04
H397	*S. Cholerasuis*	Blood	Chanthaburi	2007	ND	-	CTX-M-14	Yes	108-kb(FII$_s$)	C35-04
H478	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CTX-M-14	Yes	212-kb, 112-kb(FII^s)	C35-04
H399	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CTX-M-14	Yes	118-kb(I1)	C35-05
H582	*S. Cholerasuis*	Blood	Bangkok	2012	D87G/-	QnrS1	CTX-M-55	No	ND	C35-06
H509	*S. Cholerasuis*	Blood	Bangkok	2007	D87Y/-	-	CTX-M-14	Yes	118-kb(FII^s)	C35-07
H559	*S. Cholerasuis*	Unknown	Bangkok	2007	S83F/-	-	CMY-2	Yes	138-kb(A/C)	C35-08
H382	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	Yes	152-kb(A/C), 45-kb	C35-09
H525	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CTX-M-14	Yes	224-kb, 107-kb(1)	C35-10
H434	*S. Cholerasuis*	Blood	Ratchaburi	2007	D87Y/-	-	CTX-M-14	Yes	95-kb(I1)	C36-01
H365	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C37-01
H375	*S. Cholerasuis*	Blood	Bangkok	2007	ND	-	CMY-2	No	ND	C37-02
H480	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	Yes	207-kb(A/C)	C37-03
H407	*S. Cholerasuis*	Blood	Nonthaburi	2007	D87G/-	-	CTX-M-14	Yes	64-kb(FII), 42-kb	C38-01
H533	*S. Cholerasuis*	Blood	Chanthaburi	2007	D87G/-	-	CTX-M-14	Yes	117-kb(I1)	C38-02
H491	*S. Cholerasuis*	Blood	Chonburi	2007	ND	-	CMY-2, TEM-1	Yes	161-kb(A/C), 41-kb	C38-03
H81	*S. Cholerasuis*	Stool		2005	D87G/-	-	CTX-M-14, TEM-1	Yes	38-kb(UT)	C39-01
H412	*S. Cholerasuis*	Blood	Ratchaburi	2007	D87G/-	-	CMY-2	No	ND	C40-01
H568	*S. Cholerasuis*	Urine	Bangkok	2012	ND	-	CTX-M-14, TEM-1	Yes	120-kb(I1)	C41-01
H535	*S. Cholerasuis*	CSF	Chanthaburi	2007	ND	-	CTX-M-14	Yes	87-kb(FII)	C42-01
H386	*S. Cholerasuis*	Blood	Chanthaburi	2007	ND	-	CTX-M-14	Yes	190-kb, 107-kb(FII^s)	C43-01
H414	*S. Cholerasuis*	Blood	Bangkok	2007	D87G/-	-	CMY-2	No	ND	C44-01
H391	*S. Cholerasuis*	Blood	Ratchaburi	2007	ND	-	CMY-2	No	ND	C45-01
H79	*S. Cholerasuis*	Stool		2005	ND	-	CTX-M-14, TEM-1	Yes	117-kb(I1), 44-kb	C31-01

Ampicillin, AMP; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefotaxime, CTX; nalidixic acid, NAL; ciprofloxacin, CIP; norfloxacin, NOR; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE; trimethoprim-sulfamethoxazole, SXT. PMQR, plasmid-mediated quinolone resistance; D, Aspartic acid; G, Glycine; Y, Tyrosine; S, Serine; F, Phenylalanine; N, Asparagine; I, Isolucine; ND, not determined; -, not found.

*Plasmid sizes, replicon types, and antimicrobial resistance mechanisms transferred to the transconjugants following conjugation are underlined.

[a] Plasmid sizes, replicon types, and antimicrobial resistance mechanisms confirmed the locations by Southern blot and hybridisation are underlined and superscribed with 'a'.

