ลักษณะทางอณูชีววิทยาของการดื้อยาและปัจจัยก่อความรุนแรงของเชื้อแซลโมเนลลา เอนเทอริกาที่แยกได้จากกระบวนการผลิตสุกรและคนในเขตภาคตะวันออกเฉียงเหนือและพื้นที่ชายแดนไทย-ลาว

นางนวลอนงค์ สินวัต

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

Molecular characteristics of antimicrobial resistance and virulence factors of *Salmonella enterica* isolated from pig production and humans in Northeastern Thailand and Thailand-Laos border area

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Public Health
Department of Veterinary Public Health
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2016

Thesis Title	Molecular characteristics of antimicrobial resistance and virulence factors of *Salmonella enterica* isolated from pig production and humans in NortheasternThailand and Thailand-Laos border area
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นวลอนงค์ สินวัต : ลักษณะทางอณูชีววิทยาของการดื้อยาและปัจจัยก่อความรุนแรงของเชื้อแซลโมเนลลาเอนเทอริกาที่แยกได้จากกระบวนการผลิตสุกรและคนในเขตภาคตะวันออกเฉียงเหนือและพื้นที่ชายแดนไทย-ลาว (Molecular characteristics of antimicrobial resistance and virulence factors of *Salmonella enterica* isolated from pig production and humans in NortheasternThailand and Thailand-Laos border area) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร.รุ่งทิพย์ ชวนชื่น, หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาคุณลักษณะการดื้อยาและปัจจัยที่เกี่ยวข้องกับความรุนแรงของเชื้อแซลโมเนลลาที่แยก ได้จากกระบวนการผลิตสุกรและคนในเขตจังหวัดภาคตะวันออกเฉียงเหนือของประเทศไทยและจังหวัดในเขตพื้นที่ชายแดนไทย-ลาว การศึกษานี้ประกอบด้วย 3 โครงการวิจัย ได้แก่ โครงการวิจัยที่ 1 อุบัติการณ์และลักษณะทางอณูชีววิทยาของเชื้อแซลโมเนลลาดื้อต่อยาหลายชนิดพร้อมกันที่แยกได้จากสุกร เนื้อสุกร และคนในพื้นที่ชายแดนไทย-ลาว โดยจำนวนตัวอย่างทั้งสิ้น 1,187 ตัวอย่างเป็นตัวอย่างที่แยกได้จากสุกร ซากสุกร และคนงานในโรงฆ่าสัตว์ เนื้อสุกรและคนขายเนื้อสัตว์ ในตลาดค้าปลีก และผู้ป่วยจากโรงพยาบาลในจังหวัดที่มีพื้นที่ชายแดนติดต่อกัน ได้แก่ จังหวัดหนองคาย อุบลราชธานี มุกดาหารของประเทศไทย และจังหวัดเวียงจันทน์ และสะหวันนะเขตของประเทศลาว ในระหว่างปี 2556-2557 พบ 469 ตัวอย่าง (39.5%) ให้ผลบวกต่อเชื้อแซลโมเนลลาจากจำนวนตัวอย่างทั้งหมดที่เก็บจากพื้นที่ไทยลาว ซีโรวาร์ที่พบมากในพื้นที่ฝั่งไทย คือ *S*. Typhimurium (32.9%) และ *S*. Rissen (20.3%). ซีโรวาร์ที่พบมากในพื้นที่ฝั่งลาว คือ *S.* Anatum (24.1%) และ *S*. Typhimurium (20.5%) เชื้อส่วนใหญ่ (98.2%) สามารถดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกันได้ (multidrug resistance, MDR) Class 1 integrons ที่มี gene cassette array ชนิด *dfrA12-aadA2* ถูกพบมากที่สุด (19.2%) พบการปรากฏของยีน *qnrB*, *qnrS*และ *aac(6')-Ib-cr* ที่ 0.9%, 6.4%, 0.2% ของเชื้อจากพื้นที่ไทยลาวตามลำดับ พบเชื้อแซลโมเนลลาที่ให้ผลบวกต่อ ESBL และมียีน $bla_{ctx\text{-}M14}$ (2.4 %) จากเชื้อที่แยกได้จากสุกรในจังหวัดมุกดาหารประเทศไทย โครงการวิจัยที่ 2 ทำการศึกษาลักษณะทางอณูชีววิทยาของการดื้อยาของเชื้อแซลโมเนลลา เอนเทอริกาที่แยกได้จากเนื้อสุกร เนื้อไก่ และคนในเขตภาคตะวันออกเฉียงเหนือ ประเทศไทย โดยเชื้อแซลโมเนลลาจำนวน 221 isolates ที่แยกได้จากคน เนื้อสุกรและเนื้อไก่จากพื้นที่ 5 จังหวัดในเขตภาคตะวันออกเฉียงเหนือ (กาฬสินธุ์ ขอนแก่น เลย หนองคาย ร้อยเอ็ด) ระหว่างปี 2553-2554 ถูกนำมาใช้ในการศึกษานี้ ซีโรวาร์ที่พบมาก คือ *S*. Rissen (15.8%) และ *S*. Anatum (14.9%) เชื้อส่วนใหญ่ (95.9%) สามารถดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกันได้ พบ gene cassette array ชนิด *dfrA12-aadA2* มากที่สุด (40%) นอกจากนี้พบ SGI-1 like gene cluster ในเชื้อ *S*. Anatum ที่แยกได้จากเนื้อสุกร การเปลี่ยนแปลงแบบ Single amino acid substitutions ที่ตำแหน่ง Ser83 และ Asp 87 ในโปรตีน GyrA พบมากที่สุดในเชื้อที่ดื้อต่อยาซิโปรฟลอกซาซิน (1.8%). สำหรับยีนบนพลาสมิดที่เกี่ยวข้องกับการดื้อต่อยาควิโนโลน ตรวจพบยีน *qnrB* และ *qnrS* ที่ 1.8% และ 4% ของเชื้อทั้งหมด ตามลำดับ การปรากฏของยีน *spvC*, *pefA* และ *rck* ถูกตรวจพบที่ 8.1%, 1.8% and 1.4% ของเชื้อที่มาจากภาคตะวันออกเฉียงเหนือ ตามลำดับ และโครงการวิจัยที่ 3 การศึกษาการกลายพันธุ์ที่ตำแหน่ง quinolone resistant determining regions ของยีน DNA gyrase and topoisomerase IV ของเชื้อแซลโมเนลลา เอนเทอริกาดื้อต่อยานาลิดิซิก แอซิดที่แยกได้จากเนื้อไก่ เนื้อสุกรและคน เชื้อแซลโมเนลลา เอนเทอริกาที่ดื้อต่อยานาลิดิซิก แอซิด จำนวน 28 isolates แยกมาจากเนื้อไก่ เนื้อสุกร และคน ตรวจหาการกลายพันธุ์ในตำแหน่ง the quinolone resistant determining regions (QRDRs) ของยีน *gyrA*, *gyrB*, *parC* และ *parE* พบการเปลี่ยนแปลงของกรดอะมิโนแบบ single point mutation จำนวน 4 จุด (C248A, C248T, G259T, G259A) ซึ่งทำให้เกิดการเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง Ser83Tyr, Ser83Phe, Asp87Tyr และ Asp87Asn บนโปรตีน GyrA ของเชื้อแซลโมเนลลาดื้อต่อยานาลิดิซิก แอซิดจำนวน 11 isolates พบการเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง Ser 83 มากที่สุด (28.5%) ไม่พบการกลายพันธุ์บนยีน *gyrB, parC* และ *parE*. จากผลการศึกษาพบอุบัติการณ์ของเชื้อแซลโมเนลลาชนิด multidrug resistant ในอัตราที่สูงจากตัวอย่างที่มาจากคน สัตว์ที่ใช้เป็นอาหารและผลิตภัณฑ์จากสัตว์ ในพื้นที่จังหวัดของประเทศไทยและลาว แสดงให้เห็นว่ามีแพร่การกระจายและหมุนเวียนของยีนดื้อยาเกิดขึ้น ดังนั้นการเฝ้าระวังเชื้อดื้อยาอย่างต่อเนื่องมีความจำเป็นทั้งในระดับชาติ และนานาชาติ

ภาควิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5475404031 : MAJOR VETERINARY PUBLIC HEALTH
KEYWORDS: ANTIMICROBIAL RESISTANCE / LAOS PDR / SALMONELLA ENTERICA / THAILAND

NUANANONG SINWAT: Molecular characteristics of antimicrobial resistance and virulence factors of *Salmonella enterica* isolated from pig production and humans in NortheasternThailand and Thailand-Laos border area. ADVISOR: ASSOC. PROF. RUNGTIP CHUANCHUEN, D.V.M.,M.Sc.,Ph.D., pp.

In this study, we aimed to characterize antimicrobial resistance and virulence factors in the *Salmonella* isolates from pig production and humans in Northeastern provinces of Thailand and Thailand-Laos border provinces. This study comprises three projects. Project 1 demonstrated high prevalence and molecular characteristics of multidrug resistant *Salmonella* in pigs, pork and humans in Thailand-Laos provinces. A total of 1,187 samples were collected from pigs, pig carcasses and workers in slaughterhouses; pork and butchers in retail markets and patients in hospitals in the provinces with cross border points including NongKhai, Ubon Ratchathani, Mukdahan of Thailand and Vientiane and Savannakhet in Laos during 2013-2014. Among these samples, 469 samples (39.5%) were positive to *Salmonella enterica.* The predominant serovars in Thailand provinces were *S*. Typhimurium (32.9%) and *S*. Rissen (20.3%). In Laos isolates, the predominant serovars were S. Anatum (24.1%) and *S*. Typhimurium (20.5%). Most isolates (98.2%) were multidrug resistance (MDR). Class 1 integrons carrying *dfrA12-aadA2* gene cassette array were most commonly observed (19.2%). The presence of *qnrB*, *qnrS* and *aac(6')-Ib-cr* was identified in 0.9%, 6.4%, 0.2% of the isolates, respectively. ESBL-producing *Salmonella* strains carrying $bla_{\text{CTX-M14}}$ (2.4%) were identified in pig samples from Mukdahan province, Thailand. Project 2 described characterization of antimicrobial resistance in *Salmonella enterica* isolated from pork, chicken meat and humans in Northeastern Thailand. A total of 221 *Salmonella* isolates obtained from humans, raw pork and raw chicken in five Northeastern provinces of Thailand (Kalasin, Khon Kean, Loei, Nong Khai, Roi Et) during 2010-2011 were used in this project. The predominant serovars were *S*. Rissen (15.8%) and *S*. Anatum (14.9%). Most isolates (95.9%) were MDR. The *dfrA12-aadA2* gene cassette array was commonly identified in the isolates from Northeastern region (40%). SGI-1 like gene cluster was observed in a serovar Anatum from pork. Single amino acid substitutions at position Ser 83 and Asp 87 in GyrA were most commonly observed in ciprofloxacin-resistant strains (1.8%). Of the plasmid-mediated quinolone resistance genes, only *qnrB and qnrS* genes were detected in 1.8% and 4% of all *Salmonella* isolates, respectively. The presence of *spvC*, *pefA* and *rck* was identified in 8.1%, 1.8% and 1.4% in the isolates, respectively. Project 3 described mutations in quinolone resistance-determining region in DNA gyrase and topoisomerase IV genes in nalidixic–acid resistant *Salmonella enterica* isolated from chicken meat, pork and humans. Twenty-eight nalidixic acid-resistant *Salmonella* isolates originated from chicken meat, pork and humans were examined for mutation in the quinolone determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes. Four single-point mutations in *gyrA* (i.e. C248A, C248T, G259T, G259A) leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr and Asp87Asn in GyrA, respectively, were identified in 11 nalidixic acid-resistant strains. Amino acid change at position Ser83 was most frequently identified (28.5%). No mutations were observed in *gyrB, parC* and *parE*. Overall, our findings demonstrated the high prevalence of MDR *Salmonella* in humans, food-producing animals and their products in Thailand and Laos provinces. The circulation of their resistance determinants was highlighted in this study. The results indicate the need continuing antimicrobial resistance monitoring at nation, regional and global levels.

Department: Veterinary Public Health Student's Signature .....
Field of Study: Veterinary Public Health Advisor's Signature .....
Academic Year: 2016

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere appreciation and gratitude to my advisor Assoc. Prof.Dr. Rungtip Chuanchuen for her thoughtful guidance and continuous support during my entire Ph.D.program, for her patience and advice during the writing of this dissertation. Her advice on research as well as on my student life has been invaluable.

I would like to thank my thesis committee members, Prof. Dr. Alongkorn Amonsin, Assoc. Prof. Dr. Suphachai Nuannualsuwan, Dr. Taradon Luangtongkum and Assoc. Prof. Dr. Sunpetch Angkititrakul for their comments and advice.

I am grateful to all staffs and friends in Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for all their support and help during my research.

I would like to thank Royal Golden Jubilee PhD program (Thailand Research Fund) and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) for financial support.

Finally, and most importantly, I would like to thank my family, especially my husband and my daughter for their patience and unconditional support throughout my life and my studies.

CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP	ampicillin
Bp	base pair(s)
^{o}C	degree (s) Celcius
CAZ	ceftazidime
CHL	chloramphenicol
CEF	cefoperazone
CIP	ciprofloxacin
CPO	cefpodoxime
CTX	cefotaxime
CS-PCR	conserved-segment polymarse chain reaction
DNA	deoxyribonucleic acid (s)
GEN	gentamicin
Kb	kilobase (s)
IP	integrons profile
MIC	minimal inhibitory concentration
RFLP	restriction fragment-length polymorphism
STR	streptomycin
SPC	spectinomycin
SUL	sulfamethoxazole
TET	tetracycline
TRI	trimethoprim

CHAPTER I

1.1 Importance and Rationale

Salmonellosis is one of the most important food-borne diseases caused by *Salmonella enterica.* Over 90 million cases of gastroenteritis caused by this pathogen occur worldwide each year (Majowicz et al., 2010). In the United States, there are approximately 1 million cases of non-typhoidal *Salmonella* report every year (Scallan et al., 2011). According to the European Union report, salmonellosis was also observed in humans and 88,715 salmonellosis cases were confirmed in EU countries in 2014 (EFSA, 2015) In Southeast Asia, the reports on the prevalence of *Salmonella* infection are still limited in some countries and there is still a lack of the official report at the regional level. However, it has been estimated that around 22.8 million cases were infected with *Salmonella* and 37,600 deaths each year in Southeast Asia (Majowicz et al., 2010).

Antimicrobial agents are commonly used to treat *Salmonella* infection in humans and animals. In the last two decades, emergence of antimicrobial resistance in *Salmonella* has significantly increased worldwide, including the countries in Southeast Asia (Miko et al., 2005).The extensive use of antimicrobials in both humans and veterinary medicine is a significant factor contributing to the development of antimicrobial resistance (AMR) in *Salmonella*. This phenomenon has become a serious public health concern because *Salmonella* can develop resistance to many antibiotics simultaneously, so called multidrug resistance (MDR). The prevalence of MDR-*Salmonella* has rapidly increased over the past decade in many countries (Casin et al., 1999) because resistance genetic traits can transfer and exchange among

bacterial population by horizontal or vertical transfer. Resistant bacteria developing in humans, animals or the environment can disseminate from one species to the other or from one geographic location to another. AMR problem does not recognize geographical, humans, animals or ecological border (WHO, 2015).

Therefore, "one health" concept that recognized as multi-sectoral responsible for health of humans, animals and the environmental system can help to develop keys for control and prevention strategies for AMR at national and international level (WHO, 2015).

Thailand is located on the mainland of Southeast Asia region which shares land borders with four neighboring countries including Lao PDR, Cambodia, Myanmar and Malaysia. Thailand and Laos boundary is the longest common border that is approximately 1,800 kilometres in length and covers 5 provinces of Northern region (Chiang Rai, Payao, Nan, Uttaradit and Phitsanulok) and 6 provinces of Northeastern regions of Thailand (Loei, Nong Khai, Nakhon Phanom, Mukdahan, Amnat-Charoen, and Ubonratchathani). Cross-border trade has been promoted by Thai government. The border trade market has boomed and yielded massive economic benefits. This leads to development of land transportation between Thailand and Laos that facilitates travel and movement across the border for both humans and animals (Supatn, 2012). Thailand is an important agriculture country in Southeast Asia. The livestock industry including pigs and poultry products is one of agriculture sector which has a significant impact on Thailand's economic growth (Charoensook et al., 2013). Thailand is also the important exporter of livestock products to Lao PDR (OAE, 2015). In 2015 total cross-border trade values from Thai-Laos were 130,000 million baht of which 6,720 million baht was generated from livestock products (DFT, 2015)

and the highest trade value was from Nong Khai, Mukdahan and Ubonratchathani (DFT, 2015).

Exports from Thailand to Laos are mostly higher than those from Laos to Thailand (Supatn, 2012). Among livestock-related merchandises, pigs and pork have been exported from Thailand to Laos because the domestic production in Laos is insufficient for the increasing demand (Polly et al., 2009). In Laos, fattened pigs are usually grown from piglets imported from Thailand by either commercial-pig producing companies or individual traders (Polly et al., 2009). Live pigs including piglets and fattening pigs are usually exported from Thailand to Laos due to the higher price at Laos. Concurrently, some pigs are smuggled through the border and slaughtered illegally in poor hygienic slaughterhouses. Such illegal movement of pigs and pork products occurs repeatedly and is easily possible despite intense investigation. This is mainly due to the typical structure of the long border line of Thailand and *Laos* that is formed by the Mekong River (Supatn, 2012). However, price of Thai pigs has been increased since 2008. This made importations of piglets from Thailand to Laos less attractive and resulted in increasing numbers of piglets sold from Laos to Thailand (Polly et al., 2009). Pork is commonly shifted from Laos to sell in Thai border as well. Frozen suckling piglets are frequently transported in refrigerated trucks by Vietnamese traders from Vietnam through Savannakhet province, Laos. Some of them are sold in Laos and the remainders will be continuously shipped to Thailand (Polly et al., 2009).

It is evident that pigs serve as a reservoir of *Salmonella enterica* (Mughini-Gras et al., 2014).Therefore, legal and illegal cross-border movement of pigs and pig products between Thai-Laos boundary may promote distribution of AMR *Salmonella*

and their resistance determinants. In addition, the movement of people or international travelling may also a major risk factor leading the rapid dissemination and circulation of this pathogen in this region. Concurrently, this evidence is not limited to the border area. This could adversely affect other parts of two countries and even other neighboring countries.

Addressing the rising public health risk on antimicrobial resistance in *Salmonella,* monitoring of antimicrobial resistance phenotype and genotype in *Salmonella* is required to provide a useful data for future strategies of control and prevention program for Thailand- Laos and other countries. However, data on the current situation and genotypic characteristic of AMR *Salmonella* isolated from these countries is still limited. Therefore, the aim of this study was to investigate the prevalence and characterize of AMR in *Salmonella* isolates from pigs, pork and humans in the Thailand-Laos border provinces. The obtained data will be the first report on the situation of AMR in Thailand-Laos border area and reinforce awareness concerning the use of antimicrobials in humans and livestock animals in Asian country.

The objectives in this study have been accomplished. The research was conducted as planned with the successful results and part of dissertation has been published in international journals (Appendix A).

1.2 Keywords

Keywords

Antimicrobial resistance, Lao PDR, Pigs, *Salmonella enterica*, Thailand

1.3 Literature Review

1.3.1 General characteristics of *S. enterica*

Salmonella species is a gram-negative bacterium and divided into two main species ; *Salmonella enterica* and *Salmonella bongori*. *S. enterica* can be classified into 6 subspecies and *S. enterica* subspecies *enterica* are mostly associated with human and animal infection or known as food-borne pathogens. Classification of *Salmonella* strains is based on O antigens (lipopolysaccharide, LPS) and H antigens (flagella protein) according to the Kauffmann–White scheme. There are over 2,500 serovars recognized (Jones et al., 2008).

1.3.2. *S. enterica* infection

Salmonella enterica is a food-borne pathogen and currently a major public health problem worldwide. Enteric disease associated with non-typhoidal *Salmonella* infection is commonly found in human and clinical signs such as fever, nausea, vomiting, diarrhea and acute septicemia may occur. The incubation period differs from 4 hrs to 72 hrs following ingestion of contaminated food or contact with animals (Chen et al., 2013). Animals, especially food animals, play an important role as reservoir for dissemination of *Salmonella* to humans.

In general, *Salmonella* infection in pigs is asymptomatic except *S*. Cholereasuis. *Salmonella* colonize tonsil and gut systems of healthy pigs that serve as *Salmonella* carriers and sources of pig carcasses contamination during slaughtering process such as unhygienic slaughtering process, improper re-packaging or grinding process at the retail market. (Mürmann et al., 2009; Baer et al., 2013).

1.3.3. Antimicrobial resistance in *S. enterica*

Salmonella enterica usually causes self-limiting gastrointestinal illness. Therefore, antimicrobial treatment may not be necessary. However, antimicrobial treatment is required in patients with life-threatening infection. MDR *Salmonella* in human has been increasingly reported (Vo et al., 2010; Boxstael et al., 2012; Perez-Moreno et al., 2013). Fluoroquinolones and third-generation of cephalosporin are drugs of choice for *salmonella* infection in human (Hur et al., 2012). Recently, resistance rates to these antimicrobials significantly increase worldwide and several relevant resistance mechanisms including QRDR, PMQR and ESBLs have been observed (Hur et al., 2012).

In food animal production, the purposes of antimicrobial use include infection treatment, disease prevention and growth promotion. Antimicrobial agents are commonly mixed in feed and distributed to animals in large amounts. Such extensive use of antimicrobials may lead to development and distribution of antimicrobial resistance in bacteria that could be transferred to human via food chain (Molbak, 2005). Currently, use of antibiotic growth promoters in livestock have been banned in many countries including European Union and Thailand (Angulo et al., 2004).

