Genetic characteristics of antimicrobial resistance of *Salmonella enterica* and *Escherichia coli* isolated from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces

Miss Suthathip Trongjit

A Dissertation Submitted in Partial Fulfillment of the Requirements
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ลักษณะทางพันธุกรรมของการดื้อยาของซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไล ที่แยกได้จากไก่เนื้อ สุกร และผลิตภัณฑ์จากสัตว์ในเขตพื้นที่จังหวัดสระแก้ว, บันเตียเมียนเจย และเสียมเรียบ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Genetic characteristics of antimicrobial resistance of *Salmonella enterica* and *Escherichia coli* isolated from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces
By	Miss Suthathip Trongjit
Field of Study	Veterinary Public Health
Thesis Advisor	Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.....
Dean of the FACULTY OF VETERINARY SCIENCE

(Professor ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D.)

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Chairman

(Professor ALONGKORN AMONSIN, D.V.M., Ph.D.)

.....
Thesis Advisor

(Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.)

.....
Examiner

(Doctor TARADON LUANGTONGKUM, D.V.M., Ph.D.)

.....
Examiner

(Doctor SAHARUETAI JEAMSRIPONG, D.V.M., MPVM., Ph.D.)

.....
External Examiner

(Associate Professor Sunpeth Angkittitrakul, D.V.M., M.Sc., Ph.D.)

สุธาทิพย์ ตรองจิตต์ : ลักษณะทางพันธุกรรมของการดื้อยาของซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไล ที่แยกได้จากไก่เนื้อ สุกร และผลิตภัณฑ์จากสัตว์ในเขตพื้นที่จังหวัดสระแก้ว, บันเตียเมียนเจย และเสียมเรียบ. (Genetic characteristics of antimicrobial resistance of *Salmonella enterica* and *Escherichia coli* isolated from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces) อ.ที่ปรึกษาหลัก : ศ. สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น

การศึกษาวิจัยครั้งนี้มีวัตถุประสงค์เพื่อศึกษาอุบัติการณ์และคุณลักษณะการดื้อยาของซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไล ที่แยกได้จาก ไก่เนื้อ สุกร และผลิตภัณฑ์จากปศุสัตว์ในเขตพื้นที่จังหวัดชายแดนไทย-กัมพูชา และการศึกษาคุณลักษณะการดื้อต่อยาปฏิชีวนะที่มีความสำคัญทางการแพทย์ในเอสเชอริเชีย โคไลที่แยกได้จากสุกรในจังหวัดที่มีการเลี้ยงสุกรหนาแน่นของประเทศไทย จากจำนวนตัวอย่างทั้งสิ้น 941 ตัวอย่าง เป็นตัวอย่างที่แยกได้จากไก่เนื้อและสุกรในโรงฆ่าสัตว์ เนื้อสุกรและเนื้อไก่จากเขียงในตลาดค้าปลีกในจังหวัดสระแก้ว ประเทศไทย จำนวน 554 ตัวอย่าง และเป็นตัวอย่างจากพื้นที่จังหวัดบันเตียเมียนเจยและเสียมเรียบ ประเทศกัมพูชา จำนวน 387 ตัวอย่าง ในระหว่างปี พ.ศ. 2557 ถึง 2558 พบซัลโมเนลลาจำนวน 345 เชื้อ เป็นซัลโมเนลลาที่แยกได้จากตัวอย่างในเขตพื้นที่ประเทศไทยจำนวน 145 เชื้อ (23%) และจากเขตพื้นที่ประเทศกัมพูชาจำนวน 200 เชื้อ (47%) ซีโรวาร์ที่พบมากในเขตพื้นที่ฝั่งไทยและกัมพูชา คือ *S.* Typhimurium (29%) และ *S.* Rissen (29%) ตามลำดับ พบซัลโมเนลลาจากเขตพื้นที่ประเทศไทยร้อยละ 34 และประเทศกัมพูชาร้อยละ 52 ของเชื้อทั้งหมด มีความสามารถดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน Class 1 integrons ที่มี gene cassette ชนิด *dfrA12-aadA2* ตรวจพบมากที่สุด (61.1%) พบซัลโมเนลลาที่มีความสามารถในการผลิตเอนไซม์บีตา-แลคทาเมสชนิดฤทธิ์ขยายจำนวน 6 เชื้อ และตรวจพบยีน $bla_{CTX-M-55}$, bla_{TEM-1} และ bla_{CMY-2} จากจำนวนตัวอย่างทั้งหมดพบเอสเชอริเชีย โคไลจำนวน 667 เชื้อ เป็นเอสเชอริเชีย โคไลที่แยกได้จากตัวอย่างในเขตพื้นที่จังหวัดสระแก้ว ประเทศไทย (381 เชื้อ) และจากตัวอย่างในเขตพื้นที่จังหวัดบันเตียเมียนเจยและเสียมเรียบ ประเทศกัมพูชา (286 เชื้อ) พบความชุกของเอสเชอริเชีย โคไล ที่แยกได้จากเนื้อไก่และเนื้อสุกรในโรงฆ่าและจากตลาดค้าปลีกในเขตพื้นที่ประเทศไทย ร้อยละ 57.1 และจากตัวอย่างในเขตพื้นที่ประเทศกัมพูชา ร้อยละ 58.8 โดยพบว่าเอสเชอริเชีย โคไลส่วนมากดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน (75.3%) การศึกษา Class 1 integrons พบการปรากฏของยีนชนิด *dfrA12-aadA2* (41.5%) มากที่สุดในตัวอย่างจากไทย แต่พบ *dfrA1-aadA1* (70.8%) มากที่สุดในตัวอย่างจากกัมพูชา การศึกษา ESBL พบว่าเอสเชอริเชีย โคไลจำนวน 16 เชื้อ สามารถผลิตเอนไซม์บีตาแลคทาเมสชนิดฤทธิ์ขยายและมีการปรากฏของยีน $bla_{CTX-M-15}$ bla_{TEM-1} และbla_{CMY-2} การศึกษาในเอสเชอริเชีย โคไลจำนวน 454 เชื้อ ที่แยกได้จากสุกรสุขภาพดีจำนวน 354 เชื้อ (ลูกสุกร 83 เชื้อ สุกรขุน 142 เชื้อ และสุกรแม่พันธุ์ 129 เชื้อ) และเชื้อจากสุกรป่วยจำนวน 100 เชื้อ มาจากสุกรในเขตจังหวัดของประเทศไทย ระหว่างปี พ.ศ. 2550 ถึง 2561 ถูกนำมาศึกษาเพื่อหาความชุกและกลไกที่ควบคุมการดื้อต่อยาโคลิสตินและการสร้างเอนไซม์บีตา-แลคทาเมสชนิดฤทธิ์ขยาย พบเชื้อส่วนมากที่แยกได้จากสุกรสุขภาพดี (41%) และสุกรป่วย (73%) ดื้อต่อยาโคลิสติน ตรวจพบยีน *mcr-3* เป็นยีนที่พบมากที่สุดในเชื้อจากสุกรสุขภาพดี (37.9%) และสุกรป่วย (70%) พบการปรากฏของยีน *mcr* มากกว่า 1 ชนิด ได้แก่ *mcr-1/mcr-3* และ *mcr-2/mcr-3* และตรวจพบมากที่สุดในเชื้อจากลูกสุกร (23%) พบเชื้อที่มีความสามารถในการผลิตเอนไซม์บีตา-แลคทาเมสชนิดฤทธิ์ขยายในสุกรป่วย (44%) มากกว่าสุกรสุขภาพดี (19%) ($P < 0.05$) พบยีน ESBL ชนิด $bla_{CTX-M-14}$ (54.5%) และ$bla_{CTX-M-55}$ (42.9%) มากที่สุด เอสเชอริเชีย โคไลที่ให้ผลบวกต่อ ESBL และมีการปรากฏร่วมของยีน *mcr* (80.4%) มีความสามารถดื้อต่อยาปฏิชีวนะหลายชนิดพร้อมกัน พบการถ่ายทอดของยีน β-lactamase (bla_{TEM-1} และ $bla_{CTX-M-55}$) และ *mcr-3* เกิดขึ้นได้พร้อมกัน การศึกษาลักษณะทางอณูชีววิทยาและกลไกการดื้อยาของเอสเชอริเชีย โคไลที่มีการปรากฏร่วมของยีน ESBL และ *mcr* genes โดยใช้เทคนิคการหาลำดับเบสของสารพันธุกรรมทั้งหมด (WGS) พบว่ายีน *mcr-1.1* จากสุกรป่วย จำนวน 2 ตัวอย่าง อยู่บนพลาสมิดชนิด IncI2 และมีความเหมือนเท่ากับ 98% เมื่อเปรียบเทียบกับพลาสมิด pHNSHP45 ตรวจพบ *mcr-2.1* และ ในเชื้อจากสุกรขุน ที่มีความเหมือนเท่ากับ 100% กับยีนบนพลาสมิด pKP37-BE พบการปรากฏของยีน *mcr-3.1* ทั้ง 3 ตัวอย่างที่มี core segment ΔTn*As2-mcr-3.1-dsbA* และมีความเหมือนกับพลาสมิดต้นแบบ pWJ1 (85%-100%) โดยสรุปจากผลการศึกษาพบอุบัติการณ์ของซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไลที่ดื้อต่อยาหลายชนิดในอัตราที่สูงจากตัวอย่างที่มาจากไก่เนื้อ สุกร และผลิตภัณฑ์จากสัตว์ในเขตพื้นที่จังหวัดชายแดนไทย-กัมพูชา สุกรเป็นแหล่งสะสมที่สำคัญของแบคทีเรียที่ดื้อต่อโคลิสติน และ ESBL การส่งเสริมนโยบายการควบคุมการใช้ยาปฏิชีวนะอย่างสมเหตุสมผลจึงเป็นเรื่องที่สำคัญ การวางแผนยุทธศาสตร์ในการตรวจติดตามและเฝ้าระวังเชื้อดื้อยาในระดับประเทศควรศึกษาลักษณะปรากฏของการดื้อยาร่วมกับการศึกษาลักษณะทางพันธุกรรมของการดื้อยา เพื่อให้เข้าใจถึงปัญหาและพัฒนาระบบการควบคุมเชื้อดื้อยาได้อย่างมีประสิทธิภาพ ข้อมูลที่ได้สามารถนำไปใช้เป็นส่วนหนึ่งของการเฝ้าระวังเชื้อดื้อยาทั้งในระดับภูมิภาค และระดับนานาชาติต่อไป

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Suthathip Trongjit : Genetic characteristics of antimicrobial resistance of *Salmonella enterica* and *Escherichia coli* isolated from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces . Advisor: Prof. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

This research study aimed to investigate the incidence and characterize antimicrobial resistance (AMR) in *Salmonella enterica* and *Escherichia coli* isolated from broilers, pigs and their meat products in Thailand–Cambodia border provinces. Resistance to clinically importance antimicrobials was additionally examined in the *E. coli* isolates from other provinces with high pig production in Thailand. A total of 941 samples were collected from broilers and pigs at slaughterhouses and their carcasses at local fresh markets in Sa Kaeo, Thailand (n=554) and Banteay Meanchey and Siem Reap, Cambodia (n=387) during 2014-2015. Three hundred forty-five *Salmonella* isolates were collected from Thailand (n = 145; 23%) and Cambodia (n = 200; 47%). Serovars Typhimurium (29%) and Rissen (29%) were most frequently found among the Thai and Cambodian isolates, respectively. Multidrug resistance (MDR) was detected in 34% and 52% of the isolates from Thailand and Cambodia, respectively. Class 1 integrons containing *dfrA12-aadA2* cassette array was most prevalent (61.1%). Six isolates were ESBL producers, of which bla_{TEM-1}, $bla_{CTX-M-55}$ and bla_{CMY-2} were found. A total of 667 commensal *E. coli* isolates were obtained from Sa Kaeo, Thailand (n=381) and Banteay Meanchey and Siem Reap, Cambodia (n=286). The prevalence of *E. coli* in pig and broiler carcasses from slaughterhouses and fresh markets was 57.1% in Thailand and 58.8% in Cambodia. The majority were MDR (75.3%). The *dfrA12-aadA2* cassette array (41.5%) was predominant class 1 integrons-gene cassette in Thai isolates, whereas the *dfrA1-aadA1* cassette array (70.8%) was most common in Cambodian isolates. Sixteen *E. coli* isolates were confirmed to be ESBL producers, of which $bla_{CTX-M-15}$, bla_{TEM-1} and bla_{CMY-2} were detected. Four hundred fifty-four *E. coli* isolates from healthy pigs (n=354; piglets, n=83; fattening pigs, n=142 and sows, n=129) and sick pigs (n=100) collected in Thailand during 2007-2018 were determined for the prevalence and investigated for the molecular mechanisms underlying colistin resistance and ESBL production. The healthy pig (41%) and sick pig (73%) isolates were commonly resistant to colistin. The *mcr-3* gene was most predominant in the healthy (37.9%) and sick pig (70%) isolates. Coexistence of *mcr-1/mcr-3* and *mcr-2/mcr-3* was observed and common in the piglet isolates (23%). The ESBL producers were more frequently detected in sick pig (44%) than healthy pig isolates (19.2%) ($P<0.05$). The $bla_{CTX-M-14}$ (54.5%) and $bla_{CTX-M-55}$ (42.9%) genes were the predominant ESBL gene in this study. Most ESBL producers (80.4%) additionally carried *mcr* and all were MDR. Co-transfer of ß-lactamase genes (bla_{TEM-1} and $bla_{CTX-M-55}$) and *mcr*-3 was detected. Characterization of three *E. coli* co-harboring ESBL and *mcr* genes from sick pigs by using WGS approach revealed two *mcr-1.1* gene cassettes that were located on IncI2 plasmid and had plasmid backbone identical to (98% identity) pHNSHP45. The *mcr-2.1* gene carrying contig in fattening pig showed 100% identity to pKP37-BE. All three *mcr.3.1* carrying contigs contained a core segment ΔTn*As2-mcr-3.1-dgkA* and had high nucleotide similarity (85-100%) to the original plasmid pWJ1. In summary, the results demonstrated the high prevalence of MDR *Salmonella* and *E. coli* in broilers, pigs and their meat products in Thailand–Cambodia border provinces. Pigs are the important reservoirs of colistin-resistant and ESBL-producing strains. It indicates that the restrictive policies on prudent use of antimicrobials in pigs and other food animals should be encouraged. The implementation of national AMR monitoring and surveillance at phenotypic and genotypic level is required to elucidate the root causes and burden of AMR. The data can be combined for regional and global perspective.

Field of Study: Veterinary Public Health Student's Signature .....

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Suthathip Trongjit

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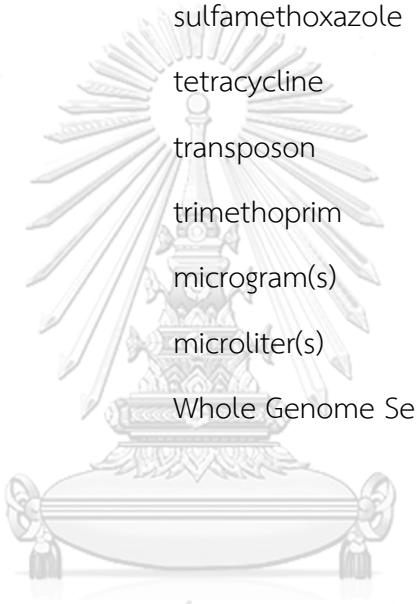
aad	aminoglycoside adenyltransferase
AMP	ampicillin
AMR	antimicrobial resistance
Bp	base pair(s)
bla	ß-lactamase
℃	degree(s) Celcius
CAZ	ceftazidime
CIP	ciprofloxacin
CHC	chloramphenicol
CLSI	Clinical & Laboratory Standards Institute
COL	colistin
CPD	cefpodoxime
CTX	cefotaxime
dfrA	dihydrofolate reductase
DNA	deoxyribonucleic acid(s)
EFSA	European Food Safety Authority
e.g.	exempli gratia, for example
ESBL	extended-spectrum ß-lactamase
GEN	gentamicin
i.e.	id est, that is
IP	integrons profile
IS	insertion sequences
Kb	kilobase(s)
LB	Luria Bertani
mcr	mobilized colistin resistance

MDR	multidrug resistance
MHA	Mueller Hinton Agar
MIC	minimum inhibitory concentrations
ml	milliliter(s)
mM	milimolar(s)
PCR	Polymerase chain reaction
STP	streptomycin
SUL	sulfamethoxazole
TET	tetracycline
Tn	transposon
TRI/TMP	trimethoprim
µg	microgram(s)
µl	microliter(s)
WGS	Whole Genome Sequencing



LIST OF PUBLICATIONS

Parts of this dissertation have been published in the following articles:

1. Trongjit, S., Angkittitrakul, S. and Chuanchuen, R., 2016. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. Microbiology and immunology, 60(9), pp.575-585. doi: 10.1111/1348-0421.12407

2. Trongjit, S., Angkititrakul, S., Tuttle, R.E., Poungseree, J., Padungtod, P. and Chuanchuen, R., 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces. Microbiology and immunology, 61(1), pp.23-33. doi: 10.1111/1348-0421.12462

CHAPTER I

1.1 Importance and Rationale

Antimicrobial resistance (AMR) has become one of the greatest threats to public health globally. It is mainly due to the extensive use of antimicrobials in diverse fields i.e. human and veterinary medicine and as well as food animal production. In food animal production, antimicrobials are widely used for curing clinical diseases, growth promotion and prophylactic treatment (McEwen and Fedorka-Cray, 2002; American Veterinary Medical Association, 2004; Goutard et al., 2017). WHO has predicted the projected global antimicrobial consumption in food animal production sector increases by 67% (from 63,151 ± 1,560 tons to 105,596 ± 3,605 tons) in 2030. In low- and middle-income countries, especially those in Southeast Asia antimicrobial consumption increase by 46% in 2030 (Van Boeckel et al., 2015). This is due to the raising consumer's demand for livestock products and shifts in production systems to largescale intensive farming operations where antimicrobials are increasingly used to maintain animal healthy and productivity (Van Boeckel et al., 2015). In 2030, antimicrobial consumption in chickens and pigs in Asia is expected to increase by 129% and 124%, respectively (Van Boeckel et al., 2015). These fast-growing antimicrobial consumption in Asia countries establishes a serious challenge because the widespread use of antimicrobials in livestock may contribute to the emergence of AMR and generate impact on public health threats.

Evidently, the emergence and spread of resistant bacteria is a result of over- or mis-use of antimicrobial agents in any sectors (i.e. humans, animals and environment). Among these, livestock and their meat products including pigs, poultry, pork and chicken meat have been considered as important reservoirs of multi-drugs resistant (MDR) foodborne pathogens that possibly transmit to humans

though the food chain (Padungtod et al., 2008). In the US, approximately 48 million cases of foodborne pathogen infections with 128,000 hospitalizations and 3,000 deaths has reported annually (FDA, 2017). In EU, European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported 5,146 foodborne outbreaks affecting 48,365 people in 2018 (EFSA&ECDC, 2019). In South East Asia, there is a poor track record on foodborne illnesses, however the report of WHO in the South-East Asia region estimated more than 150 million infected people and 175,000 deaths annually (WHO, 2016). Of all foodborne diseases, diarrheal and invasive infection caused by nontyphoidal *Salmonella enterica* is most common and MDR *Salmonella* in humans has been drastically reported (Van Boxstael et al., 2012; Perez-Moreno et al., 2013). In addition to MDR *Salmonella*, MDR commensal *E. coli* has been increasingly isolated from food animals (Lay et al., 2012; Adenipekun et al., 2015). These commensal *E. coli* are generally harmless and found in the intestines of people and animals contributing to intestinal health. However, commensal *E. coli* may serve as reservoirs of resistance genes that could be transferred to pathogenic bacteria in either humans or animals.

Antimicrobial agents are commonly used to treat salmonellosis and *E. coli* infection in both human and animals e.g. fluoroquinolone, 3rd generation cephalosporin and colistin. In the past few years, emergence of antimicrobial resistance to clinically important antimicrobial drugs has been increasingly reported not only in clinical medicine, but also widely in livestock production e.g. resistance to 3rd cephalosporins in extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae (Paterson and Bonomo, 2005) and colistin resistance in *E. coli* and *Salmonella* (Liu et al., 2016; Yang et al., 2016; Wu et al., 2018a). These increasing prevalence of colistin-resistant and ESBL-producing *Salmonella* and *E. coli* have become a serious public health treat and raised particular concern regarding the

compromise therapeutic efficacy and limitation of antimicrobial drugs available for infection treatment in the future.

AMR is a complicated and multifaceted problem that threatens human and animal health and economy at national, regional and global levels. AMR does not recognize geographic or human–animal borders, resulting in global dissemination. Resistant bacteria originating from humans, animals or the environment can move from one to another and from one country to another without barriers (WHO, 2016). Therefore, addressing the raising threat of AMR requires a "One Health approach" that needs cooperation of multisectoral responsibility for health of human, animal and ecosystem. This is one of the major hindrances of development a global control to facilitate the management and interventions for preventing AMR spread. Therefore, monitoring and surveillance of AMR is essential to reveal the cost and causes of this issue. According to EU's recommendation, bacteria strains that should be included for AMR monitoring and surveillance system in food-producing animals are zoonotic bacteria, such as *Salmonella* and *Campylobacter* and indicator bacteria, such as *E. coli* and *Enterococcus* (EFSA, 2012). Pathogenic bacteria cause direct hazard to human and animal health, whereas commensal bacteria cause indirect effects by serving as potential reservoirs for AMR determinants that could be transferred to pathogenic bacteria (EFSA, 2012).

Thailand and Cambodia share common border approximately 800 kilometers in length and covers seven provinces of Thailand (i.e. Ubolrachathani, Sisaket, Surin, Burirum, Sa Kaeo, Chantaburi and Trad). There are currently six permanent border trade routes between these two countries, where millions of people and animals travel across each year (Paitoonpong, 2007; Krainara and Routray, 2015). Border trade between Thailand and Cambodia has boomed since 2005 (Manarungsan, 2010). The financial trade value of Thailand and Cambodia worths more than 50% of the total

border trade income between the two countries (Manarungsan, 2010). Among all border trade routes, Rongkrue market at Aranyaprathet district in Sa Kaeo province is the most popular trading area with the highest trading value (Manarungsan, 2010). The border trade activities mainly include tourism, importation and exportation. The variety of items are available for trading, of which trade of food animals and their meat products is common. However, illegal trafficking of livestock and illegal slaughtering practice with low hygienic practice frequently occurs (Knips, 2004; Tornimbene and Drew, 2012).

In Cambodia, the continuous growth of the population has led to a higher demand for meat. However, food animal production in this country is still not efficient and the meat productivity does not meet the domestic demand. Cambodia has started to import pigs and pork from neighboring countries, mainly Thailand and Vietnam since 2002 (Tornimbene and Drew, 2012). The price of imported pigs is generally cheaper than pigs raised in Cambodia, however, the direction of fattening pig trade can be changed and depends upon pig market price (Tornimbene and Drew, 2012). For poultry production, commercial farm run by foreign-private companies has been increasing. However, the importation of poultry products from neighboring, including Thailand is common. The backyards widely distribute throughout the country as well (VSF, 2004; Burgos et al., 2008). In addition, people movement including tourists and villagers has routinely occurred at the border. Cambodians frequency cross the border to seek for employment and medical service in Thailand (Paitoonpong, 2007). It was reported 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; Vlieghe et al., 2012).

As a result, the massive movement across the border of humans, livestock and livestock products in either legal and illegal manners may increase the

emergence and distribution of MDR foodborne pathogens. This could generate adverse effects that are not limited to the border area but may affect the other parts of the countries and other world regions. Up to date, the occurrence of antimicrobial resistance and genotypic characteristic of AMR in *Salmonella* and commensal *E. coli* in these bordering areas are still limited. As phenotypic monitoring generates limited information on AMR, genotypic examination is required to expand the knowledge. Thus, this study investigated the occurrence and characteristics of AMR bacteria associated with livestock and their meat products in the Thailand-Cambodia border provinces. The data obtained can be applied for development the guideline of prudent and responsible use of antimicrobial agents and supporting the regulation of animal and human trade at the area of Thailand-Cambodia border provinces. Besides these valuable data could be applicable for improving awareness and understanding of antimicrobial resistance in other Asia countries and encouragement judicious use among health and veterinary sectors and animal farming practice through professional education, training and certification to minimize the emergence and spread of AMR.

Keywords: Antimicrobial resistance, Banteay Meanchey, *Escherichia coli*, Sa Kaeo, *Salmonella*, Siem Reap

1.2 Literature Review

1.2.1 *Salmonella* spp.