7. The result of the investigation of the genetic relationship among antimicrobial-resistant plasmids by RFLP using *EcoR*I, *Sca*I, *Hind*III, and *Hindc*II

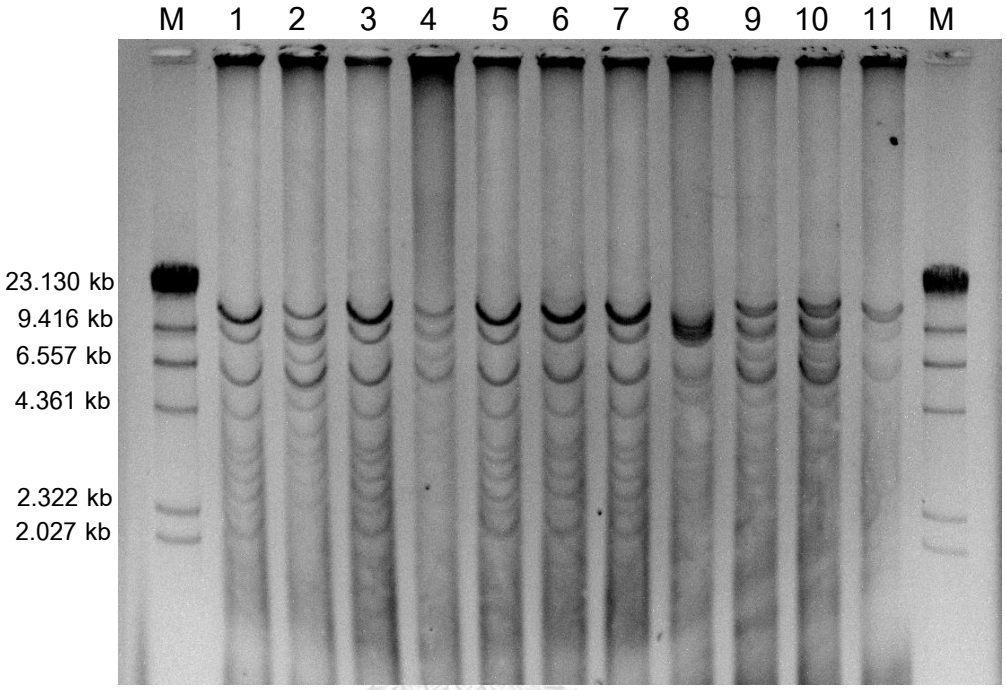
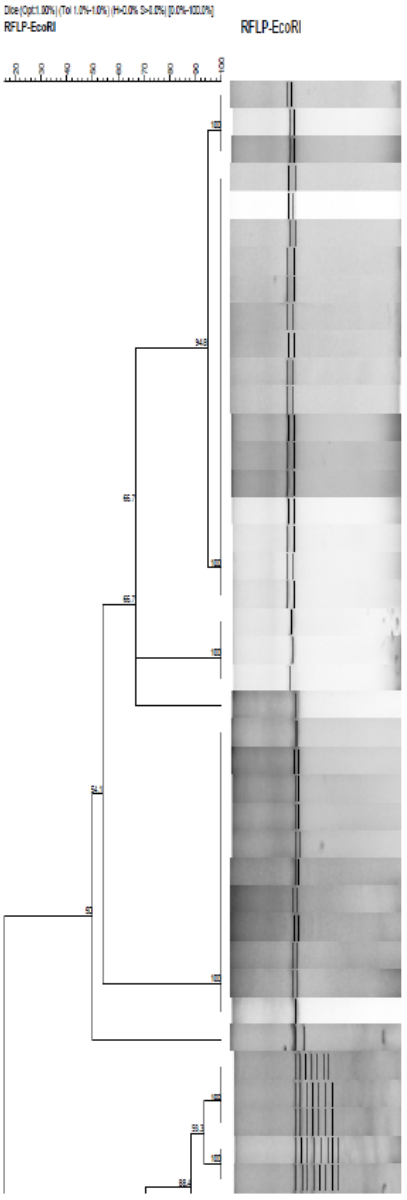