Antimicrobial resistance in *Salmonella* isolated from pigs especially resistance to traditional antimicrobials such as tetracycline, ampicillin, streptomycin has been increasingly reported (Kim et al., 2011; Wannaprasat et al., 2011; Bolton et al., 2013) . While link between antimicrobial resistance in food animals and humans was previously described, pigs play a major role as reservoir for MDR *Salmonella* (Molbak et al., 1999; Angulo et al., 2004). Cross-

contamination among pigs and pig carcasses in slaughterhouse could promote with spread of antimicrobial resistance from pigs to humans. One of the evidences was the outbreak of quinolone-resistant *Salmonella* Typhimurium DT104 in Denmark in 1998. It was revealed that pork served as a major source of the resistant *Salmonella* in the outbreak (Molbak et al., 1999).

In Thailand, MDR-*Salmonella* have been frequently observed in humans, pigs and pig products. MDR-*Salmonella* isolated from patients and pork was resistant to different antimicrobials classes. (e.g. ampicillin, gentamicin, tetracycline, trimethoprim, streptomycin and spectinomycin) (Wannaprasat et al., 2011). Data on MDR-*Salmonella* in Northeastern Thailand were reported for example all *Salmonella* isolates from patients, pork and chicken meat in Khon Kean province resistant to ampicillin, chloramphenicol, gentamicin, sulfamethoxazole-trimethoprim, tetracycline, streptomycin and sulfamethoxazole (Angkititrakul et al., 2005). For recent report, *Salmonella* isolates from swine and pork in Sa kaew province were highly resistant to tetracycline (69%), ampicillin (50%), sulfamethoxazol-trimethoprim(36%) and streptomycin (31%) (Pulsrikarn et al., 2012).

Data on MDR-*Salmonella* in Laos is limited. The first related report was *Salmonella* isolated from buffaloes and pigs in Vientiane in 2008. The study showed that *Salmonella* isolates from pigs were resistant to ampicillin, amoxicillin-clavulanic acid, chloramphenicol, nalidixic acid, tetracycline, streptomycin and sulfamethoxazole-trimethoprim (Boonmar et al., 2008).The latest study reported that *Salmonella* isolated from meat including beef, pork

and buffalo meat in Pakse, Champasak Province exhibited resistance to ampicillin, tetracycline and streptomycin (Boonmar et al., 2013).

1.3.4 Genetic of antimicrobial resistance in *S. enterica*

1.3.4.1 Class 1 integrons

Integrons are genetic DNA elements. Their significance in antimicrobial resistance arises from possession gene cassettes, particularly antimicrobial resistance genes in variable region. Of all integrons type, class 1 integrons are the most commonly found type in *Enterobacteriaceae* including *Salmonella*. These genetic elements can be located on plasmid and associated with widespread of resistance genes in bacteria by horizontal transfer. Many resistance-gene cassettes can integrate into variable region of the same integrons. This links to co-selection for many antimicrobial resistance genes simultaneously by a single antibiotic (Chuanchuen et al., 2008). Existing data supports that integrons play a major role in spreading of MDR *Salmonella* (Hsu et al., 2006; Meng et al., 2011).

Class 1 integrons consist of two conserve segments (5' and 3' CS). The 5' conserve segment contains an integrase (Klionsky et al.) gene with recombination site (*attI*). The 3' conserve segment consist of *qacEΔ1*, *sul1 and orf5* encoding quaternarium ammonium compound resistance, sulphonamide resistance and unknown function, respectively (Fluit and Schmitz, 2004). Variable region is located between 5' and 3' conserve segments and usually carries resistance gene cassettes. Downstream of each resistance gene cassette contains a short sequence or 59-base element or *attC* site where a new resistance cassette inserted (figure 1).

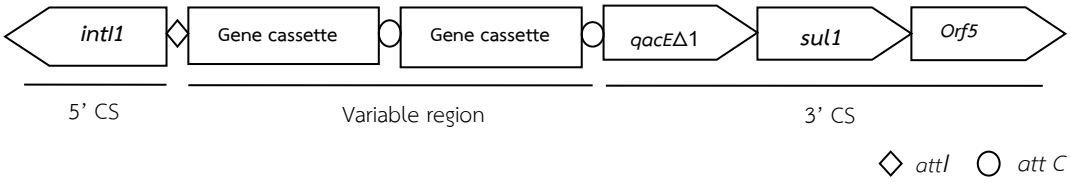


Figure 1 Structure of class 1 integrons. Class 1 integrons comprise two conserve segments (5' and 3' CS). Variable region is located between 5' and 3' conserve segments and usually carries resistance gene cassettes. *intI1*, integrase 1 gene; *attI*, recombination site; *qacEΔ1*, quaternary ammonium compound resistance; *sul1*, sulphonamide resistance; *orf5,* unknown function; *attC*, 59-base element.

1.3.4.2 Salmonella Genomic Island 1 (SGI1)

Class 1 integrons can integrate into *S. enterica* chromosome, particularly in *Salmonella* Genomic Island 1 (SGI1). SGI1 is a 43-kb genomic island located between site-specific of 3' end of *thdF* (*attB*) and *int2* that was firstly found in *S.* Typhimurium. SGI1 and its variants in other *Salmonella* serovar are located between of 3' end of *thdF* and *yidY* (Doublet et al., 2005). SGI1 region consists of complex class 1 integrons with multiple antimicrobial resistance gene clusters (Mulvey et al., 2006).

In addition, SGI1 can excise by itself into extrachromosomal circular form. This form can be transferred from SGI1 *Salmonella* strain to non-SGI1 *Salmonella* or *Escherichia coli* by conjugative mobilization (Doublet et al., 2005). Therefore, SGI1 plays a role in spreading of antimicrobial resistance clusters among bacterial population.

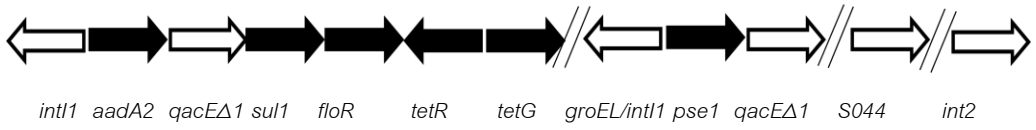


Figure 2 Structure of *Salmonella* genomic island 1 (SGI1). SGI1 is a 43-kb genomic island located between site-specific of 3' end of *thdF* and i*nt2* or *yidY*. Complex of class 1 integrons is located on 13-kb segment near 3' end of SGI1. Black arrows represent antimicrobial resistance genes. (Mulvey *et al*., 2006).

1.3.4.3 Quinolone resistance

Quinolone resistance is usually associated with chromosomal mutations in *gyrA* and *parC.* These mutations occur in Quinolone Resistance Determining Region (QRDR). Recently, plasmid-mediated quinolone resistance was shown to play an important role in fluoroquinolone resistance and has been increasingly emerged worldwide (Stephenson et al., 2010).

1. Mutation in *gyrA* and *parC*

Mutations in both DNA gyrase and topoisomerase IV are common resistance mechanisms to quinolones in bacteria. Topoisomerase II (DNA gyrase) and Topoisomerase IV are enzymes for DNA replication of bacteria. DNA gyrase is a tetramer composing two GyrA and two GyrB subunits, which encoded by *gyrA* and *gyrB*, respectively. Topoisomerase IV contain two subunits (ParC and ParE) encoded by *parC* and *parE*, respectively (Blondeau, 2004). Quinolones form drug-enzyme-DNA-complex, resulting in inhibition of bacterial DNA synthesis. DNA gyrase is a major target enzyme for quinolone action in gram-negative bacteria, while topoisomerase IV tends to be a major target in gram-positive bacteria (Hopkins et al., 2005). In *Salmonella*, mutations in *gyrA* is usually involved in quinolone resistance. These mutations are usually clustered between amino acid 67 and 122 in quinolone resistance determining region (QRDR). The mutations in this region lead to reduced drug affinity for enzyme-DNA complex (Seminati et al., 2005). A single amino acid substitution at Serine 83 or Aspartate 87 is most frequently observed in *gyrA* (Griggs et al., 1996). Mutation of *parC* at codon 80 is commonly observed. A study showed that mutation in *parC* alone did not

affect quinolone susceptibility level. The *Salmonella* strain harboring both of *parC* and *gyrA* mutation exhibited high-level of quinolone resistance (Hopkins et al.,2005).

2 Plasmid-mediated quinolone resistance (PMQR)

Recent studies described plasmid-mediated quinolone resistance (PMQR) and demonstrated that these plasmids contribute to quinolone resistance and are involved in distribution of fluoroquinolone resistance genes by horizontal transfer. The common genes on PMQR include *qnr*, aac *(6')-Ib-cr* and *qepA*. Qnr is a pentapeptide protein that protects DNA gyrase from quinolone inhibition and confers decrease susceptibility to nalidixic acid (Strahilevitz et al., 2009). AAC (6')-Ib-cr is a variant aminoglycoside acetyltransferase that can reduce ciprofloxacin activity. QepA is a plasmid mediated-quinolone efflux pump that increases the level of fluoroquinolone resistance including ciprofloxacin, norfloxacin and enrofloxacin (Yamane K et al., 2007).

1.3.4.4 Extended-Spectrum ß Lactamases

ESBLs are designed as ß-lactamases that hydrolyze ß-lactam ring of ß-lactams, resulting in pan-resistance to ß-lactams. However, these enzymes may be inhibited by ß-lactamase inhibitors (Paterson and Bonomo, 2005). Plasmid-borne mediated ß-lactamase genes have been observed in *Salmonella* isolated from humans and food-producing animals including pigs and poultry (Riano et al., 2006). Several types of ESBLs have been described, for example Temoniera (TEM) that is commonly found; Sulfhydryl variable (SHV) that is mediated resistance to broad-spectrum penicillins and new

cephalosporin; and CTX-M-types that is capable of hydrolyzing cefotaxime. Some types of CTX-M can hydrolyze ceftazidime and promote resistance to cephalosporin (Sturenburg and Mack, 2003).

1.3.5. *Salmonella* Virulence plasmids

Pathogenicity of *Salmonella* is associated virulence factors and some of virulence genes are plasmid-borne. Virulence plasmids found in *Salmonella* vary from 50 to 285 kb in size (Villa and Carattoli, 2005). Several *Salmonella* serovars such as Choleraesuis, Dublin, Enteritidis, Typhimurium are known to harbor serovar-specific plasmid, of which the most common one is the *spv* operon containing five genes, *spvRABCD* (Chu and Chiu, 2006).In previous study, the *spv* operon increases ability of *Salmonella* to grow in the reticulo endothelial cells from mice and enhances severity of extraintestinal infection (Chu and Chiu, 2006). Plasmid-encoded fimbriae (*pef*) locus carrys four genes including *pefB, pefA, pefC and pefD* and is found in serovars Choleraesuis, Enteritidis and Typhimurium. *S*. Typhimurium harboring *pef* locus could adhere to mouse intestinal epithelium (Baumler et al., 1996). The other virulence plasmid, *rck* gene was located on virulence plasmid of *S*. Typhimurium and *S.* Enteritidis and associated with high-level serum resistance and adhesion to epithelial cell line (Rychlik et al., 2006).

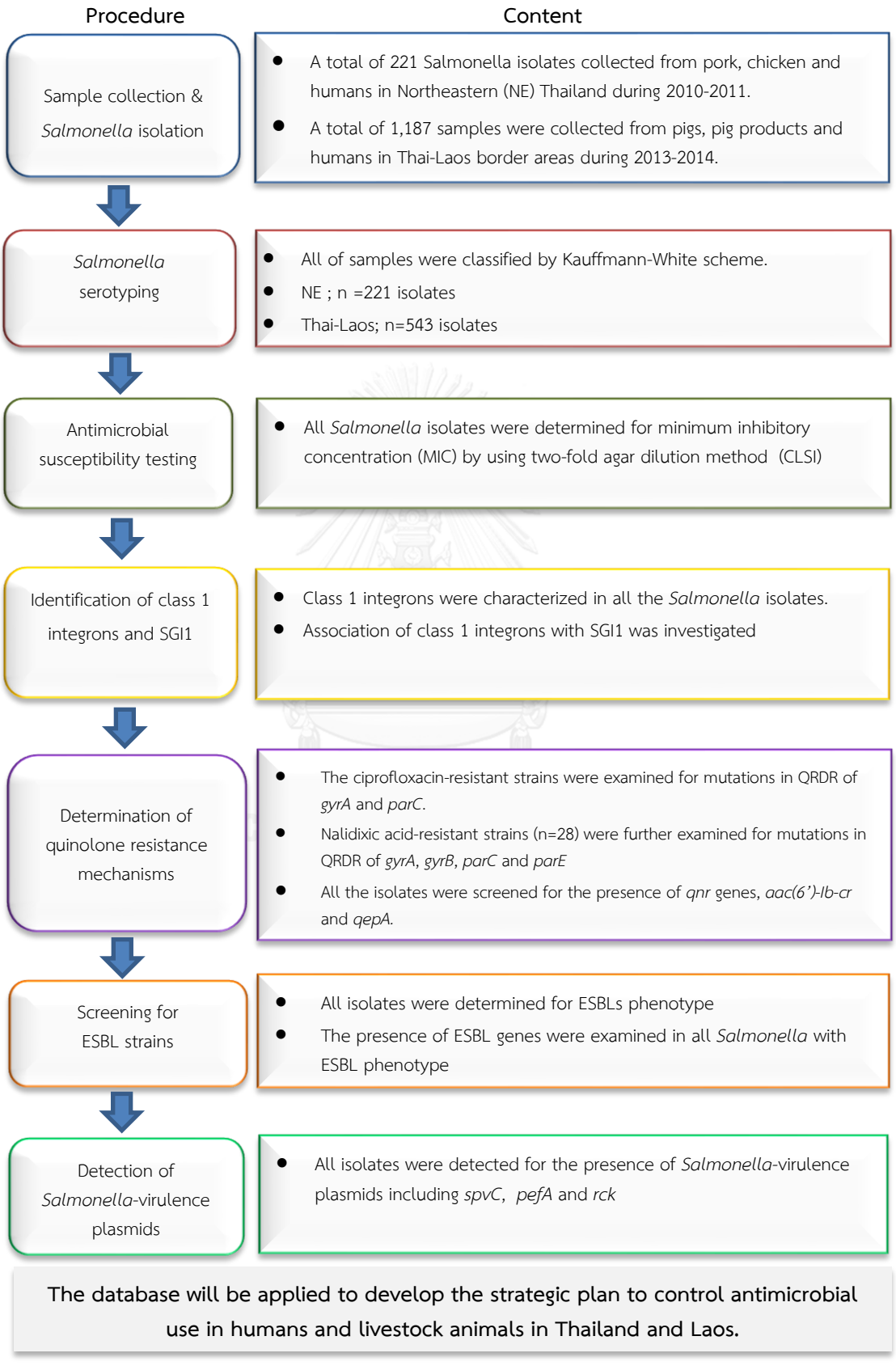
A novel combination between antimicrobial resistance gene and virulence plasmid was identified in *S*. Typhimurium (Guerra et al., 2002; Villa and Carattoli, 2005). Such plasmid evolution was caused by extensive antibiotic use in humans and animals. In this case, a single use of antibiotic

may co-select for both resistant and virulent *Salmonella* strain, resulting in more severe infection and a serious public health issue.

1.4 Research Objectives

1. To monitor antimicrobial resistance among *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area.
2. To characterize of antimicrobial resistance of *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area.
3. To examine plasmid-borne virulence factors and their correlation to antimicrobial resistance in *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area.

1.5 Research outline



1.6 Advantages of this study

1.6.1 Novel knowledge

- Occurrence and antimicrobial resistance rate of *Salmonella* isolated from humans, pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area were obtained.
- Data on genetic characteristics of antimicrobial resistance in *Salmonella* isolated from, pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area were obtained.
- Data on *Salmonella* virulence plasmid in pigs, pig products and humans were reported.
- Links between AMR phenotype/genotype and virulence plasmid in pigs, pig products and humans was obtained.

1.6.2 Application of knowledges

- Information on antimicrobial resistance from this project will be used to support development of guideline for prudent antimicrobial use in animals in Thailand and neighboring countries.
- The data can be used to educate veterinarians and related personnel to raise concern on antibiotic use and minimized antibiotic resistance problems.
- It is to monitor the newly AMR determinants that could emerge at the border of Thailand and neighboring countries.
- The bacterial strains, plasmid and technology can be used for future studies
- The data will be used to support the regulation of animal and animal products movement at the area of Thailand and Laos border.

CHAPTER II

High prevalence and molecular characteristics of multidrug resistant *Salmonella* in pigs, pork and humans in Thailand-Laos provinces

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Journal of Medical Microbiology (2016)

High prevalence and molecular characteristics of multidrug resistant *Salmonella* in pigs, pork and humans in Thailand-Laos provinces

3.1 ABSTRACT

This study aimed to examine occurrence and antimicrobial resistance characteristics of *Salmonella* from pigs, pork and humans in Thailand and Laos provinces. The samples were collected from pigs, carcasses and workers in slaughterhouses; retail pork and butchers in fresh markets and patients in hospitals in Thailand (n=729) and Laos (n=458). A total of 295 of 729 samples (34.6%) collected in Thailand and 253 of 458 (47.4%) samples collected in Laos were positive for *Salmonella*. Five-hundred and forty eight *Salmonella* isolates from Thailand (n=295) and Laos (n=253) were further analyzed. Serovar Typhimurium was the most common serotype in Thai (34%) and Laos (20.6%) samples. Approximately 2.4% of Thai isolates produced extended-spectrum-β-lactamase (ESBL). All the ESBL producers possessed $bla_{\text{CTX-M-14}}$, some of which were horizontally transferred. Class 1 integrons were common in Thai (31.9%) and Laos (39.1%) isolates but none was associated with SGI1. The resistance cassette *dfrA12-aadA2* was the most common while the least common was *aadA2-linG* (n=1). The *dfrA12-aadA2* gene cassette in 5 isolates and *aadA2-linG* were located on conjugative plasmid. Three pork isolates were fluoroquinolone-resistant and carried an amino acid substitute Ser-83-Tyr in GyrA. The *qnrS* gene was found in 7.1% and 5.5% of the Thai and Laos isolates respectively, while *qnrB* was carried in another Laos isolates (1.9%). All ESBL producers carried *qnrS*. In conclusion, MDR *Salmonella* were common in pigs, pork and human samples in this region. The bacteria carried mobile genetic elements and

resistance genes on conjugative plasmids that could be readily transferred to other bacterial species.

Keywords: class 1 integrons, extended-spectrum β-lactamases, Lao PDR, *Salmonella enterica,* Thailand

3.2 INTRODUCTION

Salmonella enterica is a major food-borne pathogen that causes diseases in humans and animals worldwide. According to recent global estimates, 93.8 million cases of nontyphoidal *Salmonella* gastroenteritis occur annually, producing up to 155,000 deaths (Majowicz et al., 2010). In Southeast Asia, It is estimated that 22.8 million cases with 37,600 deaths occurred each year (Majowicz et al., 2010). Currently, this public health concern has become complicated by rapid emergence and spread of *Salmonella* strains resistant to clinically important antimicrobial agents.

Antimicrobial resistance (AMR) in *Salmonella* is often linked to the acquisition of resistance genes associated with mobile genetic elements, particularly class 1 integrons (Partridge et al., 2009). At the same time, extended-spectrum β-lactamase (ESBL) producing-*Salmonella* particularly serovars Typhimurium, have increasingly emerged (Wong et al., 2014). Collectively, the emergence of these resistance characteristics have become a serious public health problem as 3rd -generation cephalosporins are the drugs of choice for treatment of invasive infections or severe diarrhea (EFSA, 2012). Even more concerning, class 1 integrons and ESBL genes are commonly associated with conjugative plasmids that are potentially transferred intra- and interspecies. This could lead to emergence and spread of multidrug-resistant (MDR) *Salmonella* superbugs.

Mainland Southeast Asia comprises 6 countries, including Lao PDR, Cambodia, Myanmar, Thailand, Vietnam, and West Malaysia, which are closely connected to other world regions, including India and China. For countries with land connections,

cross-border economic activities are evident and yield enormous monetary benefits (Paitoonpong, 2006). Due to regional economic integration (known as *ASEAN Economic Community (AEC)),* the rapid growth of *cross-border* trade is expected.

Globalization of travel and commerce has been implicated as a risk factor for the emergence and spread of antimicrobial-resistant bacteria and resistance determinants (Senok et al., 2012). Thailand and Laos share a common border approximately 1,800 km in length with up to 36 regulated-crossing points (Polly et al., 2009). The largest T*hailand/Laos* connecting points are between Nongkhai/Vientiane, Mukdaharn/Sacannakhet and Ubonratchathani/Pakse provinces. Pigs and their meat products are commonly traded commodities between these two countries (Knips, 2004), while unsanctioned or unregulated slaughter facilities still exist. This leads to frequent unhygienic slaughter of pigs in and around the Thailand-Lao PDR border causing a major concern for public health authorities. These concerns are further compounded by the illegal movement of pigs in and out between Thailand and Laos (Cocks et al., 2009). Such legal and illegal border activities, including movement of people, goods and services, could lead to greater risk of cross-border transmission of resistant-bacterial pathogens and resistance determinants (Kaferstein et al., 1997).