1.2.1.1 General characteristics of *Salmonella* spp.

Salmonella species are Gram-negative flagellated rod-shaped bacterium which is about 2-3 x 0.4-0.6 µm in size. They are non-capsulated, non–spore forming, facultative anaerobic bacteria, which is classified in the family Enterobacteriaceae. The genus *Salmonella* is divided into two main species including

Salmonella enterica and *Salmonella bongori*. *Salmonella enterica* can be categorized into 6 subspecies, however *S. enterica* subspecies *enterica*, known as a food-borne bacteria pathogen, is recognized as a major cause of salmonellosis in human and warm-blooded animals (Pui, 2011). Regarding *Salmonella* classification, according to Kauffmann-White scheme three main antigenic determinants consisted of somatic O antigens (lipopolysaccharide, LPS), H antigens (flagella protein) and virulence (Vi) capsular K antigens. Currently, there are over 2,500 *Salmonella* serovars identified (Jones et al., 2008).

1.2.1.2 Pathogenesis of *Salmonella* infection

Salmonellosis is one of the major foodborne disease in humans and recently non-typhoidal salmonellosis has increased worldwide. An estimated 93.8 million cases with 155,000 deaths of gastroenteritis resulting from non-typhoidal *Salmonella* occur globally each year (Majowicz et al., 2010). Gastroenteritis due to non-typhoidal *Salmonella* infection in humans always relates to ingestion of water or food contaminated with *Salmonella* through an animal reservoir. The clinical symptoms of salmonellosis in human are fever, abdominal pain, diarrhea, nausea, and vomiting. *Salmonella* septicemia may occur in some cases. The incubation period ranges between 6–72 hours (usually 12–36 hours) after ingestion of *Salmonella*. The patients can recover without specific treatment in many cases. However, young children and those who are immunocompromised may show severe clinical symptoms; therefore, antimicrobials are required for lifesaving.

Meat products from pigs, poultry and cattle have been identified containing various *Salmonella* serotypes, however poultry related foods have been recognized as a major vehicle of *Salmonella* outbreak (Pui, 2011). Thus, foods of animal origin are an importance reservoir for salmonellosis in human.

1.2.2 *Escherichia coli*

1.2.2.1 General characteristics of *Escherichia coli*

Escherichia coli (*E. coli*) is a gram-negative, facultative anaerobic, rod-shaped bacteria. This organism belongs to the family Enterobacteriaceae and is ubiquitous in the lower intestinal tract of humans and warm-blooded animals. The optimal temperature for growth is 37 ºC and optimal pH is 6-7. Eosin methylene blue and MacConkey agar are considered to be selective and differential media used for differentiating *E. coli* from non-lactose fermenting gram-negative organisms.

1.2.2.2 Pathogenesis of *Escherichia coli* infection

Although, most of the *E. coli* strains are harmless, some *E. coli* strains cause pathogenic foodborne disease in human. Pathogenesis of *E. coli* can occur locally in the intestinal tract and may spread throughout the body. The common diseases in humans are gastroenteritis, urinary tract infections, and neonatal meningitis. Enteric *E. coli* infection can be classified into 6 pathotypes based on their pathogenic profiles (e.g., Enterotoxigenic *E. coli* (ETEC), Entero-pathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC) (Stenutz et al., 2006). ETEC is a major pathogen causing colibacillosis in newborn livestock that causes significantly economic loss worldwide. Moreover, ETEC commonly causes traveler diarrhea in the developing countries (Nagy and Fekete, 2005). EHEC O157:H7 is the common outbreak strain causing hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans (Robins-Browne and Hartland, 2002). While the harmless *E. coli* plays as normal gut flora to prevent colonization of pathogenic bacteria in the intestine. Presently, the global concern of commensal *E. coli* has been increasing because they

may act as reservoir of resistant genes and resistant determinants that could be transmitted to pathogenic bacteria.

1.2.3 Antimicrobial resistance in *Salmonella* and *Escherichia coli*

Emerging antimicrobial resistance in *Salmonella* spp. has been a global concern and now is widespread in both developed and developing countries (Threlfall, 2002). In the past few decades, the highly virulent and antibiotic-resistant *Salmonella*, causing greater morbidity and mortality in humans have been increasingly reported (Divek V T Nair, 2018). Elimination of *Salmonella* from its reservoir hosts is challenging, and food producing animals often server as reservoirs of the pathogen. Currently, non-typhoidal *Salmonella* causes the highest number of illnesses, hospitalizations, and deaths associated with foodborne illness (Scallan et al., 2011). In the United State, there are about 1,200,000 illnesses annually, and among these approximately 100,000 infections are result of antibiotic-resistant *Salmonella*, including those that are resistant to clinically-important antibiotics i.e. ceftriaxone (36,000 illnesses/year) and ciprofloxacin (33,000 illnesses/year) (Divek V T Nair, 2018). Apart from that *Salmonella* has been reported resistance to various antimicrobial agents such as tetracycline, sulfonamide, streptomycin, kanamycin and chloramphenicol. However, resistance to ß-lactam and fluoroquinolone antibiotics are particularly concerning because they are antimicrobial drugs of choice for treatment of *Salmonella* infection (Hur et al., 2012; Economou and Gousia, 2015).

Commensal *E. coli* from healthy animals can cause indirect effect on human and animal health by serving as potential reservoirs for antimicrobial resistance and resistant determinants. Moreover, commensal *E. coli* can be an indicator of what antimicrobials were used in animal production.

In food animal production, antimicrobial agents are administered to animals for many reasons, including disease treatment, prevention, control, and growth

promotion. Some studies indicated that the use of antimicrobials through feeding at sub-therapeutic administration in animal production over a long period could lead to an increase in the prevalence of resistance bacteria (Gullberg et al., 2011). Nowadays, many counties in EU and Thailand have restricted the use of antimicrobials mixed in animal feed as a growth promoter (EU, 2003; Elliott, 2015).

In Thailand, MDR *Salmonella* has been commonly reported in humans, livestock and their associated meat products. Sixty-three percent of *Salmonella* isolates from pork and patients were MDR (Wannaprasat et al., 2011). In addition, *Salmonella* strains obtained from poultry and swine were resistant to different antimicrobial classes (e.g. ampicillin, chloramphenicol, ciprofloxacin, gentamicin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim) (Khemtong and Chuanchuen, 2008). Up to 98% of the *E. coli* isolates from healthy pigs in Thailand were multidrug resistant (Lay et al., 2012). This emergence of several *Salmonella* serotypes and commensal *E. coli* resistant to multiple antibiotics in food animals are considered a significant food safety hazard.

The information of MDR *Salmonella* and *E. coli* in Cambodia is limited. In 2011, the surveillance in Cambodian retail markets indicated that the major bacteria contamination in poultry carcasses was *Salmonella* serovars which exhibited a high antimicrobial resistance rate (Lay et al., 2011).

1.2.4 Genetic of antimicrobial resistance in *Salmonella* and *E. coli*

1.2.4.1 Class 1 integrons

Integrons are genetic DNA elements that play a major role in the development of MDR in Enterobacteriaceae including *Salmonella* and *E. coli*. Even though most integrons were first identified in human clinical isolates, they have been known in several other sources including food animal origins (Domingues et al., 2012).

There are two groups of integrons that have been recognized: super integrons and mobile integrons. Super integrons are found on bacterial chromosomes, whereas mobile integrons are located on transposons, plasmids and chromosomes. Currently, five classes of mobile integrons have been identified, of which class 1 integrons have been reported as one of the major contributions of antibiotic resistance and are mainly found in gram-negative bacteria (Fluit and Schmitz, 2004; Mazel, 2006). Presently, over 80 gene cassettes have been characterized in this class (Mazel, 2006).

The structure of class 1 integrons consists of two conserve segments (CS), 5' and 3' CS separated by a variable region (Fig. 1). The 5'conserve segment contains the *intI* gene encoding integrase enzyme, an *attI* site where gene cassettes are integrated and a promoter region. The 3'conserve segment composes of a quaternary ammonium compound resistance gene (*quaEΔ1*), sulfonamide resistance gene (*sul1*) and an open reading frame 5 (*orf5*) of unknown function (Domingues et al., 2012). The variable region is located between these two segments in which one or more gene cassettes may present at the same time.

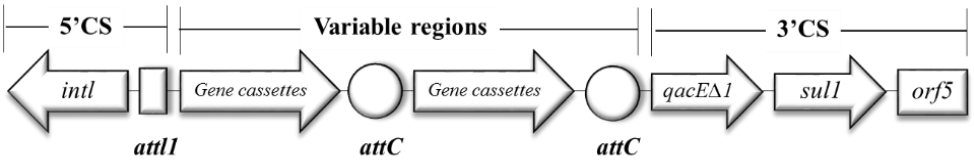


Figure 1. General structure of class 1 integrons. Class 1 integrons consists of two conserve segments (5' and 3' CS) separated by a variable region where gene cassettes are inserted by using a site-specific recombination mechanism. Genes are as follows: *intI1*, integrase 1 gene; *attI*, recombination site; *qacEΔ1*, quaternary ammonium compound resistance; *sul1*, sulphonamide resistance; *orf5*, gene of unknown function.

1.2.4.2 Extended-Spectrum ß Lactamases

Extended-spectrum ß-lactamases (ESBLs) are enzymes that mediate resistance to oxyimino-cephalosporins such as aztreonam, cefotaxime, ceftazidime and older penicillin and cephalosporins. However, the mechanisms of resistance by hydrolyze ß-lactam ring of ß-lactams antibiotics are inhibited by ß-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). The widespread of ESBLs have significantly increased in many Enterobacteriaceae species, particularly in *E. coli* and *Salmonella* spp. These enzymes probably involve bacterial chromosomes, but many ESBLs genes are located on plasmids and are capable of being transferred between organisms. Presently over 200 types of ESBLs have been described among various species of the Enterobacteriaceae family (Hijazi et al., 2016). The most common reports of ESBL genes are bla_{CTX-M}, bla_{TEM} and bla_{SHV} (Barguigua et al., 2011).

In Thailand, the high proportion of ESBLs producing strain in *E. coli* isolates from healthy pigs (76.7%) and broilers (40%) were reported (Boonyasiri et al., 2014). Thus, the significant rate of ESBLs producing *E. coli* in the food chain might be a

reservoir of ESBLs spreading to humans. *Salmonella* isolates carrying ESBLs have been increasingly concerned because cephalosporins are considered the antibiotic of choice for treatment of salmonellosis in children. Several ESBL genes have been found among ESBL producing *Salmonella* (Winokur et al., 2000; Mulvey et al., 2003).

1.2.4.3 Colistin resistance mechanism

Bacteria have several means to protect themselves from exposure to cationic antimicrobial peptides i.e. polymyxin B and polymyxin E (colistin). For example, alteration of lipopolysaccharides (LPSs) by covalent modifications of the lipid A moiety of LPS via adding of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N). Regarding colistin- resistant *Salmonella* and *E. coli,* one principal mechanism underlying this modification is the mutations in the PmrA/PmrB (two-component system-mediated LPS modifications), which can cause their constitutive overexpression, resulting in the modification in lipid A of LPS and reducing the binding of colistin (Quesada et al., 2015). In addition to chromosomal mutation, in 2015 when a plasmid-mediated resistance, *mcr-1* gene was described for the first time in China, that reached global concern as its high horizontal gene transfer potential (Liu et al., 2016).

1.2.4.3.1 Chromosomally mediated colistin resistance mechanism (two component system, pmrAB)

Chromosomal mediated colistin resistance relates to a two-component regulatory system, which are PmrA (response regulator) and PmrB (sensor histidine kinase). The mutations in the *pmrA* and *pmrB* genes cause overexpression of *arnBCADTEF* and *pmrCAB* operons that mediate the synthesis and transfer of PEtN and L-Ara4N to lipid A, respectively leading to alteration of LPS and a reduction in colistin-affinity to the external surface of gram-negative bacteria (Fig. 2) (Quesada et

al., 2015). The major dissemination of this mechanism is through clonal transmission of resistance isolates (Kluytmans-van den Bergh et al., 2016). However, mutation of *pmrA* and *pmrB* genes are rarely reported in *Salmonella* and *E. coli* (Quesada et al., 2015). In *Salmonella*, the missense mutations in the *pmrA* gene resulting in an amino acid substitution of R81H and R81C among *S.* Typhimurium strains have been identified (Sun et al., 2009). Three missense mutations i.e. S39I and R81S in PmrA and V161G in PmrB have been found in colistin-resistant *E. coli* isolates from pigs (Quesada et al., 2015).

1.2.4.3.2 Plasmid-mediated colistin resistance mechanism, *mcr* genes

The recent report of a plasmid-mediated colistin resistance gene, *mcr-1* gene in *E. coli* and *Klebsiella pneumoniae* from animals, food stuff and human origins in China has demonstrated a horizontal transfer mechanism for colistin resistance (Liu et al., 2016). The *mcr-1* encodes a phosphoethanolamine (pEtN) transferase enzyme that modifies the pEtN moiety of lipid A of bacteria cells (Fig. 2), resulting in alteration of the colistin target (Liu et al., 2016). After the emergence of plasmid-mediated colistin resistance was reported due to the high potential on horizontal colistin resistance gene transfer to other bacteria, presently *mcr-1* was the major public health concern on colistin resistance mechanisms. The *mcr-1* has been found in many bacteria species i.e. *E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *E. cloacae* from various sources across different parts of the world i.e. Asia, US and EU countries (Beceiro et al., 2014; Skov and Monnet, 2016). In July 2016, the *mcr-2* gene, encoding phosphoethanolamine transferase (pEtN) to lipid A, was reported in *E. coli* isolates from pigs, and cattle in Belgium (Xavier et al., 2016). Then shortly, *mcr-3* gene was reported in *E. coli* isolates from a pig in China (Yin et al., 2017). Up to date, nine *mcr* variants (*mcr-1*, *-2*, *-3*, *-4*, *-5*, *-6*, *-7*, *-8* and *-9*) have been described and disseminated globally (Xavier et al., 2016;

Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; AbuOun et al., 2018; Wang et al., 2018; Yang et al., 2018; Carroll et al., 2019). However, *Salmonella* has been reported to harbor *mcr-1*, *mcr-4*, *mcr-5* and *mcr-9*. In addition, *E. coli* has been considered as a reservoir for the *mcr* genes by carrying *mcr-1* to *mcr-5* and occasionally for coexistence of more than one *mcr* gene (Gharaibeh and Shatnawi, 2019).

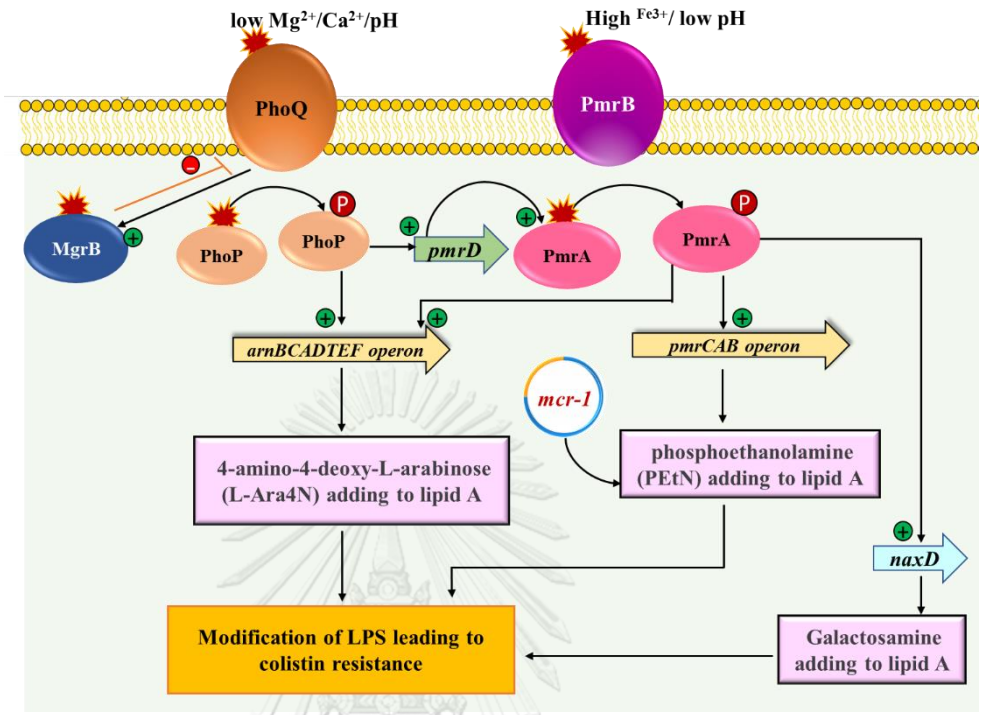


Figure 2. Mechanisms of LPS modification involved in polymyxin resistance in Gram-negative bacilli.

In *Salmonella* spp. and *E. coli*, the sensing of various stress conditions, such as the presence of cationic compounds (polymyxins), low Mg^{2+} and Ca^{2+} concentrations, acidic pH, and high Fe^{3+} concentrations, by the histidine kinases PhoQ and PmrB activates the two-component systems (TCSs) PhoP-PhoQ and PmrA-PmrB, respectively. Subsequent activation of the arnBCADTEF and pmrCAB operons leads to the synthesis and addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) to lipid A, respectively. In addition, PmrAB is activated by PhoP-PhoQ via the product of the pmrD gene, which in turn activates pmrA for activation of the arnBCADTEF operon. Inactivation of MgrB (a negative regulator of the PhoP-PhoQ system) by amino acid substitutions leads to overexpression of the phoP-phoQ operon as well, causing activation of the pmrHFIJKLM operon, thus leading to the production of L-Ara4N. In addition, a recently identified *mcr-1* gene, encoding phosphoethanolamine transferase, has been shown to be the main mechanism of polymyxin resistance (Modified from (Ezadi et al., 2019)).

1.2.5 Plasmid mediated antimicrobial resistance (R-plasmid)

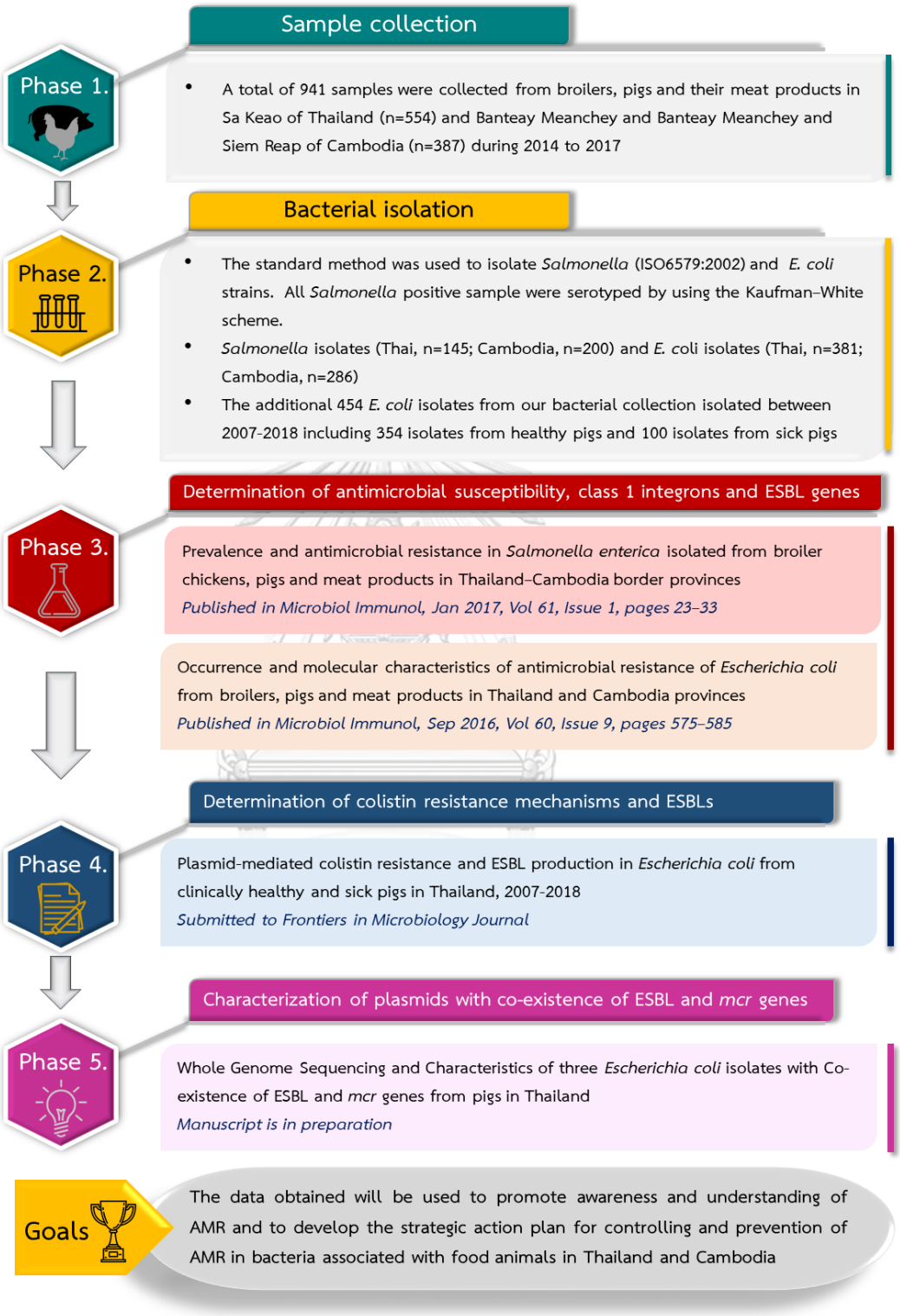
Bacteria acquire antimicrobial resistance genes through two principal ways i.e. chromosomal mutation and the acquisition of mobile genetic elements such as plasmids or transposons (Mokracka et al., 2012). The R-plasmids are extrachromosomal circular DNA molecules that replicate independently of the host genome and can transfer horizontally between bacteria by conjugation. R-plasmids can carry resistance genes encoding resistance to one or more antibiotics that play an important role in the evolution and disseminating of resistance genes among the most worrisome clinical pathogens. This free movement of R-plasmids has been considered certainly as the most important drivers of rapid global spread in bacterial families such as Enterobacteriaceae and widespread of MDR bacteria (Rozwandowicz et al., 2018). Unfortunately, the coexistence of multiple-important antimicrobial classes on the same conjugative plasmid have been identified e.g. the co-occurrence of *mcr-1* and $bla_{\text{CTX-M-1}}$ genes together with genes conferring resistance to sulfonamides and tetracyclines on a large conjugative plasmid in *E. coli* from cattle in France (Haenni et al., 2016) and plasmid co-harboring *mcr-1* and $bla_{\text{CTX-M-55}}$ in *Salmonella* in chicken in China (Yang et al., 2016). Thus, the acquisition of a single R-plasmid can simultaneously acquire resistance to multiple antimicrobials. This indicates the biggest threat to global health and serves as a key catalyst for the spread of MDR bacteria.

1.3 Research objectives

1.3.1 To determine the occurrence of antimicrobial resistance among *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces.

1.3.2 To characterize antimicrobial resistance of *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces.

1.4 Research outline



1.5 Advantages of this study

1.5.1 Novel knowledge

- Occurrence and antimicrobial resistance rates of *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces will be obtained.
- Data on genetic characteristics of antimicrobial resistance in *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces will be obtained.

1.5.2 Application of knowledges

- Information on antimicrobial resistance from this project can be used to support development of guidelines for prudent antimicrobial use in animals in Thailand and neighboring countries.
- The data will be applied to support the control of animal and animal product movement in Thailand and between Thai-Cambodia border provinces.
- The data can be used to educate veterinarians and related personnel and raise concern on antimicrobial use in food animals.
- The newly AMR determinants that could emerge at the border of Thailand and neighboring countries will be monitored.
- The results from plasmid characterization and plasmid mapping will describe the co-occurrence and co-selection of resistance genes on a single-plasmid and can be applied for future study.

CHAPTER II

Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces.

Suthathip Trongjit[1], Sunpetch Angkititrakul[2], R. Emerson Tuttle[3], Jiratchaya Poungseree[1], Pawin Padungtod[3] and Rungtip Chuanchuen[1]

[1]Research Unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330

[2]Research Group for Prevention Technology in Livestock, Faculty of Veterinary Medicine, Khon Kaen University, Khon Khan 40000 Thailand

[3]Division of Global Health Protection, Thailand Ministry of Public Health–US Centers for Disease Control and Prevention Collaboration, Nonthaburi 11000 Thailand

Microbiology and Immunology Journal (2017)

Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces.

2.1 ABSTRACT

This study aimed to examine the prevalence and antimicrobial resistance (AMR) of *Salmonella* isolates from broiler chickens, pigs and their associated meat products in the Thailand–Cambodia border provinces. A total of 941 samples were collected from pigs and broiler chickens at slaughterhouses and from carcasses at local fresh markets in Sa Kaeo, Thailand (n = 554) and Banteay Meanchey, Cambodia (n = 387) in 2014 and 2015. From these samples, 345 *Salmonella* isolates were collected from Sa Kaeo (n = 145; 23%) and Banteay Meanchey (n = 200; 47%) and assayed for antimicrobial susceptibility, class 1 integrons and extended-spectrum β-lactamase (ESBL) genes. Serovars Typhimurium (29%) and Rissen (29%) were the most common serotypes found in Thai and Cambodian isolates, respectively. Multidrug resistance was detected in 34% and 52% of isolates from Sa Kaeo and Banteay Meanchey, respectively. The majority of the Thai isolates were resistant to ampicillin (72.4%), whereas most Cambodian isolates were resistant to sulfamethoxazole (71%). Eleven isolates from Sa Kaeo and 44 from Banteay Meanchey carried class 1 integrons comprising resistance gene cassettes. The most common gene cassette array was *dfrA12-aadA2* (61.1%). Six isolates were ESBL producers. The β-lactamase genes found included $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$. Some of these class 1 integrons and ESBL genes were located on conjugative plasmid. In conclusion, multidrug-resistant *Salmonella* are common in pigs, chickens and their products in the Thailand–Cambodia border provinces. Our findings indicate that class 1 integrons play a role in spread of AMR in the strains in this study.