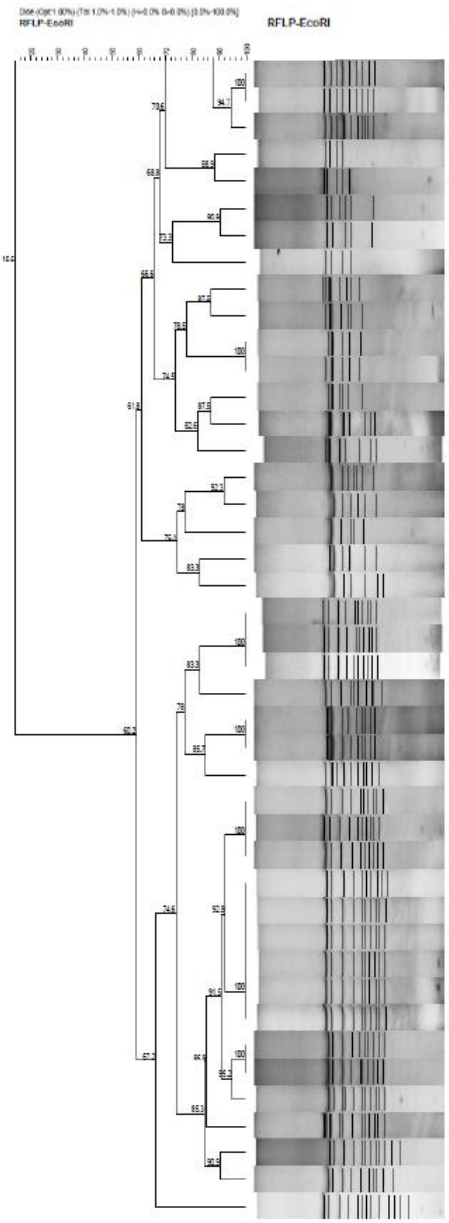
Figure 26. The result of RFLP of plasmid carrying $bla_{CTX\text{-}M\text{-}14}$ gene using *EcoR*I

M, Lambda DNA-*Hind*III Digest; 1, plasmid from H399U; 2, plasmid from H401U; 3, plasmid from H507U; 4, plasmid from H528U; 5, plasmid from H79U; 6, plasmid from H526U; 7, plasmid from H584U; 8, plasmid from H435U; 9, plasmid from H509U; 10, plasmid from H393U; 11, plasmid from H91U; M, Lambda DNA-*Hind*III Digest. The number of isolates with alphabet U represents their transconjugants. Lane 1, 3, and 5-7 represent for the plasmid RFLP type E21-02.