The strategic objectives of the AMR global action plan include increasing awareness, knowledge and understanding of AMR (WHO, 2015). Accurate information about the extent and impact of AMR is indispensable for development and evaluation of interventions. However, little is known about the current situation, distribution, diversity, and AMR of *Salmonella* in Southeast Asian

countries. This study was designed to investigate the prevalence of *Salmonella* in pigs, pork and humans in the Thailand-Laos border provinces and to further conduct molecular characterization of AMR in recovered *Salmonella* isolates. The obtained data will strengthen the knowledge and evidence base of AMR in the region and contribute to the objective of the AMR Global Action Plan.

3.3 METHODS

3.3.1 Samples collection

A total of 1,187 samples were collected from Thai (n=729) and Laos (n=458) provinces (Table 1) between October 2013 to September 2014. The number of samples collected was calculated based on a true prevalence of 50%, at 95% confidence level and 10% error. The sampling sites were located in Thailand/Laos connecting provinces including Nongkhai/Vientiane and Mukdaharn/Sacannakhet. The samples were additionally collected in Ubonratchathani, Thailand. Samples were collected 4 times at 3-4 month interval from each sampling site. For each trip, the sampling was performed in both connecting provinces of Thailand and Laos. The sampling sites were selected to be located within 3 hrs (15-20 km) from the shipping carriers to ensure that the samples would arrive in the laboratory within 24 hrs after sampling. Due to logistical constraints, randomized sampling was not possible. The sampling sites included one municipal pig slaughter house, one municipal fresh market and one municipal hospital in each province covering crossing points with the largest volume traffic between Thailand and Laos, including Nongkhai, Mukdaharn and Ubonratchathani provinces of Thailand and

Vientiane and Savannakhet provinces of Laos. In Thailand, the sampled slaughterhouses (n=3) were large-scale facilities with a throughput of 80 or more pigs per day. In Laos, one participating plant was a large-scale pig slaughterhouse with a throughput of 200 or more animals per day, while the others were small-scale with a throughput of 50 or less animals per day. The slaughter facilities and markets were chosen so that animals and their harvested meat could be tracked and sampled at each point in the supply chain.

During processing, pigs were stunned, bleeded, dehaired, eviscerated, broken down into six cuts of meat and transported directly to fresh market. Fecal samples (n=314) were collected from pigs by rectal swab after bleeding but before the scalding process. These feces samples are now termed “the pig samples”. The sample collection was designed to obtain feces comparable to those of the living animals and to avoid the effect of heat generated by scalding hot water on bacteria. It was also to avoid interrupting the slaughtering process because the carcasses were subjected to de-hair instantly after scalding. The carcasses of the sampled animals (n=321) were followed through the slaughter facility and sampled (at least 50 cm^2 area) again after evisceration. The sampled area was the muscle around the neck area. Since the carcass was hung upside down, liquid from other body parts dripped downward to the neck area. Corresponding retail-raw meat samples (n=292) were collected from the same carcasses after arriving at the fresh market. All carcass and retail-raw meat samples were obtained using sterile swabs.

Humans stool samples were collected from workers of the slaughter facilities (n=88) and butchers (n=58) at the retail meat shops participating in this study as well

as from patients with diarrhea (n=114) of local hospitals. The slaughterhouse workers and butchers provided their own stool samples, while nurses or technicians collected samples from patients. Due to low numbers of human samples, efforts were made to obtain most possible participants. Research protocols involving human subjects were approved by the ethics committee, Faculty of Medicine, Khon Kaen University (the authorization ID, HE572136).

3.3.2 *Salmonella* isolation and identification

The *Salmonella* strains were isolated using the standard protocol ISO6579:2002 (E) (ISO, 2002). Five colonies were initially collected from each positive sample and subjected to serotyping by slide agglutination based on the Kaufman-White scheme using commercially available antiserum (S&A Reagents Lab Ltd., Bangkok, Thailand) (Popoff and LeMinor, 1992). One colony of each serotype was collected from each positive sample. All bacterial strains were stored as 20% glycerol stocks at -80°C until further analyzed.

3.3.3 Antimicrobial susceptibility testing and screening for ESBL strains

Antimicrobial susceptibility profiles were examined by determination of Minimum inhibitory concentrations (MICs) using two-fold agar dilution (CLSI, 2013). The antimicrobials and corresponding MIC breakpoints were ampicillin (AMP, 32 µg/ml), chloramphenicol (CHL, 32 µg/ml), ciprofloxacin (CIP, 4 µg/ml), gentamicin (GEN, 8 µg/ml), streptomycin (STR, 32 µg/ml), sulfamethoxazole (SUL, 512 µg/ml), tetracycline (TET, 16 µg/ml) and trimethoprim (TRI, 16 µg/ml). The antimicrobials

were chosen on the basis of their use in the region and also to represent drugs in different classes. Susceptibility to cefotaxime (CTX, 30μg), cefpodoxime (CPO, 10μg) and ceftazidime (CAZ, 30μg) was detected using the disk diffusion method (CLSI, 2013). Isolates resistant to at least one cephalosporin antibiotic were subsequently confirmed for ESBL production using the combination disk method, which compared the inhibition zones created by cefotaxime and cefotaxime (30μg)/clavulanic acid (10μg) disks as well as ceftazidime and ceftazidime (30μg)/clavulanic acid (10μg) disks (Oxoid, Hamshire, England). A difference of ≥5 mm between the inhibition zone of the clavulanic acid and corresponding ESBL disks was interpreted as positive ESBL phenotype. *Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC®27853 and *Staphylococcus aureus* ATCC® 29213 were used to verify the quality and accuracy of the testing procedure.

3.3.4 DNA extraction, PCR analysis and DNA sequencing

DNA templates used for PCR were prepared by the boiled whole cell lysate method (Levesque et al., 1995). Chromosomal DNA was extracted using the GF-1 Nucleic Acid extraction kit (Vivantis®, Selangor Darul Ehsan, Malaysia). Plasmid DNA was extracted using QIAprep Mini-spin kit (Qiagen®, Hilden, Germany) and PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA, USA). PCR primers used in this study are listed in Table 1. All PCR amplifications were performed using Red dye PCR Master Mix Genei (Merck™, Germany). The PCR reactions were performed in 20 μl volume containing 10 ng DNA, 0.4 μM each forward and reverse primer and 10 μl 2x Red dye PCR Master Mix. For nucleotide sequencing analysis, PCR amplicons were purified using Nucleospin® Gel and PCR clean up (Mccherey-Nagel, Düren, Germany)

and then submitted to First Base Laboratories, Selangor Darul Ehsan, Malaysia for sequencing. The DNA sequences obtained were compared to GenBank database using the Blast algorithm available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

All *Salmonella* isolates were screened for *intI1* using established laboratory protocols, as previously described (Chuanchuen et al., 2007; Ekkapobyotin et al., 2008). Gene cassettes inserted in variable regions were determined using conserved segment (CS)-PCR (Levesque et al., 1995). The CS-PCR amplicons with the same size were subjected to restriction fragment-length polymorphism (RFLP) analysis using *Eco*RI and *Hind*III. The digested-PCR products yielding with the same RFLP patterns were considered identical.

Detection of *Salmonella* genomic island 1 (SGI1), and its variants within the chromosome was performed in all isolates carrying class 1 integrons with resistance gene cassettes (*n* = 106). The left (*thdF*) and right (*S044–yidY*) junction of SGI1 were detected with specific primers as described previously (Doublet et al., 2003).

All the ciprofloxacin-resistant *Salmonella* strains (n=3) were examined for mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* (Chuanchuen and Padungtod, 2009) using PCR and DNA sequencing. The *gyrA* and *parC* QRDRs obtained from two ciprofloxacin-susceptible strains were included as controls.

The presence of plasmid mediated quinolone resistance (PMQR) including *qnr* (*qnrA, qnrB, qnrS*), *aac(6')-Ib-cr* and *qepA* genes was determined in all *Salmonella*

*i*solates as described previously (Park et al., 2006; Yamane et al., 2008; Stephenson et al., 2010). *Escherichia coli qnr*-positive strains served as positive controls (Wu et al., 2008).

Genes encoding β-lactamases, including bla_{TEM}, bla_{SHV}, bla_{CMY-1} , bla_{CMY-2} (Hasman et al., 2005), bla_{CTX-M} group I, bla_{CTX-M} group II, bla_{CTX-M} group III and bla_{CTX-M} group IV were additionally examined (Pitout et al., 2004) and bla_{PSE} (Li et al., 2013) were detected by PCR and analyzed by nucleotide sequencing. All isolates were screened by PCR for plasmid-encoded virulence factors (*spvC, rck* and *pefA*) (Chiu and Ou, 1996; Skyberg et al., 2006)

Table 1 Primer used in this study

Gene	Primer	Primer sequences	Tm (°C)	reference
Class 1 integrons				
intI1	intF	CCTGCACGGTTCGAATG	50	Chuanchuen *et al*,
	intR	TCGTTTGTTCGCCCAGC		
Variable region	5'-CS	GGCATCCAAGCAGCAAG	50	Levesque *et al*, 1995
	3'-CS	AAGCAGACTTGACCTGA		
SGI1				
thdF	*thdF*-F	ACACCTTGAGCAGGGCAAG	55	Doublet *et al*, 2003
	thdF-R	AGTTCTAAAGGTTCGTAGTCG		
S044-int2	S044-1	TGACGAGCTGAAGCGAATTG	55	Doublet *et al*, 2003
	S044-2	AGCAAGTGTGCGTAATTTGG		
S044-yidY	S044-1	TGACGAGCTGAAGCGAATTG	50	Doublet *et al*, 2003
	yidY	ACCAGGGCAAAACTACACAG		
sul1- floR	sulTER	AAGGATTTCCTGACCCTG	50	Doublet *et al*, 2003
	F3	AAAGGAGCCATCAGCAGCAG		
floR-tetR	F4	TTCCTCACCTTCATCCTACC	56	Doublet *et al*, 2003
	F6	TTGGAACAGACGGCATGG		
tetR- tetG	tetR	GCCGTCCCGATAAGAGAGCA	55	Doublet *et al*, 2003
	tetG	GAAGTTGCGATTGGTCTGCG		
groEL- pse1	groEL	TTCTGGTCTTCGTTGATGCC	55	Doublet *et al*, 2003
	pse1	CATCATTTCGCTCTGCCATT		
QRDR				
gyrA	gyrA-F	GCTGAAGAGCTCCTATCTGG	57	Chuanchuen and
	gyrA-R	GGTCGGCATGACGTCCGG		
parC	parC-F	GTACGTGATCATGGATCGTG	57	Chuanchuen and
	parC-R	TTCCTGCATGGTGCCGTCG		
PMQR				
qnrA	qnrA-F	ATTTCTCACGCCAGGATTTG	53	Stephenson *et al*, 2010
	qnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB	qnrB-F	GATCGTGAAAGCCAGAAAGG	53	Stephenson *et al*, 2010
	qnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS	qnrS-F	ACGACATTCGTCAACTGCAA	53	Stephenson *et al*, 2010
	qnrS-R	TAAATTGGCACCCTGTAGGC		
qepA	qepA-F	GCAGGTCCAGCAGCGGGTAG	60	Yamane *et al*, 2008
	qepA-R	CTTCCTGCCCGAGTATCGTG		
aac(6')-Ib-cr	aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	54	Park *et al,* 2006
	aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		

Table 1 (Continued)

Gene	Primer	Primer sequences	Tm (°C)	reference
Virulence gene				
spvC	spvC-F	ACTCCTTGCACAACCAAATGCGGA	56	Chiu and Ou, 1996
	spvC-R	TGTCTTCTGCATTTCGCCACCATCA		
pefA	pefA-F	GCGCCGCTCAGCCGAACCAG	66	Skyberg *et al*, 2006
	pefA-R	GCAGCAGAAGCCCAGGAAACAGTG		
rck	rck-F	TCGTTCTGTCCTCACTGC	50	Guerra *et al*, 2002
	rck-R	TCATAGCCCAGATCGATG		
ESBL				
bla_{TEM}	TEMup	GCGGAACCCCTATTT	50	Hasman *et al*., 2005
	TEMdown	TCTAAAGTATATATGAGTAAACTTGGTCT		
bla_{SHV}	SHVup	TTCGCCTGTGTATTATCTCCCTG	50	Hasman *et al*., 2005
	SHVdown	TTAGCGTTGCCAGTGYTG		
bla_{CTX-M} gr. I	CTX-M 1F	GACGATGTCACTGGCTGAGC	55	Pitout *et al*., 2004
	CTX-M 1R	AGCCGCCGACGCTAATACA		
bla_{CTX-M} gr. II	CTX-M 2F	GCGACCTGGTTAACTACAATCC	55	Pitout *et al*., 2004
	CTX-M 2R	CGGTAGTATTGCCCTTAAGCC		
bla_{CTX-M} gr. III	CTX-M 3F	CGCTTTGCCATGTGCAGCACC	55	Pitout *et al*., 2004
	CTX-M 3R	GCTCAGTACGATCGAGCC		
bla_{CTX-M} gr. IV	CTX-M 4F	GCTGGAGAAAAGCAGCGGAG	62	Pitout *et al*., 2004
	CTX-M 4R	GTAAGCTGACGCAACGTCTG		
bla_{PSE}	PSEup	GCTCGTATAGGTGTTTCCGTTT	50	Batchelor *et al*.,2005
	PSEdown	CGATCCGCAATGTTCCATCC		
bla_{CMY-1}	CMY1up	GTGGTGGATGCCAGCATCC	51	Hasman *et al*., 2005
	CMY1down	GGTCGAGCCGGTCTTGTTGAA		
bla_{CMY-2}	CMY2up	GCACTTAGCCACCTATACGGCAG	51	Hasman *et al*., 2005
	CMY2 down	GCTTTTCAAGAATGCGCCAGG		

3.3.5 Conjugation study

The *Salmonella* isolates carrying class 1 integrons with resistance gene cassette (n=106) and/or bla genes (n=13) were used as donor strains in conjugation

experiments (Chen et al., 2004; Khemtong and Chuanchuen, 2008). The rifampicin-resistant derivatives of E. coli K12 strain MG1655 were used as recipient strains. Transconjugants were selected on Colinstant Chromogenic agar (Scharlau, Barcelona, Spain) supplemented with 32 μg/ml of rifampicin and one of the following antibiotics ampicillin (100 μg/ml), streptomycin (50 μg/ml), trimethoprim (25 μg/ml) or cefoperazone (2 μg/ml). Transconjugants were confirmed to be E. coli by growing on MacConkey agar (Difco) or Eosin Methylene Blue agar (Difco) and assayed for antimicrobial susceptibility. Plasmid DNA was extracted from representative transconjugants and examined for the presence of class 1 integrons, the corresponding resistance gene cassettes and/or ESBL genes using PCR as described above.

3.3.6 Statistical analysis

The significance ($p < 0.05$) of the occurrence of *Salmonella* spp. and antimicrobial resistance genes/cassettes in various sample types, populations and locations were determined by using Pearson's Chi-square test (SPSS, version 20.0).

3.4 RESULTS

Prevalence of *Salmonella*

In this study, 469 samples (39.5%) collected were positive for *Salmonella*. The prevalence of *Salmonella* varied between sample from different sources and different locations (Table 2). When considering pig-associated samples (i.e. feces, carcasses and retail pork), the overall prevalence of *Salmonella* in Laos provinces (47.4%) was significantly higher than Thai provinces (34.6%, $p<0.05$). The prevalence

of *Salmonella* in carcasses at Laos pig slaughter facilities (53.3%) was significantly higher ($p<0.05$) than sampled carcasses in Thailand (30.9%). There was a notable, but not significant difference, in the prevalence of *Salmonella* in retail pork from Thai (60.5%) and Laos (72.3%) fresh markets ($p>0.05$).

In analyzing samples obtained from Thai provinces, the prevalence of *Salmonella* was significantly higher ($p<0.05$) in retail pork at fresh markets than pigs and pig carcasses at slaughter. There was no difference in the prevalence between pigs and carcass samples ($p>0.05$). Similarly, in Laos provinces, the prevalence of *Salmonella* was significantly higher ($p<0.05$) in retail pork samples collected at fresh markets than in source animals and carcasses. However, unlike Thai samples, the prevalence of *Salmonella* was significantly higher ($p<0.05$) in pig carcasses than pigs.

In humans, the overall prevalence of *Salmonella* was not significantly different ($p>0.05$) between Thai (12.8%) and Laos (16.3%) provinces. The *Salmonella* prevalence in the slaughterhouse workers in Thailand (13.4%) was not significantly different from those in Laos (25%, $p>0.05$). Similarly, the prevalence of *Salmonella* was not different between patients in Thai and Laos hospitals ($p>0.05$). A comparison of butchers cannot be made due to the limited number of samples obtained from Laos.

Table *2*. Prevalence of *Salmonella enterica* in Thailand-Laos porivinces (n=1,187)

Category	Source	Sample	Sample type	Positive samples				p-value
				Thailand		Laos		
				Total	No. (%)	Total	No. (%)	
Pigs & pig products	Slaughterhouse	Pigs	Rectal swab	185	63(34)	129	50(38.7)	0.4622
		Pig carcass	Carcass swab	184	57(30.9)	137	73(53.3)	9.158e-05
	Fresh market	Pork	Pork swab	180	109(60.5)	112	81(72.3)	0.0543
			Subtotal	549	229(41.7)	378	204(54%)	0.000308
Human	Slaughterhouse	Worker	Stool sample	52	7(13.4)	36	9 (25)	0.2719
	Fresh market	Butcher	Stool sample	50	2(4)	8	0	1
	Hospital	Patients	Stool sample	78	14(17.9)	36	4(11.1)	0.5129
			Subtotal	180	23(12.8)	80	13(16.3)	0.5798
			Grand total	**729**	**252(34.6)**	**458**	**217(47.4)**	**1.462e-05**

Salmonella serotype

Five hundred-forty eight *Salmonella* isolates were obtained from the 469 *Salmonella*-positive samples including 295 isolates from Thai provinces and 253 from Laos provinces. Twenty three and 27 different serovars of *Salmonella* were identified in Thailand and Laos samples, respectively (Table 3). The most common serotype in Thai isolates was Typhimurium, followed by Rissen. Serovars Typhimurium, Rissen, Kedougou and Weltreveden were present in all sample types. In Laos isolates, the most common serotypes were Typhimurium and Anatum. These two serovars together with Rissen and Stanley were found in all sample types.

Antimicrobial susceptibility

Multidrug resistance, defined resistance to 3 or more different classes of antibiotics was found in samples of all sources from both countries (Table 4). Of the 548 resistant isolates analyzed, 98.2% were found to be MDR. The isolates were most commonly resistant to suphonamides (98.3%), trimetroprim (49.5%), ampicillin (91%), tetracycline (92.5%), spectinomycin (76%) and streptomycin (84.7%), while infrequently resistant to nalidixic acid (10.9%) and ciprofloxacin (0.5%). Resistance to gentamicin (22.4%), ceftazidime (2.4%), cefotaxime (4.4%) and cepodoxime (4.4%) was found at low rate only in Thai isolates. A total of 89 antimicrobial resistance patterns of the *Salmonella* isolates from Thai and Laos were observed in this study (Table12). The most common antimicrobial resistance patterns were AMP-SPC-STR-SUL-TET-TRI (19.7%) for Thai isolates and AMP-SPC-STR-SUL-TET-TRI (29.2%) and AMP-STR-SUL-TET (22.9%) for Laos isolates.