KEYWORDS: antimicrobial resistance, Cambodia, *Salmonella enterica*, Thailand.

2.2 INTRODUCTION

The widespread and indiscriminate use of antimicrobial agents in veterinary medicine, including food animal production, is one of the major contributors to development and spread of AMR (Marshall and Levy, 2011). Pigs and poultry are known to act as important reservoirs of resistant *Salmonella* that can be transmitted to humans via several routes (e.g., direct contact, food consumption, spreading of manure). It has been shown that resistance encoding genes are often associated with integrons, class 1 integrons being the commonest type to mediate MDR in gram-negative bacteria including *Salmonella* (Hsu et al., 2006). In recent years, resistance of *Salmonella* to extended spectrum cephalosporins has increased dramatically worldwide (EFSA, 2015). This resistance is predominantly linked to plasmid-mediated ß-lactamase genes, the most commonly identified type in *Salmonella* obtained from animal and human origin being ESBL (Clemente et al., 2013).

Countries with land border neighbors are common in Southeast Asia, where cross-border trade has dramatically increased. Continued rapid growth is expected as a result of regional–economic integration or the ASEAN Economic Community. Thailand and Cambodia are neighboring countries that share a long common border and have six permanent land crossings for importation and exportation. These sites account for more than 50% of the total trade income between the two countries and are focal points for millions of people and animals from diverse geographical regions to pass through each year (Manarungsan, 2010). Food animals and meat products are amongst the most frequently such goods and illegal trafficking and slaughtering of livestock is a predominant problematic issue. In the border areas close to Thailand, pigs may be illegally imported by farmers who cross the border to buy piglets, the direction of such pig trade depending on pig-market prices in the two countries (Tornimbene and Drew, 2012). These pigs are mostly killed in illegal

slaughterhouses with poor hygiene. Because there are no modern slaughterhouses for pigs in Cambodia, pigs are mostly slaughtered in simple, traditional, relatively unhygienic ways.

The emergence and spread of AMR bacteria have been identified as a global public health priority and underscores the importance of surveillance system for AMR monitoring. However, only a few studies have investigated the prevalence and genetic characteristics of AMR in bacteria associated with food animals in Southeast Asia (Ekkapobyotin et al., 2008; Khemtong and Chuanchuen, 2008; Vo et al., 2010; Van et al., 2012), where awareness and understanding of AMR is still limited (Chuanchuen et al., 2014).

In this study, we analyzed the prevalence, serotype, antimicrobial resistance, class 1 integrons and ESBL encoding genes in *Salmonella* isolates obtained from broiler chickens, pigs and their associated meat products in the Thailand–Cambodia border provinces.

2.3 MATERIALS AND METHODS

2.3.1 Samples and *Salmonella* isolation

During the seven months from July 2014 to January 2015, 941 samples were collected, including 554 samples from Sa Kaeo province, Thailand and 387 samples from Banteay Meanchey province, Cambodia.

In Sa Kaeo, the sampling sites included three private pig slaughterhouses, one private broiler slaughterhouse and the municipal fresh market. The pig slaughterhouses are small-scale facilities that provide meat for local consumption. The poultry slaughter plants produce poultry meat for both local consumption and exporting to Cambodia. All pigs killed in the slaughterhouses had been raised by

local farmers, whereas all broilers originated from commercial poultry-production farms. Sources of carcasses at the local fresh markets varied. In Banteay Meanchey, samples were obtained from one municipal pig slaughterhouse, one municipal broiler slaughterhouse and one municipal fresh market. All pigs and broilers at slaughterhouses were from commercial production farms that provide meat for local consumption only.

Samples were collected on four occasions from each sampling site. The pigs slaughtered were from many different farms; on each trip, fecal samples were taken from the rectums of all pig carcasses that were at the pig slaughterhouse after bleeding but before the scalding process had begun. Samples were also taken from each pig carcass by swabbing an area of approximately 50 cm^2 before they were delivered directly to the fresh markets after slaughter. Broiler slaughterhouse samples were collected from chicken by rectal swab after bleeding but before defeathering. Additionally, broiler carcass samples were obtained by carcass swab before they were shipped to fresh markets. Refusal by owners prevented sampling of broiler carcasses at poultry slaughterhouses in Cambodia. Samples were obtained by swabbing both pig and broiler carcasses at the fresh markets. All collected samples were stored in an icebox and transported to a laboratory within 2 hrs. of collection for immediate processing or held in refrigerator at 4°C until processing, which was completed within 48 hrs. of collection.

Standard method ISO6579:2002 (E) was used to isolate Salmonella strains (ISO, 2002). Three colonies of *Salmonella* were selected from each positive sample. Each isolate was serotyped by slide agglutination based on the Kaufman–White scheme using a commercially available antiserum (ECDC 2012; S&A Reagents Lab, Bangkok, Thailand). Only a single colony of each serotype was collected from each positive sample.

2.3.2 Antimicrobial susceptibility testing and screening for ESBL strains

Minimum inhibitory concentrations against eight antimicrobial agents were determined for all *Salmonella* isolates by agar dilution according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2013). Antimicrobials tested were (clinical breakpoints in parenthesis) ampicillin (32 µg/mL), chloramphenicol (32 µg/mL), ciprofloxacin (4 µg/mL), gentamicin (8 µg/mL), streptomycin (32 µg/mL), sulfamethoxazole (512 µg/mL), tetracycline (16 µg/mL) and trimethoprim (16 µg/mL). Susceptibilities to ceftazidime (30 µg) cefotaxime (30 µg) and cefpodoxime (10 µg) were determined by a disk diffusion method (CLSI, 2013).

Salmonella isolates that were resistant to at least one cephalosporin antibiotic were further tested for ESBL production using a combination disk method in which the zones for cefotaxime and cefotaxime (30 µg) / clavulanic acid (10 µg) disks and ceftazidime and ceftazidime (30 µg) / clavulanic acid (10 µg) disks were compared (Oxoid, Hamshire, UK). A difference of ≥ 5 mm between the inhibition zone of the clavulanic acid and that of the corresponding ESBL disks was taken to confirm a positive ESBL phenotype. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains.

2.3.3 DNA manipulation, PCR amplification and DNA sequencing

Whole cell DNA was prepared as previously described and used as DNA template for PCR (Levesque et al., 1995). All PCR amplifications were performed using a PCR master mix (GeNei MasterMix; Merck, Munich, Germany) according to the manufacturer's instructions. Plasmid DNA was extracted using a QIAprep Mini-spin kit (Qiagen, Hilden, Germany). DNA concentrations were measured using a Nanodrop

ND-1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). For DNA sequencing analyses, the PCR products were gel purified using a Nucleospin Gel and PCR clean up (McCherey–Nagel, Duren, Germany) and submitted for sequencing at First Base Laboratories (Selangor Darul Ehsan, Malaysia). The obtained DNA sequence was BLAST compared with the GenBank database (NCBI, 2014).

2.3.4 Detection of class 1 integrons and mapping of gene cassettes

Presence of integrons was determined by PCR amplification of the class 1 integrase-specific *intI1* gene in all the *Salmonella* isolates (Chuanchuen et al., 2007; Ekkapobyotin et al., 2008). The presence of inserted gene cassettes in variable regions in all *intI1*-positive isolates was determined by using conserved segment PCR (Levesque et al., 1995). The PCR products were gel purified and submitted for sequencing. Clone-specific-PCR amplicons of the same size were analyzed by restriction enzyme digestion using restriction endonucleases *EcoR*I, *BamH*I and *Xba*I and resolved by gel electrophoresis. Amplicons with the same restriction patterns were considered identical.

2.3.5 Detection of ESBL-encoding genes

The presence of genes encoding ß-lactamases was investigated by PCR and nucleotide sequencing in all ESBL-producing isolates. The ß-lactamase genes and primers included:

bla_{TEM} (TEMup, 5´-GCGGAACCCCTATTT-3´; TEMdown 5´-TCTAAAGTATATATGAGTAAACTTGGTCT-3´), bla_{SHV} (SHVup, 5´-TTCGCCTGTGTATTATCTCCCTG-3´; SHVdown, 5´- TTAGCGTTGCCAGTGYTG-3´), bla_{CMY-1} (CMY1up, 5´-GTGGTGGATGCCAGCATCC-3´; CMY1down, 5´-GGTCGAGCCGGTCTTGTTGAA-3´), bla_{CMY-2}(CMY2up,5´-GCACTTAGCCACCTATACGGCAG-3’; CMY2down,5’-GCTTTTCAAGAATGCGCCAGG-3´), bla_{CTX-M} (CTXMup, 5´-CGATGTGCAGTACCAGTAA-3´; CTXMdown, 5´- AGTGACCAGAATCAGCGG-3´) and bla_{PSE} (PSEup, 5´-GCTCGTATAGGTGTTTCCGTTT-3´; PSEdown, 5´-CGATCCGCAATGTTCCATCC-3´)

(Batchelor et al., 2005; Hasman et al., 2005; Li et al., 2013). All PCR amplicons were submitted for DNA sequencing.

2.3.6 Conjugation transfer of class 1 integrons and ESBL-encoding genes.

Conjugation experiments were conducted with the *Salmonella* isolates carrying class 1 integrons (n = 55) and the ESBL-positive strains (n = 4) as the donor strains and rifampicin-resistant *E. coli* K12 strain MG1655 (MIC = 256 mg/mL) as the recipient strain (Chen et al., 2004). Transconjugants were selected on LB agar (Difco, BD Diagnostic Systems, Maryland, USA) containing 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 tested for resistance to antimicrobial agents corresponding to the resistance profile of the donor and for class 1 integrons and ESBL genes by PCR, as described above.

2.3.7 Statistical analysis

Statistical analyses were performed with SPSS version 20.0. The significance ($P < 0.05$) of differences between prevalence of *Salmonella* spp. and between antimicrobial resistance occurrence in various populations, locations and sample types was determined using Pearson's $\chi 2$ test or Fisher's exact test. The significance of trends was determined by the Mantel–Haenzel test (STATA SE12, College Station, TX, USA).

2.4 RESULTS

2.4.1 *Salmonella* prevalence and serovars

The prevalence of *Salmonella* varied between locations and sample sources (Table 1). Overall, the total prevalence of *Salmonella* was significantly higher in pigs, broilers, and their meat products from Banteay Meanchey province Cambodia than in

those from Sa Kaeo province, Thailand ($P < 0.001$). The prevalence of *Salmonella* was higher in pig rectal swabs and pig carcasses from slaughterhouses, and in pig and broiler carcasses from markets, in Banteay Meanchey than in Sa Kaeo ($P < 0.008$). There was no significance difference between Sa Kaeo and Banteay Meanchey in *Salmonella* prevalence in broilers at slaughterhouses ($P = 0.205$).

In Sa Kaeo, *Salmonella* contamination rates in both pig and broiler carcasses at fresh markets were significantly higher than in those originating from the same kinds of animals at the slaughterhouses ($P < 0.001$). The prevalence of *Salmonella* was higher in pig carcass samples at slaughterhouses than in the rectal swab samples ($P < 0.014$). The prevalence of *Salmonella* from broiler rectal swabs was significantly higher than in carcass swabs taken at the slaughterhouse ($P = 0.007$).

In Banteay Meanchey, the prevalence of *Salmonella* in broiler carcasses at fresh markets was higher than in rectal swabs taken at slaughterhouses ($P < 0.001$). The contamination rates of pig carcasses at both slaughterhouses and fresh markets were higher than that of pig rectal swabs at slaughterhouses ($P < 0.001$).

A total of 345 *Salmonella* isolates comprising 45 serotypes were cultured from Sa Kaeo (n = 145) and Banteay Meanchey (n = 200). The Thai isolates were composed of 17 serotypes, of which Typhimurium was the most common (29%) (Table 2). Twenty-eight serotypes were identified among the Cambodia isolates, serovar Rissen being the most common serotype (57, 28.5%).

Table 1. Prevalence of *Salmonella* by location and sample type in Thailand and Cambodia (n = 941)

			Positive samples				*P*-value
			Thailand		Cambodia		
Species	**Location**	**Sample type**	Total	No. (%)	Total	No. (%)	
Pigs	Slaughterhouse	Pigs	100	3 (3)	90	17 (18.9)	0.000
		Pig carcass	88	11 (12.5)	20	20 (100)	0.000
	Market	Pig carcass	87	49 (56.3)	90	64 (71.1)	0.041
		Sub total	275	63 (22.9)	200	101 (50.5)	0.000
Chicken	Slaughterhouse	Broiler	90	10 (1.1)	100	6 (6)	0.205
		Broiler carcass	84	1 (1.2)	ND	ND	-
	Market	Broiler carcass	105	69 (65.7)	87	72 (82.8)	0.008
		Sub total	279	80 (28.7)	187	80 (42.8)	0.002
		Total	554	143 (25.8)	387	179 (46.3)	0.000

ND, not determined.

Table 2. Serotypes of *Salmonella* from various sources in Thailand-Cambodia provinces (n = 345)

Salmonella serotype	Thailand (n=145)						Cambodia (n=200)				
	Slaughterhouse				Fresh market		Slaughterhouse			Fresh market	
	Pigs (n=8)	Pig carcass (n=12)	Broiler (n=4)	Broiler carcass (n=2)	Pork (n=49)	Chicken meat (n=70)	Pigs (n=21)	Pig carcass (n=30)	Broiler (n=6)	Pork (n=72)	Chicken meat (n=71)
Typhimurium	2 (1.4)	2 (1.4)	3 (2)		23 (15.9)	12 (8.3)	3 (1.5)	3 (1.5)			6 (3)
Rissen		9 (6.2)			21 (14.5)		3 (1.5)	6 (3)		47 (23.5)	1 (0.5)
Corvallis						29 (20)					11 (5.5)
Enteritidis	1 (0.7)			1 (0.7)		19 (13)		1 (0.5)	2 (1)		6 (3)
Duesseldorf	4 (2.8)			1 (0.7)		1 (0.7)					3 (1.5)
Kentucky		1 (0.7)				2 (1.4)			4 (2)		4 (2)
Yehuda						2 (1.4)					
Gombe					2 (1.4)						
Monschaui						2 (1.4)					
Meunster					1 (0.7)						
Welterreden						1 (0.7)	1 (0.5)				4 (2)
Albany			1 (0.7)								
Menden	1 (0.7)										
Stanleyville					1 (0.7)						
Stuttgart					1 (0.7)						
Schwarzengrund						1 (0.7)					
Wongata						1 (0.7)					
Agona											28 (14)
Derby							3 (1.5)	6 (3)		13 (6.5)	

Table 2. Serotypes of *Salmonella* from various sources in Thailand-Cambodia provinces (n = 345) (continued)

Salmonella serotype	Thailand (n=145)						Cambodia (n=200)				
	Slaughterhouse				Fresh market		Slaughterhouse			Fresh market	
	Pigs (n=8)	Pig carcass (n=12)	Broiler (n=4)	Broiler carcass (n=2)	Pork (n=49)	Chicken meat (n=70)	Pigs (n=21)	Pig carcass (n=30)	Broiler (n=6)	Pork (n=72)	Chicken meat (n=71)
Parathyphi							3 (1.5)	3 (1.5)			
Indiens										1 (0.5)	6 (3)
Anatum							1 (0.5)	1 (0.5)		2 (1)	
Stanley								2 (1)		2 (1)	1 (0.5)
Lexington							3 (1.5)				1 (0.5)
Newmexico							2 (1)	1 (0.5)			
Doncaster								1 (0.5)		1 (0.5)	
Singapore								2 (1)			
Warragul							1 (0.5)	1 (0.5)			
London										1 (0.5)	
Schleissheim								1 (0.5)			
Fufu							1 (0.5)				
Wandsworth								1 (0.5)			
Norwish								1 (0.5)			
Give										1 (0.5)	
Braenderup										1 (0.5)	
Chincol										1 (0.5)	
Mbandaka										1 (0.5)	
Mowanjum										1 (0.5)	

2.4.2 Antimicrobial resistance of *Salmonella* isolates

Of the 345 *Salmonella* isolates tested, 311 (90%) were resistant to at least one antimicrobial agent and 154 (45%) were MDR (resistant to three or more antimicrobial agents) (Appendix B). There was no significant difference in the overall proportion of any antibiotic resistance between Banteay Meanchey and Sa Kaeo (P = 0.531). The proportion of MDR was significantly higher in Banteay Meanchey (52%) than Sa Kaeo (34%; P = 0.001). The highest proportions of resistance were to ampicillin (70.7%) and sulfamethoxazole (69.5%). Resistance rates to chloramphenicol, trimethoprim, tetracycline and streptomycin were 10.7%, 29.6%, 26% and 28%, respectively. Resistance rates to ceftazidime (4.3%), cefotaxime (3.8%), cefpodoxime (4.9%), gentamicin (2.9%) and ciprofloxacin (0.6%) were low.

The majority of the isolates from Sa Kaeo exhibited resistance to ampicillin (72.4%) and sulfamethoxazole (68.0%) (Fig. 3). Eighty nine percent were resistant to at least one antimicrobial, of which 34.5% were MDR, the most frequent resistance pattern being ampicillin/ sulfamethoxazole (27.6%) (Appendix B). The overall resistance rate was significantly higher for pig and pig carcass isolates than for broiler and broiler carcass isolates (P < 0.001) (Fig. 3).

Among the *Salmonella* isolates from Banteay Meanchey, 91% were resistant to at least one antimicrobial agent and 52% were MDR. The most frequent resistance observed was to sulfamethoxazole (71%), followed by ampicillin (69.5%). The most common resistance pattern was ampicillin/sulfamethoxazole/ trimethoprim (10%). The resistance rate was significantly higher for broiler and broiler carcass isolates than for pig and pig carcass isolates (P = 0.000) (Fig. 3). The proportion of *Salmonella* isolates with resistant to ceftazidime, cefotaxime and

cefpodoxime in Thailand (2 – 4%) was not significantly lower than in Cambodia (5 – 6%) (P = 0.474).

Among 19 *Salmonella* isolates (5.5%) that were resistant to at least one cephalosporin tested, six were found to be ESBL producers, including two isolates from Sa Kaeo (both serovar Rissen from fresh market pig carcasses) and four from Banteay Meanchey (two serovar Parathyphi, a serovar Derby from pigs in slaughterhouse and a serovar Agona from a fresh market broiler carcass) (Table 3).

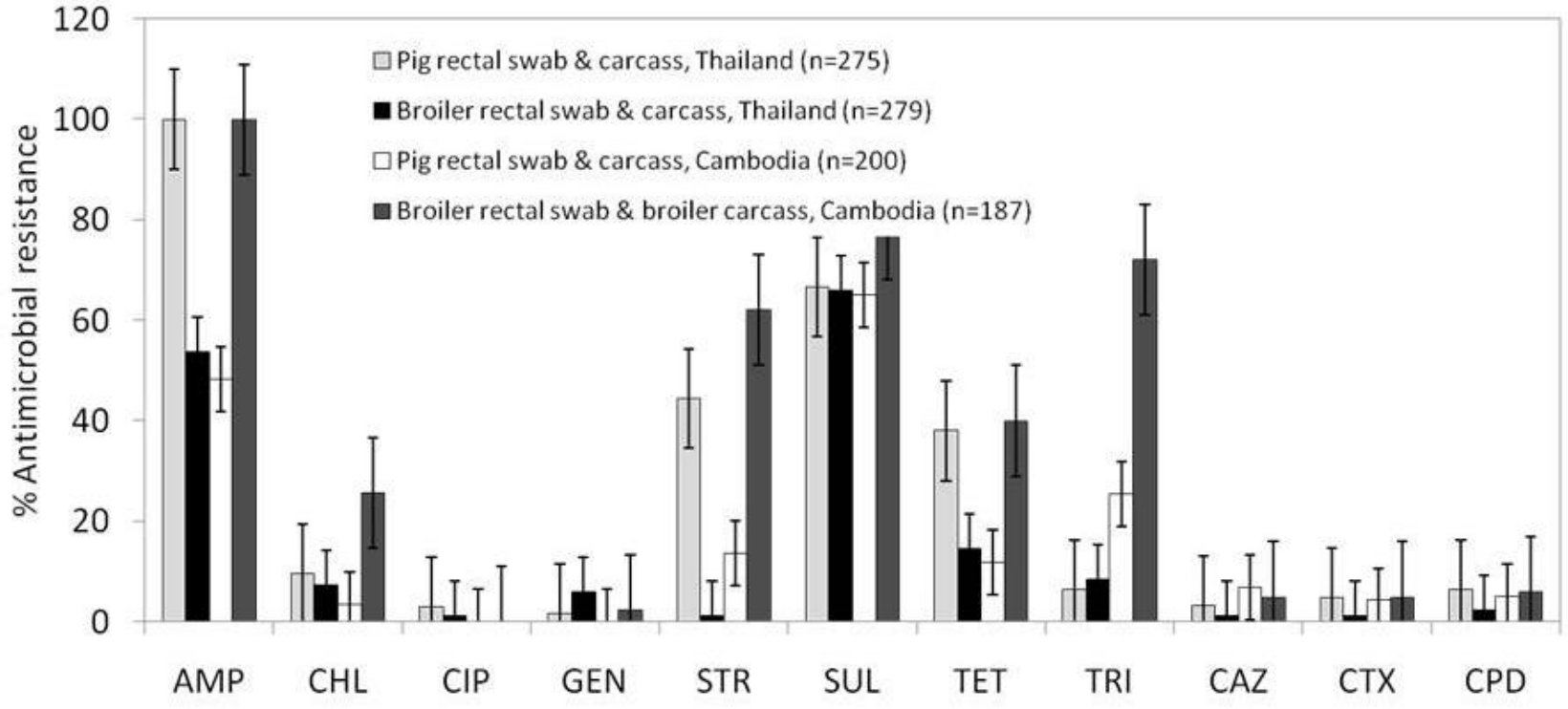


Figure 3. Prevalence of antimicrobial resistance in *Salmonella enterica* from broilers, pigs and their meat in the Thailand–Cambodia border region (n = 667). AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim.

Table 3. Characteristics of ESBL-positive *Salmonella* from Thailand and Cambodia (n = 6)

Country	Serovars (No.)	Source	Resistance pattern	ESBL gene	Class 1 integron
Thailand	Rissen (2)	Pig carcass from	AMP-CIP-CHC-STP-SUL-TET-CAZ-CPD (1)	-	-
		fresh market	AMP-CHC-GEN-STP-SUL-TET-CAZ-CTX-CPD (1)[†]	bla_{TEM-1}, $bla_{CTX-M-55}$	-
Cambodia	Agona (1)	Broiler carcass	AMP-STP-SUL-TRI-CAZ-CTX-CPD[†]	bla_{CMY-2}	-
		from fresh market			
	Paratyphi (2)	Pigs	TRI-CAZ-CTX-CPD (1)[†]	bla_{TEM-1}, $bla_{CTX-M-55}$	-
			CAZ-CTX-CPD (1)[‡]	bla_{TEM-1}, $bla_{CTX-M-55}$	-
	Derby (1)	Pigs	TET-CAZ-CTX-CPD	bla_{TEM-1}	Empty

[†]Capable of transfer of all ESBL genes identified

[‡]Transfer only $bla_{CTX-M-55}$

AMP, ampicillin; TRI, trimethoprim; SUL, sulfamethoxazole; GEN, gentamicin; TET, tetracycline; STP, streptomycin; CHC, chloramphenicol; CIP, ciprofloxacin; CAZ, ceftazidime; CTX, cefotaxime; CPD, cefpodoxime

2.4.3 Characteristics and transfer of class 1 integrons

The *intI1* gene was present in 31% of all the *Salmonella* isolates, including 17.2% of the Thai isolates and 41.0% of the Cambodian isolates. Sixteen percent of all the *Salmonella* isolates carried class 1 integrons comprising diverse arrays of resistance gene, including 8% (11/145) of the isolates from Sa Kaeo and 22.0% (44/200) of the isolates from Banteay Meanchey (Table 4). The majority of the class 1 integrons-carrying isolates from Sa Kaeo were from pigs and pig carcass (55%; 6/11). Similarly, most of the class 1 integron-carrying isolates in Banteay Meanchey were from pigs and pork (93%; 41/44). Three resistance gene cassettes (*aadA2*, bla_{PSE-1} and *dfrA12-aadA2*) were found, the most frequently identified array being *dfrA12-aadA2* (61.8%).