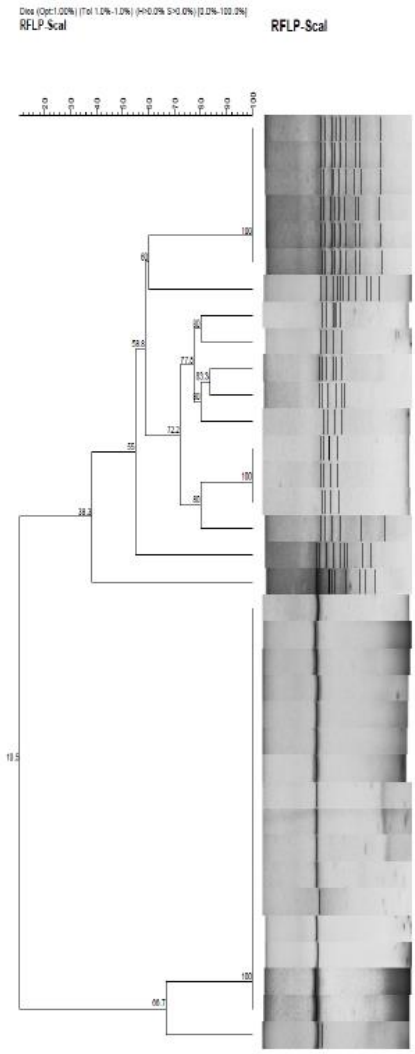
Transconjugant	Beta-lactamase	Transferable plasmid	Inc type	Resistance pattern	RFLP type
H512U	CMY-2	146	A/C	CRO GEN CHL TE	E01-01
H521U	CMY-2	188	A/C	CRO GEN CHL TE	E01-01
H405U	CMY-2	177	A/C	CRO GEN CHL TE	E01-01
H513U	CMY-2	137	A/C	CRO GEN CHL TE	E01-02
H378U	CMY-2	153	A/C	CRO GEN CHL TE	E01-02
H552U	CMY-2	168	A/C	CRO GEN CHL TE	E01-02
H536U	CMY-2	160	A/C	CRO GEN CHL TE	E01-02
H546U	CMY-2	156	A/C	CRO GEN CHL TE	E01-02
H503U	CMY-2	133	A/C	CRO GEN CHL TE	E01-02
A291U	CTX-M-55	57	FII	CRO	E01-02
H431U	CMY-2	152	A/C	CRO GEN CHL TE	E01-02
H498U	CMY-2	169	A/C	CRO GEN CHL TE	E01-02
H417U	CMY-2	144	A/C	CRO GEN CHL TE	E01-02
H400U	CMY-2	170	A/C	CRO GEN CHL TE	E01-02
H480U	CMY-2	207	A/C	CRO GEN CHL TE	E01-02
H494U	CMY-2	154	A/C	CRO GEN CHL TE	E01-02
H544U	CMY-2	133	A/C	CRO GEN CHL TE	E01-02
H543U	CMY-2	132	A/C	CRO GEN CHL TE	E01-02
H524U	CMY-2	180	A/C	CRO GEN CHL TE	E01-02
H580U	CMY-2	103	A/C	CRO GEN CHL TE	E02-01
H491U	CMY-2	161	A/C	CRO GEN CHL TE	E02-01
H527U	CMY-2	114	A/C	CRO GEN CHL TE	E02-01
H93U	CMY-2	141	A/C	CRO GEN CHL TE	E03-01
H559U	CMY-2	138	A/C	CRO GEN CHL TE	E04-01
H406U	CMY-2	124	A/C	CRO GEN CHL TE	E04-01
H542U	CMY-2	148	A/C	CRO GEN CHL TE	E04-01
H522U	CMY-2	164	A/C	CRO GEN CHL TE	E04-01
H382U	CMY-2	152	A/C	CRO GEN CHL TE	E04-01
H126U	CTX-M-55	172	A/C	CRO GEN CHL TE	E04-01
H579U	CMY-2	160	A/C	CRO GEN CHL TE	E04-01
H144U	CTX-M-55 TEM-1	186	A/C	CRO GEN CHL TE	E04-01
H395U	CMY-2	137	A/C	CRO CHL TE	E04-01
H85U	CMY-2	129	A/C	CRO GEN CHL TE	E04-01
H612U	CTX-M-55	70	FII	CRO GEN CHL	E04-01
H81U	CTX-M-14 TEM-1	38	UT	CRO	E05-01
H572U	CTX-M-14	128	FIIS	CRO	E06-01
H515U	CTX-M-14	127	FII	CRO GEN	E06-01
H393U	CTX-M-14	120	FIIS	CRO GEN	E06-01
H509U	CTX-M-14	118	FIIS	CRO	E06-02
H410U	CTX-M-14	99	FII	CRO GEN	E06-02

Table 31. The Result of RFLP using *EcoR*I, plasmid sizes, Inc groups, the antimicrobial susceptibility profiles, and resistance mechanisms. The number of isolates with alphabet U represents their transconjugants. Ceftriaxone, CRO; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE



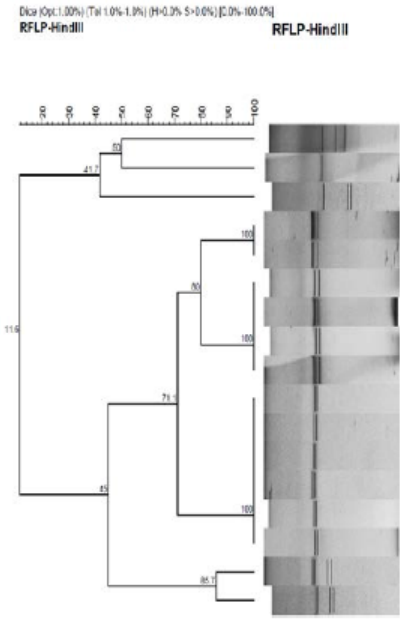
RFLP-EcoRI	Transconjugant	Beta-lactamase	Transferable plasmid	Inc type	Resistance pattern	RFLP typ
	H409U	CTX-M-14	118	FII	CRO	E06-03
	H497U	CTX-M-14	119	FIIS	CRO GEN	E06-03
	H476U	CTX-M-14	118	FIIS	CRO	E06-04
	H528U	CTX-M-14	127	FII	CRO GEN	E07-01
	H128U	CTX-M-55	264	A/C	CRO CHL TE	E07-02
	H157U	CTX-M-15	107	I1	CRO GEN	E08-01
	H531U	CTX-M-14	102	FIIS	CRO GEN	E08-02
	H91U	CTX-M-14	108	I1	CRO GEN	E09-01
	H532U	CTX-M-14	97	FIIS	CRO GEN	E10-01
	H508U	CTX-M-14	97	FII	CRO GEN	E10-02
	H402U	CTX-M-14	97	FII	CRO	E11-01
	H407U	CTX-M-14	64	FII	CRO	E11-01
	H535U	CTX-M-14	87	FII	CRO	E12-01
	H435U	CTX-M-14	102	FIIS	CRO GEN	E12-02
	H474U	CTX-M-14	107	FIIS	CRO GEN	E13-01
	H441U	CTX-M-14	99	FIIS	CRO	E14-01
	H397U	CTX-M-14	108	FIIS	CRO GEN	E14-02
	H525U	CTX-M-14	107	FII	CRO	E15-01
	H478U	CTX-M-14	112	FIIS	CRO GEN	E16-01
	H386U	CTX-M-14	107	FIIS	CRO GEN	E17-01
	H384U	CTX-M-14	102	I1	CRO GEN	E18-01
	H370U	CTX-M-14	113	I1	CRO GEN	E18-01
	H534U	CTX-M-14	113	I1	CRO GEN	E18-01
	H568U	CTX-M-14	121	I1	CRO GEN	E19-01
	H430U	CTX-M-14	83	FII	CRO	E20-01
	H381U	CTX-M-14	108	FII	CRO GEN	E20-01
	H401U	CTX-M-14	119	FII	CRO	E20-02
	H533U	CTX-M-14	117	I1	CRO GEN	E21-01
	H434U	CTX-M-14	95	I1	CRO GEN	E21-01
	H145U	CTX-M-55	108	FII	CRO GEN	E21-01
	H399U	CTX-M-14	118	I1	CRO GEN	E21-02
	H507U	CTX-M-14	92	I1	CRO GEN	E21-02
	H79U	CTX-M-14	117	I1	CRO GEN	E21-02
	H526U	CTX-M-14	103	I1	CRO GEN	E21-02
	H584U	CTX-M-14	134	I1	CRO GEN	E21-02
	H481U	CTX-M-14	91	I1	CRO GEN	E21-02
	H143U	CTX-M-55	184	A/C	CRO GEN	E21-03
	H127U	CTX-M-55 TEM-1	183	A/C	CRO GEN	E21-03
	H144U	CTX-M-55 TEM-1	186	A/C	CRO GEN CHL TE	E21-03
	H577U	CTX-M-14	136	I1	CRO GEN	E21-04
	H125U	CTX-M-55	71	I1	CRO GEN	E21-05
	H150U	CTX-M-55	255	FII	CRO GEN CHL TE	E21-06
	H123U	CTX-M-55	263	UT	CRO GEN CHL TE	E22-01

Table 31. The Result of RFLP using *EcoR*I, plasmid sizes, Inc groups, the antimicrobial susceptibility profiles, and resistance mechanisms (continuous). The number of isolates with alphabet U represents their transconjugants. Ceftriaxone, CRO; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE



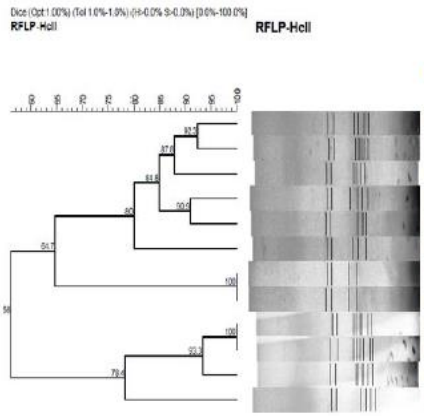
Transconjugant	Beta-lactamase	Transferable plasmid	Inc type	Resistance pattern	RFLP type
H546U	CMY-2	156	A/C	CRO GEN CHL TE	S01-01
H431U	CMY-2	152	A/C	CRO GEN CHL TE	S01-01
H378U	CMY-2	153	A/C	CRO GEN CHL TE	S01-01
H542U	CMY-2	148	A/C	CRO GEN CHL TE	S01-01
H406U	CMY-2	124	A/C	CRO GEN CHL TE	S01-01
H93U	CMY-2	141	A/C	CRO GEN CHL TE	S01-01
H527U	CMY-2	114	A/C	CRO GEN CHL TE	S02-01
H521U	CMY-2	188	A/C	CRO GEN CHL TE	S03-01
H512U	CMY-2	146	A/C	CRO GEN CHL TE	S04-01
H554U	CMY-2	137	A/C	CRO GEN	S05-01
H536U	CMY-2	160	A/C	CRO GEN CHL TE	S06-01
H498U	CMY-2	169	A/C	CRO GEN CHL TE	S07-01
H513U	CMY-2	137	A/C	CRO GEN CHL TE	S08-01
H491U	CMY-2	161	A/C	CRO GEN CHL TE	S08-01
H85U	CMY-2	129	A/C	CRO GEN CHL TE	S08-01
H405U	CMY-2	177	A/C	CRO GEN CHL TE	S09-01
H494U	CMY-2	154	A/C	CRO GEN CHL TE	S10-01
H126U	CTX-M-55	172	A/C	CRO GEN CHL TE	S11-01
H81U	CTX-M-14 TEM-1	38	UT	CRO	S12-01
H542U	CMY-2	148	A/C	CRO GEN CHL TE	S12-01
H395U	CMY-2	137	A/C	CRO CHL TE	S12-01
H580U	CMY-2	103	A/C	CRO GEN CHL TE	S12-01
H503U	CMY-2	133	A/C	CRO GEN CHL TE	S12-01
H480U	CMY-2	207	A/C	CRO GEN CHL TE	S12-01
H417U	CMY-2	144	A/C	CRO GEN CHL TE	S12-01
H546U	CMY-2	156	A/C	CRO GEN CHL TE	S12-01
H513U	CMY-2	137	A/C	CRO GEN CHL TE	S12-01
H579U	CMY-2	160	A/C	CRO GEN CHL TE	S12-01
H400U	CMY-2	170	A/C	CRO GEN CHL TE	S12-01
H612U	CTX-M-55	70	FII	CRO GEN CHL	S12-01
H536U	CMY-2	160	A/C	CRO GEN CHL TE	S12-01
H559U	CMY-2	138	A/C	CRO GEN CHL TE	S12-01
H144U	CTX-M-55 TEM-1	186	A/C	CRO GEN CHL TE	S12-01
A291U	CTX-M-55	57	FII	CRO	S12-01
H405U	CMY-2	177	A/C	CRO GEN CHL TE	S13-01

Table 32. The Result of RFLP using *ScaI*, plasmid sizes, Inc groups, the antimicrobial susceptibility profiles, and resistance mechanisms. The number of isolates with alphabet U represents their transconjugants. Ceftriaxone, CRO; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE



Transconjugant	Beta-lactamase	Transferable plasmid	Inc type	Resistance pattern	RFLP type
H612U	CTX-M-55	70	FII	CRO GEN CHL	H01-01
A291U	CTX-M-55	57	FII	CRO	H02-01
H81U	CTX-M-14 TEM-1	38	UT	CRO	H03-01
H405U	CMY-2	177	A/C	CRO GEN CHL TE	H04-01
H400U	CMY-2	170	A/C	CRO GEN CHL TE	H04-01
H546U	CMY-2	156	A/C	CRO GEN CHL TE	H05-01
H417U	CMY-2	144	A/C	CRO GEN CHL TE	H05-01
H513U	CMY-2	137	A/C	CRO GEN CHL TE	H05-01
H503U	CMY-2	133	A/C	CRO GEN CHL TE	H05-01
H559U	CMY-2	138	A/C	CRO GEN CHL TE	H06-01
H579U	CMY-2	160	A/C	CRO GEN CHL TE	H06-01
H542U	CMY-2	148	A/C	CRO GEN CHL TE	H06-01
H480U	CMY-2	207	A/C	CRO GEN CHL TE	H06-01
H580U	CMY-2	103	A/C	CRO GEN CHL TE	H06-01
H536U	CMY-2	160	A/C	CRO GEN CHL TE	H06-01
H395U	CMY-2	137	A/C	CRO CHL TE	H07-01
H480U	CMY-2	207	A/C	CRO GEN CHL TE	H07-02

Table 33. The Result of RFLP using *Hind*III, plasmid sizes, Inc groups, the antimicrobial susceptibility profiles, and resistance mechanisms. The number of isolates with alphabet U represents their transconjugants. Ceftriaxone, CRO; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE



Transconjugant	Beta-lactamase	Transferable plasmid	Inc type	Resistance pattern	RFLP type
H400U	CMY-2	170	A/C	CRO GEN CHL TE	HC01-01
H552U	CMY-2	164	A/C	CRO GEN CHL TE	HC01-02
H503U	CMY-2	133	A/C	CRO GEN CHL TE	HC01-03
H543U	CMY-2	132	A/C	CRO GEN CHL TE	HC02-01
H544U	CMY-2	133	A/C	CRO GEN CHL TE	HC02-02
H580U	CMY-2	103	A/C	CRO GEN CHL TE	HC03-01
H382U	CMY-2	152	A/C	CRO GEN CHL TE	HC04-01
H559U	CMY-2	138	A/C	CRO GEN CHL TE	HC04-01
H417U	CMY-2	144	A/C	CRO GEN CHL TE	HC05-01
H522U	CMY-2	164	A/C	CRO GEN CHL TE	HC05-01
H524U	CMY-2	180	A/C	CRO GEN CHL TE	HC05-02
H579U	CMY-2	160	A/C	CRO GEN CHL TE	HC06-01

Table 34. The Result of RFLP using *Hinc*II, plasmid sizes, Inc groups, the antimicrobial susceptibility profiles, and resistance mechanisms. The number of isolates with alphabet U represents their transconjugants. Ceftriaxone, CRO; gentamicin, GEN; chloramphenicol, CHL; tetracycline, TE

APPENDIX E

DNA CODON

One- and Three-Letter symbols for the amino acids

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Trytophan
Y	Tyr	Tyrosine
Z	Glx	Gln or Glu

APPENDIX F

SALMONELLA SEROTYPE

Serogroup	Serotype
B	Agona
	Brandenburg
	Derby
	Heidelberg
	I 4,[5],12:i:-
	Kiambu
	Paratyphi B
	Reading
	Saintpaul
	Sandiego
	Schwarzengrund
	Stanley
	Typhimurium
C1	Bareilly
	Braenderup
	Choleraesuis
	Hartford
	Infantis
	Mbandaka
	Montevideo
	Ohio
	Oranienburg

	Tennessee
	Thompson
	Virchow
C2	Blockley
	Hadar
	I 8,20:-:z6
	Litchfield
	Manhattan
	Muenchen
	Newport
C3	Kentucky
D1	Berta
	Dublin
	Enteritidis
	Javiana
	Panama
	Typhi
E1	Anatum
	Meleagridis
	Muenster
	Uganda
E4	Senftenberg
F	Rubislaw

G1	Poona
	Worthington
G2	Cubana
	Havana
	Mississippi
K	Cerro
	IIIa 18:z4,z32:-
L	Minnesota
O	Adelaide
R	Johannesburg

VITA

Miss Sirirat Luk-in was born on April 29, 1985 in Bangkok, Thailand. She graduated with the Bachelor degree of Science (Medical Technology) from the Faculty of Allied Health Sciences, Chulalongkorn University in 2006. She graduated with the Master degree of Science from Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University in 2010. She is currently a PhD student in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2012.