Table 3. Predominant **s**erotypes of *Salmonella enterica* from various sources in Thailand-Laos provinces (n=1,187)

Salmonella serotype	Thailand						Lao PDR					
	Slaughterhouse			Fresh market		Hospital	Slaughterhouse			Fresh market		Hospital
	Pig (n=70)	Carcass (n=61)	Worker (n=7)	Pork (n=139)	Butcher (n=2)	Patients (n=16)	Pig (n=59)	Carcass (n=82)	Worker (n=12)	Pork (n=96)	Butcher (n=0)	Patients (n=4)
Agona						2				1		
Afula							2					
Anatum	3	1		15			9	25	1	16		
Bardo			1									
Bovismorbificans				2								
Derby				4			4	3	1	10		1
Dessau							2					
Eindegi							1	6		2		
Give	1	2		3				1	1	1		
Havana										2		
Hvittingfoss	0	1	2	1				1				
Kedougou	11	4		11		1						
Lexington									1			
London								1	1			2
Panama		1		2	1		1					

Table 3 (Continued)

Salmonella serotype	Thailand						Lao PDR					
	Slaughterhouse			Fresh market		Hospital	Slaughterhouse			Fresh market		Hospital
	Pig (n=70)	Carcass (n=61)	Worker (n=7)	Pork (n=139)	Butcher (n=2)	Patients (n=16)	Pig (n=59)	Carcass (n=82)	Worker (n=12)	Pork (n=96)	Butcher (n=0)	Patients (n=4)
Paratyphi		1		1								
Preston										2		
Rissen	15	15	2	25		3	7	11	1	17		1
Reading							1					
Saintpaul		6		7								
Singapore									1			
Stanley	1	6		9			11	10	1	13		
Tsevie										2		
Typhimurium	33	18	1	44	1	6	14	19	1	18		
Urbana				2								
Vejle									1			
Welteverden	4	6	1	9		3	5	3	2	1		
Worthington	1									10		
Total	295						253					

Table 4. Antimicrobial resistance rates of *Salmonella enterica* in pigs, pig carcasses, pork and humans in Thailand-Laos provinces (n=1,187)

Antimicrobial Agent	No. of isolates (%)										
	Thailand					Laos					Overall
	Pig (n=70)	Carcass (n=61)	Pork (n=139)	Human* (n=25)	Total (n=295)	Pig (n= 59)	Carcass (n=82)	Pork (n=96)	Human† (n=16)	Total (n=253)	(n= 548)
MDR	70(100)	60(98.4)	136(97.8)	23(92)	289(98)	56(95)	81(98.8)	96(100)	16(100)	249(98.4)	538 (98.2)
Ampicillin	70(100)	59(96.7)	134(96.4)	17(68)	280 (95)	56(95)	75(91.5)	78(81.3)	10(62.5)	219(86.6)	499(91)
Tetracyclines	64(91.4)	56(91.8)	127(91.4)	21(84)	268(90.8)	53(89.8)	80(97.6)	90(93.8)	16(100)	239(94.5)	507(92.5)
Nalidixic acid	4(5.7)	8(13.1)	16(11.5)	4(16)	32(10.8)	1(1.7)	2(2.4)	21(21.9)	4(25)	28(11)	60(10.9)
Ciprofloxacin	0	0	1(0.7)	0	1(0.3)	0	0	2(2)	0	2(0.8)	3(0.5)
Ceftazidime	2(2.9)	1(1.6)	4(2.9)	0	7(2.4)	0	0	0	0	0	7(1.3)
Cefoperazone	1(1.4)	5(8.2)	7(5)	2(8)	15(5)	0	1(1.2)	5(5.2)	0	6(2.4)	21(3.8)
Cefotaxime	0	5(8.2)	8(5.8)	0	13(4.4)	0	0	0	0	0	13(2.4)
Cepodoxime	0	5(8.2)	8(5.8)	0	13(4.4)	0	0	0	0	0	13(2.4)
Gentamicin	15(21.4)	14(23)	37(26.6)	0	66(22.4)	0	0	3	0	0	69(12.6)
Streptomycin	52(74.3)	46(75.4)	113(81.3)	17(68)	228(77.3)	50(84.7)	79(96.3)	93(96.9)	14(87.5)	236(93.2)	464(84.7)
Spectinomycin	61(87.1)	53(86.9)	112(80.6)	22(88)	248(84)	34(57.6)	55(67)	67(69.8)	13(81.3)	169(66.8)	417(76)
Chloramphenicol	21(30)	22(36)	59(42.4)	6(24)	108(36.6)	3(5)	10(12.2)	11(11.5)	4(25)	28(11)	136(24.8)
Sulfamethoxazole	70(100)	61(100)	139(100)	25(100)	295(100)	54(91.5)	82(100)	92(95.8)	16(100)	244(96.4)	539(98.3)
Trimethoprim	39(55.7)	32(52.5)	52(37.4)	11(44)	134(45.4)	26(44)	38(46.3)	63(65.6)	10(62.5)	137(54.1)	271(49.5)

ESBL production and ESBL genes

Thirteen *Salmonella* (2.4%) isolates collected from Thai provinces produced ESBL enzymes, including 4 serovar Saintpaul and a serovar Weltreverden from pig carcasses; 7 serovar Saintpaul and a serovar Typhimurium from retail pork. None of the isolates from Laos provinces produced ESBL. All ESBL-producing strains yielded PCR amplicon of bla_{CTX-M} group IV that was confirmed to be $bla_{CTX-M-14}$ by sequencing. The $bla_{CTX-M-14}$ gene in all isolates could be horizontally transferred to *E. coli* with the exception of one isolate (a serovar Weltreverden) from a pig carcass and 2 isolates (serovar Saintpaul) from retail pork.

Class 1 integrons, resistance gene cassettes and SGI1

Ninety-four *Salmonella* isolates (31.9%) collected in Thailand provinces were positive for *intI1*. Among the *intI1*-positive strains, 45 isolates (47.9%) carried resistance gene cassettes in variable regions, including *dfrA12-aadA2* (n=44) and *aadA2-linG* (n=1) (Table 5). Ninety-nine *Salmonella* isolates (39.1%) collected in Laos possessed *intI1*, of which 61.6% harbored resistant gene cassette *dfrA12- aadA2*. Five isolates carrying class 1 integrons containing *dfrA12-aadA2* (n=4) or *aadA2-linG* (n=1) were capable of horizontal transfer to *E. coli* (Table 6). None of the *Salmonella* isolates were found to carry SGI1.

Table 5. Class 1 integrons of *Salmonella enterica* in pigs, pork and humans in Thailand-Laos provinces (n=1,187)

Gr.	Amp size(bp)	Gene cassettes	Thailand		Laos	
			Serotype	Sample type (n)	Serotype	Sample type (n)
I	2000	*dfrA12-aadA2*	Anatum(3)	Pig(1)[*(1)], Pig carcass(1), Pork(1)	Anatum(24)	Pig (7), Pig carcass(9), Pork(8)[*(1)]
			Rissen(40)	Pig(10), Pig carcass (12), Pork(15), Worker (1), Patient(2)[*(1)]	Rissen(34)	Pig(7), Pig carcass (10), Pork(15), Worker(1), Patient(1)
			Typhimurium (1)	Pork(1)	Regent(1) Stanley(1) Weltreverden(1)	Pork(1) Pork (1)[*(1)] Carcass (1)
II	2000	*aadA2-linG*	Give(1)	Pig(1)[*(1)]	-	-

[*]Capable of transfer of integrons. Number of isolates is in parenthesis

Mutation(s) of the QRDRs in *gyrA* and *parC* and PMQR genes

Three pork isolates were resistant to fluoroquinolones (MIC for nalidixic acid and ciprofloxacin are in parenthesis, respectively), including one serovar Give from Thai (>256 μg/ml, 4 μg/ml) as well as one Serovar Give (>256 μg/ml, 8 μg/ml) and serovar Anatum (>256 μg/ml, 8 μg/ml) from Laos. All of the isolates carried a single point mutation C-248-A in QRDR of *gyrA* leading to an amino acid substitute Ser-83-Tyr in GyrA. Additionally, all carried a single point mutation, C-283-G, in *parC* leading to an amino acid change Thr-57-Ser in ParC. The latter was also found in the ciprofloxacin susceptible strains. The *qnrS* gene was identified in 7.1% of isolates from carcasses (n=6) and retail pork (n=15) from Thai provinces and 5.5% of carcasses (n=4), retail pork (n=6) and patients (n=4) from Laos provinces (Table 6).

The *qnrB* gene was additionally found in 1.9% of Laos isolates sourced from pigs (n=1) and retail pork (n=4). MICs for nalidixic acid (8->128 μg/ml) and ciprofloxacin (0.25-8 μg/ml) varied for the PMQR-carrying *Salmonella* isolates. One serovar Give collected from retail pork in Thai province carried *qnrS,* which and also had a base pair change C-248-A in *gyrA* (MIC for nalidixic acid =>256 μg/ml; ciprofloxacin = 4 μg/ml). All of the ESBL producers carried *qnrS* (n=13). None of the isolates carried *qnrB* and *qnrS* simultaneously.

Plasmid-associated virulence genes

None of the *Salmonella* isolates in this study were found to carry *spvC*, *pefA* and *rck* genes.

Table 6. Plasmid-mediated quinolone resistance (PMQR) in *Salmonella enterica* in pigs, pork and humans in Thailand-Laos provinces(n=1,187)

Country (n)	Gene	No. of positive isolates (%)	Source	Serovars*	MIC(μg/ml) Nal, Cip (n)
Thailand (295)	*qnrS*	23(7.8)	Carcasses(n=6)	Saintpaul(5)†(4)	32,0.5(4); 16,0.5(1)
				Weltreverden(1)†(1)	8,0.25(1)
			Pork(n=15)	Bovismorbifican(2)	32,1(2)
				Cuckmere(1)	>128,2(1)
				Give(3)‡(1)	>128,2(2); >256,
				Rissen(1)	4(1)
				Saintpaul(7)†(7)	64,4(1)
					32,1(1); 32,0.5(3);
				Typhimurium(1)†(1)	16,0.5(3)
			Patient(2)	Agona(2)	32,0.5(1)
	aac(6')-Ib-cr	1(0.3)	Worker(1)	Rissen(1)	16,1(2)
					16,1(1)
Laos (253)	*qnrB*	5(1.9)	Pig(n=1)	Derby(1)	8,0.03(1)
			Pork(n=4)	Derby(3)	64,1(2); 32,1(1)
				Preston(1)	64,1(1)
	qnrS	14(5.5)	Pig carcass	Derby(2)	8,0.03(2); 8,0.5(1)
			(n=4)	Hvittingfoss(1)	32,1(1)
				Typhimurium(1)	8,0.03(1)
			Pork(n=6)	Agona(1)	32,0.5(1)
				Anatum(1)	>256,4(1)
				Derby(1)	64,1(1)
				Give(1)	>256,8(1)
				Havana(1)	64,1(1)
				Rissen(1)	32,0.5(1)
			Patient(n=4)	London(2)	16,0.5(1); 8, 0.5(1)
				Derby(1)	64,1(1)
				Rissen(1)	8,0.5(1)

*Number of strains is indicated in parenthesis.

†ESBL producing strains(number of isolates)

‡Carry a single point mutation C-248-A in QRDR of *gyrA*(number of isolates)

3.5 DISCUSSION

In this study, the overall prevalence of *Salmonella* in slaughterhouses and fresh markets in Laos provinces is significantly higher than that in Thai provinces ($p<0.05$). The prevalence of *Salmonella* was also significantly higher ($p<0.05$) in samples collected from pig carcasses in Laos slaughterhouses than those in Thailand. This may reflect differences in hygiene practices within the pig production chain of these two countries. In Thailand, pig production has shifted to *intensive* commercial industry (Falvey, 2000). Abattoirs are generally licensed and operate with modern technology under good hygienic standards for abattoir (ACSF, 2006) and for animal welfare (ACSF, 2010). Still, provincial slaughterhouses with inadequate hygiene exist and are of particular concern. In contrast, most pigs in Laos are raised on family farm (Boonmar et al., 2008) and the small scale abattoirs with low hygienic practice in slaughtering process are still common (Bastiaensen et al., 2011). The samples obtained for this study were collected from a limited number of sites (one slaughterhouse, one fresh market and one hospital in each province), which must be considered when interpreting this data. Additional surveillance should be performed in other locations to further support the findings of this study.

The prevalence of *Salmonella* from pig samples collected at slaughter facilities in Thailand (34%) and Laos (38.7%) are lower than previous reports from Vientiane, Laos (Boonmar et al., 2008) but higher than reports from Northern Thailand (Padungtod and Kaneene, 2006) and Sakaew, Cambodia (Pulsrikarn et al., 2012). In this study, the prevalence of *Salmonella* in each country was higher in the fresh markets compared to in the slaughterhouse. The general protocol for slaughter and processing included evisceration at the slaughter facility, direct transportation of

the carcass to the local fresh market, followed by cutting the carcass into retail meat portions and cleaning by market butchers. It is likely that *Salmonella* cross-contamination could occur during transportation and during carcass breakdown and cleaning at the retail shop from contaminated equipment and uncontrolled environmental conditions.

A small percentage of workers tested at slaughter facilities were positive for *Salmonella* in both Thailand (13.4%) and Laos (25%), suggesting that these workers could possibly serve as carriers for the pathogen and that an occupational risk exists for these individuals. Pigs can be asymptomatic carriers capable of shedding *Salmonella* for prolonged periods of time, which can increase during times of stress such as transportation and processing at the slaughter facility. Interestingly, although the prevalence of *Salmonella* in retail meat samples was higher than in pigs or carcasses, the butchers sampled from Thai fresh markets had a relatively low prevalence of *Salmonella* (4%) compared to slaughter facility workers. None of the Laotian butchers were *Salmonella*-positive, likely due to the limited number of the study participants. It is possible that the opportunities for infection during the butchering process are diminished compared to activities performed during slaughter. In hospitalized patients undergoing treatment for diarrhea, the percentage of *Salmonella*-positive samples was moderately higher in both Thailand (17.9%) and Laos (11.1%) compared to a previous report, which found a prevalence of 7% in hospitalized children in Northern Thailand (Padungtod and Kaneene, 2006). Dietary intake and livestock contact was not analyzed in this study, therefore, it cannot be concluded that the source of *Salmonella* infection in these patients was related to pork consumption or exposure to pigs.

The most common serotypes among Thai isolates were Typhimurium and Rissen, while Typhimurium and Anatum were most common in Laos. Even though the same serovar was present in both Thailand and Laos, it cannot be concluded that these two countries are sharing a pool of *Salmonella* from pig source and the study of genetic relatedness is required. A previous survey suggested that Anatum and Derby were typical characteristics of *Salmonella* in Laotian pigs (Sumalee Boonmar, 2013). However, in this study, serovar Derby was less common (4/59). Serovar Weltevreden has been increasingly reported in human salmonellosis (Makendi et al., 2016) but was found to a lesser extent in this study. These results indicate variations of *Salmonella* serovars in South East Asian countries. As the serotypes of *Salmonella* vary by geographic locations (Boonmar et al., 2008), additional factors could affect the different-predominant serovars e.g. a time factor, limited sample sites and certain serovars more likely to carry resistance genes leading to a shift in prevalent serotypes.

The majority (98.2%) of the *Salmonella* isolates in this study were MDR. This may reflect the extensive use of antimicrobials in pig production in these two countries or co-selection/cross-resistance by other substances. Interestingly, cephalosporin resistance was found in Thai isolates (2.4-5%), while almost all the Laos isolates were susceptible to these drugs (0-2.4%). Most pig farms in Laos are run by families. One interpretation of these data is that cephalosporins are cost prohibitive for most family-owned Laotian pig farms and the drugs are less often used, thereby limiting the selection pressure for resistance genes (Boonmar et al., 2008). In addition, gentamicin resistance in Thai isolates was higher than previous reports in pork from the same country (Angkititrakul et al., 2005; Sinwat et al., 2015)

but more similar to that in Vietnam (Thai et al., 2012). In contrast, almost all the Laos isolates were susceptible to gentamicin. This variation is likely associated with different type of antibiotics commonly used in pig production in different locations even in the same counties. The findings underscore the necessities of monitoring antimicrobial use in this region.

Overall, similar rate of class 1 integrons was found among the *Salmonella* isolates from Thai (31.9%) and Laos (39.1%) provinces. The *dfrA12-aadA2* gene cassette was predominant and found in all sample types, indicating its circulation and the presence of its selective pressure in pig production chains. This is supported by the observation that some class 1 integrons carrying the *dfrA12-aadA2* were located on horizontally-transferred plasmid. As it is often difficult to determine the direction of gene transfer in clinical settings, spread of MDR *Salmonella* and their resistance determinants from humans (especially workers at slaughterhouse) to meat products should not be neglected.

The *aadA2-linG* gene cassette combination (encoding for resistance to streptomycin/spectinomycin - lincomycin) was detected in one MDR isolate, serovar Give, from a pig in Thailand. Lincomycin has been frequently used in combination with spectinomycin for treatment of swine dysentery and respiratory infection in pigs in Thailand (Juttupornpong et al., 1996). However, its use has been diminished due to increasing resistance. This could explain the presence of the *aadA2-linG* gene cassette in *Salmonella* from pig samples in this study.

In this study, ESBL-producing *Salmonella* isolates were detected only in pig carcasses and retail pork from Thai provinces and all were MDR. It has been demonstrated that ESBL genes are often located on large plasmids carrying many other resistance genes (Paterson, 2000). As a result, ESBL-producing enterobacterial isolates are frequently resistant to broad spectrum of antibiotics, in agreement with this study. This observation is of particular concern, even at a low rate, since use of antimicrobials may co-select for both ESBL and other resistance determinants. In previous reports, CTX-M type ESBL-producing isolates were frequently found among bacterial species over a wide geographical area (Bonnet, 2004), in agreement with this study where nearly all ESBL-producing strains (n=10) carried $bla_{\text{CTX-M-14}}$. The $bla_{\text{CTX-M-14}}$ gene was previously found in S. Enteritridis from a 4-year old boy in Japan (Izumiya et al., 2005); patients in Argentina (Jure et al., 2010) and patients in Hongkong (Jin and Ling, 2006). The gene was also found in other serovars, including Infantis from broiler in Japan (Kameyama et al., 2012) and Typhimurium from patients in Hongkong (Jin and Ling, 2006). These results highlight the horizontal transfer of the gene, in agreement with our results.

The prevalence of three PMQR determinants (i.e. *qnrB*, *qnrS* and *aac(6')-Ib-cr*) in this study were similar to previous reports in food animals from Japan (Asai et al., 2010) and humans in France (Cattoir et al., 2007). In contrast, it was higher than previous reports in Vietnamese retail meat (Thai and Yamaguchi, 2012) and food animals from South Korea (Kim et al., 2011), where no PMQR genes were found. As the presence of Qnr determinants may accelerate mutations in DNA gyrase and topoisomerase IV (Kim et al., 2011), only one serovar Give harbored *qnrS* in combination with a single base pair change in *gyrA* (Nal, >256 μg/ml; Cip, 4 μg/ml).

Many PMQR isolates in this study exhibited low nalidixic and ciprofloxacin resistance levels (as low as 8 and 0.03 μg/ml, respectively).This finding contradicts previous studies suggesting that the presence of *qnr* genes mediated reduced susceptibility to ciprofloxacin and nalidixic acid (Hopkins et al., 2008; Ferrari et al., 2013).The data suggest that PMQR-carrying isolates could be missed if resistance to nalidixic acid and ciprofloxacin were exclusive criteria used to select isolates for further genetic screening.

Coexistence of *qnrS* and $bla_{CTX\text{-}M\text{-}14}$ was observed in all ESBL-producing isolates exhibiting low ciprofloxacin MIC (0.5-1 μg/ml) and varied nalidixic MIC (8-32 μg/ml). Although PMQR determinants did not convey high quinolone resistance to these isolates, it potentially influenced co-selection for β-lactamase-encoding genes (Cremet et al., 2011). However, this study did not determine if *qnrS* and $bla_{CTX\text{-}M\text{-}14}$ were co-localized on the same plasmid. The *aac(6)Ibcr* gene was previously shown to contribute to an increased-ciprofloxacin MIC (Wong et al., 2014) and was more common than other PMQR determinants in ESBL-producing *Enterobactericeae* (Warburg et al., 2009). In contrast, none of the ESBL-producing *Salmonella* in this study possessed *aac(6)Ibcr*.

In conclusion, these data demonstrated high prevalence of multidrug resistant *Salmonella* in pigs, pork and humans within the Thai and Laos border provinces. To our best knowledge, this is the first report regarding genotypic characteristics of AMR in *Salmonella* isolated from pig production chain and patients in Laos and South East Asia. This study highlights the need for surveillance program as well as control and prevention strategic plan for AMR in bacteria of food animal origin at national

and regional level. The epidemiological data will help to understand the root cause of AMR, provide information to guide the interventions that are essential for the region and evaluate the success of the interventions.

Conclusion

The data in this chapter show high prevalence of multidrug resistant *Salmonella* in pigs, pork and humans within the Thai and Laos border provinces. The *dfrA12-aadA2* gene array was predominant in this study. Coexistence of *qnrS* and $bla_{\text{CTX-M-14}}$ was observed in all ESBL-producing isolates. This is the first report regarding phenotypic and genotypic characteristics of AMR in *Salmonella* isolated from pig production chain and patients in Thailand and Laos connecting provinces. This study indicates the need for surveillance and monitoring program as well as control and prevention strategic plan for AMR in bacteria of food-producing animal at national and global level. The epidemiological data will help to better understand AMR problem, provide information to develop the public health policy, guideline of responsible use of antimicrobials and raise awareness of antimicrobial used in humans and livestock animals in these countries and worldwide

CHAPTER III

Characterization of antimicrobial resistance in *Salmonella enterica* isolated from pork, chicken meat and humans in Northeastern Thailand

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FOODBORNE PATHOGENS AND DISEASE (2015)

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2.1 ABSTRACT

A total of 221 *Salmonella enterica* from raw pork (n=64), raw chicken (n=80) and humans (n=77) were characterized for antimicrobial resistance phenotypes and genotypes and virulence plasmid associated genes. Most *Salmonella* isolates (95.9%) were multidrug resistant and exhibited high resistance to sulfamethoxazole (96.4%), streptomycin (93.2%), spectinomycin (76.5%), tetracycline (73.3%), ampicillin (70.1%) and trimethoprim (60.2%). Forty-one percent of all isolates were *intI1*-positive, of which 60% carried class 1 integrons with variable region ranging in size from 0.2 to 2.0 kb. Six integron profiles (IP-I to IP-VI) were defined. The *dfrA12-aadA2* cassette was most prevalent (66.7%). Class 1 integrons with the *dfrA12-aadA2* cassette in 5 pork isolates could be horizontally transferred. Three pork isolates carried *Salmonella* genomic island 1 (SGI1), of which a serovar Anatum harbored SGI1 gene cluster located between *thdF* and *int2*. Two single point mutations i.e. G-259-T and C-248-T in *gyrA* leading to Asp-87-Tyr and Ser-83-Phe substitutions in GyrA, respectively were detected. Of all plasmid-mediated quinolone resistance (PMQR) genes tested, only *qnrS* (4.1%) and *qnrB* (1.8%) were found. The virulence plasmid associated genes including *spvC, pefA* and *rck* were identified in 8.1%, 1.8% and 1.4% of all *Salmonella* isolates, respectively.

Keywords: antimicrobial resistance, class 1 integrons, virulence plasmid, *Salmonella enterica*

2.2 INTRODUCTION

Multidrug resistant (MDR) *Salmonella enterica* has increasingly emerged worldwide as a result of the extensive use of antibiotics in human and veterinary medicine (Khemtong and Chuanchuen, 2008; Ogasawara et al., 2008). Several approaches have been taken to understand emergence and spread of antimicrobial resistance (AMR), of which monitoring and surveillance program is a key tool for tracking drug resistance, measuring its health and economic impacts, and designing targeted solutions and must provide comparable data on antimicrobial prevalence. However, the program does not exist or is ineffective in many countries. From a public health perspective, *Salmonella* is one of the targeted zoonotic agents for antimicrobial resistance monitoring in animals and food (EFSA, 2012).