Horizontal transfer of class 1 integrons carrying *dfrA12-aadA2* was demonstrated in six class 1 integrons positive isolates (10.9%), including four from Sa Kaeo (four serovar Rissen from fresh market pig carcasses) and two from Banteay Meanchey (a serovar Anatum from a pig in the slaughterhouse and a serovar Rissen from a fresh market pig carcass). All the *E. coli* transconjugants were confirmed to have *intI1* and resistance gene cassettes identified in the corresponding donors.

2.4.4 Presence and transfer of ESBL genes

Two serovar Rissen Thai isolates were ESBL-positive strains, only one of which (from a fresh market pig carcass) was found to harbor bla_{TEM-1} and $bla_{CTX-M-55}$ on conjugative plasmid (Table 3). As to the ESBL-positive strains from Cambodia, two serovar Parathyphi isolates from pigs carried bla_{TEM-1} in addition to $bla_{CTX-M-55}$. One of the isolates was able to transfer both genes to *E. coli*, whereas the other could only transfer $bla_{CTX-M-55}$. The bla_{TEM-1} gene was only found in a Derby serovar from a pig and was not transferable. The latter additionally carried empty class 1 integrons.

One serovar Agona isolate from a broiler carcass carried *bla*$_{CMY-2}$ on a conjugative plasmid. All of the ESBL genes detected in this study were outside the variable regions of class 1 integrons.

Table 4. Characterization of class 1 integrons in *Salmonella* from Thailand and Cambodia (n = 55)

			Thailand			Cambodia		
IP	**Size (bp)**	**Gene cassettes**	No. (%)	Serotype (No.)	Source[†] (No.)	No. (%)	Serotype (No.)	Source[†] (No.)
I	1000	*aadA2*	-	-	-	13 (29.5%)	Derby (11)	Ps (2), Pc (3), Pf (6)
							Rissen (2)	Ps (2)
II	1300	bla_{PSE-1}	6 (54%)	Albany (1)	Bs (1)	2 (4.5%)	Agona (2)	Bf (2)
				Typhimurium (2)	Bs (2)			
				Duesseldorf (3)	Ps (1), Bs (1), Bf (1)			
III	2000	*dfrA12-aadA2*	5 (46%)	Rissen (5)[‡] (4)	Pf (5)	29 (66%)	Rissen (18)	Ps (1)[‡], Pc (3), Pf (14)
							Derby (2)	Ps (1), Pf (1)
							Anatum (1)	Ps (1)[‡]
							Warragul (1)	Ps (1)
							Doncaster (1)	Pc (1)
							Enteritidis (1)	Pc (1)
							Welterreden (1)	Ps (1)
							Parathyphi (1)	Ps (1)
							Give (1)	Pf (1)
							Typhimurium (2)	Ps (1), Bf (1)

[†]Ps, pig from slaughterhouse; Pc, pig carcass from slaughterhouse; Pf, pork from fresh market; Bs, broiler from slaughterhouse; Bc, broiler carcass from slaughterhouse; BF, broiler carcass from fresh market.

[‡]Capable of transfer of class 1 integrons.

The superscript number in parentheses indicates the number of isolates with the corresponding letter.

2.5 DISCUSSION

One of the main findings of this study was the extensive prevalence of *Salmonella* contamination in pigs, broilers and their meat in the Thailand–Cambodia border area. Several factors may be implicated, including subclinical carriers being induced to actively shed bacteria through the stress induced by transport and lairage (Mannion et al., 2012) and a lack of biosecurity measures in both slaughterhouses and fresh markets. In this study, the prevalence of *Salmonella* was much higher in samples collected at fresh markets than in those collected from slaughterhouses, which is discordant with the findings of previous findings (Padungtod and Kaneene, 2006). It was also much higher than previously reported (Padungtod and Kaneene, 2006). These discrepancies may be related to the evisceration process and potential cross-contamination between carcasses at fresh markets. The chicken carcasses were chilled in cold water after mechanical defeathering at the slaughterhouse and then shipped to local retail markets, where they were eviscerated and cleaned to provide visceral organs that were sold separately (Hsu et al., 2006). In contrast, evisceration of pigs occurred at the slaughterhouse; however, they were cleaned and cut up at the markets, which could have increased the risk of cross contamination (Carrasco et al., 2012). Another possible explanation for the conflict in findings between this and previous studies is the poorer hygiene practices in fresh markets in the study area. In general, hygiene practices in fresh markets vary by location. Hygiene and safety standards are higher in big cities and their surrounds, whereas in provincial fresh markets, sanitation practices are not routinely carried out and market facilities tend to be less stringently regulated (Knips, 2004). Meat is usually delivered in open vehicles without cooling systems and unsold meat is kept in an icebox overnight for reselling the following day. Of interest, the prevalence of *Salmonella* at slaughterhouses in this study is lower than previously reported for Thailand

(Vadhanasin et al., 2004; Padungtod and Kaneene, 2006). It has been suggested that contamination of meat products cannot be completely prevented (Bolton et al., 2013). Slaughter practices in Sa Kaeo and Banteay Meanchey differ from those in more developed countries and have shifted in recent years toward being more industrialized (Chantong and Kaneene, 2011).

Serovar Typhimurium was most prevalent among the samples from Thailand, particularly in pig carcasses from fresh markets. This finding differs from that of a previous study in Northern Thailand in which only 2/109 *Salmonella* isolates from pigs were serovar Typhimurium and the most common serovar was Weltevreden (Padungtod and Kaneene, 2006). Possible explanations for these discrepancies include the geographically different sampling sites and the approximately 10 years interval between the two studies.

Salmonella from pigs, broilers and their associated meat products at retail markets were resistant to multiple antimicrobial agents, particularly ampicillin, sulfonamides and tetracycline, in agreement with previous studies (Wannaprasat et al., 2011). These findings are also consistent with those of our previous study of *E. coli* isolates from the same samples (Trongjit et al., 2016) and may be attributable to the various antimicrobials used in food animals in these two countries (Chuanchuen et al., 2014). The presence of resistance to chloramphenicol, banned in Thailand since 1996, may be attributable to co-selection and co-resistance. Resistance to 3rd-generation cephalosporins is currently particularly concerning because of their importance as drugs of choice for treating Salmonellosis in humans. Fortunately, we detected only a low prevalence of *Salmonella* isolates resistant to ceftazidime, cefotaxime and cefpodoxime in Thailand (2–4.1%) and Cambodia (4.5–6%), in agreement with a previous study (Padungtod and Kaneene, 2006). These

findings are similar to those for *E. coli* isolates from the same source in our previous study (Trongjit et al., 2016).

ESBL-producing *Salmonella* have been increasingly reported (Morris et al., 2006; Choi et al., 2015; Irenge et al., 2015); however, ESBLs are less common in nontyphoidal *Salmonella* than other Gram-negative bacteria (Hsu et al., 2006). This is in agreement with our present study in which only 2.4% of the *Salmonella* isolates were ESBL producers; there was a similarly low prevalence in *E. coli* isolates in our previous study (Trongjit et al., 2016). Use of cephalosporins in food animals is not prohibited in these countries; however, the higher cost of these classes of antibiotics may prohibit their use and at least partially explain the low prevalence of ESBL producers recovered in these countries.

Fluoroquinolone and quinolones have been used in both human and veterinary medicine in both Cambodia and Thailand. Fortunately, we found a low prevalence of resistance to this class of antibiotic among Thai isolates, and we did not find any such resistance in the Cambodian isolates. An association between ESBL production and ciprofloxacin resistance has previously been demonstrated (Dobiasova et al., 2013; Matsumura et al., 2013), however, we did not identify this link in the present study.

Overall, the frequency of class 1 integrons was lower than in previous studies in Thailand (Wannaprasat et al., 2011; Sinwat et al., 2015). The gene cassette we most frequently found in the present study was *dfrA12-aadA2*, which is in agreement with our study of *E. coli* isolates (Chuanchuen et al., 2014) and previous studies from other countries (Mannion et al., 2012). These results confirm the global spread of this gene cassette and are supported by our finding that class 1 integrons carrying *dfrA12-aadA2* were conjugally transferred. In our previous study, we also commonly

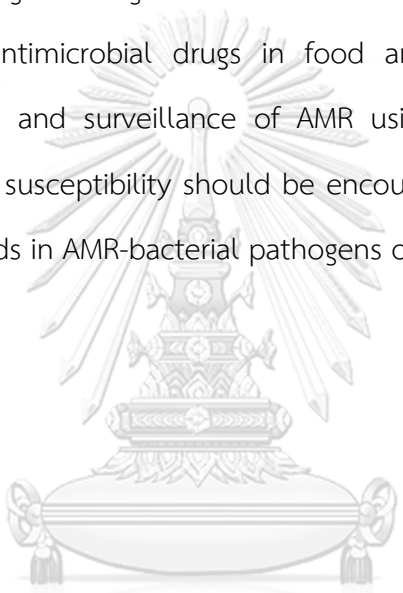
found the *dfrA1-aadA1* cassette array among *E. coli* isolates (Trongjit et al., 2016). In contrast, we did not find this gene cassette array in the *Salmonella* isolates in this study; the reason for this discrepancy remains unclear. In addition, we detected class 1 integrons more frequently among the isolates from Cambodia. To the best of our knowledge, this is the first report of class 1 integrons in *Salmonella* isolates from Cambodia. In addition, it should be noted that we found class 1 integrons more frequently in the pig and pig carcass isolates. The reason for this remains unclear but it may reflect more frequent use of antimicrobials in pig production in the countries studied. Non-antibiotic selection pressure for class 1 integrons has previously been demonstrated in bacterial strains in a quaternary compounds-polluted environment (Gaze et al., 2005). However, such role of the compounds was not pursued in the pig farming environment.

Several ESBL genes have been reported in *Salmonella* from multiple animal food sources globally through various surveillance programs (Aarestrup et al., 2010). In contrast, we detected only $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ in the Thai pig carcass isolates in this study; we also found $bla_{\text{CMY-2}}$ in the Cambodian isolates. Most of the isolates carried two of the ß-lactamase genes tested, in agreement with previous studies demonstrating that a single *Salmonella* isolate usually carries more than one ß-lactamase gene (Usha et al., 2008). In agreement with previous studies (Yang et al., 2014), the $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$ genes were found to reside on conjugative plasmids, confirming the potential spread of these b-lactamase genes to other bacteria via horizontal transfer.

In addition, $bla_{\text{PSE-1}}$ gene cassette was the second most common inclusion gene cassette within a class 1 integrons. However, none of the *Salmonella* isolates carrying class 1 integrons with $bla_{\text{PSE-1}}$ yielded a positive result in the phenotypic ESBL screening test and we detected no mutations in this gene (data not shown). The

reason of this observation is unclear. It is possible that the presence of ESBL genes in these strains may not produce enough ESBLs to yield positive results with the clinical breakpoints used in this study.

In summary, we found a high prevalence of AMR *Salmonella* in pigs, chicken and their products along the Thailand–Cambodia border provinces. This finding highlights the need to improve hygiene practices including the effectiveness of cleaning and disinfecting in slaughterhouses and fresh markets. Judicious use of medically important antimicrobial drugs in food animals should be promoted. In addition, monitoring and surveillance of AMR using standardized methods for sampling and assessing susceptibility should be encouraged to enable monitoring of the burden of and trends in AMR-bacterial pathogens over time.



CHAPTER III

Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces

Suthathip Trongjit[1], Sunpetch Angkititrakul[2] and Rungtip Chuanchuen[1]

[1]Research Unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330

[2]Research Group for Prevention Technology in Livestock, Faculty of Veterinary Medicine, Khon Kaen University, Khon Khan 40000 Thailand

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Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces

3.1 ABSTRACT

Nine hundred and forty-one samples were collected in Sa Kaeo, Thailand (n = 554) and Banteay Meanchey, Cambodia (n = 387) from July 2014 to January 2015. A total of 667 *Escherichia coli* isolates (381 isolates from Sa Kaeo and 286 isolates from Banteay Meanchey) were obtained and examined for antimicrobial susceptibility, class 1 integrons, ESBL genes and horizontal transfer of resistance determinants. Prevalence of *E. coli* in pig and broiler carcass samples from slaughterhouses and fresh markets was 36–85% in Sa Kaeo and 11–69% in Banteay Meanchey. The majority of these isolates were multidrug resistant (75.3%). Class 1 integrons were common in both Thai (47%) and Cambodian (62%) isolates, of which four resistance gene cassette arrays including *aadA1*, *dfrA1-aadA1*, *dfrA12-aadA2* and *aadA2-linF* were identified. Class 1 integrons in two broiler isolates from Sa Kaeo (dfrA12-aadA2) and one broiler isolate from Banteay Meanchey (*dfrA1-aadA1*) were horizontally transferable. Sixteen isolates were confirmed to be ESBL-producing strains with ESBL gene $bla_{\text{CTX-M15}}$, broad spectrum β-lactamase gene $bla_{\text{TEM-1}}$ and the AmpC gene $bla_{\text{CMY-2}}$ being detected. The $bla_{\text{TEM-1}}$ gene was most prevalent and located on a conjugative plasmid.

KEYWORDS: antimicrobial resistance, Cambodia, *Escherichia coli*, Thailand

3.2 INTRODUCTION

Antimicrobial resistance (AMR) is a serious concern in human and veterinary medicine. Infections with AMR bacterial pathogens have increased globally and led to the rise of medical expenses, treatment failure and increased morbidity and mortality. Surveillance of AMR in zoonotic agents and indicator bacteria of commensal flora represents an important step in developing an effective control and prevention action plan to combat AMR (EFSA, 2012). For this reason, commensal *Escherichia coli* from healthy food animals are suggested as indicator species for AMR in Gram-negative bacteria

It is well known that resistance genes in bacteria are often linked to mobile genetic elements, especially integrons that are capable of capturing and mobilizing genes (Partridge et al., 2009). Within this group of genetic elements, class 1 integrons are the most frequent type identified in Enterobacteriae (Hsu et al., 2006). Simultaneously, ESBL-producing Enterobactericeae has rapidly increased worldwide and acquisition of ESBL genes has been increasingly recognized (EFSA&ECDC, 2012). Both class 1 integrons and ESBL genes are commonly located on transferable plasmids that are potentially transferred intra- and interspecies, leading to the emergence and spread of MDR bacterial strains.

Escherichia coli are ubiquitous in the intestinal tract of humans and animals. Even though commensal *E. coli* is usually non-pathogenic, it is one of the most frequent bacteria of the Enterobacteriaceae family associated with extraintestinal infections (Mellata, 2013). Concern has been raised that commensal *E. coli* in animals may serve as a reservoir of resistance determinants that could be transferred to pathogenic bacteria in either humans or animals (Szmolka and Nagy, 2013). Plasmid carrying several resistance genes including genes encoding ESBL has

increasingly emerged in *E. coli* isolates of animal origin (Szmolka and Nagy, 2013). Of particular concern is that this resistant *E. coli* may contaminate carcasses during the lairage, slaughter, and marketing processes, contributing to the spread of AMR.

Globalization travel has been implicated as a risk factor for the emergence and dissemination of antimicrobial-resistant bacteria and resistance determinants (Senok et al., 2012). Thailand and Cambodia share a common border with six permanent land crossings for tourism, importation and exportation, accounting for more than 50% of the total trade income of the two countries (Manarungsan, 2010). Millions of people from different world regions, live animals and meat products pass through these border crossings each year. A variety of goods is available for trading, of which food animals and their meat products make up a significant portion of trade volume. However, illegal trafficking of livestock and ensuing illegal slaughter practices with poor hygiene frequently occurs. Travelers who choose land routes may get sick with bacterial pathogen(s) prior to traveling or during their trip. They may require medical treatment from local health services or buy antibiotics over the counter at pharmacies. Such antibiotic treatment may be suboptimal and provides a selective advantage for resistant bacteria. This may lead to emergence of novel AMR genotypes and spread of AMR pathogens across geographical borders, creating a public health impact not only locally, but also throughout the region and the globe as a whole.

Increasing prevalence of MDR- and ESBL-producing *E. coli* has been recognized worldwide. However, only a few reports have attempted to elucidate this public health crisis in food animals in developing countries, including those in South-East Asia. Based on the available data, AMR in bacteria from food animals in Asia was reviewed (Chuanchuen et al., 2014). Existing data are mostly dedicated to the resistance phenotype in bacterial pathogens, especially *Salmonella*. Awareness

and understanding of the threat of AMR development and spread is limited and few countries in the region have systems in place to monitor it. Therefore, the present study aimed to investigate prevalence and characteristics of AMR in commensal *E. coli* isolates from broilers, pigs and their meat in the area along the Thailand–Cambodia border. AMR phenotype, class 1 integrons, ESBL and localization of resistance determinants were analyzed.

3.3 MATERIALS AND METHODS

3.3.1 Samples and bacterial isolation

In this cross-sectional study, a total of 941 samples (554 samples from Sa Kaeo province, Thailand and 387 samples from Banteay Meanchey province, Cambodia) were collected from July 2014 to January 2015. The Thai samples were obtained from three small private pig slaughterhouses, one private broiler slaughterhouse and one municipal fresh market. Cambodian sampling sites included one municipal pig slaughterhouse, one municipal broiler slaughterhouse and one municipal fresh market.

At slaughterhouses, the samples included: (i) swine fecal sample (n=190) obtained by rectal evacuation after bleeding but prior to scalding; (ii) broiler rectal swab (n=190) obtained after bleeding but prior to defeathering; (iii) swine carcass swab (n=285) obtained by swabbing an area of approximately 50 cm^2 on each fresh carcass prior to delivery to market; and (iv) broiler carcass swabs (n=276) obtained in a similar manner prior to shipment to market. Due to owner's disallowance, chicken carcass swab samples were not obtained from poultry slaughterhouses in Banteay Meanchey. At fresh markets, the samples included carcass swab from both pig and broiler carcasses. The sample collection was done four times from each sampling site. As a result of logistical constraints, random sampling was not possible. The samples were processed by standard methods for *E. coli* isolation (Quinn et al., 1994).

The *E. coli* isolated were streaked on MacConkey agar (Difco, Sparks, MD, USA). Five colonies resembling *E. coli* were randomly selected and confirmed on eosin methylene blue agar and biochemical tests (Carter and Cole Jr, 2012).

A single colony from each positive sample was collected and stored as 20% glycerol stock at - 80°C.

3.3.2 Determination of antimicrobial susceptibility and ESBL phenotype

Antimicrobial minimum inhibitory concentrations (MIC) were determined using the agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2013). The following antimicrobials were tested (their recommended breakpoints are in parentheses): ampicillin (32 µg/mL), chloramphenicol (32 µg/mL), ciprofloxacin (4 µg/mL), gentamicin (8 µg/mL), streptomycin (32 µg/mL), sulfamethoxazole (512 µg/mL), tetracycline (16 µg/mL) and trimethoprim (16 µg/mL). The disk diffusion method was carried out to detect susceptibility to cefotaxime (30 µg), cefpodoxime (10 µg) and ceftazidime (30 µg). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. MDR was defined as isolates being resistant to three or more different classes of antibiotic (O'Mahony et al., 2005).

Isolates resistant to at least one cephalosporin tested were subsequently confirmed for ESBL production using the combination disk method (CLSI, 2013). This compared the inhibition zones given by cefotaxime and cefotaxime (30 µg)/clavulanic acid (10 µg) disks, and ceftazidime and ceftazidime (30 µg)/clavulanic acid (10 µg) disks (Oxoid, Hampshire, England). Isolates that showed a difference of $\geq$ 5 mm between the inhibition zone of the clavulanic acid and the corresponding ESBL disks were considered a positive ESBL phenotype.

3.3.3 DNA isolation, PCR and DNA sequence analysis

Whole-cell lysate was used as DNA template for PCR (Levesque et al., 1995) unless otherwise indicated. Plasmid DNA was extracted using a NucleoSpin® Plasmid

DNA Purification Kit (Macherey-Nagel, Düren, Germany) or the alkaline lysis procedure as previously described (Liou et al., 1999). Chromosomal DNA was prepared using a GF-1 Bacteria DNA Extraction Kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia). All PCR amplifications were carried out using PCR master mix of GeNei™ MasterMix (Merck, Munich, Germany) according to the manufacturer's instructions. The PCR products were purified with Nucleospin® Gel and PCR clean up (Macherey-Nagel) and were sequenced at First Base Laboratories (Selangor Darul Ehsan, Malaysia). The resulting DNA sequences were compared to the GenBank Database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov).

All the *E. coli* isolates were characterized for class 1 integrons. The *intI1* gene was first screened (Chuanchuen et al., 2007; Ekkapobyotin et al., 2008). Then, all the *intI1*-positive strains were determined for inserted gene cassettes in the variable region using conserved segment PCR (CS-PCR) and DNA sequencing (Levesque et al., 1995).

All ESBL-positive *E. coli* were investigated for ESBL genes as previously described, including bla_{TEM}, bla_{SHV} (Hasman et al., 2005), bla_{CTX-M} (Batchelor et al., 2005) and bla_{PSE} (Li et al., 2013). Presence of AmpC ß-lactamase gene bla_{CMY-1} and bla_{CMY-2} was also examined (Hasman et al., 2005). All the amplicons were gel purified and sequenced using PCR primers.

3.3.4 Conjugation experiments

The *E. coli* isolates carrying class 1 integrons with resistance gene cassettes (n = 45) and the ESBL-positive *E. coli* (n = 16) were used as donors and SE12Rif^r, the spontaneous rifampicin-resistant derivatives of *Salmonella enteritidis* SE12 (MIC = 256 µg/mL), was used as recipient in the filter mating conjugation experiments (Chen et al., 2004). SE12 is susceptible to all antimicrobial agents tested and does not carry

plasmid and class 1 integrons. Transconjugants were confirmed to be *Salmonella* on Xylose Lysine Deoxycholate agar (Difco) and tested for antimicrobial susceptibility. Transfer of resistance determinants were confirmed by PCR as described above. For the isolates carrying two different class 1 integrons: *dfrA12-aadA2* and *aadA2-linF* (n = 3), chromosomal DNA and plasmid were extracted and used for PCR template to detect intl1 and variable region to localize the integrons. PCR amplicons of variable regions were subjected to DNA sequencing analysis as described above.

3.3.5 Statistical analysis

The chi-squared test (χ^2) with SPSS version 20.0 was used to analyze the data. A *P*-value of < 0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 *E. coli* prevalence in pig and broiler carcasses

Five hundred and sixty-one samples that originated from pig carcasses at slaughterhouses and fresh markets (n = 285) and broiler carcasses at slaughterhouses and fresh markets (n = 276) were included for determination of *E. coli* prevalence (Table 5). The prevalence of *E. coli* in the pig samples in Sa Kaeo was significantly higher than that in Banteay Meanchey ($P < 0.05$) but the opposite was true regarding chicken samples with the prevalence being significantly higher in Banteay Meanchey than in Sa Kaeo ($P < 0.05$). The prevalence of *E. coli* in the chicken carcasses from fresh markets in Banteay Meanchey was significantly higher than that in Sa Kaeo ($P < 0.05$) but there was no difference between the countries regarding the prevalence from pig carcass samples ($P = 0.642$).

Table 5. Prevalence of *Escherichia coli* obtained from pig and chicken carcasses at slaughterhouses and their meat products from fresh markets in Sa Kaeo, Thailand and Banteay Meanchey, Cambodia (n = 561)

			Thailand		Cambodia		
Animal Species	Source	Sample type	Total	No. (% Positive)	Total	No. (% Positive)	*P*-value
Pigs	Slaughterhouse	Carcass swab	88	75 (85.2)	20	11 (55)	0.005
	Fresh market	Carcass swab/ Pork	87	31 (35.6)	90	36 (40)	0.642
		Subtotal	175	106 (60.6)	110	47 (42.7)	0.004
Chicken	Slaughterhouse	Carcass swab	84	61 (72.6)	ND	ND	-
	Fresh market	Carcass swab/ Chicken meat	105	41 (39.8)	87	69 (79.3)	0.000
		Subtotal	189	102 (60)	87	69 (79.3)	0.000
No. tested			364	208 (57.1)	197	116 (58.8)	0.721

ND, not determine

3.4.2 Antimicrobial resistance rates

A total of 667 *E. coli* isolates (381 isolates from Sa Kaeo and 286 isolates from Banteay Meanchey) were determined for antimicrobial susceptibility, the prevalence of class 1 integrons and ESBL genes. These included the *E. coli* isolates from pig rectal swabs (85 isolates from Sa Kaeo and 82 isolates from Banteay Meanchey), broiler rectal swabs (88 isolates from Sa Kaeo and 88 isolates from Banteay Meanchey) and those from pig and broiler carcasses at slaughterhouses and fresh markets (n = 324) (Table 5).

Ninety-eight percent of the isolates from Sa Kaeo were resistant to at least one antimicrobial agent and 77.4% were MDR. Resistance rates to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, cefotaxime, cefpodoxime and ceftazidime were 91%, 14.8%, 5%, 58.4%, 40.9%, 63.4%, 45.7%, 76.1%, 11%, 10% and 9%, respectively. When considering the sample source, resistance to ampicillin (87%, 94%), trimethoprim (82%, 71%) and sulfamethoxazole (67%, 66%) was most common among the pig rectal swab/pig carcass isolates and the broiler rectal swab/carcass isolates, respectively (Fig. 4). The most common resistance patterns AMP-SUL-TRI-TET was most common (12.3%) (Appendix B). The resistance rates in the *E. coli* isolates from pig rectal swabs/pig carcasses from slaughterhouses and pig carcasses from fresh markets (99.5%) were not different from broiler rectal swabs/carcasses from slaughterhouses and broiler carcasses in fresh markets (97.4%) ($P < 0.05$).

Among the Banteay Meanchey isolates, up to 93% were resistant to at least one antimicrobial agent and 72.4% were MDR. Resistance rates to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, streptomycin, sulfamethoxazole,

tetracycline, trimethoprim, cefotaxime, cefpodoxime and ceftazidime were 86%, 22.4%, 0.7%, 6.6%, 32.8%, 74.8%, 13%, 75.5%, 7%, 7% and 8.4%, respectively.

When considering the sample source, resistance to ampicillin (78%, 93%), trimethoprim (64.3%, 84%) and sulfamethoxazole (70.5%, 78.3%) was most common among the pig rectal swab/carcass isolates and the broiler rectal swab/carcass isolates, respectively (Fig. 4). Resistance patterns AMP-SUL-TRI (16.8%) and AMP-SUL-TRI (16.8%) were most common. Resistance in the isolates from samples originating from broilers was higher than that in isolates from pigs ($P < 0.05$). The resistance rates in the *E. coli* isolates from broiler rectal swabs/carcasses from slaughterhouses and broiler carcasses in fresh markets (96.8%) were significantly higher than those from pig rectal swabs/pig carcasses from slaughterhouses and pig carcasses from fresh markets (88.4%) ($P < 0.05$).

When comparing between the two countries, resistance rates in the *E. coli* isolates from Sa Kaeo (98.4%) were significantly higher than those from Banteay Meanchey (93%) ($P < 0.05$). The AMR rate was significantly higher in the pig rectal swab samples from Sa Kaeo (98.8%) than those from Banteay Meanchey (87.8%). Similarly, the AMR rate was significantly higher in the pig carcass swab samples from Sa Kaeo (100%) than in those from Banteay Meanchey (89.4%) ($P < 0.05$). Resistance rates of the broiler rectal swab/carcass isolates (97.4% in Sa Kaeo and 96.8% in Banteay Meanchey) in the two provinces were not different.

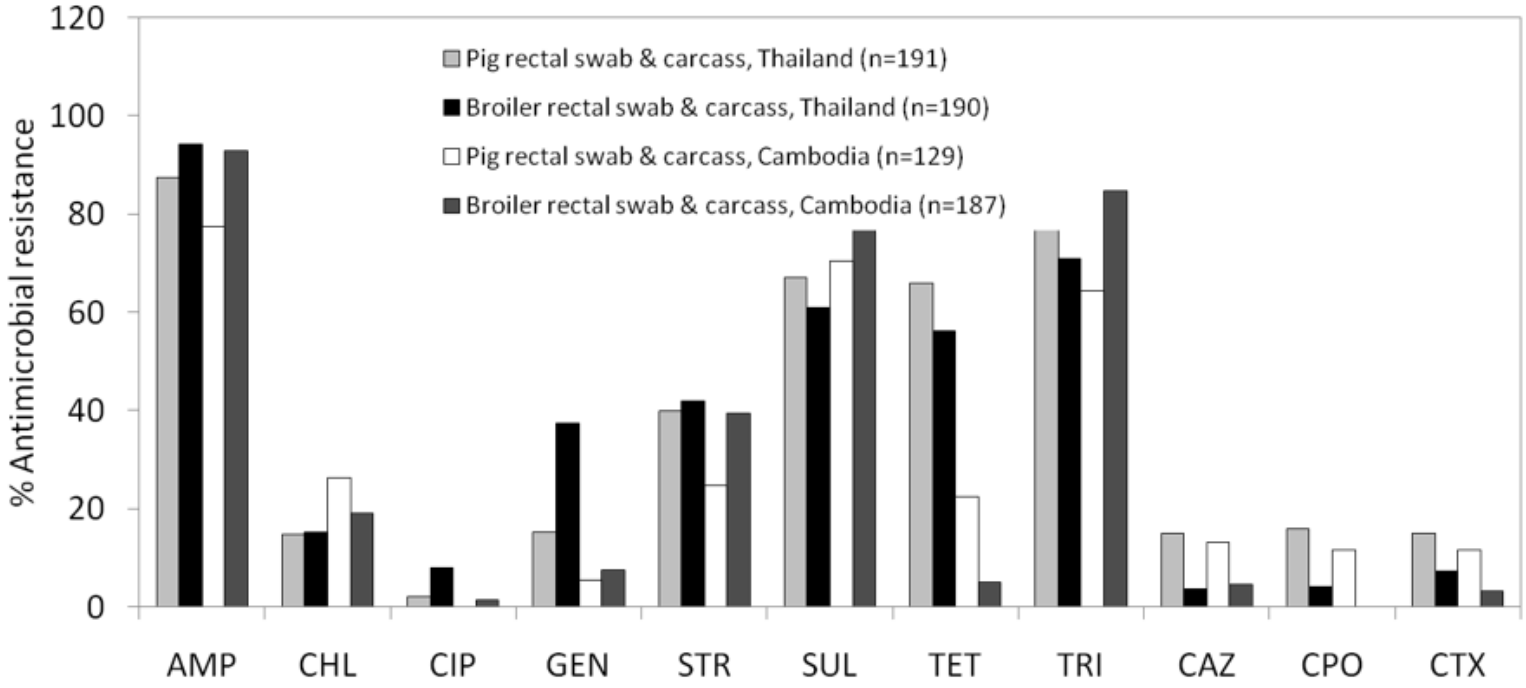


Figure 4. Distribution of antimicrobial resistance in *Escherichia coli* from broilers, pigs and their meat products in the Thailand-Cambodia border region (n = 667). AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; TRI, trimethoprim; SUL, sulfamethoxazole; TET, tetracycline.

3.4.3 Presence and transfer of class 1 integrons

Fifty-three percent of all isolates were positive to *intl1*, including 179 isolates (47%) from Sa Kaeo and 176 isolates (62%) from Banteay Meanchey. Of the Thai isolates, the prevalence of class 1 integrons from pig rectal swabs, pig carcasses from slaughterhouses, pig carcasses from fresh markets, broiler rectal swabs, broiler carcasses from slaughterhouses, and broiler carcasses from fresh markets were 11.5%, 9.7%, 4.5%, 10.8%, 6.3%, and 4.2%, respectively. For the Cambodian isolates, the prevalence of class 1 integrons was highest in samples obtained from broiler rectal swabs (23%), followed by those from pig rectal swabs (15%), from broiler carcasses at fresh markets (14.7%), from pig carcasses at fresh markets (5.9%), and from pig carcasses at slaughterhouses (2.8%).

Of the *intl1*-positive isolates, 41 isolates (23%) from Sa Kaeo and 24 isolates (14%) from Banteay Meanchey carried resistance gene cassettes. Four resistance gene cassette arrays were identified, including *aadA1*, *dfrA1-aadA1*, *dfrA12-aadA2* and *aadA2-linF* (Table 6). The *dfrA12-aadA2* cassette array (41.5%, 17/41) was most common among the isolates from Sa Kaeo, whereas the *dfrA1-aadA1* cassette array (70.8%, 17/24) was most common in the isolates from Banteay Meanchey. Two isolates from broiler rectal swabs and one isolate from broiler carcasses in Sa Kaeo carried two class 1 integrons: *dfrA12-aadA2* and *aadA2-linF*. PCR results showed that both integrons were located on plasmid. No CS-PCR products were observed when chromosomal DNA was used as DNA templates.

Table 6. Characterization of class 1 integrons in *Escherichia coli* from Sa Kaeo, Thailand and Banteay Meanchey, Cambodia[†]

IP	Size (bp)	Gene cassettes	Thailand			Cambodia		
			Total	Resistance pattern	Source	Total	Resistance pattern	Source
I	1000	*aadA1*	11	GEN-STP-TRI-TET-CAZ-CTX-CPD (2)	Ps(1), Pc(1)	2	AMP-STP-TET (1)	Ps(1)
			(26.8%)	AMP-GEN-STP-SUL-TRI-TET (2)	Cf(2)	(8.3%)	AMP-CHC-GEN-STP-SUL-TRI (1)	Cs(1)
				AMP-STP-TET (2)	Ps(1), Pc(1)			
				AMP-STP-SUL-TRI-TET (2)	Pc(2)			
				GEN-STP-TRI-CAZ-CPD (1)	Ps(1)			
				AMP-STP-SUL-TRI-TET (1)	Pc(1)			
				AMP-CHC-GEN-STP-SUL-TRI-TET (1)	Cf(1)			
II	1500	*dfrA1-aadA1*	13	AMP-STP-TRI (2)	Cc(1), Cf(1)	17	AMP- STP-SUL-TRI (6)[(1)‡]	Cs(6)
			(31.7%)	AMP-GEN-STP-SUL-TRI (2)	Cs(2)	(70.8%)	AMP-STP-SUL-TRI (6)	Cf(1), Cs(5)
				AMP-GEN-STP-SUL-TRI-TET (3)	Ps(2), Cf(1)		AMP- STP-SUL-TRI-TET (1)	Ps(1)
				STP-SUL-TR I(1)	Ps(1)		AMP-CHC-STP-SUL (1)	Cf(1)
				AMP-STP-SUL-TR I(1)	Cc(1)		STP-SUL-TRI (1)	Cf(1)
				AMP-STP-SUL-TRI-TET (1)	Ps(1)		AMP- STP-TRI (1)	Cf(1)
				AMP-CHC-GEN-STP-SUL-TRI-TET (1)	Cc(1)		AMP-GEN- STP-SUL-TRI (1)	Cf(1)
				AMP-STP-SUL-TRI-TET (1)	Cf(1)			
				AMP-GEN-STP-SUL-TRI-TET (1)	Cf(1)			

Table 6. Characterization of class 1 integrons in *Escherichia coli* from Sa Kaeo, Thailand and Banteay Meanchey, Cambodia† (continued).

IP	Size (bp)	Gene cassettes	Thailand			Cambodia		
			Total	Resistance pattern	Source	Total	Resistance pattern	Source
III	2000	*dfrA12-aadA2*	14 (34.2%)	STP-TRI (2)	Pf(1), Cs(1)	5 (20.8%)	AMP- STP-SUL-TRI (2)	Cs(5)
				AMP-STP-SUL-TRI (2)	Cs(1), Cc(1)		AMP-CHC- STP-SUL-TRI (1)	
				AMP-CHC-GEN-STP-SUL-TRI-TET (3)	Cc(2), Ps		AMP-STP-SUL-TRI (1)	
				AMP-CIP-CHC-STP-SUL-TRI-TET (1)	(1)		AMP-CHC-GEN- STP-SUL-TRI (1)	
				STP-SUL-TRI (1)	Ps(1)			
				AMP-CIP-CHC-GEN-STP-SUL-TRI-TET (1)‡	Cs(1)			
				AMP-GEN-STP-SUL-TRI-TET (1)	Cs(1)			
				AMP-STP-SUL-TRI-TET (1)	Cf(1)			
				AMP-STP-SUL-TRI-TET (1)	Cf(1)			
				AMP-STP-SUL-TRI-TET (1)	Pc(1)			
					Cs(1)			
IV	2000, 2100	*dfrA12-aadA2, aadA2-linF*	3 (7.3%)	AMP-GEN-STP-SUL-TRI (3)	Cs(2), Cc(1)	-	-	-

-, Not found., †n = 65., ‡Capable of horizontal transfer. Value in the superscript parenthesis is the number of isolates. AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim. Pc, pig carcass from slaughterhouse; Pf, pork from fresh market; Ps, pigs from slaughterhouse; Cc, chicken carcass from slaughterhouse; Cf, chicken meat from fresh market; Cs, chicken from slaughterhouse.

Only two broiler isolates, including one Thai isolate carrying class 1 integrons with *dfrA12-aadA2* and one Cambodian isolate harboring class 1 integrons with *dfrA1-aadA1*, yielded *Salmonella* transconjugants. Efficiency of conjugation is shown in Table 7. PCR amplification using the plasmids of the transconjugants showed that the Thai and Cambodian isolates produced an amplicon of approximately 2000 and 1500 bp, which were confirmed to be *dfrA12-aadA2* gene and *dfrA1-aadA1*, respectively, by DNA sequencing. Presence of resistance gene cassettes corresponded to those in their *E. coli* donors. The transconjugants were resistant to trimethoprim and streptomycin. The results confirmed that the two broiler isolates were able to transfer class 1 integrons.

Table 7. Efficiencies of conjugation between *Escherichia coli* carrying class 1 integrons with *dfrA12-aadA2* or *dfrA-aadA1* and *Salmonella* Enteritidis SE12Rif^r (n = 2)

E. coli/ gene cassette	Count of SE12 Rif^r (CFU/mL)†	Ratio of *E. coli* donor: SE12Rifr‡	Transconjugants (CFU/mL)§	Efficiency¶
dfrA12-aadA2	8.3×10^8	7 : 4	3.6×10^7	4.3×10^{-2}
dfrA1-aadA1	8.3 x 108	7 : 4	4.8×10^7	5.8×10^{-2}

Average of two replicates‡

Ratio of *E. coli* donor: *S.* Enteritidis SE12Rif^r recipient cells in each conjugation§

Average number of transconjugants in duplicate plates (200 mL was plated)¶

Average number of transconjugants per recipient

3.4.4 ESBL phenotype and ß-lactamase encoding genes

Sixteen isolates were confirmed to be ESBL-producing strains (2.4%, 16/667), including 11 isolates from Sa Kaeo (pig rectal swabs, three; pig carcasses from slaughterhouse, six; pig carcasses from fresh markets, one; broiler rectal swabs, one; and five isolates from Banteay Meanchey (all sourced from pig rectal swabs) (Table 8). All the ESBL-producing strains were MDR.

Of all ESBL genes tested, only $bla_{CTX\text{-}M\text{-}15}$ was found. The gene was detected in four isolates from Sa Kaeo but none from Banteay Meanchey. The $bla_{TEM\text{-}1}$ gene encoding for broad-spectrum ß-lactamase was most common (n = 12). The AmpC gene $bla_{CMY\text{-}2}$ was detected (n = 2). One ESBL producer from a pig carcass in a fresh market in Sa Kaeo and another isolate from pig rectal swab from Banteay Meanchey carried $bla_{TEM\text{-}1}$/$bla_{CTX\text{-}M15}$ and $bla_{TEM\text{-}1}$/$bla_{CMY\text{-}2}$, respectively (Table 8). Two isolates from pig carcasses at slaughterhouse in Sa Kaeo and one isolate from pig rectal swab in Banteay Meanchey carried $bla_{TEM\text{-}1}$ on conjugative plasmid.

Table 8. Characteristics of the ß-lactamase positive *Escherichia coli* isolates (n = 16)

Country	Source	Resistance pattern	ß-lactamase gene	Class 1 integrons
Thailand (n=11)	Pig (3)	AMP-TRI-CAZ	$bla_{\text{TEM-1}}$	ND
		AMP-CIP-CHC-STP-SUL-TRI-TET-CAZ-CTX-CPD	$bla_{\text{CTX-M-15}}$	ND
		AMP-TRI-CAZ-CTX-CPD	$bla_{\text{CTX-M-15}}$	ND
	Pig carcass at slaughterhouse (6)	AMP-CHC-SUL-TRI-TET-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$	Empty
		AMP-STP-SUL-TRI-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$ †	ND
		AMP-SUL-TRI-CAZ-CTX-CPD (2)	$bla_{\text{TEM-1}}$ †	ND
		AMP-SUL-TRI-TET-CAZ-CTX-CPD	$bla_{\text{CMY-2}}$	ND
		AMP-SUL-TRI-CAZ-CTX-CPD	$bla_{\text{CTX-M-15}}$	ND
	Pig carcass at fresh market (1)	AMP-CHC-SUL-TRI-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$	Empty
	Broiler (1)	AMP-CHC-GEN-SUL-TRI-TET-CAZ-CTX-CPD	$bla_{\text{CTX-M}}$	Empty
Cambodia (n=5)	Pig (5)	AMP-SUL-TRI-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$	Empty
		AMP-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$	ND
		AMP-CHC-STP-SUL-TRI-TET-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$	ND
		AMP-SUL-TRI-CAZ-CTX	$bla_{\text{TEM-1}}$ †	ND
		AMP-STP-SUL-TRI-CAZ-CTX-CPD	$bla_{\text{TEM-1}}$, $bla_{\text{CMY-2}}$	Empty

Empty means class 1 integrons with no gene cassette insert.

†Capable of horizontal transfer.

AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim.

ND, no class 1 integrons detected.

3.5 DISCUSSION

Pig production in Thailand has been developed to an intensive pig industry with medium- to large-scale producers (Falvey, 2000). There are a number of private or government-owned abattoirs with modern technology and good hygienic standards in the country. Slaughtering is generally done in modern slaughterhouses, municipal abattoirs, or simple local government slaughterhouses. Most abattoirs have undergone good manufacturing practices for abattoirs (ACSF, 2006a) and for animal welfare (ACSF, 2010). However, insufficient hygiene at provincial slaughterhouses still occurs and are a major concern. In Cambodia, the majority of pigs are slaughtered in simple traditional ways with low hygienic practices in the slaughtering process as a result of a lack of modern slaughterhouses for pigs (Tum, 2008).

Of the six border crossings, the crossing point between Sa Kaeo, Thailand and Poipet, Cambodia is a major trade route with much tourist activity. However, most Cambodians in Poipet routinely cross the border to buy live animals, fresh meat and meat products from Thai markets. Therefore, in the present study, the sample collection was done in Banteay Meanchey, the nearest town to Poipet, to ensure local sources of samples as much as possible.

One of the main findings in this study was the high prevalence of *E. coli* in pig and broiler carcasses, which indicates the role of food of animal origin as a major reservoir for *E. coli* transmission to humans. The prevalence of *E. coli* in broiler carcasses from fresh markets in Banteay Meanchey was significantly higher than was found in Sa Kaeo ($P < 0.05$). Typically, meat in fresh markets either in Sa Kaeo or Banteay Meanchey are sold in the open air at ambient temperature. However, Thai fresh markets are under a monthly-cleaning routine. Vendors are trained and are

aware of hygiene and sanitation. Raw carcasses are delivered from slaughterhouses to the butcher shop by mini-truck and the unsold remnants are stored in a freezer or refrigerator. In contrast, in fresh markets in Banteay Meanchey where facilities tend to be less extensively regulated, the hygiene and sanitation practices may not be very strict or routinely carried out (Knips, 2004). Meat is usually delivered in open buckets on tricycles and the unsold meat is stored in an icebox overnight for sale again the following morning. This may explain the different prevalence of *E. coli* in broiler carcasses from fresh markets. Regardless, the present study points out the microbial contamination status of retail meat in fresh markets that may spread to humans. The application of hygiene practices is therefore essential. Regulatory authorities should spread awareness about basic hygiene principles to venders and consumers. It is important to provide sanitation and food hygiene training to meat handlers and venders and to ensure that hygienic practice is carried out correctly and routinely.

The antimicrobials tested in this study were those commonly used in food animals in Asia (Chuanchuen et al., 2014). It is interesting to observe resistance to chloramphenicol in the Thai isolates even though the drug has been banned from use in food animals. It was previously suggested that this phenomenon may be a result of co-selection and/or cross-resistance generated by other antibiotics (Bischoff et al., 2005). Regardless, observation of the chloramphenicol-resistant isolates suggests that removal of certain antimicrobial selection pressures may not completely eliminate AMR.

In the present study, the widespread nature of MDR *E. coli* isolates among food animals and meat products in Thai-Cambodian border provinces was highlighted. Such high AMR rates were similar to those previously reported in both countries (Diarrassouba et al., 2007; Lay et al., 2012). This is not surprising because a

variety of antimicrobial agents have been widely used in food animal production in Thailand and Cambodia (Chuanchuen et al., 2014). As fluoroquinolones and cephalosporins are the drug classes of choice for the treatment of bacterial infections, *E. coli* resistant to these antimicrobials could present a challenge to human and animal therapeutic interventions and marks a relevant public health implication (Yang et al., 2009). In addition, food animals are now recognized as important reservoirs of ESBL-producing *E. coli* (Kluytmans et al., 2013). Fortunately, in the present study, the prevalence of ciprofloxacin-resistant and ESBL-producing *E. coli* was rather limited. The majority of the ESBL producers found in this study were from pig rectal swabs and pig carcasses (15/16). This may imply the more frequent use of cephalosporins in pig production. Raising pigs in backyards by families or by smallholders is common in this region (Tornimbene and Drew, 2012). Many antibiotics are widely used in this pig production system because disease prevention is not efficient. In contrast, the poultry production sector has undergone a shift in structure and operation, from a backyard activity to an industrial activity. Farm size has significantly increased, together with improved feed technology, animal husbandry, farm management and prevention and control of diseases. Evidently, antimicrobial use in poultry production is controlled more strictly and under supervision by veterinarians.

The frequency of class 1 integrons in pig rectal swabs (11.5% in Sa Kaeo and 15.4% in Banteay Meanchey) and broiler rectal swabs (10.8% in Sa Kaeo and 22.7% in Banteay Meanchey) was lower than in previous studies in the same countries (Diarrassouba et al., 2007; Lay et al., 2012) and others (Nógrády et al., 2006). Concurrently, class 1 integrons in the pig and broiler carcass isolates in fresh markets in both countries were limited, in agreement with previous reports from Japan (Ahmed et al., 2009) and Vietnam (Van et al., 2007). This suggests that non-class 1

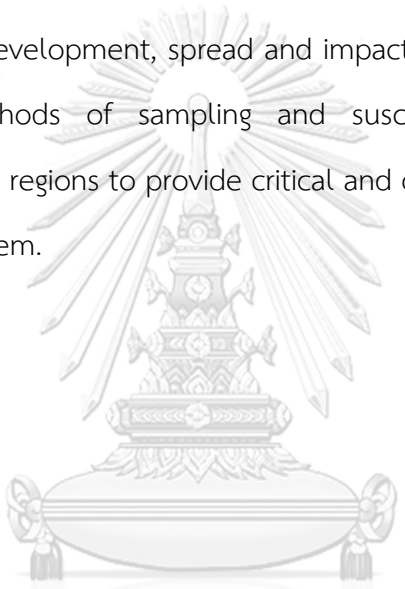
integrons-borne resistance determinants are responsible for resistance phenotype in the majority of *E. coli* in this study. Overall, *dfrA1-aadA1* and *dfrA12-aadA2* were the most prevalent gene cassettes and could be transferred from *E. coli* to *Salmonella*, in agreement with previous studies (Thong and Modarressi, 2011). The explanation could be that streptomycin and trimethoprim/sulfamethoxazole combination have been widely used in the region and this could create specific selection pressure for the acquisition and maintenance of a streptomycin and trimethoprim resistance cassette by class 1 integrons. In the Thai isolates, the *dfrA12-aadA2*, *dfrA1-aadA1* and *aadA1* gene cassettes were found at a similar frequency. In contrast, *dfrA1-aadA1* was solely dominant among the Cambodian isolates. This supports the notion that the occurrence of a particular combination gene cassette varied with the geographical area (Yu et al., 2003). It was proposed that gene cassettes become stably integrated over a long period and transfer of the entire integron, via a plasmid or transposon, is more common than single-gene mobilization or integration within the integron (Martinez-Freijo et al., 1998). This may explain the large numbers of *dfrA1-aadA1* that were also located in conjugative plasmids in the Cambodian isolates. In addition, the presence of different *dfr* types may have been caused by differences in types of trimethroprim and/or antibiotic therapy regimens used in each country. The combinations of *dfrA12-aadA2* and dfrA1-aadA1 were previously found in *E. coli* from food animals and their derived products (Sunde and Norström, 2006; Lay et al., 2012) and also identified in *Salmonella* from different sources in different countries (Hsu et al., 2006; Vo et al., 2010). These results indicate the exchange of integrons or gene cassettes intra- and inter-species and suggest that the import and export of livestock and their products may contribute to the distribution of class 1 integrons or bacterial host strains to different world regions.

Three *E. coli* isolates from chicken and chick carcasses in slaughterhouses in Sa Kaeo carrying class 1 integrons with *dfrA12-aadA2* additionally harbored an *aadA2-linF* cassette array in another class 1 integron. The *aadA2-linF* cassette array is a rare combination that was formerly observed in an *E. coli* strain isolated from the bloodstream of human patients (Kor et al., 2013) and *Salmonella* isolated from poultry (Van Essen-Zandbergen et al., 2007). These data confirm the horizontal transfer of the resistance determinants among *E. coli* from different hosts and between *E. coli* and *Salmonella*. It is interesting to observe that these strains carried two copies of the same streptomycin resistance encoding gene, *aadA2*, located within different integrons that were found on the plasmid. The coexistence of multiple plasmids carrying class 1 integrons with different gene cassettes or that of multiple integrons on the same plasmid was previously reported in *Salmonella* (Tosini et al., 1998). However, plasmid analysis was not pursued in this study and it is warranted to provide further understanding of the acquisition and spread of integrons.