Northeastern Thailand, the largest region with the most population of the country, shares common border with Laos PDR to the north and east and Cambodia to the southeast, where millions of people and animals travel across each year (Murshid et al., 2005). Thailand's cross border trade with neighboring countries has boomed and contributed to a significant amount of income. Livestock (including pigs, poultry and cattle) and their products are the outstanding-cross border merchandises that are traded legally and illegally (Murshid et al., 2005). Such re-location at the border undoubtedly has contributed to emergence and distribution of various bacterial pathogens including MDR *Salmonella* and their resistance determinants. This would affect not only the border area but also other parts of the two countries and eventually other parts of the world. While *Salmonella* is the second most common cause of diarrhea in Thailand, chicken and pig products are

important exporting goods (Bodhidatta et al., 2002). AMR in *Salmonella* has been widely studied in Thailand but this is not always the case in neighboring countries. Report showed that diarrhea and food poisoning are the most common diseases among Cambodian patients who came for treatment in Thai hospitals along the border (Lay et al., 2011). Septicemic salmonellosis caused by MDR *Salmonella*, particularly *S.* Choleraesuis, has increased in Cambodia as well (Lay et al., 2011). In Laos PDR, *Salmonell*a was commonly found in pigs and pork carcass in slaughter houses (Boonmar et al., 2008). However, data on antimicrobial resistance is still limited.

Up to date, the prevalence, characteristics and transfer of antimicrobial resistance in *Salmonella* has been extensively studied but mostly limited to developed countries. Therefore, this study aimed to i) determine antimicrobial susceptibility; ii) characterize class 1 integrons and the SGI1 variants; and iii) examine the presence of virulence plasmid associated genes in the *Salmonella* isolates from pork, chicken meat and humans in Northeastern Thailand.

2.3 METHODS

2.3.1 Bacterial strains

A total of 221 *Salmonella* isolates from raw pork (n = 64), raw chicken meat (n=80), and humans (n=77) were obtained from samples collected in 5 provinces in Northeastern Thailand, including Kalasin, Khon Kaen, Loei, NongKhai and Roi Et during 2010-2013 (Table 7). Sample collection was performed at the largest municipal

slaughterhouses with the highest number of slaughtered pigs or broilers per day and at local markets receiving the carcasses from the selected slaughterhouses. Pig carcass swab and broiler carcass were taken at the end of the slaughter line before transporting to retail markets. Retail pork cuts and broiler carcass were obtained from the selected local markets. All the human isolates were originated from rectal swab of diarrhea patients at Khon Kaen hospital. However, dietary history (including chicken or pork consumption prior to illness onset) and information on food animal rearing was not available. The *Salmonella* strains were isolated using the standard methods as described in ISO6579:2002 (E) (ISO, 2002) at Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University and were subjected to serotyping at Center of Antimicrobial Resistance in Foodborne Pathogens (in cooperation with WHO), Faculty of Veterinary Science, Chulalongkorn University. Only one colony of each serotype was collected from each positive sample and stored as 20% glycerol stocks at -80°C. Three *Escherichia coli* strains carrying *qnrA, qnrB* or *qnrS* were used as positive controls (Wu et al., 2008).

Table 7. *Salmonella* serovars from pork, chicken and human (n=221)

Serotype	Source (No, %)			
	Pork (n=64)	Chicken (n=80)	Human (n=77)	Total (n=221)
Agona	0	6 (7.5)	1 (1.3)	6 (2.7)
Albany	1 (1.6)	1 (1.3)	1 (1.3)	2 (0.9)
Altona	0	4 (5.0)	0	4 (1.8)
Amsterdam	0	9 (11.3)	16 (20.8)	9 (4.1)
Anatum	24 (37.5)	3 (3.8)	1 (1.3)	33 (14.9)
Bovismorbificans	0	0	3 (3.9)	2 (0.9)
Brunei	0	0	15 (19.5)	1 (0.5)
Corvalis	5 (7.8)	6 (7.5)	0	15 (6.8)
Derby	0	1 (1.3)	1 (1.3)	4 (1.8)
Enteritidis	0	2 (2.5)	1 (1.3)	4 (1.8)
Give	1 (1.6)	0	2 (2.6)	3 (1.4)
Kedougou	0	1 (1.3)	1 (1.3)	2 (0.9)
Lexington	0	2 (2.5)	1 (1.3)	6 (2.7)
Lille	0	0	1 (1.3)	1 (0.5)
Manhattan	0	2 (2.5)	1 (1.3)	2 (0.9)
Newport	0	1 (1.3)	1 (1.3)	1 (0.5)
Panama	1 (1.6)	0	1 (1.3)	4 (1.8)
Rissen	20 (31.3)	13 (16.3)	0	35 (15.8)
Saintpaul	0	0	16 (20.8)	1 (0.5)

Table 7. (Continued)

Serotype	Source (No, %)			
	Pork (n=64)	Chicken (n=80)	Human (n=77)	Total (n=221)
Senftenberg	1 (1.6)	0	1 (1.3)	2 (0.9)
Schwarzengrund	0	19 (23.8)	1 (1.3)	20 (9.0)
Singapore	0	3 (3.8)	0	3 (1.4)
Stanley	6 (9.4)	2 (2.5)	16 (20.8)	24 (10.9)
Typhimurium	0	0	1 (1.3)	1 (0.5)
Virchow	1 (1.6)	5 (6.3)	3 (3.9)	9 (4.1)
Welteverden	1 (1.6)	0	15 (19.5)	16 (7.2)
Worthington	3 (4.7)	0	0	3 (1.4)
ser 4,512:b	0	0	1 (1.3)	1 (0.5)
ser 4,5,12	0	0	1 (1.3)	1 (0.5)
ser 1,4,5,12	0	0	2 (2.6)	2 (0.9)
ser 4,21	0	0	1 (1.3)	1 (0.5)
ser 4,12:I	0	0	1 (1.3)	1 (0.5)
ser 9,12:1,5	0	0	1 (1.3)	1 (0.5)
ser 4,12	0	0	1 (1.3)	1 (0.5)

2.3.2 Antimicrobial susceptibility test

Minimum Inhibitory Concentrations (MICs) was determined using a two-fold agar dilution (CLSI, 2008). Ten antimicrobials were as follows: ampicillin (AMP), cefoperazone (CEF), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN),streptomycin (STR), spectinomycin (SPC), sulfamethoxazole (SUL), tetracycline (TET), trimethoprim (TRI) were tested. All antibiotics were purchased from Sigma-

Aldrich (St. Louis, MO, USA). *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 served as control strains.

2.3.3 General DNA manipulations

Template DNA for PCR were whole cell DNA prepared by boiling lysate procedure (Levesque et al., 1995) except when indicated. Chromosomal DNA was isolated using the GF-1 Nucleic Acid extraction kit (Vivantis®, Selangor Darul Ehsan, Malaysia). Plasmid DNA was obtained by using QIAprep Mini-spin kit (Qiagen®, Hilden, Germany). PCP products were purified using Nucleospin® Gel and PCR clean up (Mccherey-Nagel, Düren, Germany).

2.3.4 Identification of class 1 integrons, SGI and virulence plasmid associated genes

All PCR reactions were performed by using Red dye PCR Master Mix Genei (Merck™, Germany). Class 1 integrons were characterized in all the *Salmonella* isolates using CS-PCR as previously described (Levesque et al., 1995; Chuanchuen et al., 2007). CS-PCR amplicons were gel purified and submitted for nucleotide sequencing at First Base Laboratories, (Selangor Darul Ehsan, Malaysia). The DNA sequences obtained were compared with the published data in the GenBank database available at www.ncbi.nlm.nih.gov.

Association of class 1 integrons with SGI1 was investigated by PCR in all the *Salmonella* isolates carrying class 1 integrons with resistance gene cassettes (Doublet et al., 2003; Khemtong and Chuanchuen, 2008). The left (*thdF*) and right (*S044–yidY*) junction of SGI1 were first determined and the organization of antibiotic-resistance

gene clusters for SGI1-like element was examined using different combinations of primers.

The presence of *Salmonella*-virulence plasmids including *spvC*, *pefA* and *rck* was screened in all isolates (Chiu and Ou, 1996; Guerra et al., 2002; Skyberg et al., 2006). Plasmid DNA was extracted from the *spvC, pefA* or *rck* -positive *Salmonella* strains and used as templates for PCR amplification to confirm plasmid localization of the genes.

2.3.5 **Detection of mutation(s) in the QRDR of the *gyrA* and *parC***

Mutation(s) in the QRDR of the *gyrA* and *parC* were examined in all the ciprofloxacin-resistant *Salmonella* strains, including the chicken (n=2) and the human (n=4) isolates by nucleotide sequencing analysis using the forward and reverse primers as previously described (Chuanchuen and Padungtod, 2009). The QRDRs of *gyrA* and *parC* obtained from two ciprofloxacin-susceptible strains were included as controls. All the *Salmonella i*solates were screened for the presence of *qnr* genes (*qnrA, qnrB* and *qnrS*), *aac(6')-Ib-cr* and *qepA* (Park et al., 2006; Yamane et al., 2008; Stephenson et al., 2010).

2.3.6 Conjugation experiments

Filter mating assay was performed as previously described (Khemtong and Chuanchuen, 2008). All *Salmonella* isolates with class 1 integrons carrying resistance gene cassettes served as donors (n=45) and the spontaneous rifampicin-resistant derivatives of *E.coli* K-12 MG1655 (MG1655 rif^r, MIC=256 µg/ml) were recipients. In *E.*

coli transconjugants, transfer of class 1 integrons and resistance gene cassettes was confirmed by PCR using plasmid DNA as described above.

2.4 Results

Antimicrobial resistance of the *Salmonella* isolates

The overall resistance rates to sulfamethoxazole, streptomycin, spectinomycin, tetracycline, ampicillin, trimethoprim, chloramphenicol, gentamicin, cefoperazone and ciprofloxacin were 96.4%, 93.2%, 76.5%, 73.3%, 70.1%, 60.2%, 36.7%, 36.7%, 10.4% and 3.2%, respectively. All the *Salmonella* isolates were resistant to at least one antibiotic (Figure 3). Most (95.9%) exhibited multidrug resistance (being resistant to at least 3 different classes of antimicrobial agents). AMR patterns commonly found are shown in Table 8.

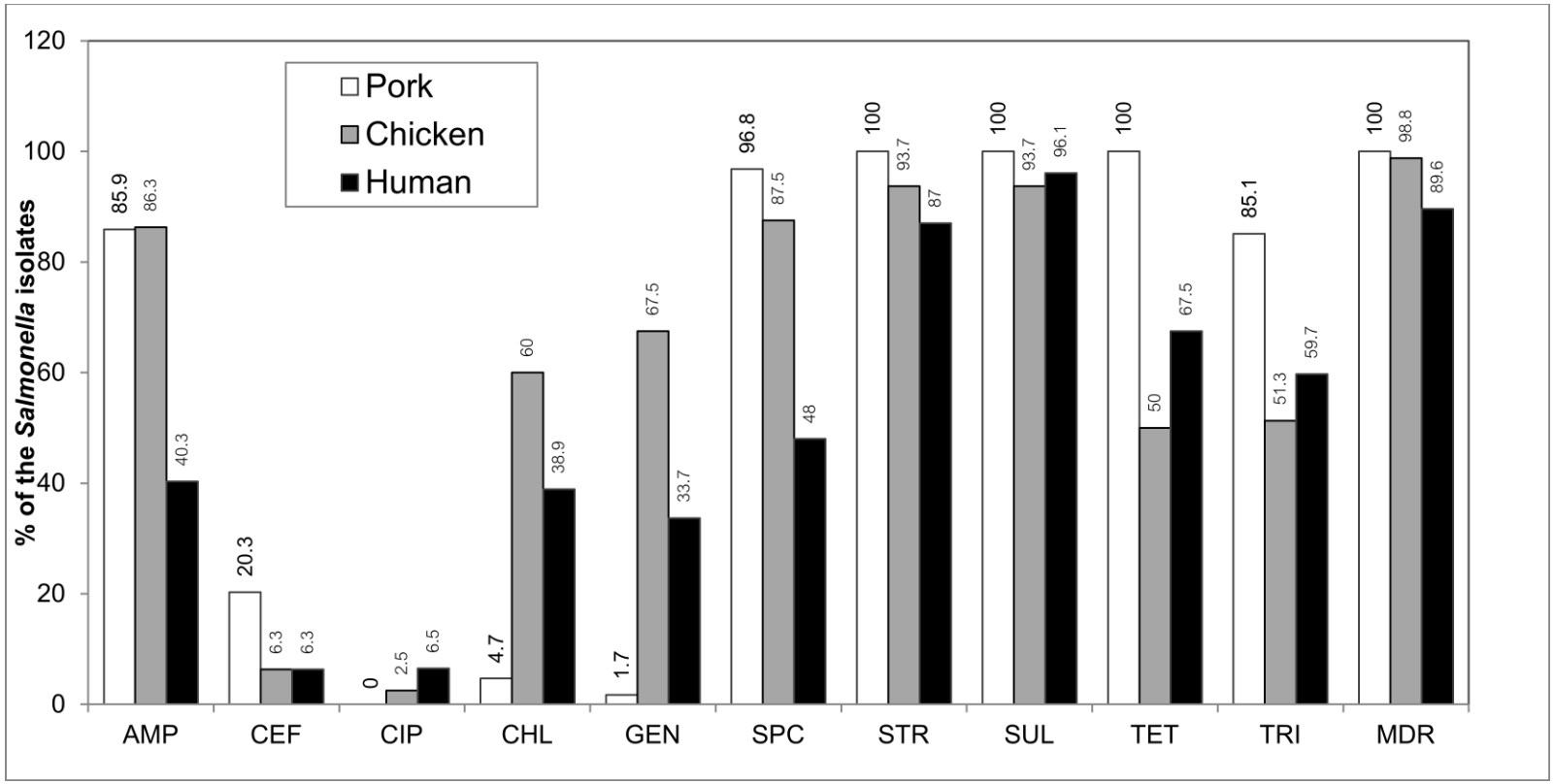

Figure 3. Frequency of resistance to 10 antimicrobial agents in *Salmonella (*n=221) from retail pork (n=64) retail chicken (n=80) and humans(n=77). AMP, ampicillin ; CEF, cefoperazone; CIP, ciprofloxacin; CHL, chloramphenicol; GEN, gentamicin ; SPC, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline, TRI, trimethoprim; MDR, multidrug resistant

Table 8. Antimicrobial resistance pattern of *Salmonella* from pork, chicken andhuman (n=221)

Species	Antimicrobial resistance pattern[a]	No. of isolates (%)
Pork (n=64)	AMP-SPC-STR-SUL-TET	7(10.9)
	AMP-SPC-STR-SUL-TET-TRI[b(10), e(12)]	27(42.1)
	AMP-CEF-SPC-STR-SUL-TET-TRI	6(9.3)
Chicken (n=80)	AMP-SPC-STR-SUL	6(7.5)
	AMP- CHL-GEN- SPC-STR-SU[d(10)]	14(17.5)
	AMP- CHL-GEN-SPC-STR-SUL-TET-TRI[b(6)]	14(17.5)
Human (n=77)	STR-SUL	4(5.1)
	SPC-STR-SUL[f(3)]	4(5.1)
	STR- SUL-TET-TRI	4(5.1)
	AMP- GEN- SUL-STR-TET-TRI	4(5.1)
	AMP- CHL-GEN-SPC-STR-SUL-TET-TRI	9(11.6)

[a]Only the antimicrobial resistance patterns represented by at least 5 isolates are shown.

[b]The most common resistance pattern in serovars Rissen from pork (n=10)

[c] The most common resistance pattern in serovars Rissen from chicken (n=6)

[d] The most common resistance pattern in serovars Schwarzengrund from chicken (n=5)

[e] The most common resistance pattern in serovars Anatum from pork (n=12)

[f] The most common resistance pattern in serovars Weltreverden from human (n=3)

Class 1 integrons, gene cassettes and their horizontal transfer

Forty-one percent of the *Salmonella* isolates was positive to *intI1* (i.e. pork, 26/64; chicken, 49/80; and human, 15/77), of which 60% carried class 1 integrons with variable region ranging in size from 0.2 to 2.0 kb. Six integron profiles (IP-I to IP-VI) were defined (Table 9). The most prevalent resistance genes were *dfrA12* (66.7%) and *aadA2* (75.9%) that were combined to the *dfrA12-aadA2* cassette array (66.7%). Co-existence of two distinct integrons carrying different gene cassettes i.e. *aadA2* and bla_{PSE-1} was identified in 4 isolates (IP-II)

Nine isolates from pork and humans (4.1%) carried class 1 integrons with the 200 bp fragment of an internal segment of *purG* encoding enzyme phosphoribosylformylglycinamide synthetase (IP-VI). All the isolates additionally yielded a 750 bp PCR fragment of a partial *codB* gene mediating cytosine permease that was not located within class 1 integrons as determined by nucleotide sequencing.

Only class 1 integrons with the *dfrA12-aadA2* cassette array in 5 isolates from retail pork (i.e. 3 serovars Anatum, a serovar Virchow and a serovar Seftenberg) could be horizontally transferred to *E. coli*.

Table 9. Class 1 integrons in *Salmonella* from pork, chicken and human (n=221)

IP[a]	Amplicon size (kb)	Inserted gene cassettes[f]	Serotype (number)[b]			
			Pork (n=64)	Chicken (n=80)	Human (n=77)	Total (n=221)
I	2.0	*dfrA12-aadA2*	Anatum (7)[c (3)] Corvallis (1) Rissen (4)[d(2)] Senftenberg (1)[c] Stanley (1) Virchow (1)[c]	Anatum (2) Rissen (13) Schwarzengrund (3)	Give (2) Schwarzengrund (1)	36(16.3)
II	1.0, 1.2	*aadA2, bla*$_{PSE-1}$	Anatum (1)[f]	Anatum (1)	Derby (1)	4(1.8)
III	1.0	*aadA4*	-	Albany (1)		3(13.4)
IV	1.2	*bla*$_{PSE-1}$	Albany (1)[e]	-	Panama (3)[d(1)]	1(0.5)
V	1.0	*aadA2*	Anatum (1)[e]	-	-	1(0.5)
VI	0.2	*purG*	-	Amsterdam (1) Agona (1) Corvallis (1) Kedougou (1)	Corvallis (1) Kedougou (1) Rissen (1) Weltreverden (2)	9(4.1)
Total			18 (28.1)	24(30)	12(15.6)	54(24.4)

[a] The integron profiles (IPs) were defined by the number and the size of the PCR amplicons and resistance gene cassettes identified.

[b] Number of strains is indicated in parenthesis.

[c] Successful class 1 integrons transfer (numbers of isolates with transferred class 1 integrons).

[d] Carry both *spvC* and class 1 integrons (numbers of isolates)

[e] Positive to *thdF* and *S044–yidY* of SGI1

[f] Positive to *thdF* and *int2* and termed SGI1-like elements

[g] *bla*$_{PSE-1}$ encodes β-lactam resistance, *dfrA12* encodes trimethoprim resistance; *aadA2* encodes streptomycin and spectomycin resistance; *aadA4* encodes streptomycin and spectomycin resistanc

Presence of SGI1-like gene cluster in *S. enterica* Anatum

Only three pork isolates carrying class 1 integrons with resistance gene cassettes were positive to *thdF.* Two isolates (i.e. serovars Anatum and Albany) carried *thdF* and *S044–yidY* (IP-IV and IP-V). The others (i.e a serovar Anatum) was positive to *thdF* but not *S044–yidY* (IP-II). The latter harbored *int2* and all SGI1 antibiotic resistance gene clusters located between *thdF* and *int2*.

Mutations within *gyrA* and *parC* QRDRs and the presence of PMQR genes

Two point mutations in *gyrA* (i.e. G-259-T and C-248-T, leading to Asp-87-Tyr and Ser-83-Phe substitutions in GyrA, respectively) were detected. Four ciprofloxacin-resistant *Salmonella* strains (MIC=4 μg/ml) carried mutations in *gyrA,* including a serovar Virchow from chicken and 2 serovars Enteritidis and a serovar Rissen from humans. A G-283-C change in *parC* leading to a Val-95-Leu mutation in ParC was additionally found. This amino acid substitution was also identified in two ciprofloxacin-susceptible *Salmonella* control strains and therefore, could be a result of sequence variation.

Nine *Salmonella* isolates harbored *qnrS*, including a serovar Stanley (CIP MIC=4 μg/ml) from humans; a serovar Anatum (CIP MIC=4 μg/ml), 5 serovars Covallis (CIP MIC=2 μg/ml) from poultry and 2 serovars Covallis (CIP MIC=2 μg/ml) from pork. The *qnrB* gene was found in 2 pork isolates, a serovar Rissen (CIP MIC=0.125 μg/ml) and a serovar Stanley (CIP MIC=0.125 μg/ml). None carried both PMQR genes and mutation in *gyrA* QRDRs.

Occurrence of *Salmonella* virulence plasmids

Overall, the prevalence of *Salmonella* plasmid virulence genes was low (i.e. *spvC*, 8.1%; *pefA*, 1.8% and *rck,* 1.4%). All the isolates yielded PCR products of the corresponding genes when using plasmid DNA as templates. Three *Salmonella* isolates i.e. 2 serovars Rissen from pork (IP-1) and a serovar Panama from human (IP-III) carried both class 1 integrons and *spvC* (Table 10) but none of class 1 integrons were located on conjugative plasmid. The latter was confirmed by PCR using the *E. coli* transconjugant plasmid DNA.