In this study, the $bla_{\text{TEM-1}}$ gene encoding broad spectrum ß-lactamase was most frequently found, in agreement with the findings that the gene was horizontally transferred to *Salmonella*. CTX-M were noted as dominant ESBL genes in previous studies (Tong et al., 2015) but their limited prevalence was observed in this study. In addition, the AmpC $bla_{\text{CMY-2}}$ gene was detected at low frequency. This plasmid-mediated *ampC*-like gene was previously found to spread among *E. coli* and *Salmonella* in food animals, retail meat and humans (Yan et al., 2004; Liu et al., 2007). The different type and frequency of ESBL genes present is likely as a result of different geographical areas, different types of cephalosporins used, and different antimicrobial selective pressure (Trang et al., 2013). The latter is supported by the

observation that all the ESBL producers were multi-resistant, suggesting the possible co-selection of resistance genes.

In summary, the present study demonstrates the high contamination rate of MDR *E. coli* in pig and broiler carcasses in Thai-Cambodian border provinces and confirms the role of commensal *E. coli* as carriers of class 1 integrons and genes encoding broad-spectrum ß-lactamase that have the potential for horizontal transfer. Similar research and surveillance should be conducted in every country to improve understanding of the development, spread and impact of AMR. Use of standardized and harmonized methods of sampling and susceptibility testing should be encouraged in all world regions to provide critical and comparable information to the global surveillance system.



CHAPTER IV

Plasmid-mediated colistin resistance and ESBL production in *Escherichia coli* from clinically healthy and sick pigs in Thailand, 2007-2018

Suthathip Trongjit[1], Pornchalit Assavacheep[2], Sukuma Samngamnim[2], Tran Hoang My[3], Vo Thi Tra An[3], Shabbir Simjee[4] and Rungtip Chuanchuen [1]

[1]Research Unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[2]Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

[3]Ministry of Education and training, Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam

[4]Global Regulatory & Technical Advisor, Microbiology & Antimicrobials, Elanco Animal Health Inc, Basingstoke, England

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Plasmid-mediated colistin resistance and ESBL production in *Escherichia coli* from clinically healthy and sick pigs in Thailand, 2007-2018

4.1 ABSTRACT

This study aimed to determine the percentage of colistin resistant and ESBL-producing *Escherichia coli* from clinically sick and healthy pigs and understand the molecular mechanisms underlying colistin resistance and ESBL production. A total of 454 *E. coli* isolates from healthy pigs (n=354; piglets, n=83; fattening pigs, n=142 and sows, n=129) and sick pigs (n=100) were examined for antimicrobial susceptibility, chromosomal and plasmid-mediated colistin resistance mechanisms and ESBL genes. The healthy (41%) and sick pig (73%) isolates were commonly resistant to colistin. Three *mcr* genes including *mcr-1* (10.4%), *mcr-2* (1.1%) and *mcr-3* (45%) were detected, of which *mcr-3* was most frequently detected in the healthy (33%) and sick pig (57%) isolates. Coexistence of *mcr-1/mcr-3* and *mcr-2/mcr-3* was observed in piglets (23%), fattening pig (3.5%) and sick pig (13%) isolates. The percentage of ESBL-producing *E. coli* was significantly higher in the sick pigs (44%) than the healthy pigs (19.2%) (P = 0.00). The $bla_{\text{CTX-M}}$ group was most prevalent (98.5%), of which $bla_{\text{CTX-M-14}}$ (54.5%) and $bla_{\text{CTX-M-55}}$ (42.9%) were predominant. The $bla_{\text{TEM-1}}$ (68.8%) and $bla_{\text{CMY-2}}$ (6.3%) genes were identified in ESBL-producers. All ESBL producers were multidrug resistant and the majority from piglets (97%), fattening pigs (77.3%) and sick pigs (82%) carried *mcr* gene (s). ESBL producers from piglets (n = 5) and sick pig (n=1) simultaneously transferred $bla_{\text{TEM-1}}$ (or $bla_{\text{CTX-M-55}}$) and *mcr-3* to *Salmonella*. In conclusion, pigs are important reservoirs of colistin-resistant *E. coli* that also produced ESBLs, highlighting the need for prudent and effective use of antimicrobials in pigs and other food-producing animals.

KEYWORDS: colistin resistance, *Escherichia coli*, ESBL, *mcr*, pig, Thailand.

4.2 INTRODUCTION

In recent times antimicrobial resistance (AMR) has rapidly increased and become one of the greatest threats to public health globally. The highest-increasing rates of AMR have been reported in low and middle-income countries, especially those in Southeast Asia (Van Boeckel et al., 2015). Extensive use of antimicrobials in either human medicine or animal farming is considered a major contributor to emergence and spread of AMR (Van Boeckel et al., 2015). In livestock production, the purposes of antimicrobials are either to treat infections, control or promote growth (McEwen and Fedorka-Cray, 2002; American Veterinary Medical Association, 2004; Goutard et al., 2017). Different countries have different policies and regulations with respect to antibiotic growth promoter (AGP). For example, Thailand phased in AGP ban in 2011 and implemented total ban in 2015 (MOAC, 2015). The US FDA prohibited the use of medically important antibiotics for AGP in 2017 but not for non-medically important ones (Scott et al., 2019). Consumer's demand for livestock products has risen globally and is effectively driving antimicrobial consumption in food animals to maintain animal health and increase productivity some of these actions are consequently resulting in increasing levels of AMR (Van Boeckel et al., 2015). The emergence of multi-drug resistant *E. coli* has been frequently reported not only in clinical medicine but also in livestock production. Particular concern has been raised to the dissemination of *E. coli* resistant to clinically important antibiotics (i.e. colistin and newer generation cephalosporins) that may diminish antibiotics of choice for infection treatment in the near future.

Colistin is a cationic polypeptide antibiotic belonging to the class of polymyxin with a narrow antibacterial spectrum activity against Gram-negative bacteria. Although colistin is considered as a last resort antibiotic for treatment of serious infections caused by carbapenemase-producing Enterobacteriaceae in human,

its usage continues to be restricted due to its side effects (e.g. neurotoxicity and nephrotoxicity) (Poirel et al., 2017) and replaced by less toxic antibiotics, (e.g. aminoglycosides, quinolones, and ß-lactams). In veterinary medicine, colistin has been commonly used in pig production for preventing and controlling the clinical outcomes of *E. coli* infection e.g. neonatal diarrhea, post-weaning diarrhea and edema disease by giving either in feed or in water (Chauvin et al., 2002). However, its use in animals has been limited since 2016 as a consequence of the rising report of colistin resistance among the bacterial isolates from livestock, especially pig production (Liu et al., 2016). Colistin resistance in *E. coli* can be associated with mutations in chromosomal genes i.e. *pmrA* and *pmrB* (Quesada et al., 2015). In 2016, the presence of transferable plasmid-mediated colistin resistance, *mcr-1,* was detected in Enterobacteriaceae isolated from food animals, foodstuffs and humans in China and has posed a worrying threat to public health worldwide (Liu et al., 2016). To date, several variants of plasmid-mediated colistin resistance genes (e.g. *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5*) have been identified (Xavier et al., 2016; Carattoli et al., 2017; Yin et al., 2017). The *mcr-1* gene is globally distributed in many bacterial species isolated from various sources (Skov and Monnet, 2016; Poirel et al., 2017). While *mcr-2* and *mcr-4* have been mainly identified in European countries (Xavier et al., 2016; Carattoli et al., 2017), *mcr-3* has been reported in *E. coli* from a variety of sources across Europe and Asia (Hernandez et al., 2017; Yin et al., 2017; Fukuda et al., 2018; Zhang et al., 2018). It is well noted that the *mcr-1* prevalence in bacteria isolated from food animals, especially swine, was higher than that from humans (Liu et al., 2016).

ESBLs are enzymes conferring resistance to oxyimino cephalosporins (e.g. cefotaxime, ceftazidime and ceftriaxone) and oxyimino-monobactam aztreonam. Most ESBL encoding genes are located on conjugative plasmids (Khan et al., 2017;

Kim et al., 2018; Ramos et al., 2020). ESBLs have been increasingly reported among Enterobacteriaceae, particularly *E. coli* from food animals. The latter are considered an important reservoir of *E. coli* resistant to last-line antibiotics that can spread to humans via food chain.

ESBL-producing *E. coli* carrying *mcr-1* have been isolated from food animals and humans (Wu et al., 2018a). Co-existence of *mcr-1* with an ESBL gene on plasmids e.g. bla_{VIM-1} (Poirel et al., 2016) and bla_{CTX-M1} (Haenni et al., 2016)) was previously demonstrated in clinical *E. coli* isolates. A former study in China reported the increasing prevalence of ESBL- producing *E. coli* in chicken origin from 2008 to 2014, of which the *mcr-1* gene was more prevalent in ESBL producers than non-ESBL-producers (Wu et al., 2018a). To date, many studies have investigated the emergence and dissemination of plasmid involved in colistin and cephalosporin resistance in livestock production and role of food animals as potential reservoirs of resistant bacteria and resistance genes was highlighted (Dominguez et al., 2018; Wu et al., 2018a; Zhang et al., 2019b). However, the knowledge of colistin and cephalosporin resistance associated with livestock in Asia, including Thailand, is still limited. Commensal *E. coli* from healthy pigs may serve as potential reservoirs of AMR genes and potentially contaminate pork and pork products. Thus, the aims of this study were to determine the percentage of ESBL production and colistin resistance and the distribution of ESBL and plasmid-mediated colistin resistance genes in *E. coli* isolated from clinically healthy as well as sick pigs.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial isolates

A total of 454 *E. coli* isolates were obtained from two bacterial culture stocks isolated between 2007-2018 as described below. All *E. coli* strains were isolated by

using standard method as previously described (Quinn et al., 1994). One *E. coli* colony from each positive sample was collected and stored in 20% glycerol at -80ºC.

4.3.1.1 Isolates from healthy pigs

Isolates were obtained from the bacterial stock of Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University (n=354). These isolates were originated from fecal samples collected from clinically healthy pigs, confirmed by farm veterinarians, piglets at 4-8 weeks of age (n=83), fattening pigs at 9-18 weeks of age (n=142) and sow at 37-45 weeks of age (n=129) between 2007-2018 as part of our AMR studies. A yearly distribution of the isolates is shown in figure 5. The samples originated from farms located in Central and Northeast Thailand including Aungthong, Chachoengsao, Chonburi, Kanchanaburi, Ratchaburi, Suphanburi, Nakhonratchasima, Burirum, and Udonthani regions. These provinces have high densities of pig population, with farm sizes varying from small scale (51-500 pigs) to large scale (>5,000 pigs). One sample was collected from one pig house in each farm by farm veterinarians.

4.3.1.2 Isolates from sick pigs

The isolates were obtained from the strain collection of Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University (n=100). All were isolated from fecal swab samples routinely collected from sick pigs at 2-21 weeks old displaying clinical signs of diarrhea during 2011-2018 (Fig 5). Farm veterinarians collected and submitted samples for clinical diagnosis at Veterinary Diagnostic Laboratory (VDL), Livestock Animal Hospital, the Nakornpathom campus. The farms from which these samples were obtained were located in Central (i.e. Nakornpathom, Saraburi and Suphanburi), Eastern (i.e. Chachoengsao and Chonburi),

Western (i.e. Kanchanaburi and Ratchaburi) and Southern (i.e. Trang) regions of Thailand. Antibiotic use history was not available.

4.3.2 Test for antimicrobial susceptibility and extended spectrum β-lactamase (ESBL) production

Antimicrobial susceptibility was determined against 8 antimicrobial agents using agar dilution method (CLSI, 2013). Antimicrobials tested, concentration ranges and clinical breakpoints, in parenthesis, are as follows: ampicillin (0.5-512 µg/mL, 32 µg/mL), chloramphenicol (0.5-512 µg/mL, 32 µg/mL), ciprofloxacin (0.125-256 µg/mL, 4 µg/mL), gentamicin (0.25-256 µg/mL, 8 µg/mL), streptomycin (0.5-1024 µg/mL, 32 µg/mL), sulfamethoxazole (0.5-1024 µg/mL, 512 µg/mL), tetracycline (0.5-512 µg/mL, 16 µg/mL) and trimethoprim (0.25-512 µg/mL, 16 µg/mL). Phenotypic resistance to colistin (0.25-128 µg/mL,) was tested by using two-fold agar dilution method (CLSI, 2013) and MIC results was interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for Enterobacteriaceae (MIC > 2 µg/mL) (EUCAST, 2017). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 served as quality control strains.

Detection of ESBL production was performed by disk diffusion method using antibiotics (quantity of antibiotic, zone diameter breakpoint) as follows: cefotaxime (30ug, ≤ 27 mm), cefpodoxime (10ug, ≤ 17 mm) and ceftazidime (30ug, ≤ 22 mm) (CLSI, 2013). The antibiotic disks were obtained from Oxoid (Oxoid™, Hamshire, England). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 served as quality control strains.

The *E. coli* isolates exhibiting resistance to at least one cephalosporin tested were phenotypically confirmed for ESBL production using the combination disk

method including cefotaxime and cefotaxime (30 mg)/clavulanic acid (10 mg), and ceftazidime and ceftazidime (30 mg)/clavulanic acid (10 mg) (Oxoid™, Hamshire, England). The inhibition zone difference of ≥ 5 mm in the combination with clavulanic acid versus the inhibition zone in the cephalosporin alone was interpreted as positive for ESBL production (CLSI, 2013).

4.3.3 DNA isolation, PCR and DNA sequencing analysis

DNA template for PCR was prepared by whole cell boiled lysates as previously described (Levesque et al., 1995). All PCR amplifications were performed using TopTaq™ Master Mix Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instruction. Primers used in this study are listed in Table 9. PCR products were separated on 1.5% agarose gel electrophoresis (Sigma-Aldrish®) in 1XTris-acetate/EDTA (TAE) buffer. The gels were stained in RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, NJ, USA) and visualized using the Omega Fluor™ Gel Documentation System (APLEGEN™ Gel Company, CA, USA). The PCR products were purified using Nucleospin® Gel and PCR clean up (Macherey-Nagel, Düren, Germany) and submitted for DNA sequencing at First Base Laboratories (Selangor Darul Ehsan, Malaysia). The DNA sequences obtained were compared with the reference sequence available at GenBank Database using the Blast algorithm (http://www.ncbi.nlm.nih.gov).

4.3.3.1 Detection of mutations in the *pmrAB* system

Twenty colistin-resistant *E. coli* isolates from healthy pigs (n=10) and sick pigs (n=10) were arbitrarily selected (n=20) for PCR amplification of *pmrA* and *pmrB* genes (Quesada et al., 2015). Five colistin-susceptible isolates from healthy pigs were included as control. The PCR amplicons were gel purified and submitted for DNA

sequencing using PCR primers. DNA sequences were compared to those of *E. coli* K12 (U00096.2) available at GenBank database.

4.3.3.2 Detection of plasmid-mediated colistin resistance determinants and ß-lactamase genes

All *E. coli* isolates (n=454) were screened for the presence of *mcr* genes by PCR using specific primers, including *mcr-1* (MCR1-IF and MCR1-IR) (Liu et al., 2016), *mcr-2* (MCR2-IF and MCR2-IR) (Xavier et al., 2016), *mcr-3* (MCR3-IF and MCR3-IR) (Yin et al., 2017) *and mcr-4* (MCR4-IF and MCR4-IR) (Carattoli et al., 2017). All the ESBL positive-isolates (n=112) were examined for the presence of ß-lactamase genes using specific primers, including bla_{CTX-M} (bla_{CTX-M}_FW and bla_{CTX-M}_RW), bla_{PSE-M}(bla_{PSE-M}_FW and bla_{PSE-M}_RW), bla_{SHV} (bla_{SHV}_FW and bla_{SHV}_RW), bla_{TEM} (bla_{TEM}_FW and bla_{TEM}_RW), bla_{CMY-1} (bla_{CMY-1}_FW and bla_{CMY-1}_RW) and bla_{CMY-2} (bla_{CMY-2}_FW and bla_{CMY-2}_RW) (Batchelor et al., 2005; Hasman et al., 2005; Li et al., 2013).

The identification of bla_{CTX-M} groups was performed in all bla_{CTX-M} positive isolates by multiplex PCR using specific primers for CTX-M group1 (Multi CTXMGp1_FW and Multi CTXMGp1_RW), CTX-M group 2 (Multi CTXMGp2_FW and Multi CTXMGp2_RW), CTX-M group 8/25 (CTX-M group 8/25_FW and CTX-M group 8/25_RW) and CTX-M group 9 (CTX-M group 9_FW and CTX-M group 9_RW) (Sabate et al., 2000; Dallenne et al., 2010). All the isolates positive to CTX-M group 1 were further examined for $bla_{CTX-M15}$ (CTX-M15_SFW and CTX-M15_SRW) (Muzaheed et al., 2008). The PCR amplicons of bla_{TEM} and bla_{CTX-M} were subjected to direct sequencing and their subtypes were analyzed by BLAST search.

4.3.4 Conjugation experiments

Biparental filter mating method was performed to test transferability of *mcr* and ESBL genes. All the *E. coli* isolates carrying *mcr* and/or ESBL genes served as

donors and the spontaneous rifampicin-resistant *Salmonella* Enteritidis (SE12Rif^R, rifampicin MIC=256 µg/ml), was used as recipient (Chen et al., 2004). The *Salmonella* transconjugants were confirmed on Xylose Lysine Deoxycholate agar (Difco, MD, USA) containing 32 µg/mL rifampicin and an appropriate antibiotic (i.e. 100 µg/mL ampicillin, or 2 µg/mL colistin). Transfer of *mcr* and ESBL genes was confirmed by PCR as described above.

4.3.5 Statistical analysis

Comparisons of the association between antimicrobial resistance phenotype and resistance encoding gene were performed by using Pearson's chi-squared test (χ^2) (SPSS, version 22.0). A *P*-value of < 0.05 was considered statistically significant. Odds ratios with 95% confidence intervals (CIs) were calculated.

Table 9. Primers used in this study.

PCR and primer names	Sequence (5' – 3')	Amplicon size (bp)	References
Mcr genes			
MCR1-IF	CGGTCAGTCCGTTTGTTC	309	(Liu et al., 2016)
MCR1-IR	CTTGGTCGGTCTGTA		
MCR2-IF	TGTTGCTTGTGCCGATTGGA	619	(Xavier et al., 2016)
MCR2-IR	AGATGGTATTGTTGGTTGCTG		
MCR3-IF	TTGGCACTGTATTTTGCATTT	542	(Yin et al., 2017)
MCR3-IR	TTAACGAAATTGGCTGGAACA		
MCR4-IF	ATTGGGATAGTCGCCTTTTT	487	(Carattoli et al., 2017)
MCR4-IR	TTACAGCCAGAATCATTATCA		
ß-lactamase and ESBL genes			
bla$_{CTX-M}$_FW	CGATGTGCAGTACCAGTAA	585	(Batchelor et al., 2005)
bla$_{CTX-M}$_RW	AGTGACCAGAATCAGCGG		
bla$_{PSE-M}$_FW	GCTCGTATAGGTGTTTCCGTTT	575	(Li et al., 2013)
bla$_{PSE-M}$_RW	CGATCCGCAATGTTCCATCC		
bla$_{TEM}$_FW	GCGGAACCCCTATTT	964	(Olesen et al., 2004)
bla$_{TEM}$_RW	TCTAAAGTATATATGAGTAAACTTGGTCT		
bla$_{SHV}$_FW	TTCGCCTGTGTATTATCTCCCTG	854	(Hasman et al., 2005)
bla$_{SHV}$_RW	TTAGCGTTGCCAGTGYTG		
bla$_{CMY-1}$_FW	GTGGTGGATGCCAGCATCC	854	
bla$_{CMY-1}$_RW	GGTCGAGCCGGTCTTGTTGAA		
bla$_{CMY-2}$_FW	GCACTTAGCCACCTATACGGCAG	856	
bla$_{CMY-2}$_RW	GCTTTTCAAGAATGCGCCAGG		
Multiplex CTX-M group 1 and group 2			(Dallenne et al., 2010)
MultiCTXMGp1_FW	TTAGGAARTGTGCCGCTGYA[a]	688	
MultiCTXMGp1_RW	CGATATCGTTGGTGGTRCCAT[a]		
MultiCTXMGp2_FW	CGTTAACGGCACGATGAC	404	
MultiCTXMGp2_RW	CGATATCGTTGGTGGTRCCAT[a]		
CTX-M group 8/25_FW	AACRCRCAGACGCTCTAC[a]	326	
CTX-M group 8/25_RW	TCGAGCCGGAASGTGTYAT[a]		
CTX-M group 9_FW	GTGACAAAGAGAGTGCAACGG	850	(Sabate et al., 2000)
CTX-M group 9_RW	ATGATTCTCGCCGCTGAAGCC		
CTX-M15_SFW	CACACGTGGAATTTAGGGACT	876	(Muzaheed et al., 2008)
CTX-M15_SRW	GCCGTCTAAGGCGATAAACA		

[a] Y = T or C; R = A or G; S = G or C; D=A, G, or T

4.4 RESULTS

4.4.1 Antimicrobial susceptibility

4.4.1.1 Healthy pigs

Of the 354 *E. coli* isolates from healthy pigs, 78% were resistant to at least one antimicrobial agent, including 96.5% (137/142) of the isolates from fattening pigs and all the isolates from piglets and sows. Most isolates (i.e. all piglet isolates, 99.2% of the sow isolates and 94.4% of the fattening pig isolates) were MDR (resistant to at least three antimicrobial agents in different classes). However, there was no significant difference of MDR proportion among the *E. coli* isolates from different groups of the healthy pigs. Overall, the percentage of colistin-resistant *E. coli* was 40.7%.

Colistin-resistance was predominant among the piglet isolates (95.2%), follow by the isolates from fattening pigs (43.7%) and sows (2.3%). The colistin resistance rate in the piglet isolates was significantly higher than that in the sow and fattening pig isolates ($p<0.05$).

Sixty-eight (19.2%) *E. coli* isolates from the healthy pigs were confirmed to be ESBL-producers, including the isolates from piglets (45.8%, n=38), fattening pigs (15.5%, n=22) and sows (6.2%, n=8). Resistance to ceftazidime, cefotaxime and cefpodoxime was highest in piglets (27.7%, 49.4%, 49.4%) followed by fattening pigs (9.2%, 15.5%, 16.2%) and sows (2.3%, 6.2%, 6.2%), respectively (Fig 6). The percentage of ESBL-producing isolates was significantly higher in piglets than the others ($p < 0.05$). Similarly, the percentage of ESBL producers in fattening pigs was significantly higher when compared with that in sows ($p < 0.05$). The isolates from piglets, sows and fattening pigs were most frequently resistant to tetracycline (98.8%,

100%, 92.3%), ampicillin (96.4%, 96.1%, 81%) and chloramphenicol (92.8%, 57.4%, 92.3%), respectively (Fig 6).

4.4.1.2 Sick pigs

All the *E. coli* isolates from sick pigs were resistant to at least one antimicrobial agent and up to 99% were MDR. Resistance to colistin was found in 73 % of the isolates. Forty-four *E. coli* isolates (44%) in this group were ESBL-producers, that exhibited resistance to ceftazidime (53%), cefotaxime (53%) and cefpodoxime (37%). The percentage of ESBL-producing *E. coli* isolates was significantly higher in sick pigs than healthy pigs ($p<0.05$). The majority of the sick pig isolates were resistant to tetracycline (100%) and ampicillin (97%) (Fig 6). Distribution of Minimum Inhibitory Concentrations (MICs) and resistance percentages for the *E. coli* isolates from healthy pigs and sick pigs are shown in table 10.