Table 10 The virulence plasmid associated genes in *Salmonella* from pork, chicken and human (n=221)

Gene	Positive serovars (No.)			Total no. (%)
	Pork (n=64)	Chicken (n=80)	Human (n=77)	
spvC	Rissen (5)	Enteritidis (1)	Anatum (1), Enteritidis (2), Typhimurium (1), Weltevreden (2), ser 1,,4,5,12 (1), Lexington (1), ser 4,12:i (1), ser 4,12 (2), Panama (1)	18 (8.1)
pefA	-	Enteritidis (1)	Enteritidis (2),Typhimurium (1)	4(1.8)
rck	-	Enteritidis (1)	Enteritidis (1), Typhimurium (1)	3(1.4)
Total no. (%)	5 (7.8)	3 (3.8)	17 (22.1)	25(11.3)

2.5 Discussion

In general, the majority of *Salmonella* serotypes can affect different hosts but some are host specific. In this study, some serotypes was predominantly found in particular hosts such as serovars Rissen in pork and chicken, serovars Schwarzengrund in chicken, serovars Anatum in pork and serovars Welterverden in humans (Table 1). These findings suggest potential host-specific population dynamics of *Salmonella*.

The data in this study highlighted a high rate of AMR among the *Salmonella* isolates from pork, chicken meat and diarrhea patients in Northeastern Thailand. The frequency of MDR isolates was higher than a previous report in the Mekong Delta, Vietnam (Ogasawara et al., 2008). The observation of ciprofloxacin resistance in the chicken and human isolates, even at low rate, raises particular concern because fluoroquinolone is the recommended drug for invasive Salmonellosis. These results may suggest the wide use of the antimicrobials in either animals or humans in Thailand. However, it should be noted that spread of AMR bacteria may be a result of non-antibiotic selective pressure e.g. disinfectants, heavy metals (Langsrud et al., 2004).

Overall, the prevalence of class 1 integrons was high (40.7%). It is comparable to our previous study in the pork and human isolates from Northern Thailand (Wannaprasat et al., 2011) but higher than that reported in other countries (Thong and Modarressi, 2011). It is interesting to observe that class 1 integrons were more common among the chicken isolates than the pork and human isolates. The reason for this observation, however, remains unclear. The pork and chicken isolates were originated from 5 provinces, while the human isolates were from a province. This source difference together with the possible sampling bias may limit the ability to compare the resistance profile among the isolates.

A particular concern was the presence of the same class integrons (i.e. IP-I, II and VI) in the pork, chicken and human isolates, suggesting circulation and horizontal transfer of these resistance determinants in the food chain. Therefore, the food chain serves as a potential route for the introduction of animal and environment

associated antibiotic resistant bacteria into the human. However, transmission of antimicrobial resistance from animals to humans can take through other routes e.g. direct handling, close contact, the spreading of slurry.

Class 1 integrons with incomplete *purG* (IP-VI) were previously identified as PCR artifact (Lindstedt et al., 2003) or as a small integron specific to serovar Typhimurium in (Daly et al., 2000). In this study, the partial *purG* gene was part of class 1 integron but not serovar-specific. All the isolates in IP-VI had an additional-incomplete *codB* fragment that was found to be a PCR artifact by DNA sequencing. In contrast, this defective *codB* gene was part of class 1 integrons in our previous study (Khemtong and Chuanchuen, 2008).

Almost half of the *intI1*-positive isolates (40%) carried empty integrons. These are likely due to excision of the previously-inserted gene cassettes when the antimicrobial selective pressure is diluted (Rosser and Young, 1999) and are available for new-emerging resistance genes. Therefore, minimized antimicrobial use may significantly reduce the integrons with resistance gene cassettes and their horizontal transfer among bacteria, leading to reduction of resistant bacteria.

It is of particular interest to observe a serovar Anatum from pork carrying SGI1-like elements with SGI1-gene cluster located between *thdf* and *int2*. This is in agreement with a previous study reporting a serovar Meleagridis containing SGI1-like gene cluster with the right junction *int2* retron phage (Ebner et al., 2004). The *int2* gene is part of retron sequence that was found only in S. Typhimurium (Doublet et al., 2003). Therefore, identification of SGI1 like elements on a non Typhimurium isolate confirmed that SGI1 genes are (partly) mobile (Ebner et al., 2004) and this

could be a possible route for spreading of multidrug resistance. Horizontal transfer of class 1 integrons and the presence of SGI-1 were limited to the pork isolates in this study. A possible explanation may be the different selective pressure created by different antibiotics used in different food animals and humans.

In this study, mutations were observed only in *gyrA* QRDRs. Two point mutations in GyrA i.e. Asp-87-Tyr and Ser-83-Phe was previously identified in *Salmonella* (Wang et al., 2015). It was shown that those with mutation at position 87 exhibited higher ciprofloxacin resistance than those with mutation at position 83 (Wang et al., 2015). In contrast, all 4 isolates with mutation in *gyrA* QRDRs had the same ciprofloxacin MIC value (i.e. 4 μg/ml). Still, it cannot conclude that the single mutation in *gyrA* QRDRs was a sole mechanism responsible to the MIC value observed and other uncharacterized mechanisms may exist.

In this study, ciprofloxacin MIC of the *qnr*-carrying isolates varied and two pig isolates carrying *qnrB* had low ciprofloxacin MIC (0.125μg/ml). This supports that impact of PMQR genes on ciprofloxacin MIC level varies and their presence does not always cause high level of fluoroquinolone resistance (Robicsek et al., 2006). The PMQR determinants are likely to provide co selection of resistance genes for other antibiotics. Two ciprofloxacin-resistant isolates had neither *gyrA* mutations nor PMQR determinants, indicating existence of other resistance mechanisms (e.g. the efflux pumps and decreased membrane permeability).

The *spvC* gene was most frequently found (8.1%). This is not surprising because the *spv* operon is common to all *Salmonella* virulence plasmids (Zou et al., 2012). The *pefA* and *rck* genes were rare and limited to serovars Enteritridis and

Typhimurium. This is most likely due to the serovar specificity of these two genes (Skyberg et al., 2006). It should be also noted that virulence plasmid associated genes can be presented on chromosome (Boyd and Hartl, 1998). The isolate with the genes on genomic DNA (if any) would also yield a positive result. Some *Salmonella* carried both class 1 integrons and *spvC* but not on the same conjugative plasmid, suggesting the presence of multiple plasmids or chromosomal integration of the virulence plasmid.

In summary, the results in this study highlight the high rates of MDR phenotype and class 1 integrons among the *Salmonella* isolates from pork, chicken and humans. It suggests that antimicrobials should be appropriately used in food animals and humans to preserve the efficacy of the existing drugs and to minimize the risk of transfer of resistant foodborne pathogens to humans.

Conclusion

The results in this study highlight the high rates of MDR phenotype and class 1 integrons among the *Salmonella* isolates from pork, chicken and humans. The most prevalent resistance genes were *dfrA12-aadA2* cassette array. A serovar Anatum carried SGI1-like element. The prevalence of *Salmonella* plasmid virulence genes in this study was low. The *spvC* gene was the predominant *Salmonella* plasmid virulence followed by *pefA* and *rck*. Our finding suggests that antimicrobials should be properly used in food-producing animals and humans to preserve the efficacy of the existing drugs and to reduce the risk of transfer of resistant foodborne pathogens to humans.

CHAPTER IV

Mutations in quinolone resistance-determining region in DNA gyrase and topoisomerase IV genes in quinolone-resistant *Salmonella enterica* isolated from chicken meat, pork and humans

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The Journal of Veterinary Medical Science (Submitted)

Mutations in the QRDRs of DNA gyrase and topoisomerase IV genes in nalidixic acid and ciprofloxacin-resistant *Salmonella enterica* isolated from chicken meat, pork and humans

ABSTRACT

Twenty-eight nalidixic acid-resistant *Salmonella* isolates originated from chicken meat, pork and humans were examined for mutation in the quinolone determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes. Four single-point mutations in *gyrA* (i.e. C248A, C248T, G259T, G259A) leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr and Asp87Asn in GyrA, respectively, were identified in 11 quinolone-resistant strains. Amino acid change at position Ser83 was most frequently identified. Resistance to nalidixic acid was not always associated with ciprofloxacin resistance. The presence of mutations in GyrA did not well correlate with ciprofloxacin resistance phenotype. No mutations were observed in *gyrB, parC* and *parE*. A serovar Typhimurium resistant to nalidixic acid did not carry any mutations in QRDR of all genes tested. The results highlight the high frequency of mutation in *gyrA* in the quinolone-resistant isolates and the existence of alternative-quinolone resistance mechanisms.

KEYWORDS: DNA gyrase, fluoroquinolone resistance, nalidixic acid resistance, *Salmonella enterica,* topoisomerase IV

Non typhoidal *Salmonella enterica* infections *are a* major threat to global public health and is commonly associated with consumption of contaminated food of animal origin (e.g. chicken meat, pork). Salmonellosis is usually self-limited and does not require antibiotic therapy. However, antibiotic treatment may be required for young children or elderly people. Fluoroquinolones have been recommended by WHO as empirical treatment for multidrug resistant enteric fever (WHO, 2007) and are often used to treat invasive *Salmonella* infection in humans (Cremet et al., 2011). Particular concern is that the increasing use of fluoroquinolones may lead to the emergence and spread of fluoroquinolone- resistant *Salmonella*. Recently, fluoroquinolone-resistant *Salmonella* has been increasingly reported (Lertworapreecha et al., 2013, Wang et al., 2015).

Quinolones are broad-spectrum antibiotics with four generations, of which nalidixic acid is the first-generation drug exhibiting a narrow-spectrum activity against Gram-negative bacteria. Ciprofloxacin is the second generation quinolone with a broad-spectrum activity against Gram-negative and Gram-positive bacteria (Fàbrega et al., 2009). These antibiotics selectively inhibit topoisomerase II (DNA gyrase) and topoisomerase IV and prevent bacterial DNA replication during growth and reproduction. DNA gyrase has two A subunits and two B subunits that are encoded by *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is composed of two C and two E subunits encoded by *parC* and *parE* genes, respectively. During DNA replication, DNA gyrase catalyses negative DNA supercoiling and topoisomerase IV is responsible for segregation daughter chromosome (Hopkins et al., 2005).

Three major mechanisms of quinolone resistance have been recognized including mutations in the quinolone-resistance-determining region (QRDR) of DNA gyrase and topoisomerase IV genes; reduced drug accumulation in bacterial cell by overexpression of efflux pump; impermeability of outer membrane and the presence of plasmid-mediated quinolone resistance genes (Jacoby, 2005). Among these, mutations in QRDR of DNA gyrase and topoisomerase IV genes are frequently present in quinolone-resistant bacteria (Wasyl et al., 2014, García-Fernández et al., 2015). QRDR is a small region located between amino acid 67-107 in GyrA and amino acid 426-447 in GyrB (Yoshida, 1991) and may be quinolone-binding site (Madurga et al., 2008). The amino acid substitutions in QRDR could result in reduced fluoroquinolone binding affinity, leading to decrease fluoroquinolones susceptibility in bacteria (Vashist et al., 2009).

Quinolone resistant *Salmonella* has been previously reported in Thailand. However, most studies focused on the resistance phenotype (Minami et al., 2010, Sanpong et al., 2010, Lertworapreecha et al 2013) and little published information on the quinolone resistance genotype is available. We now provide an update on mutations in the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes in nalidixic acid and/or ciprofloxacin resistant *Salmonella* from pork, chicken meat and humans in Thailand.

We examined a total of 28 nalidixic-resistant *Salmonella* isolates that were originated from faecal samples of humans (n= 7), pigs (n=16) and chicken (n=5) in 2010-2011 (Table1).The strains were isolated using ISO6579:2002 (E) (ISO, 2002) and serotyping at Center of Antimicrobial Resistance in Foodborne Pathogens (in cooperation with the World Health Organization), Faculty of Veterinary Science, Chulalongkorn University. These *Salmonella* isolates were previously tested against nalidixic acid (30 g) using

the CLSI guidelines for antimicrobial disc susceptibility testing (CLSI, 2008). All the isolates were stored at -80^{o}C in 20% glycerol as our strain collection.

All the isolates were determined for minimum inhibitory concentration (MIC) of nalidixic acid and ciprofloxacin by two-fold agar dilution method as described by CLSI . Clinical breakpoints for nalidixic acid and ciprofloxacin were ≥ 32 and ≥ 4 µg/ml, respectively. *Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC®27853, *Staphylococcus aureus* ATCC® 29213 were included as quality control strains.

The QRDRs of *gyrA*, *gyrB, parC and parE* genes were PCR amplified by using specific primer pairs as follows: *gyrA*, gyrA-F (5'-GCTGAAGAGCTCCTATCTGG-3')/gyrA-R (5'-GGTCGGCATGACGTCCGG-3'); *gyrB,* gyrB-F (5'-GCGCGCTCGATTTAGCCG-3')/gyrB-R (5'-TGATAGCGCAGCTTGTCCG-3'); *parC,* parC-F (5'- GTACGTGATCATGGATCGTG-3')/ parC-R (5'-TTCCTGCATGGTGCCGTCG-3') and . *parE*, parE-F (5'- GCCATCGCGAATATCAGGCG-3')/parE-R (5'-CAGTTGTTCCAGTACGCCC 3' (Chuanchuen and Padungtod, 2009). The PCR amplicons were gel purified using Nucleospin® Gel and PCR clean up (Düren, Germany) and submitted for DNA sequencing (First Base Laboratories, Selangor Darul Ehsan, Malaysia). All sequences obtained were compared with those in GenBank database using the Blast algorithm available at www.ncbi.nlm.nih.gov (Genbank accession numbers AE008801, AE008878and AE008846 for *gyrA*, *gyrB*, *parC* and *parE*, respectively).Two *Salmonella* strains susceptible to nalidixic acid and ciprofloxacin were included as control strains.

All the nalidixic acid-resistant *Salmonella* isolates had MIC value ranging from 32 to 256 µg/ml. Six isolates (4 human isolates and 2 chicken isolates) were additionally resistant to ciprofloxacin with MIC 4 µg/ml (Table11).

Table 11. MIC value of nalidixic acid and ciprofloxacin in the Salmonella isolates From humans, chicken meat and pork (n=28)

Source	Strain ID	Serotype	Sample type	Location	MIC (μg/ml)[a]	
					Nalidixic acid	Ciprofloxacin
Humans (n=7)	A43	Anatum	Rectal swab	Hospital	256	2
	A45	Weltevreden	Rectal swab	Hospital	256	0.125
	A50	Enteritidis	Rectal swab	Hospital	256	4
	A53	Enteritidis	Rectal swab	Hospital	256	4
	A54	Corvallis	Rectal swab	Hospital	32	4
	A60	Rissen	Rectal swab	Hospital	256	4
	C48	Typhimurium	Rectal swab	Hospital	128	1
Chicken(n=5)	B36	Anatum	Carcass swab	Slaughterhouse	32	4
	C64	Amsterdam	Carcass swab	Slaughterhouse	128	1
	D63	Virchow	Chicken meat	Market	128	0.5
	D70	Agona	Chicken meat	Market	128	0.125
	E21	Virchow	Chicken meat	Market	128	4
Pig (n=16)	A4	Anatum	Carcass swab	Slaughterhouse	32	0.125
	A5	Anatum	Carcass swab	Slaughterhouse	32	0.25
	A7	Albany	Carcass swab	Slaughterhouse	128	0.5
	C77	Weltevreden	Carcass swab	Slaughterhouse	32	0.125
	C78	Stanley	Carcass swab	Slaughterhouse	128	0.125
	C90	Anatum	Retail pork	Market	32	0.125
	C91	Corvallis	Retail pork	Market	32	2
	C92	Anatum	Retail pork	Market	>128	1
	D1	Corvallis	Retail pork	Market	32	2
	D5	Rissen	Retail pork	Market	32	0.125
	D10	Anatum	Retail pork	Market	128	1
	D11	Rissen	Retail pork	Market	128	1
	D54	Stanley	Retail pork	Market	128	1
	E16	Anatum	Retail pork	Market	128	1
	E37	Anatum	Retail pork	Market	128	1
	E82	Worthington	Retail pork	Market	32	1

[a]The clinical breakpoint for nalidixic acid and ciprofloxacin was32 μg/ml and 4 μg/ml, respectively.

Nucleotide sequencing analysis revealed that 11 isolates carried a single point mutation in *gyrA* including C248A, C248T, G259T and G259A leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr, Asp87Asn in GyrA, respectively (Table 12). Amino acid change at codon 83 (i.e. Ser83Phe and Ser83 Tyr) was most commonly identified (n=8). Four isolates resistant to both nalidixic acid and ciprofloxacin harbored Ser83Phe (i.e. A50, A53 and E21) or Asp87Tyr (i.e. E21). Seven *Salmonella*

isolates resistant to nalidixic acid but susceptible to ciprofloxacin carried amino acid change Ser83Tyr (i.e. A43, C64, C92 and D10), Ser83Phe (i.e. E37), Asp87Tyr (i.e. D63) or Asp87Asn (i.e. A7). Twenty-two isolates carried a single point mutation G283C in *parC* leading to Val95Leu substitution in ParC that was also found in the two susceptible-control strains. None of the isolates harbored mutations neither in *gyrB* nor *parE*.

Based upon the CLSI ciprofloxacin breakpoint, only six nalidixic acid resistant isolates in this collection were additionally resistant to ciprofloxacin. It indicates that resistance to nalidixic acid is not always associated with resistance to ciprofloxacin, consistent with previous studies (Hakanen et al., 1999, Kozoderovic et al., 2012). However, it has been suggested that CLSI interpretative criteria for ciprofloxacin (i.e. ≥4 μg/ml) should be reevaluated (Ryan et al., 2011). Several studies showed that the nontyphoidal and typhoidal *Salmonella* isolates had the ciprofloxacin MIC much lower than 4 mg/liter (Kozoderovic et al., 2012, Wasyl et al., 2014) and a breakpoint of ≥0.125 μg/ml has been recommended (Aarestrup et al., 2003). Based on the nucledtide sequencing analysis, the similar mutations in GyrA (e.g. Asp87Asn, Ser83Phe, Asp87Thr) were observed in the ciprofloxacin-susceptible and -resistant isolates. Seven isolates resistant to nalidixic acid but susceptible to ciprofloxacin carried amino acid mutation at position Ser83 and Asp87 in GyrA. It is possible that mutations may reduce the ciprofloxacin susceptibility at certain extent but not enough to bring their MIC value above 4 μg/ml. These may be another support for the requirement of the CLSI-ciprofloxacin breakpoint revision.

In addition, the isolates with high nalidixic acid MIC (128-256 μg/ml) exhibited varied ciprofloxacin MIC (0.125 to 4 μg/ml). Therefore, there was no correlation between

nalidixic acid MIC and the raised ciprofloxacin MIC, in agreement with a previous study (Kim et al.,2011).

Mutations within the QRDR of *gyrA* are considered one of the main mechanisms of quinolone resistance. Amino acid substitutions at position Ser83 and Asp87 in GyrA have been previously reported (Wasyl, 2014, Wang et al.,2015). The replacement of the hydrophilic hydroxyl group of serine by the hydrophobic residue of phenylalanine or tyrosine results in the loss of OH group of serine, leading to the loss of hydrogen-bonding interaction between amino acid residue and quinolone molecule and eventually, reduced drug binding affinity with target site (Piddock, 2002). The similar phenomenon is applied for the amino acid change at Asp87. It was previously shown that a mutation at Ser83 conferred a higher quinolone-resistance level, in comparison to that at position 87 (Ogbolu et al., 2012). However, it was not the case in this study. Further studies e.g. site-directed mutagenesis is required to better understand the contribution of mutations in these target genes.

All four *Salmonella* isolates resistant to both nalidixic acid and ciprofloxacin containing a single mutation at position Ser 83 and Asp87 had nalidixic acid MIC value of ≥ 128 μg/ml and ciprofloxacin MIC value of 4 μg/ml. This finding is consistent with a previous study showing that the *S*. Enteritidis isolates carrying Ser83Tyr had the high-nalidixic acid resistance level (MIC> 250 μg/ml) with the average ciprofloxacin MIC value of 4 μg/ml (Lindsted et al., 2004).

Previous studies demonstrated that high-level resistance level to fluoroquinolones was associated with double mutations in *gyrA* at position 83 and 87 coupled with *parC* mutation (Cui et al., 2008, García-Fernández A et al., 2015). However, double mutations in *gyrA* gene were not observed in our study. Most

quinolone-resistant strains in the present study (n=22) carried amino acid substitution Val95Leu in ParC that was also found in the quinolone-susceptible strains. Therefore, this amino acid change may be a result of biological variation among the *Salmonella* strains.

Table 12 Amino acid substitution in the QRDRs of GyrA in the quinolone-resistant *Salmonella* strains from humans, chicken meat and pork (*n*=11)

Source	Strain ID	Serotype	MIC (µg/ml)[a]		Mutation in QRDR of *gyrA*	
			Nalidixic acid	Ciprofloxacin	Nucleotide sequence	Amino acid substitution
Human(n=4)	A43	Anatum	256	2	C248A	Ser83Tyr
	A50	Enteritidis	256	4	C248T	Ser83Phe
	A53	Enteritidis	256	4	C248T	Ser83Phe
	A60	Rissen	256	4	C248T	Ser83Phe
Chicken meat (n=3)	C64	Amsterdam	128	1	C248A	Ser83Tyr
	D63	Virchow	128	0.5	G259T	Asp87Tyr
	E21	Virchow	128	4	G259T	Asp87Tyr
Pork (n=4)	A7	Albany	128	0.5	G259A	Asp87Asn
	C92	Anatum	128	1	C248A	Ser83 Tyr
	D10	Anatum	128	1	C248A	Ser83Tyr
	E37	Anatum	128	1	C248T	Ser83Phe

[a]The clinical breakpoint for nalidixic acid and ciprofloxacin was 32 µg/ml and 4 µg/ml, respectively.

Lack of mutation in *gyrB* and *parE* in this study supports that the quinolone-resistant strain of *Salmonella* with mutations in *gyrB* or *parE* were rare (Kozoderovic et al., 2012, Yang et al., 2012). In fact, the contribution of amino acid change in ParE to quinolone resistance remains unclear. In addition, some quinolone-resistant *Salmonella* isolates (n=17) lacked of mutation the QRDR of the tested genes,

suggesting the existence of alternative resistance mechanisms that were not characterized in this study e.g. efflux pump systems, plasmid-mediated quinolone resistance. This warranted further studies to elucidate the quinolone-resistance mechanisms in these *Salmonella* isolates.

In conclusion, the results of this study highlight the complex pictures of quinolone resistance mechanisms in the *Salmonella* isolate. The continuous monitoring of fluoroquinolone usage and resistance in *Salmonella* and other bacteria is indispensable.

Conclusion

The complex pictures of quinolone resistance mechanisms in the *Salmonella* isolate was highlighted in this study. Our finding indicates that resistance to nalidixic acid is not always associated with resistance to ciprofloxacin. The CLSI breakpoint of ciprofloxacin CLSI should be reevaluated. The continuous monitoring of fluoroquinolone usage and resistance in *Salmonella* and other bacteria at phenotypic and genotypic levels is necessary.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussions

Salmonellosis is a major foodborne bacterial disease worldwide. The outbreaks of *Salmonella* infection in humans are commonly associated with consumption of contaminated food product of animal origin. It is well known that pigs can be infected with and carry *Salmonella* in their intestinal tract. Therefore, pigs play a role as a reservoir for *Salmonella* contamination in their meat and finally, the food chain (Kim et al., 2011).

Antimicrobial resistance in foodborne pathogens becomes a critical issue in several regions including Southeast Asian regional countries. In 2015, the ASEAN Economic Community (AEC) was established and has 10 member states in this community. The aim of AEC is to assimilate Southeast Asia countries into a single region with free movement of merchandises, services, traveler and skilled labor. The increase of international trade and massive movement of people, food products across national boundaries is an important driver to promote the distribution of *Salmonella* and their resistance determinants throughout the region and the world.

In the present study, the overall prevalence of *Salmonella* isolated from Thailand and Laos provinces was 39.5%. This prevalence was higher than the previous report from pig production chain in Vietnam (28.4%) (Yokozawa, 2014), Belgium (14.1%) (De Busser et al., 2011), Mexico (17.3%) (Miranda et al., 2009) but lower than the report from China (71.5%) (Cai et al., 2016) and Mexico (58.1%) (Zaidi et al., 2006). Total prevalence of *Salmonella* collected from the slaughterhouses and retail markets in Laos provinces was significantly higher than the sample collected from Thai province ($p<0.05$).

The data obtained suggested that it may be due to the difference of sanitation procedure during slaughtering process and meat handling in each location and region. In Thailand, the demand of pork and pig products for both domestic and export markets has increased (Thai swine, 2013). As a result, the novel production technology under good hygienic standards for abattoir (ACSF, 2006) and for animal welfare (ACSF, 2010) has been introduced and used in farm and slaughterhouses in Thailand. However, small conventional swine farms and slaughterhouses with poor hygiene management are still at provincial area observed and needed to be improved. Conversely, swine farm in Laos are mostly owned by family or smallholders (Boonmar et al., 2008; Phengsavanh et al., 2011). Lack of good hygiene practice during slaughtering process is a common problem in this country (Bastiaensen et al., 2011). This could explain the high prevalence of *Salmonella* observed in Laos. It should be noted that the samples in this study were collected from only one slaughterhouse, one retail market in each province. Therefore, further investigation should be performed in different areas or pig farms in the country to support the finding in our study.

The prevalence of *Salmonella* was statistically higher in retail markets than that in the slaughterhouses in both Thailand and Laos ($p<0.05$). At the slaughterhouses, pig carcasses were directly transported to retail markets after slaughtering process. Then, the carcasses were cut into retail meat portions and cleaned by market butchers. Cross contamination may occur in this step due to poor hygiene practice such as unclean equipment and storage container, improper re-packaging and grinding process or environment

contamination (Mürmann et al., 2009).Our finding differed from the report in China showing no significant difference of *Salmonella* contamination rate between slaughterhouses and retail markets (Cai et al., 2016). It has been suggested that many factors can affect the prevalence in each region such as sampling site, sampling season, slaughterhouse management.

The low percentage of *Salmonella* was found in human samples in this study (17.7%). There was no statistical difference of *Salmonella* prevalence among workers, butchers in Thailand and Laos provinces ($p>0.05$). In general, workers or butchers may contract Salmonella from pigs and raw meat during slaughtering process and retailing. Therefore, these people may serve as carriers and contribute to *Salmonella* outbreak (Gómez-Aldapa et al., 2012). At the retail markets, only 4% of samples from butchers in Thailand were positive to *Salmonella,* while no positive samples were observed in butchers in Laos. Although high prevalence of *Salmonella* was detected in pork (65%), the positive *Salmonella* isolated from butchers was rarely identified. It is possibly due to the limited number of samples from butchers. The percentage of *Salmonella*-positive samples from hospitalized patients with diarrhea was moderately high in both Thailand (17.9%) and Laos (11.1%) compared to the prevalence in hospitalized children from Northern Thailand (7%) (Padungtod and Kaneene, 2006). However, it cannot be concluded that the source of *Salmonella* infection in these patients was related to pork consumption or exposure to pigs because history taking of patient including dietary consumption and livestock contact was not performed in this study.

When consider the *Salmonella* prevalence in each country, the prevalence of *Salmonella* isolates from pigs and pig carcasses at slaughterhouses in Thailand was 32.5%. This prevalence data is consistent with previous study in Khon Kaen province, Northeastern Thailand (Sithigon and Angkititrakul, 2011) but higher than that in Northern Thailand (Padungtod and Kaneene, 2006; Tadee et al., 2014). In Laos, the prevalence of *Salmonella* (46.2%) was lower than the previous reports in Vientiane, Laos (Inthavong et al., 2006; Boonmar et al., 2008). At retail markets, the prevalence of *Salmonella* in pork in Thailand (60.5%) and Laos (72.3%) was lower than that in previous studies in northeastern and southern part of Thailand (Angkititrakul et al., 2005; Lertworapreecha et al., 2013) and in Champasak province, Laos (Boonmar et al., 2013).

In this study, *S*. Anatum, *S*. Typhimurium and *S*. Rissen were predominant among *Salmonella* serovars in Northeastern Thailand and Laos. Although the same serovar was identified in both Thailand and Laos, it cannot be concluded that the *Salmonella* strains were originated from the same pig source. The study of genetic relatedness is required to elucidate this genetic relationship.

The distribution of serovars in our study was similar to previous studies showing *S*. Typhimurium, *S*. Rissen and *S*. Anatum were frequently found in pig products in different regions in Thailand, Laos (Sanguankiat et al., 2010; Boonmar et al., 2013; Lertworapreecha et al., 2013) and other countries in Asia such as China, Vietnam (Li et al., 2016; Nguyen et al., 2016). In

contrast, the outbreak of *S*. Rissen was rarely reported in the US (Pornsukarom et al., 2015) and European countries (Hugas and Beloeil, 2014).

Most of the isolates (98%) from Thailand and Laos were multidrug resistant. The high rates of resistance to sulfamethoxazole (98.3%) and tetracycline (96.9%) were observed in the isolates in both countries. The findings were consistent to previous studies from Northeastern and Central part of Thailand (Angkititrakul et al., 2005; Sanpong et al., 2010). In contrast, resistance rates to sulfamethoxazole and tetracycline in this study were higher than those in previous studies in Laos (Boonmar et al., 2008; Boonmar et al., 2013). Sulfonamide and tetracycline use for therapeutic and prophylaxis purposes in some countries in Asia (Vergne et al., 2014). Antibiotics are usually given at high doses for short periods of time to animals (prophylaxis) during pig production such as after weaning period or during transport for disease prevention (Becker, 2010). This could explain the massive use of antimicrobials as feed medication during pig production may be the leading cause of the high rates of sulfamethoxazole and tetracycline resistance (Angkititrakul et al., 2008).

Low-resistance rate to ciprofloxacin was observed in this study (0.5%). However, this could be a particular concern because quinolones are a drug of choice for treatment of *Salmonella* infection and often used to treat invasive *Salmonella* infection in humans (Cremet et al., 2011).

Resistance to cephalosporins was found in Thai isolates (2.4-10%), while almost all of the Laos isolates were susceptible to these drugs (0-2.4%). The high price of cephalosporin limit their use as feed medication in pigs

(Sanpong et al., 2010). In addition, the use of feed additive antibiotics is unpopular in pig farming system in Laos because pigs are commonly raised by smallholder farmers and the use of feed additive antibiotic may increase in production costs. (Boonmar et al., 2008). Limiting the use of cephalosporin in pig farm may be one factor for low-level cephalosporin resistance in *Salmonella*.

Gentamicin resistance in Thai isolates was higher than previous reports in pork from the same country (Angkititrakul et al., 2008; Lertworapreecha et al., 2013) but consistent to previous report in Vietnam (Thai et al., 2012). In contrast, almost all the Laos isolates were susceptible to gentamicin (83.3%). The different type of antibiotics has been commonly used in pig production in different geographical locations even in the same counties. This could result in the variation in resistance rates of antibiotics. While database of type and amount of antimicrobials used in the countries is limited, the findings highlight the need of antimicrobial use monitoring in this region.

For class 1 integrons, the predominant gene cassette in *Salmonella* isolates from pigs and humans in both Thailand and Laos was *dfrA12-aadA2* encoding trimethoprim and spectinomycin/streptomycin resistance, respectively. Our finding was similar to previous study in pigs and farm environment from Thailand (Padungtod et al., 2011). This resistance gene cassette array was also identified in class 1 integrons in chicken in Ethiopia (Molla et al., 2007), pig and poultry in China (Li et al., 2013), beef cattle in Canada (Wu et al., 2011). The findings of the some integrons in different bacterial pathogens, different livestock animal species and different countries

indicate horizontal transfer and circulation of this gene cassette around the world. Some class 1 integrons did not contain resistance gene cassettes, so-called “empty integrons”. These integrons may lose their gene cassettes to environment when the selective pressure is absent or may transfer the gene cassettes to other bacterial cells. Regardless, the empty integrons are available for insertion of new resistance gene cassettes (Rosser and Young, 1999).

The presence of SGI1 gene cluster was identified in only three pork isolates from Northeastern Thailand. Interestingly, a serovar Anatum in this study carried SGI1-like element that was previously identified in a serovar Melegridis from bovine feces in USA (Ebner et al., 2004) and a serovar Typhimurium from human in Canada (Boyd et al., 2000). The element had which is part of retron sequence. This observation indicates that SGI1 can mobile and contribute to the dissemination the antimicrobial resistance among bacteria (Ebner et al., 2004).

For the isolates from Northeastern and Thailand—Laos border area, ciprofloxacin-resistant strains were examined for mutations in QRDR of *gyrA* and *parC*. Four single-point mutations in *gyrA* (i.e. C248A, C248T, G259T, G259A) leading to amino acid substitutions Ser83Tyr, Ser83Phe, Asp87Tyr and Asp87Asn in GyrA, respectively were observed in quinolone-resistant strains, in agreement with previous studies showing that mutations in QRDR of *gyrA* are commonly observed in quinolone-resistant Gram negative bacteria including quinolone-resistant *Salmonella* strains (Ogbolu et al., 2012; Wasyl et al., 2014). We also additionally selected nalidixic acid-resistant *Salmonella* strains

from Northeastern Thailand (n=28) to further examine for contribution of mutations in quinolone determining region (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* genes. One of major finding indicates that resistance to nalidixic acid is not always associated with resistance to ciprofloxacin, consistent with previous studies (Hakanen et al., 1999; Kim et al., 2011).

. The prevalence of *qnrB* (0.9%) and *qnrS* (5.6%) in our findings was lower than previous reports in food animals samples from China (*qnrB*; 16%, *qnrS*; 66%) (Lin et al., 2015). In contrast, it was higher than previous reports of *qnrB* (0%) and *qnrS* (0%) in Vietnamese retail meat (Thai and Yamaguchi, 2012) and food animals samples from Brazil (*qnrB*; 0.7%, *qnrS*; 0.7%) (Ferrari et al., 2011). The prevalence of *qnr* gene family varying from less than 1% to more than 50%, depending on the bacteria species, geographical location and their resistance mechanisms (Rodriguez-Martinez et al., 2011). In this study, a serovar Give from pork carried the *qnrS* gene in combination with a single amino acid substitution in *gyrA* (MIC nalidixic acid, >256 μg/ml; ciprofloxacin, 4 μg/ml). This finding is in agreement with a previous study in France reporting that their bacterial strains carried both mutations in QRDR and PMQR determinants (Cremet et al., 2011). However, the contribution of each mechanism to the resistance remains unclear. This is worth further investigation to identify the individual and combined effect.

In the present study, the *qnr*-carrying isolates exhibited low resistance levels to nalidixic acid and ciprofloxacin (as low as 8 and 0.03 μg/ml, respectively). This finding consistent with previous report in *Salmonella* isolates from humans and animal products in Thailand (Cavaco et al., 2007).

The report revealed that most *Salmonella* strains carrying *qnrS* exhibited decreased susceptible to ciprofloxacin (MIC >0.06 μg/ml) but susceptible or intermediate to nalidixic acid (MIC 8-16 μg/ml). The presence of *aac(6')-Ib-cr* was identified in only one *Salmonella* isolate (0.18%) exhibiting low level resistance to nalidixic acid and ciprofloxacin (MIC 16 and 1 μg/ml, respectively). In contrast to previous study indicating high prevalence *aac(6')-Ib-cr* (18.8%) was identified in ciprofloxacin-resistant *Enterobacteriaceae* strains in China (Ma et al., 2009). The data from our study suggests that the strains-carrying PMQR did not certainly show full resistance to nalidixic acid. Therefore, the use of nalidixic acid and ciprofloxacin as exclusive criteria to screen fluoroquinolone resistance may be not appropriate (Cavaco et al., 2007). However, It has been suggested that CLSI interpretative criteria for ciprofloxacin (i.e. ≥4 μg/ml) should be reevaluated (Ryan et al., 2011). Several studies showed that the nontyphoidal and typhoidal *Salmonella* isolates had the ciprofloxacin MIC much lower than 4 mg/liter (Kim et al., 2011; Wasyl et al., 2014) and a breakpoint of ≥0.125 μg/ml has been recommended (Aarestrup et al., 2003).

All ESBL-producing *Salmonella* strains harboring $bla_{\text{CTX-M14}}$ were detected in pig carcasses and pork in this study (2.4%). The $bla_{\text{CTX-M14}}$ gene is commonly located in plasmid carrying multiple-resistance genes. Therefore, ESBL-producing strains usually exhibited multiple -resistance to other antimicrobials (Rodriguez-Martinez et al., 2011; Zhao et al., 2014). This is supported by our observation that all ESBL-producing strains in this study exhibited multidrug-resistant phenotype. The emergence and spread of

bla$_{CTX-M14}$ among *Salmonella* isolates has been previously described in *S*.Typhimurium from patients in Hongkong (Jin and Ling, 2006), *S*.I4,[5],12:i:- from pork in Portugal (Clemente et al., 2013) or S. Infantis from broiler in Japan (Kameyama et al., 2012). The widespread of *bla*$_{CTX-M14}$ among bacterial pathogens has raised serious public health and veterinary concern. Since it could be result in limitation in drug of choice of treatment of *Salmonella* infection in the future.

The combination between *qnrS* and *bla*$_{CTX-M14}$ in all ESBL-producing *Salmonella* strains was found in this study (2.37%). Many studies previously showed the association between ESBL-producing strains and PMQR in *Enterobacteriaceae* (Cremet et al., 2011). Co-localization of *bla*$_{CTX-M14}$ and PMQR on the same plasmid may occur. This possibly lead to co-selection for β-lactamase-encoding genes by a single antibiotic use (Cremet et al., 2011), allowing the spread of multiple resistance determinants. However, the co-localization of *qnrS* and *bla*$_{CTX-M-14}$ genes was not examined in this study. In addition, *aac(6')-Ib-cr* in ESBL-producing *Salmonella* strains was not found in this study. The link between *aac(6')-Ib-cr* and *bla*$_{CTX\ M14}$ have been reported in previous study (Warburg et al., 2009). Even PMQR did not conduct high-level quinolone resistance to these isolates, it possibly influence co-selection for β-lactamase-encoding genes (Cremet et al., 2011). It is suggested that a widely use of fluoroquinolone may enhance the development of fluoroquinolone resistance mechanism and the emergence of ESBL-producing bacterial strains.

The overall prevalence of *Salmonella* plasmid virulence genes in this study was low (8.1%). The *spvC* gene was the predominant *Salmonella* plasmid virulence followed by *pefA* and *rck*. Our finding is in agreement with previous studies reporting that the *spvC* gene was commonly observed among the *Salmonella* isolates from pigs in Korea (Hur et al., 2012) and from humans in the United State (Zou et al., 2012). In addition, the presence of *pef A* and *rck* genes were limited in *S*. Enteritidis and *S*.Typhimurium. The explanation could be the role of serovar specific virulence plasmid in *Salmonella* (Rychlik et al., 2006).

5.2 Conclusion and suggestion

The objectives of this study have been accomplished. The research was conducted as planned with the successful results and there was already published in international journals (Appendix). The summary of this study can be made according to the purposed objectives as follows:

Objective 1: To monitor antimicrobial resistance among *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area.

Our study presented the situation of AMR–*Salmonella* in Northeastern Thailand and 5 provinces around Thailand-Laos border area. This agrees with the reports in other parts of the world and supports that AMR is a global problem (Figure 4.) High prevalence of MDR-*Salmonella* isolated from pig, pig products and humans was observed resistance to sulfamethoxazole and tetracycline was very common.

These findings suggest that AMR surveillance and continuous monitoring program at local, national and global level are required to provide the current information and impact of AMR.

Objective 2: To characterize of antimicrobial resistance of *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border area.

In this study, phenotypic and genetic characteristic of AMR in *Salmonella* from Thailand and Laos provinces was investigated. It is evident that class 1 integrons are important genetic elements which play key role in the spread of antimicrobial resistance among the bacterial pathogens. The *dfrA12-aadA2* gene array was predominant in this study. The gene cassette has been isolate worldwide (Figure 5).

Therefore, horizontal transfer of resistance determinants is a major route for spread of AMR bacteria. Resistance to fluoroquinolones and cephalosporins is still low in this region. However, this could be a worrisome because fluoroquinolones and cephalosporins are considered drugs of choice for Salmonellosis treatment in life threatening infection. Therefore, the awareness of fluoroquinolnes and cephalosporins used in humans and animals should be concerned.