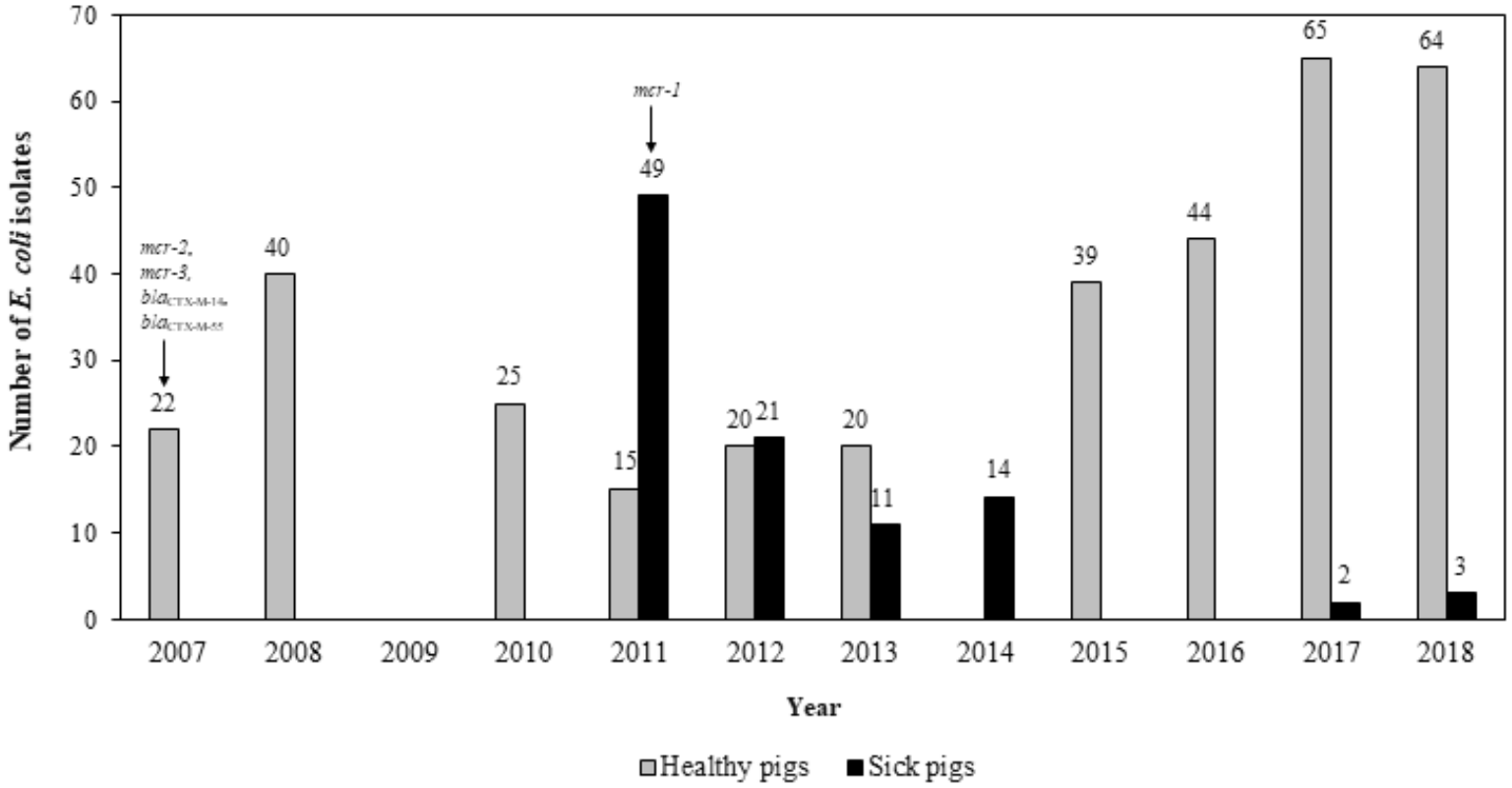


Figure 5. Yearly distribution of *Escherichia coli* from healthy pigs (n = 354) and sick pigs (n = 100) in Thailand between 2007-2018. The arrows indicates the first detection year of corresponded resistance genes.

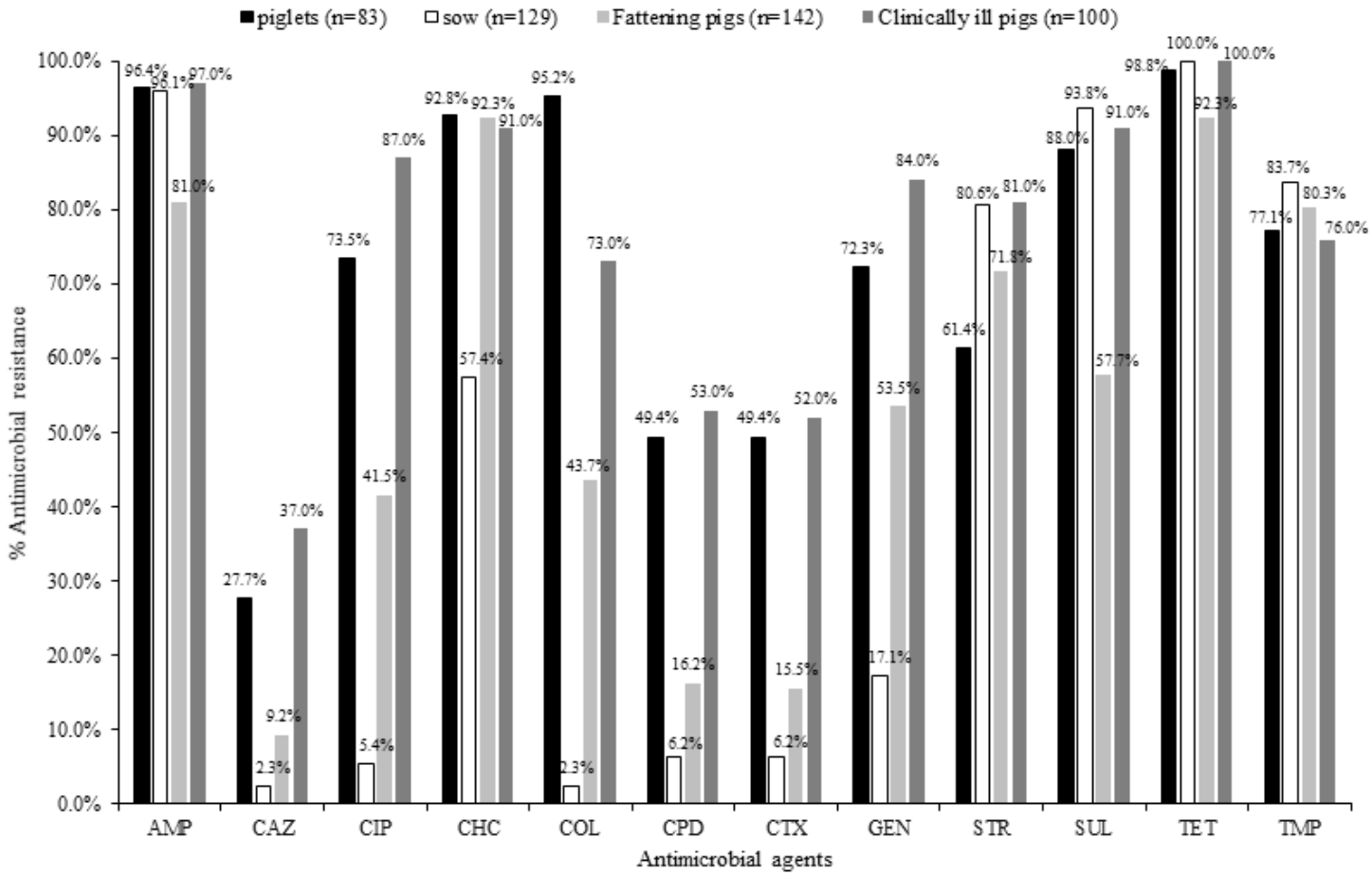


Figure 6. Distribution of antimicrobial susceptibility in *Escherichia coli* from clinically healthy pigs (n = 354) and sick pigs (n = 100) in Thailand between 2007-2018. AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CHC, chloramphenicol; COL, colistin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

Table 10. Distribution of Minimum Inhibitory Concentrations (MICs) and resistance percentages for the *E. coli* isolates from healthy pigs (n = 354) and sick pigs (n = 100).

Antimicrobials	Sources	Distributions % of MICs (µg/mL)															No. of resistance (%)
		≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024	
Ampicillin	Healthy pigs				2.8	0.6	1.4	2.8	2.3	0.6	0.3	2.0	15.5	18.4	43.2	10.2	319 (90.1)
	Sick pigs						1	2				2	11	24	28	32	97 (97)
Chloramphenicol	Healthy pigs						5.6	13.3	6.5	15.8	22.0	16.4	4.2	10.2	11.0	3.1	264 (74.6)
	Sick pigs				1	1	2	4	1	15	9	13	30	16	6	2	91 (91)
Ciprofloxacin	Healthy pigs	46.3	5.9	9.9	1.1	0.8	1.7	1.7	7.3	10.7	2.0	12.1	0.3				127 (35.9)
	Sick pigs	4	3	1	1	4	4	21	16	28	16	1	1				87 (87)
Colistin	Healthy pigs		6.5	48.6	3.1	1.1	13.6	25.7	0.8		0.6						144(40.7)
	Sick pigs			20	3	4	54	19									73 (73)
Gentamicin	Healthy pigs		2.3	13.8	23.2	8.2	4.5	3.4	3.1	7.6	5.9	7.6	11.9	8.2	0.3		170(48)
	Sick pigs				4	10	2		1	7	16	25	23	11	1		84 (84)
Streptomycin	Healthy pigs				9.3	1.1	2.0	7.6	7.3	12.7	7.9	15.3	13.8	18.4	4.5		257 (72.6)
	Sick pigs				1		1	8	9	12	5	15	33	6	2	8	81 (81)
Sulfamethoxazole	Healthy pigs					1.4	4.2	2.0	2.8	1.7		2.5	7.3	15.3	10.2	52.5	276 (78)
	Sick pigs					1	1	1	5				1	1	1	89	91 (91)
Tetracycline	Healthy pigs				0.3	2.5	0.3	0.3		1.1	11.0	20.1	39.5	23.2	1.7		342 (96.6)
	Sick pigs								1	3	7	34	55		0		100 (100)
Trimethoprim	Healthy pigs		0.3	6.2	9.0	2.0	1.1	0.8		2.3	0.6				77.7		285 (80.5)
	Sick pigs		0	0	11	7	1	5		1	1		1		5	68	76 (76)

White fields represent range of serial dilutions tested for each antimicrobial.

The MICs higher than the highest concentration tested are provided as the concentration closest above the range.

The clinical breakpoints for each antimicrobial are presented as a vertical line.

4.4.2 Presence and transfer of colistin-resistance determinants

Of all the *E. coli* isolates tested (n=454), the *mcr-1*(10.4%), *mcr-2* (1.1%) and *mcr-3* genes (45%) were identified. None of the isolates carried *mcr-4*.

Among the *E. coli* isolates from healthy pigs, the *mcr-1* (7.6%), *mcr-2* (1.4%) and *mcr-3* (37.9%) genes were detected. Four *mcr*-patterns were defined including, *mcr-1* (2.3%), *mcr-3* (31%), *mcr-1/mcr-3* (5.4%) and *mcr-2/mcr-3* (1.4%) (Table 11). Of all the isolates from healthy pigs in this collection, *mcr-1* was first detected in 2011, while *mcr-2* and *mcr-3* were found in 2007 at earliest (Figure 5). Co-existence of *mcr-1/mcr-3* and *mcr-2/mcr-3* was detected in the isolates from piglet (23%) and fattening pig (3.5%), respectively. Colistin-MIC range was 8-64 µg/mL and 0.5-16 µg/mL for the isolates carrying only *mcr-1* and only *mcr-3*, respectively. The isolates harboring *mcr-3* in combination with *mcr-1* or *mcr-2* had colistin MIC of 4 or 8 µg/mL.

Among the *E. coli* isolates from sick pigs (n=100), the *mcr-1* (20%) and *mcr-3* (70%) genes were found. Three *mcr* patterns including *mcr-1* (7%), *mcr-3* (57%) and *mcr-1/mcr-3* (13%) were observed. The colistin MIC of the isolates carrying *mcr-1* only was 4-8 µg/mL, while that of the isolates with *mcr-3* only was 1-8 µg/mL. The isolates with *mcr-1/mcr-3* had the colistin MIC of 4 or 8 µg/mL.

Based on the conjugation experiment in all *mcr*-positive *E. coli* (n=219), *mcr-3* in two isolates (one from a piglet and one from a sick pig) was horizontally transferred to *Salmonella*. All the *Salmonella* transconjugants were confirmed to carry *mcr-3* and their colistin MIC was 4 µg/mL.

Table 11. Colistin-resistance phenotype and genotype in *E. coli* isolates (n = 454) in Thailand between 2007-2018.

Source (n=454)	No. colistin-resistant isolates (%)	Colistin resistance genotype		MIC (µg/mL)
		Genes	No. (% Positive)	
Healthy pigs				
• Piglets (n=83)	79 (95.2)	*mcr-1*	7 (8.4)	8
		mcr-3	55 (66.3)	0.5 - 64
		mcr-1/mcr-3	19 (23)	4 - 8
• Lactating sows (n=129)	3 (2.3)	*mcr-1*	1 (0.8)	64
• Fattening pigs (n=142)	62 (43.7)	*mcr-2/mcr-3*	5 (3.5)	4 - 8
		mcr-3	55 (38.7)	0.5 - 16
Sick pigs (n=100)	73 (73)	*mcr-1*	7 (7)	4 - 8
		mcr-3	57 (57)	1 - 8
		mcr-1/mcr-3	13 (13)	4 - 8
Total	217 (47.8)		219 (48.2)	

4.4.3 Amino acid alterations in *pmrAB*

In comparison to *E. coli* K12, sequence variations in *pmrAB* were found in all *E. coli* tested (n=25) (Table 12). Four amino acid substitutions including S29A, E106A, G144S and E184D were identified in PmrA and five amino acid substitutions including H2R, V161G, D283G, Y358N, A360V were detected in PmrB. The amino acid changes S29A in PmrA and H2R, D283G and Y358N in PmrB were found in both colistin-resistant and susceptible *E. coli* isolates. Among the healthy pig isolates, two *mcr-3* carrying isolates (i.e. E.453 and E.454) carried G144S amino acid substitution in PmrA and additionally harbored V161G in PmrB. The colistin MIC of both isolates was 16 µg/mL. One sick pig isolate (i.e. EC.P.45, colistin MIC=8 µg/mL) carried both *mcr-3* and E06A amino acid substitution in PmrA. A colistin-susceptible isolate (i.e. GCa13, colistin MIC=0.25 µg/mL) harbored E184D amino acid substitution in PmrA that was not observed in any colistin-resistant isolates tested.

Table 12. The presence of *mcr* genes and amino acid substitution in PmrAB among colistin-resistant isolates from pigs (n = 25) in Thailand between 2007-2018.

Sources		Isolates	COL MIC (μg/mL)	*mcr* gene	PmrA	PmrB
Colistin resistant isolates	Healthy pigs	E.400[a]	8	*mcr-3*	AGC⟶GGC (S29G)	
		E.453[a]	16	*mcr-3*	AGC⟶GGC (S29G), GGC⟶AGC (G144S)	GTG⟶GGG (V161G), GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		E.454[a]	16	*mcr-3*	AGC⟶GGC (S29G), GGC⟶AGC (G144S)	GTG⟶GGG (V161G), GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		E.458[a]	8	*mcr-3*	AGC⟶GGC (S29G)	-
		E.459[a]	8	*mcr-3*	AGC⟶GGC (S29G)	-
		PLEa 3[b]	64	*mcr-3*	AGC⟶GGC (S29G)	-
		LCa 7[c]	64	*mcr-1*	-	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		PLEa 26[b]	8	*mcr-3*	AGC⟶GGC (S29G)	-
		FPEa 13[b]	8	*mcr-1*	AGC⟶GGC (S29G)	-
		FPEa 19[b]	8	*mcr-1*	AGC⟶GGC (S29G)	-
	Sick pigs	EC.P. 5	4	*mcr-1, mcr-3*	AGC⟶GGC (S29G)	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		EC.P. 9	4	*mcr-1*	AGC⟶GGC (S29G)	CAT⟶CGT (H2R), GAC⟶GGC (D283G)
		EC.P. 10	4	*mcr-1*	AGC⟶GGC (S29G)	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		EC.P. 16	4	*mcr-1*	AGC⟶GGC (S29G)	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		EC.P. 40	4	*mcr-1, mcr-3*	AGC⟶GGC (S29G)	-
		EC.P. 45	8	*mcr-3*	AGC⟶GGC (S29G), GAA⟶GCA (E106A)	-
		EC.P. 46	4	*mcr-3*	AGC⟶GGC (S29G)	-
		EC.P. 47	4	*mcr-3*	AGC⟶GGC (S29G)	-
		EC.P. 48	4	*mcr-3*	AGC⟶GGC (S29G)	-
		EC.P. 49	8	*mcr-3*	AGC⟶GGC (S29G)	-
Colistin susceptible isolates		LCa 6[d]	0.25	-	AGC⟶GGC (S29G)	-
		LCa 9[d]	0.25	-	AGC⟶GGC (S29G)	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		LCa 10[d]	0.25	-	AGC⟶GGC (S29G)	CAT⟶CGT (H2R), GAC⟶GGC (D283G), GCA⟶GTA (A360V)
		GCa 12[d]	0.25	-	AGC⟶GGC (S29G)	GAC⟶GGC (D283G), TAC⟶AAC (Y358N)
		GCa 13[d]	0.25	-	AGC⟶GGC (S29G), GAA⟶GAC (E184D)	-

[a] Fattening pigs, [b] Piglets, [c] Lactating and [d] Gestating sows

4.4.4 Presence and transfer of β-lactamase genes

One-hundred twelve ESBL-producing *E. coli* were screened for β-lactamase genes. The majority of ESBL-producing isolates were positive to bla_{CTX-M} (98.5%), of which the majority were CTX-M group 9 (54.5%), followed by CTX-M group 1 (42.9%) (Table 13). The CTX-M group 2 and CTX-M group 8/25 were not detected. DNA sequencing analysis confirmed that all CTX-M group 9 (n=61) were $bla_{CTX-M-14}$ and all CTX-M group 1(n=48) were $bla_{CTX-M-55}$. All bla_{TEM} were confirmed to be bla_{TEM-1} and were observed in 53 (78%) isolates from piglets (n=32), sows (n=7) and fattening pigs (n=14). Twenty-seven of $bla_{CTX-M-55}$ (90%) and 28 of $bla_{CTX-M-14}$ (75.7%) positive isolates simultaneously carried bla_{TEM-1}. The bla_{CMY-2} gene was found in two isolates from sows. One of the bla_{CMY-2} -positive isolate harbored bla_{TEM-1} and $bla_{CTX-M-14}$ (CMY2/TEM-1/CTX-M-14/), while the others additionally carried bla_{TEM-1} and $bla_{CTX-M-55}$ (CMY-2/TEM-1/CTX-M-55). The first detection of $bla_{CTX-M-14}$ and $bla_{CTX-M-55}$ was in the *E. coli* isolates from fattening pigs in 2007. These isolates additionally carried bla_{TEM-1}.

Among the ESBL-producing *E. coli* isolates from sick pigs (n=44), only $bla_{CTX-M-55}$ (41%) and $bla_{CTX-M-14}$ (54.5%) were identified. The bla_{TEM-1} gene was found in 54.5%, of which 10 isolates co-existed with $bla_{CTX-M-14}$ and 7 isolates co-existed with $bla_{CTX-M-55}$. The bla_{CMY-2} gene was identified in 4 isolates, of which 3 isolates simultaneously carried $bla_{CTX-M-14}$ and bla_{TEM-1} and one isolate carried $bla_{CTX-M-55}$ and bla_{TEM-1}.

Table 13. Distribution of ß-lactamase genes among *Escherichia coli* isolates from healthy and sick pigs (n = 454) in Thailand between 2007-2018

ß-lactamase genotype pattern	Healthy pigs (No. of isolates (%))			Sick pigs (n=100)	Total (n=454)
	Piglets (n=83)	Sows (n=129)	Fattening pigs (n=142)		
TEM-1	1 (1.2)	-	1 (0.7)	2 (2)	4 (3.6)
CTX-M-55	1 (1.2)	1 (0.8)	-	9 (9)	11 (9.8)
CTX-M-14	3 (3.6)	-	5 (3.5)	10 (10)	18 (16)
CTX-M-55, CTX-M-14	1 (1.2)	-	-	1 (1)	2 (1.8)
TEM-1, CTX-M-55	17 (20.5)	2 (1.6)	7 (4.9)	7 (7)	33 (29.5)
TEM-1, CTX-M-14	15 (18)	3 (2.3)	9 (6.3)	10 (10)	37 (33)
TEM-1, CMY-2	-	-	-	1 (1)	1 (0.9)
TEM-1, CTX-M-55, CMY-2	-	1 (0.8)	-	1 (1)	2(1.8)
TEM-1, CTX-M-14, CMY-2	-	1 (0.8)	-	3 (3)	4 (3.6)
Total	38 (45.8)	8 (6.2)	22 (15.5)	44 (44%)	**112**

4.4.5 Co-existence of ESBL and *mcr* genes

Up to 90 isolates (80.4%) of ESBL producers in this study (n=112) additionally harbored *mcr* genes, of which nearly 50% (n=54) were originated from healthy pigs (piglets, n= 37; fattening pigs, n=17) and 32% (n=36) were from sick pigs. Four *mcr* patterns of these ESBL producers were identified including *mcr-1* (3.6%), *mcr-3* (62.5%), *mcr-1/mcr-3* (9.8%) and *mcr-2/mcr-3* (4.5%). The *mcr-3* gene was most commonly observed in ESBL producers (76.8%) (Table 14).

Horizontal transfer of β-lactamase genes was observed in 14 *E. coli* isolates. Five piglet isolates transferred $bla_{\text{TEM-1}}$ and co-transferred *mcr-3*. One sick pig isolate was capable of transferring $bla_{\text{CTX-M-55}}$ and *mcr-3* simultaneously. Seven *E. coli* isolates including 2 isolates from piglets (one isolates with $bla_{\text{CTX-M-14}}$ and the others with $bla_{\text{CTX-M-55}}$) and 4 isolates from sick pigs (2 isolates with $bla_{\text{CTX-M-14}}$ and 2 isolates with $bla_{\text{CTX-M-55}}$) were able to transfer $bla_{\text{CTX-M}}$. One isolate from sick pig could transfer both $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1}}$ gene at the same time.

Table 14. The presence of *mcr* and β-lactamase genes in *E. coli* from healthy and sick pigs (n = 454) in Thailand between 2007-2018.

Origins	Sources[a]	Colistin resistance gene[a]	β-lactamase gene[a]
Healthy pigs	Piglets (37)	*mcr-1* (2)	$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (2)
		mcr-1/mcr-3 (5)	$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (5)
		mcr-3 (30)	$bla_{\text{CTX-M-14}}$ (3)
			$bla_{\text{CTX-M-55}}$ (1)
			$bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$ (1)
			$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (10)
			$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ (15)
	Fattening pigs (17)	*mcr-2/mcr-3* (5)	$bla_{\text{CTX-M-14}}$ (5)
		mcr-3 (12)	$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (9)
			$bla_{\text{CTX-M-14}}$ (3)
Sick pigs	Pig age 2-21 wks (36)	*mcr-1* (2)	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$ (1)
			$bla_{\text{CTX-M-14}}$ (1)
		mcr-1/mcr-3 (6)	$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (1)
			$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ (2)
			$bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$ (1)
			$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CMY-2}}$ (1)
			$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CMY-2}}$ (1)
		mcr-3 (28)	$bla_{\text{CTX-M-55}}$ (9)
			$bla_{\text{CTX-M-14}}$ (7)
			$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ (6)
			$bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ (5)
			$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CMY-2}}$ (1)

[a] Numbers in parenthesis indicate the number of positive *E. coli* isolate (s)

4.4.6 Association between AMR phenotype and genotype

The associations between resistance phenotype and genotype varied (Table 15). The positive associations between resistance phenotype and the presence of *mcr* or β-lactamase genes were as follows: CIP resistance/ CTX-M-14; STR resistance/*mcr-1*,CTX-M-55; SUL resistance/*mcr-2*,TEM-1,CTX-M-55; TET resistance/*mcr-2*, TEM-1, CTX-M-14, CTX-M-55 and TMP resistance/ *mcr-1*, *mcr-2* and CTX-M-14. The strongest positive association was observed between TET and CTX-M-55 (OR=31, 8.05-119.3) and TET and *mcr-2* (OR=9.95, 1.02-96.5).

Table 15. Associations between resistance phenotype and genotype in *Escherichia coli* from healthy pigs and sick pigs (n = 454) in Thailand between 2007-2018.