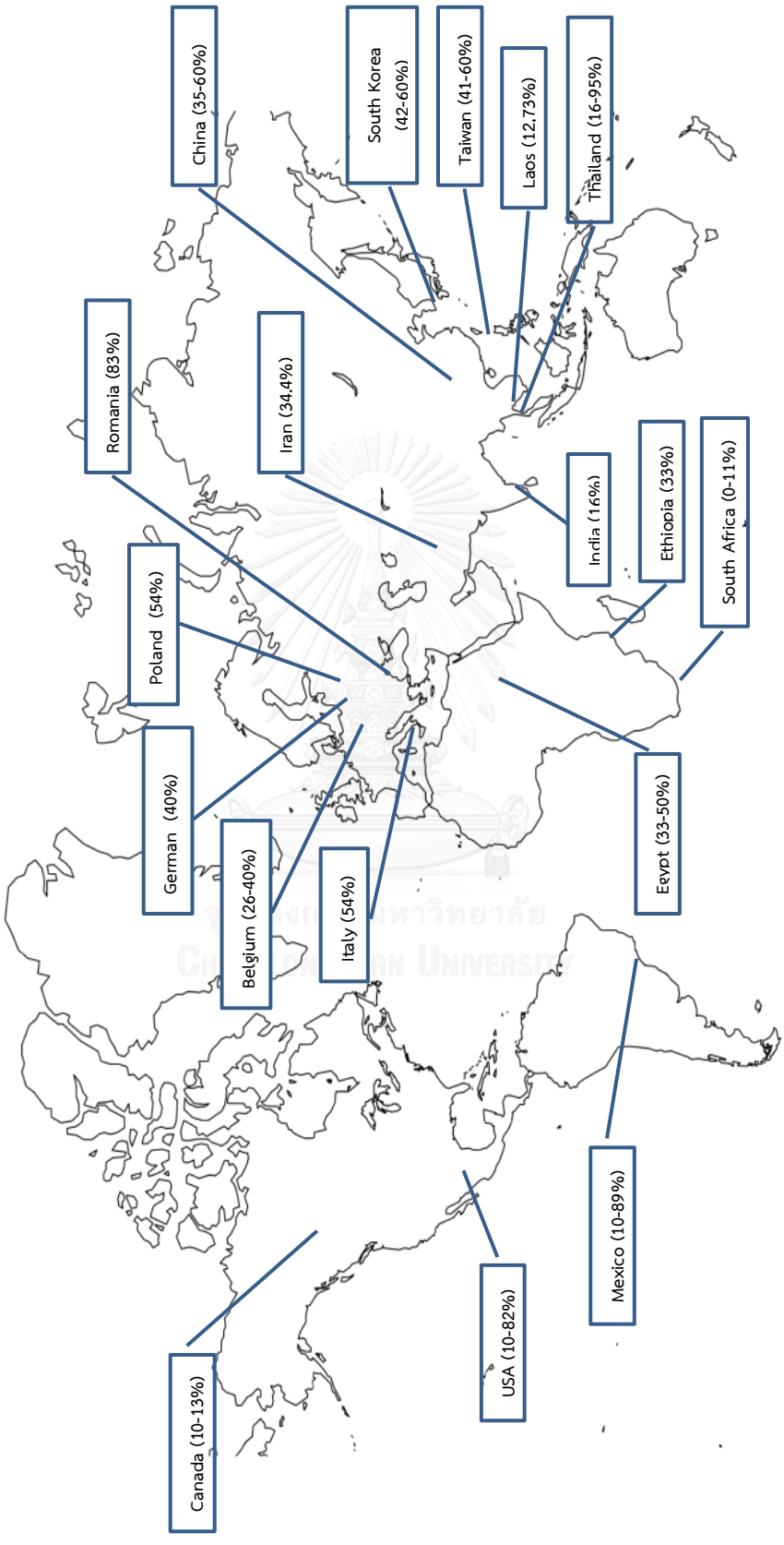

Figure 4 The prevalence of MDR-*Salmonella* in different regions of the world

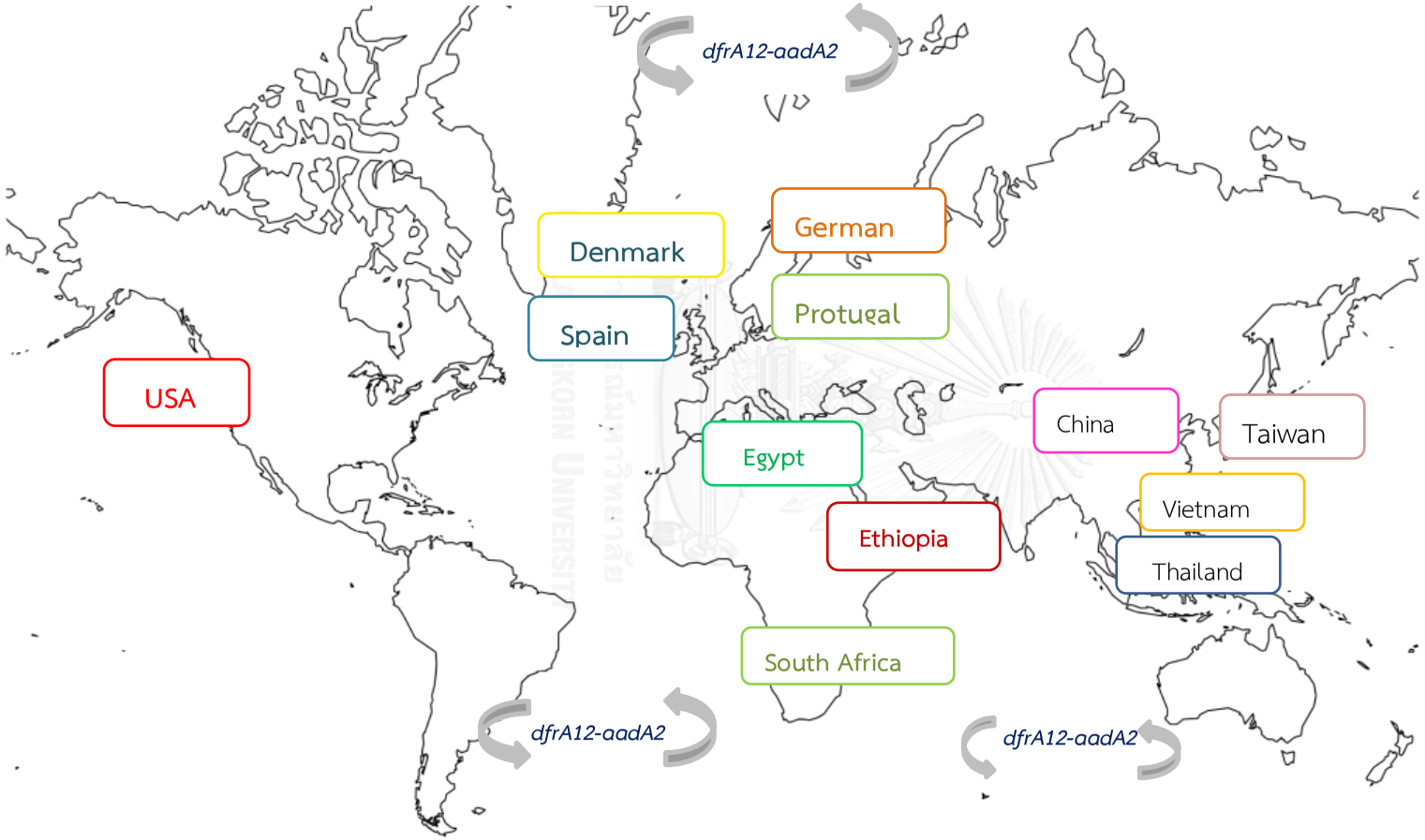

Figure 5 Worldwide distribution of class 1 integrons containing *dfrA12-aadA2* gene cassette array in *Salmonella enterica* in food animals, food of animal origins and humans

Objective 3: To examine plasmid-borne virulence factors and their correlation to antimicrobial resistance in *Salmonella* isolated from pigs, pig products and humans in Northeastern Thailand and Thai-Laos border are.

Low prevalence of virulence plasmid-associated genes was observed in Thailand and Laos border provinces. Some MDR-*Salmonella* isolates contained both class 1 integrons and virulence plasmid gene but not located on the same conjugative plasmid. The correlation between antimicrobial resistance and virulence plasmids should be examined for monitoring the emergence of new virulence plasmids and circulation of plasmids among bacteria from different environment.

Overall, the data in this study confirmed that the necessity of responsible use of antimicrobial because several antimicrobials are used in both humans and food-producing animals in Thailand and Laos. This data will help to develop the public health policy, guideline of responsible use of antimicrobials and raise awareness of antimicrobial used in humans and livestock animals in these countries and worldwide. Also, the collaboration between countries for sharing the knowledge and data of AMR in their countries is essential for the success of antibiotic control and prevention strategies for the entire region.

5.3 Further studies

1. Prevalence and genetic characteristics of antimicrobial resistance of *Salmonella* in pigs and other food-producing animals along the food chain (from farm to fork) should be investigated in a larger population and in different area in the region.

2. The genetic relatedness of the *Salmonella* isolates from humans and food-producing animals along the food chain should be performed.

3. Characterization of plasmid-mediated antimicrobial resistance in *Salmonella* isolates from humans and food-producing animals will provide useful data for better understanding of the evolution, circulation and spread of plasmids-mediated resistance genes in the region.

4. Risk assessment of antimicrobial resistance in *Salmonella* and food-borne bacterial pathogens is needed to assess the risks of illness from antimicrobial resistant bacteria in the food chain.

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APPENDICES

APPENDIX A

Part of dissertation has been published as follows:

1. Sinwat N, Angkittitrakul S, Coulson KF, Pilapil FM, Meunsene D, Chuanchuen R. 2016. High prevalence and molecular characteristics of multidrug resistant *Salmonella* in pigs, pork and humans in Thailand-Laos provinces. J Med Microbiol.
2. Sinwat N, Angkittitrakul S, Chuanchuen R.2015. Characterization of Antimicrobial Resistance in *Salmonella enterica* Isolated from Pork, Chicken Meat, and Humans in Northeastern Thailand. Food borne Pathog Dis. 2015 759-65. doi: 10.1089/fpd.2015.1946.
3. Sinwat N, Poungseree J, Angkittitrakul S, Chuanchuen R. Mutation of the quinolone determining region in DNA gyrase and topoisomerase IV genes in quinolone-resistant *Salmonella enterica* isolated from chicken meat, pork and humans. (submitted)

APPENDIX B

Antimicrobial resistance patterns in *Salmonella* isolated from Thailand and Laos (n=548)

Resistance patterns	No.of isolates(%)	
	Thailand (n=295)	Laos (n=253)
AMP		1(0.4)
SPC		1(0.4)
SUL	2(0.7)	
AMP-SUL	1(0.3)	1(0.4)
SPC-SUL	2(0.7)	
STR-SUL		1(0.4)
SUL-TET	1(0.3)	
AMP-STR-TET		3(1.2)
AMP-STR-SUL	3(1)	6(2.4)
AMP-SUL-TET	4(1.4)	1(0.4)
AMP-SPC-SUL	1(0.3)	
AMP-NAL-SUL	1(0.3)	
NAL-SPC-SUL	1(0.3)	
NAL-SUL-TET		1(0.4)
SPC-SUL-TET		1(0.4)
SPC-STR-SUL		1(0.4)
STR-SUL-TET		1(0.4)
SUL-TET-TRI	2(0.7)	
AMP-NAL-SUL-TET		1(0.4)
AMP-SPC-STR-TET		2(0.8)
AMP-SPC-SUL-TET	3(1)	2(0.8)
AMP-SPC-SUL-TRI	4(1.4)	
AMP-STR-TET-TRI		1(0.4)
AMP-SUL-TET-TRI	3(1)	
CHL-SPC-SUL-TET	1(0.3)	
CHL-SUL-TET-TRI		1(0.3)
NAL-SPC-STR-SUL	1(0.3)	

Antimicrobial resistance patterns in *Salmonella* isolated from Thailand and Laos (n=548) **(continued)**

Resistance patterns	No.of isolates(%)	
	Thailand (n=295)	Laos (n=253)
AMP-SPC-STR-SUL	5(1.7)	2(0.8)
AMP-STR-SUL-TET	18(6.1)	58(22.9)
SPC-STR-SUL-TET	3(1)	4(1.6)
SPC-SUL-TET-TRI	1(0.3)	1(0.4)
AMP-CHL-SPC-SUL-TET	3(1)	
AMP-CHL-STR-SUL-TET	2(0.7)	2(0.8)
AMP-GEN-SPC-SUL-TET	1(0.3)	
AMP-NAL-SPC-SUL-TET	1(0.3)	
AMP-NAL-STR-SUL-TET	1(0.3)	
AMP-SPC-STR-SUL-TET	40(13.6)	20(7.9)
AMP-SPC-STR-SUL-TRI	2(0.7)	1(0.4)
AMP-SPC-SUL-TET-TRI	6(2)	6(2.4)
AMP-SPC-STR-TET-TRI		1(0.4)
AMP-STR-SUL-TET-TRI	5(1.7)	6(2.4)
NAL-SPC-STR-SUL-TET		3(1.2)
SPC-STR-SUL-TET-TRI		7(2.8)
AMP-CAZ-NAL-SUL-TET-TRI	1(0.3)	
AMP-CEF-SPC-STR-SUL-TET	1(0.3)	
AMP-CHL-GEN-SPC-SUL-TET	10(3.4)	
AMP-CHL-SPC-STR-SUL-TRI	1(0.3)	
AMP-CHL-SPC-SUL-TET-TRI	4(1.4)	
AMP-CHL-STR-SUL-TET-TRI	1(0.3)	
AMP-GEN-SPC-STR-SUL-TET	2(0.7)	
AMP-NAL-SPC-STR-SUL-TRI	3(1)	
AMP-NAL-SPC-SUL-TET-TRI	1(0.3)	
CEF-SPC-STR-SUL-TET-TRI		4(1.6)
CHL-NAL-SPC-STR-SUL-TET	1(0.3)	
CHL-SPC-STR-SUL-TET-TRI		2(0.8)

Antimicrobial resistance patterns in *Salmonella* isolated from Thailand and Laos (n=548) **(continued)**

Resistance patterns	No.of isolates(%)	
	Thailand (n=295)	Laos (n=253)
NAL-SPC-STR-SUL-TET-TRI		3(1.2)
AMP-CHL-SPC-STR-SUL-TET	11(3.7)	1(0.4)
AMP-NAL-SPC-STR-SUL-TET	3(1)	1(0.4)
AMP-SPC-STR-SUL-TET-TRI	58(19.7)	74(29.2)
AMP-CEF-CHL-SPC-STR-SUL-TET		1(0.4)
AMP-CEF-SPC-STR-SUL-TET-TRI	1(0.3)	
AMP-CHL-CPD-CTX-SPC-SUL-TET	1(0.3)	
AMP-CHL-GEN-SPC-SUL-TET-TRI	4(1.4)	
AMP-CHL-GEN-SPC-STR-SUL-TET	23(7.8)	
AMP-CHL-SPC-STR-SUL-TET-TRI	15(5)	11(4.3)
AMP-GEN-SPC-STR-SUL-TET-TRI	2(0.7)	
AMP-NAL-SPC-STR-SUL-TET-TRI	5(1.7)	7(2.8)
CEF-GEN-SPC-STR-SUL-TET-TRI		1(0.4)
GEN-NAL-SPC-STR-SUL-TET-TRI		1(0.4)
AMP-CAZ-CHL-CPD-CTX-SPC-SUL-TET	1(0.3)	
AMP-CAZ-CEF-CHL-NAL-SPC-SUL-TET	1(0.3)	
AMP-CEF-CHL-SPC-STR-SUL-TET-TRI	1(0.3)	
AMP-CIP-NAL-SPC-STR-SUL-TET-TRI		1(0.4)
AMP-CHL-CIP-NAL-STR-SUL-TET-TRI	1(0.3)	
AMP-CHL-CPD-CTX-GEN-SPC-SUL-TET	1(0.3)	
AMP-CHL-GEN-SPC-STR-SUL-TET-TRI	10(3.4)	
AMP-CHL-NAL-SPC-STR-SUL-TET-TRI		8(3.2)
AMP-CHL-GEN-NAL-SPC-STR-SUL-TET-TRI	1(0.3)	1(0.4)
AMP-CHL-CPD-CTX-SPC-STR-SUL-TET-TRI	1(0.3)	
AMP-CHL-CIP-NAL-SPC-STR-SUL-TET-TRI		1(0.4)
AMP-CAZ-CEF-CHL-GEN-NAL-STR-SUL-TET	1(0.3)	
AMP-CEF-CHL-CPD-CTX-GEN-SPC-SUL-TET	1(0.3)	
AMP-CEF-CHL-GEN-NAL-SPC-STR-SUL-TET	1(0.3)	
AMP-CAZ-CEF-GEN-NAL-SPC-STR-SUL-TET-TRI	1(0.3)	

Antimicrobial resistance patterns in *Salmonella* isolated from Thailand and Laos (n=548) **(continued)**

Resistance patterns	No.of isolates(%)	
	Thailand (n=295)	Laos (n=253)
AMP-CAZ-CHL-CPD-CTX-GEN-NAL-SPC-SUL-TET	1(0.3)	
AMP-CEF-CHL-CPD-CTX-GEN-NAL-SPC-SUL-TET	1(0.3)	
AMP-CEF-CHL-CPD-CTX-GEN-SPC-STR-SUL-TET	1(0.3)	
AMP-CEF-CPD-CTX-CHL-GEN-NAL-SPC-SUL-TET	2(0.7)	
AMP-CEF-CHL-CPD-CTX-GEN-NAL-SPC-STR-SUL-TET	3(1)	
Total	**295**	**253**

Antimicrobial resistance patterns in *Salmonella* isolated from humans (n=77) in Northeastern Thailand

Antibiotic resistance patterns	**No. of isolates (%)**
GEN	1 (1.2)
AMP-SUL	1(1.2)
S TR-SUL	4(5.1)
TET-SUL	2(2.5)
AMP-STR-TRI	1(1.2)
CHL-GEN- STR	1(1.2)
CHL-STR -SUL	1(1.2)
CIP -TET-TRI	1(1.2)
SPC-STR-SUL	4(5.1)
STR-SUL- TET	2(2.5)
STR- SUL -TRI	1(1.2)
SUL-TET-TRI	1(1.2)
AMP-CHL-SUL-TET	1(1.2)
CEF- SPC-STR-SUL	1(1.2)
CHL- STR- SUL-TET	2(2.5)
CHL-STR-SUL-TRI	1(1.2)
CIP-STR-SUL-TRI	1(1.2)
GEN-SUL-TET-TRI	1(1.2)
SPC-STR -SUL- TET	2(2.5)
SPC-STR- SUL- TRI	1(1.2)
STR- SUL-TET-TRI	4(5.1)
AMP- CEF- SPC-STR-SUL	1(1.2)
AMP- GEN-SUL-TET-TRI	1(1.2)
AMP-SPC-STR -SUL-TET	1(1.2)
AMP-SPC-STR-SUL -TRI	1(1.2)
CEF -CHL- SPC-STR-SUL	1(1.2)

Antimicrobial resistance patterns in *Salmonella* isolated from humans (n=77) in Northeastern Thailand (continued)

Antibiotic resistance patterns	**No. of isolates (%)**
CHL- CIP-STR-SUL-TET	1(1.2)
CHL-GEN- SUL-TET-TRI	1(1.2)
CHL- SPC-STR-SUL-TET	2(2.5)
CHL-STR- SUL-TET-TRI	1(1.2)
CIP- STR- SUL-TET-TRI	1(1.2)
SPC-STR-SUL-TET-TRI	3 (3.9)
GEN-STR- SUL-TET-TRI	3(3.8)
AMP- CHL-SPC-STR- SUL-TET	2(2.5)
AMP- CHL -STR- SUL-TET-TRI	1(1.2)
AMP- GEN- SUL-STR-TET-TRI	4(5.1)
CHL- SPC-STR-SUL-TET-TRI	1(1.2)
AMP- CEF -CHL -STR- SUL-TET-TRI	1(1.2)
AMP-CHL-GEN- SPC-STR-SUL-TRI	1(1.2)
AMP- CHL-SPC-STR-SUL-TET-TRI	3(3.8)
AMP- GEN- SPC-STR- SUL-TET-TRI	3(3.8)
CEF-CIP-CET-GEN-SPC-STR-SUL	1(1.2)
AMP- CHL-GEN-SPC-STR-SUL-TET-TRI	9(11.6)
Total	**77**

Antimicrobial resistance patterns in *Salmonella* isolated from chicken (n=80) in Northeastern Thailand

Antibiotic resistance patterns	**No. of isolates (%)**
TET	1(1.25)
CHL-GEN-SUL	1(1.25)
SPC-STR-SUL	1(1.25)
AMP- GEN-SPC-STR	1(1.25)
AMP- GEN- STR-TRI	1(1.25)
AMP-SPC-STR-SUL	6(7.5)
AMP-SPC-STR-TRI	1(1.25)
AMP-STR- SUL-TET	3(3.75)
CEF -TET-TRI-SUL	1(1.25)
GEN- SPC-STR-SUL	3(3.75)
SPC-STR -SUL- TET	1(1.25)
AMP-CHL-GEN-SUL-TRI	1(1.25)
AMP-CHL-STR-SUL-TRI	1 (1.25)
AMP-GEN-SPC-STR-SUL	2 (2.5)
CHL- SPC-STR-SUL-TET	1(1.25)
CHL-SPC-STR-SUL-TRI	1(1.25)
GEN-SPC-STR-TET-TRI	1(1.25)
GEN- SPC-STR-SUL-TET	1(1.25)
AMP- CHL-GEN- SPC-STR-SUL	14(17.5)
AMP- CHL-SPC-STR- SUL-TET	2(2.5)
AMP- CHL-SPC-STR-SUL-TRI	1(1.25)
AMP- GEN- SUL-STR-TET-TRI	1(1.25)
AMP-SPC-STR-SUL- TET-TRI	4(5)
AMP- CHL- GEN -SPC- SUL-TET-TRI	1(1.25)
AMP- CHL-GEN-SPC-STR- SUL-TET	2(2.5)
AMP-CHL-GEN- SPC-STR-SUL-TRI	1(1.25)

Antimicrobial resistance patterns in *Salmonella* isolated from chicken (n=80) in Northeastern Thailand (continued)

Antibiotic resistance patterns	**No. of isolates (%)**
AMP- CHL-SPC-STR-SUL-TET-TRI	3(3.75)
AMP- CHL-CIP-GEN-SPC-STR- SUL-TRI	1(1.25)
AMP- CHL-GEN-SPC-STR-SUL-TET-TRI	14(17.5)
AMP- CEF-CHL-GEN-SPC-STR- SUL-TET-TRI	3(3.75)
CEF- CHL- CIP- GEN-SPC-STR- SUL- TET-TRI	1(1.25)
Total	**80**

Antimicrobial resistance patterns in *Salmonella* isolated from pig products (n=67) in Northeastern Thailand

Antibiotic resistance patterns	**No. of isolates (%)**
STR-SUL-TET	1 (1.5)
SPC-STR-SUL-TET	2(3.1)
STR-SUL-TET-TRI	1(1.5)
AMP-SPC-STR-SUL-TET	7(10.9)
NAL-SPC-STR-SUL-TET	3(4.7)
SPC-STR-SUL-TET-TRI	1(1.5)
AMP-SPC-STR-SUL-TET-TRI	27 (42.1)
AMP-NAL-SPC-STR-SUL-TET	4(6.25)
NAL-SPC-STR-SUL-TET-TRI	1(1.5)
AMP-CEF-SPC-STR-SUL-TET	2(3.1)
AMP-NAL-SPC-STR-SUL-TET-TRI	3(4.7)
AMP-CEF-SPC-STR-SUL-TET-TRI	6(9.3)
AMP-CEF-NAL-SPC-STR-SUL-TET-TRI	3(4.7)
AMP-CHL-NAL-SPC-STR-SUL-TET-TRI	1(1.5)
)AMP-CHL-CEF-NAL-SPC-STR-SUL-TET-TRI	1(1.5)
AMP-CHL-CEF-GEN-NAL-SPC-STR-SUL-TET-TRI	1(1.5)
Total	**64**

VITA

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