ABO resistance gene (n)	AMP		CAZ		CIP		CHC		COL		CPD		CTX		GEN		STR		SUL		TET		TMP	
	No[a].	*Assoc*[b].	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*	No.	*Assoc.*
mcr-1 (46)	45	0.2 (0.03-1.6)	15	0.4 (0.2-0.7)	36	0.22 (0.1-0.45)	41	0.4 (0.16-1.06)	46	-	16	0.68 (0.36-1.3)	16	0.67 (0.35-1.3)	34	0.37 (0.19-0.7)	30	1.64 (0.86-3.14)	41	0.49 (0.19-1.27)	46	-	23	4.91 (2.6-9.3)
mcr-2 (5)	5	-[c]	1	0.8 (0.1-7.3)	5	-	5	-	5	-	5	-	5	-	5	-	5	-	4	1.05 (0.12-9.6)	4	9.95 (1.02-96.5)	3	2.65 (0.44-16.2)
mcr-3 (204)	197	0.3 (0.1-0.6)	65	0.09 (0.1-0.2)	159	0.08 (0.05-0.12)	185	0.2 (0.13-0.38)	193	0.006 (0.003-0.013)	100	0.12 (0.07-0.19)	104	0.08 (0.05-0.14)	154	0.18 (0.12-0.27)	156	0.8 (0.54-1.26)	173	0.62 (0.38-1.0)	201	0.4 (0.11-1.5)	166	0.83 (0.52-1.3)
TEM-1 (81)	77	0.5 (0.2-1.5)	46	0.7 (0.04-0.12	59	0.27 (0.16-0.45)	72	0.39 (0.19-0.8)	62	0.2 (0.13-0.38)	76	0.01 (0.00-0.03)	76	0.009 (0.00-0.03)	73	0.09 (0.04-0.19)	64	0.74 (0.4-1.3)	62	1.38 (0.77-2.45)	75	4.9 (1.5-15.6)	68	0.7 (0.4-1.4)
CTX-M-14 (61)	59	-	12	0.04 (0.02-0.08)	47	2.6 (1.4-4.7)	45	1.3 (0.7-2.5)	44	0.03 (0.17-0.5)	58	0.01 (0.00-0.04)	58	0.01 (0.00-0.03)	55	0.09 (0.04-0.24)	51	0.53 (0.26-1.08)	53	0.6 (0.27-1.3)	57	3.37 (0.98-11.6)	43	1.8 (0.98-3.3)
CTX-M-55 (48)	48	-	48	0.18 0.08-0.4)	39	0.18 (0.08-0.4)	48	-	46	0.03 (0.01-0.13)	48	-	48	-	48	-	35	1.09 (0.56-2.15)	38	1.12 (0.54-2.36)	49	31 (8.05-119.3)	46	0.15 (0.04-0.6)
CMY-2 (7)	7	-	6	0.35 (0.07-1.8)	5	0.35 (0.07-1.8)	6	0.59 (0.07-4.9)	5	0.36 (0.07-1.9)	7	-	7	-	6	0.19 (0.02-1.56)	7	-	7	-	7	-	6	0.65 (0.07-5.5)

[a]No., number of isolates resistant to corresponding antimicrobial agents and carrying the relevance resistance genes.

[b]Odds ratio (OR) for significant associations between antimicrobial resistance gene and antimicrobial resistance phenotype (95% confidence interval in parenthesis). OR > 1 represents positive associations, and OR < 1 represents negative associations.

[c]No significant associations ($P \geq 0.05$).

AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CHC, chloramphenicol; COL, colistin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

4.5 DISCUSSION

The present study was conducted in *E. coli* isolates from clinically healthy and clinically sick pigs collected between 2007-2018. One significant finding was high MDR rates in the isolates from healthy (97.5%) and sick pigs (99%). It is expected that only healthy pigs are slaughtered for human consumption, but their health status does not guarantee the absence of resistant bacteria. This is because antibiotics may be previously administered to the pigs that were the source of the isolates for disease prevention and growth promotion, which may have resulted in commensal bacteria developing antibiotic resistance. As the complete ban of AGP in all animal feed was implemented in 2015, use of AGP could influence the high AMR rates observed in earlier years in this study. The highest frequency of resistance among the isolates from healthy and sick pigs was to tetracycline and ampicillin, in agreement with a previous study in *E. coli* isolated from pig farms in Thailand (Ström et al., 2017). However, it was not possible to obtain the antibiotic use history in each farm. In general, the antibiotics administered to piglets by oral route either in feed or in water for controlling gastrointestinal tract infection in piglets including polypeptides (e.g. colistin) and aminoglycosides (e.g. apramycin). Tylosin, tilmicosin and chlortetracycline were used in fattening pigs. Cephalosporins (e.g. ceftiofur and ceftriaxone) are occasionally used for treatment of respiratory diseases, lameness, and reproductive infections. It was estimated that approximately 39.7% of medicated feed was used in suckling and nursery pigs followed by fattening pigs (37.3%) and breeding pig (23%) in Thailand (Lekagul et al., 2020). Some antibiotics mixed in medicated feed used in pig production in the country are those categorized as Critically Important Antimicrobials by the WHO e.g. amoxicillin, lincomycin and colistin (WHO, 2019; Lekagul et al., 2020). Up to date, Thailand has launched law and regulations to contain AMR associated with food animals, for example, Notification of

the Ministry of Agriculture and Cooperatives that specifically prohibits the use of all antibiotics in animal feed as growth promoters was released in 2015 (MOAC, 2015). Law on "Characteristics and conditions of animal feed containing drugs prohibited from producing, importing, selling and using" was issued in 2018, of which medicated feed containing polymyxins, cephalosporins, fluoroquinolones and others are covered by this law (MOAC, 2018). A year later, regulation of antimicrobials drugs that must not be mixed in animal feed for prophylactic purposes was announced (Department of Livestock Development Ministry of Agriculture and Cooperatives, 2019). The latter included polymyxins B, colistin and other drugs in penicillin and fluroquinolone groups. Effective enforcement of these regulations is expected and the outcomes of implementation may be seen through national AMR surveillance data in coming years.

Isolates from healthy (40.7%) and sick pigs (73%) exhibited a high colistin resistance rate that was higher than in a previous study conducted in *E. coli* from healthy and diseased pigs in Japan between 2012-2013 (Fukuda et al., 2018). The highest colistin-resistance rate was found in the isolates from piglets (95.2%), followed by sick pigs (77%). This is likely because colistin has been used for treatment of gastrointestinal tract infections caused by *E. coli*, especially in post-weaning diarrhea in piglets (Lekagul et al., 2020; Poolperm et al., 2020). Colistin was commonly formulated into medicated feed for suckling and nursery pigs for the prevention of gastrointestinal tract infection in Thailand (Lekagul et al., 2020). Approximately 40 tons of colistin were mixed in medicated feed and about 87.2% were intended for piglets in Thai pig production (Lekagul et al., 2020). Such extensive use of colistin may contribute to high colistin resistance rate observed in the present study. Implementation to minimize use and encourage prudent use of colistin and other antimicrobials is mandatory.

Chromosomal mutations in the two-component regulatory system of PmrAB were previously shown to be significantly associated with colistin resistance in bacterial pathogens such as *Klebsiella pneumoniae* and *Salmonella enterica*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Bhagirath et al., 2019). However, mutations in PmrAB is rarely reported in *E. coli*. A previous study demonstrated amino acid substitutions S39I and R81S in PmrA and V161G in PmrB in colistin-resistant *E.coli* isolates from pigs in Spain (Quesada et al., 2015). However, the S39I and R81S amino acid substitutions in PmrA were not found in this study. In addition to mobile colistin resistance (*mcr*) genes, research studies focusing on the chromosomal-mediated colistin resistance and their regulatory mechanism have increased (Delannoy et al., 2017; Bhagirath et al., 2019). Some mutations (i.e. E106A and G144S in PmrA and V161G in PmrB) were observed only in colistin-resistant isolates carrying *mcr-3* in this study. However, individual contribution and cumulative effects of the genes to colistin resistance was not determined and needs further investigations. At the same time, some amino acid changes (e.g. S29G in PmrA and D283G, Y358N and H2R in PmrB) were identified in both colistin-resistant and colistin-susceptible isolates, suggesting the lack of impact on colistin resistance phenotype. Studies of other TCSs and their regulators such as PhoPQ, MgrB, and PmrD are suggested (Delannoy et al., 2017).

In this study, *mcr-3* was most predominant among the *E. coli* isolates from both healthy pigs (32.5%) and sick pigs (57%), while the lower percentage of *mcr-1* was observed in healthy pigs (7.6%) and in sick pigs (20%). These results are inconsistent to a previous study reporting that *mcr-1* was commonly detected in *E. coli* from healthy and diseased pigs in Japan (45%) and *mcr-3* was found at lower rate (8.3%) in diseased pigs (Fukuda et al., 2018). The discrepancies may be due to

difference in antimicrobial usage patterns or in the prevalence of different clones and/or plasmids.

The *mcr-1* gene is globally distributed and has been found in many bacterial species (e.g. *E. coli*, *Salmonella* spp., *Klebsiella* spp. and *Pseudomonas* spp.) from food animals, food stuff and human (Liu et al., 2016). To date, *mcr-1* is commonly screened in colistin-resistant isolates. Therefore, *mcr-1* in colistin-susceptible isolates and other *mcr* variants may be overlooked. Currently, there are still only limited report of *mcr-3*. Previous studies reported in the presence of *mcr-3* in cattle from France (Haenni et al., 2018) and Spain (Hernandez et al., 2017), pigs and chicken from China (Zhang et al., 2018) and pigs from Japan (Fukuda et al., 2018).

However, *mcr-3* appear to be common among healthy and sick pigs in this study. Further studies in different animal sources and other countries should be conducted to determine the role of this gene in the dissemination of colistin resistance. Moreover, a previous study showed that *mcr-3* was commonly located on broad-host range plasmids (i.e. IncP) and several transposases and IS elements (i.e. IS*4321*, ΔTn*As2* and ISK*pn40*) were identified in the flanking regions of *mcr-3*. This might cause wider spread and stronger transmission capabilities of *mcr-3* than *mcr-1* (Wang et al., 2019). Further genetic characterization of *mcr-3* carrying plasmid are needed to elucidate molecular mechanisms underlying dissemination of this gene.

The *mcr-2* positive isolates were detected (n=5) in fattening pigs. The *mcr-2* gene was previously reported in colistin-resistant *E. coli* from pigs in Belgium (20.8%) (Xavier et al., 2016) and China (56.3%) (Zhang et al., 2018). None of the isolates in this study carried *mcr-4*. Up to date, the report of *mcr-4* has been limited to EU countries including *Salmonella* from pigs in Italy (Carattoli et al., 2017) and *E. coli*

from pigs in Spain and Belgium (Carattoli et al., 2017; Garcia et al., 2018). These variations suggest that spread and evolution of *mcr* genes should be monitored.

Coexistence of different *mcr* variants was observed, including *mcr-1/mcr-3* (23% of piglets and 13% of sick pigs) and *mcr-2/mcr-3* (3.5% of fattening pigs). The *E. coli* carrying *mcr-1/mcr-3* were previously isolated from cattle in Spain (Hernandez et al., 2017) pig and poultry in China (Zhang et al., 2018) and humans in New Zealand (Creighton et al., 2019). The isolates carrying both *mcr-1* and *mcr-2* were previously identified in pigs in Canada (Rhouma et al., 2019). By considering the colistin MIC, all *mcr-1* harboring isolates exhibited resistance to colistin (colistin MIC 4-64 µg/mL). However, *mcr-3* can be found in colistin susceptible strains (colistin MIC 0.5-2 µg/mL), in agreement with a previous study (Fukuda et al., 2018). In addition, all the *E. coli* isolates harboring more than one *mcr* genes had colistin MIC of 4 or 8 µg/mL. Taken together, the observations indicate that the number of *mcr* derivatives is not always related to colistin resistance level. As the contribution of individual *mcr* genes, especially *mcr-3*, to colistin resistance level remains to be elucidated, monitoring *mcr* variants should be conducted in colistin-susceptible and resistant strains.

The ESBL *E. coli* of healthy pig origin (19.2%) in this study was less common than that in a previous report in the isolates obtained during 2012-2013 in the same country (Boonyasiri et al., 2014). The presence of ESBL producers in sick pigs (44%) was significantly higher than that in healthy pigs ($p<0.05$). Among the healthy pigs, the highest percentage of ESBL producers was observed in piglets (45.8%) ($p<0.05$). This is presumably associated with the common use of ß-lactam antibiotics (e.g. penicillin, amoxicillin and third-generation cephalosporins) in the suckling period for treatment of respiratory disease as suggested by a study of antimicrobial use in pigs in Germany (Van Rennings et al., 2015). The percentage of ESBL- producing *E. coli* in

sick pigs (44%) was significantly higher than that in healthy pigs (19.2%) (p<0.05). This may be a result of antibiotics previously administered to treat sick pigs. Cephalosporins are generally more expensive than other antimicrobial agents and may not be commonly used in pig production in Thailand and other countries in Southeast Asia. Currently, cephalosporins are increasingly used in pig production due to its long-lasting potency and lower doses (Callens et al., 2012). However, the presence of ESBLs may be also a result of other antimicrobial usage. This is because ESBL genes commonly colocalize on the same plasmid as other resistance genes.

The *bla* $_{CTX-M}$ gene was most prevailing ESBL genes in this study, in agreement with previous reports in Thailand (Seenama et al., 2019) and other countries in Asia e.g. China, Vietnam and Japan (Shiraki et al., 2004; Rao et al., 2014; Ueda et al., 2015). The majority of CTX-M subgroup was *bla* $_{CTX-M-14}$ of CTX-M Gr.9 (54%), followed by *bla* $_{CTX-M-55}$ of CTX-M Gr.1 (43%), in agreement with a previous study in livestock and environment in Thailand (Seenama et al., 2019; Tansawai et al., 2019).

Currently, the *bla*$_{CTX-M-55}$ gene has been increasingly reported especially in China where *bla*$_{CTX-M-55}$ is the second most frequent CTX-M variant in food-producing animals (Wu et al., 2018a). The *bla*$_{CTX-M-55}$ gene was first identified in Thailand in 2005 among ESBL-producing *E. coli* obtained from human and then was identified in clinical isolates in other countries i.e. *K. pneumonia* in China (Shi et al., 2009) and *Salmonella* spp. in the US (Sjolund-Karlsson et al., 2011). Previous studies reported that *bla*$_{CTX-M-55}$ was the major CTX-M subtype in ESBL-*E. coli* isolates from human clinical isolates, swine, farm waste and canals in Thailand (Runcharoen et al., 2017; Seenama et al., 2019). The gene was predominant in *E. coli* from livestock and pets in other Asian countries e.g. China and Hong Kong (Rao et al., 2014). The *bla*$_{CTX-M-55}$ gene was also detected in countries outside Asia but to less extent (Gallati et al., 2013).

The ß-lactamase gene, bla_{TEM-1} (72.3%) was commonly identified in this study. The gene has been frequently detected in the *E. coli* isolates from animals (Tian et al., 2009) and is commonly co-harbored with ESBL genes (Tian et al., 2009). This is in agreement with the current study where most ESBL producers (67.9%) carried TEM-1 and ESBL genes. The bla_{CMY-2} gene was detected at low frequency (5.4%). The gene was firstly identified in *K. pneumoniae* from human isolates (Jacoby and Medeiros, 1991) and are increasingly reported in different bacteria from livestock (e.g. *E. coli* from ground chicken and pig feces in Taiwan (Yan et al., 2004), *E. coli* from healthy chicken and sick animals in Spain (Briñas et al., 2003) and *Salmonella* Bredeney from turkey in UK (Liebana et al., 2004)), in agreement with this study. In addition, the isolates carrying bla_{CMY-2} coharbored $bla_{CTX-M-55}$ and $bla_{CTX-M-14}$, in agreement with previous studies (Winokur et al., 2001; Liu et al., 2007).

Most ESBL producers from piglets (97%), fattening pigs (77.3%) and sick pigs (82%) additionally carried *mcr* genes, of which the most common *mcr* gene among ESBL producers was *mcr-3*. However, a previous study in China showed that *mcr-1* was more commonly found in ESBL *E. coli* than non ESBL producers (Wu et al., 2018a). ß-lactams and colistin are bactericidal antibiotics that disrupt the outer membrane of bacterial cells. Recruiting *mcr* genes in the cell is a survival mechanism to maintain the cell wall integrity and may contribute to the increasing prevalence of ESBL producers coharboring *mcr* genes (Wu et al., 2018a). In addition, all ESBL-*mcr* carrying isolates were MDR, in agreement with a previous study (Wu et al., 2018a). These results highlight the continued need to encourage the prudent and effective use of antimicrobials in food animal production.

By using ampicillin as selectable marker, co-transfer of ß-lactamase genes (bla_{TEM-1} and $bla_{CTX-M-55}$) and *mcr* gene (*mcr-3*) was detected, suggesting co-resistance

of the gene on the same plasmid. This also suggest that distribution of *mcr* and ESBLs genes can be a result of co-selection by antibiotics in other classes.

The significant association between AMR phenotype and genotype was observed. Positive associations were identified between phenotypic resistance to CIP-CTX-M-14, STR-*mcr-1*/CTX-M-55, SUL-*mcr-2*/TEM-1/CTX-M-55, TET-*mcr-2*/TEM-1/CTX-M-14/CTX-M-55 and TMP-*mcr-1/mcr-2*/CTX-M-14. This could be possible due to co-localization of multiple resistance genes on the same plasmid. The strongest association was observed between tetracycline resistance and $bla_{\text{CTX-M-55}}$ (OR=31) or *mcr-2* (OR=9.95), in agreement with previous studies (Haenni et al., 2016). The results emphasize that emergence and spread of AMR is a dynamic issue and selective pressure of resistance to various antimicrobials are linked. Therefore, regulation of antimicrobial use should be conducted using a whole-system approach, not at individual drug level.

In conclusion, the findings emphasize the role of commensal and pathogenic *E. coli* as important reservoirs of ESBL and *mcr* genes. The use of ampicillin could select for colistin resistance, indicating that the pandemic spread of *mcr* genes can be a result of co-selection by other antimicrobial classes. Therefore, responsible use of antibiotics in food animal production should be encouraged and the complex-systematic strategic actions is required to contain AMR. Detection of ESBL production and colistin resistance at phenotypic and genotypic level should be included in national AMR surveillance program to allow epidemiological tracing of resistance trend. Further studies to characterize *E. coli* carrying different *mcr* genes and plasmid backbones of ESBL and *mcr* genes are warranted.

CHAPTER V

Whole Genome Sequencing Analysis and characterization of *Escherichia coli* with co-existence of ESBL and *mcr* genes from pigs

Suthathip Trongjit[1] and Rungtip Chuanchuen[1]

[1]Research Unit in Microbial Food Safety and Antimicrobial Resistance,

Department of Veterinary Public Health, Faculty of Veterinary Science,

Chulalongkorn University, Bangkok, Thailand

Manuscript is in preparation

Whole Genome Sequencing analysis and characterization of *Escherichia coli* with co-existence of ESBL and *mcr* genes from pigs

5.1 ABSTRACT

High-throughput Next Generation Sequencing was used to analyze the genome and transmissible plasmid of multidrug resistant *Escherichia coli* co-harboring *mcr* and ESBL genes from pigs. Three ESBL producing *E. coli* isolates carrying different combination of *mcr* were originated from healthy fattening pigs (E. 431, n=1) and sick pigs (ECP.81 and ECP.82, n=2) and subjected to Whole Genome Sequencing (WGS) using either Illumina MiSeq or HiSeq PE150 platforms. E. 431 carrying *mcr-2.1* and *mcr-3.1* belonged to serotype O142:H31 with ST 29 sequence type. ECP.81 and ECP.82 from sick pigs harboring *mcr-1.1* and *mcr-3.1* were serotype O9:H9 with ST10. Two *mcr-1.1* gene cassettes from ECP.81 and ECP.82 were located on IncI2 plasmid and had plasmid backbone identical (98% identity) to plasmid pHNSHP45. The *mcr-2.1* gene carrying contig in E. 431 showed 100% identity to plasmid pKP37-BE and with the upstream flanking sequence of IS*1595*. All three *mcr-3.1* carrying contigs contained the core segment, ΔTn*As2-mcr-3.1-dgkA*, and had high nucleotide similarity (85-100%) to the published sequence of *mcr-3.1*-carrying plasmid, pWJ1. The mobile elements i.e. IS*4321*, ΔTn*As2*, IS*Kpn40* and IS*3* were identified in the flanking regions of *mcr-3*. Several resistance genes were located on plasmid in all three isolates including genes conferring resistance to aminoglycosides (*aac(3)-IIa, aadA1, aadA2b, aph(3")-Ib, aph(3')-IIa* and *aph(6)-Id*), macrolides (*mdf(A)*), phenicols (*cmlA1*), sulphonamide (*sul3*) and tetracycline (*tet(A) and tet(M)*), of which their presence was well corresponded to resistance phenotype of the host. Single point mutations in quinolone resistant-determining regions (QRDR) of *gyrA* and *parC* resulting in amino acid substitutions including S83L and D87G in GyrA and S80I and E62K in ParC were identified. Co-transfer of *mcr-1.1*/$bla_{\text{TEM-1B}}$ and *mcr-3.1*/$bla_{\text{CTX-M-55}}$ were observed in ECP.81 and ECP.82 even though they were not located on the same plasmid. The results highlighted that application of advanced innovation technology of WGS in AMR monitoring and surveillance program provide a comprehensive information of AMR genotype that could yield invaluable benefits to development of control and prevention strategic actions plan for AMR.

KEYWORDS: colistin resistance, *Escherichia coli*, ESBLs, *mcr*, pig, WGS

5.2 INTRODUCTION

Antimicrobial resistance (AMR) in bacteria one the serious public health threats that occur worldwide. The situation has been completed with emerging and spread of bacterial pathogens that are resistant to multiple drugs as well as clinically important antibiotics (CIA) for human medicine. In the past decades, resistance to last-resort antibiotics such as carbapenems and polymyxins has been increasingly reported in Gram-negative bacteria, especially, emergence of carbapenem resistant Enterobacteriaceae (CRE) (Ellaby et al., 2019). This has raised a particular concern of limited treatment option for bacterial infections and the need for powerful antibiotics for future treatment (Biswas et al., 2012).

Colistin is considered an antibiotic of last resort for the treatment of infections with CRE in humans (Poirel et al., 2017) and classified into the Highest Priority Critically Important Antimicrobials (HPCIA) for human medicine by WHO (WHO, 2019). However, it has been widely used in livestock product, especially in pigs, for a long time. The global survey of colistin usage is varied in different countries (Gharaibeh and Shatnawi, 2019). For example, the US government has prohibited colistin use in animal production and human medicine, due to its nephrotoxicity and neurotoxicity. China is considered to be the world's highest users of colistin in agriculture, however presently colistin has been banded for using as a feed additive for animals by Chinese government. In EU, Germany, Portugal, Italy and Estonia were reported a higher colistin use than in other European countries (Gharaibeh and Shatnawi, 2019). Approximately 40 tons of colistin were used per year as medicated feed mills for prevention and/or treatment of post-weaning diarrhea (PWD) in Thailand (Lekagul et al., 2020).

Resistance to colistin is attributed to chromosomal mutations (i.e. PhoPQ two-component regulatory and *pmr*CAB operon) resulting in the modification of the lipid A of lipopolysaccharides. In 2015, plasmid-borne gene, *mcr-1,* encoding a phosphoethanolamine transferase was found to be common in Enterobacteriaceae species isolated from animals, food stuffs and patients in China (Liu et al., 2016). The report has attracted global concern of its high efficiency of horizontal transfer that places *mcr-1* as a potential public health threat. Up to date, nine different *mcr* variants (*mcr-1* to *mcr-9)* have been reported and were isolated from bacteria of different origins, including humans, livestock, wildlife, and environmental samples (Liu et al., 2016; Yin et al., 2017; Wang et al., 2018; Carroll et al., 2019). A previous study indicated that the spread of *mcr* genes occurred mainly in associated with epidemic plasmid replicon types of various (58 to 251 Kb) size (e.g. IncI2, IncHI1, IncHI2, IncP, IncFIB and IncX4) (Madec and Haenni, 2018).

Escherichia coli plays an important role as a reservoir of AMR due to its capacity to accumulate AMR genes that can be transmitted to other bacterial species through horizontal transfer. Previous studies indicated that the *E. coli* isolates, particularly the sequence types ST10 and ST155, from food animal served as an important reservoir of *mcr-1* (Yin et al., 2017; Garcia et al., 2018). Interestingly, *E. coli* with coexistence of *mcr-1* and $bla_{\text{NDM-9}}$ or $bla_{\text{CTX-M65}}$ on a single plasmid was identified (Du et al., 2016). This could be a great public health threat potentially leading to pan-drug resistance. Moreover, the IS*Apl1* insertion sequence has been considered a key element mediating translocation of *mcr-1* into diverse plasmid types and also found to be located on the chromosome by forming circular intermediates (Li et al., 2017). A previous study confirmed that the presence of mobile elements, including IS*4321*, ΔTn*As2* or IS*Kpn40*, in the flanking regions of *mcr-3* in *E. coli* (Wang et al., 2019). Therefore, genetic characterization of the plasmid backbones of *mcr* genes

and other mobile genetic elements (i.e IS and Tn) is required with the expectation to improve the understanding of the molecular mechanisms underlying dissemination of *mcr* variants.

Next generation sequencing (NGS) has appeared to be a very useful tool for epidemiological surveillance and characterization of AMR (Kwong et al., 2015). Providing comprehensive genomic data by performing in a single time, NGS has been increasingly used for genomic characterization of foodborne bacterial pathogens, identification of clonal groups in bacteria of public health importance and molecular characterization of epidemic plasmids harboring AMR or/and virulence genes (Kovac et al., 2017). We previously isolated *E. coli* with coexistence of ESBL and *mcr* genes from healthy and sick pigs. In this study, three *E. coli* co-harboring ESBL and *mcr* genes were characterized by using WGS approach.

5.3 MATERIALS AND METHODS

5.3.1 Epidemiological background of the *E. coli* collection

In this present study, three *E. coli* isolates co-expressing ESBL and *mcr* genes, i.e. E.431, ECP.81 and ECP.82, were selected from our bacterial collection. E.431 was collected in 2007 from a healthy fattening pig and found to carry *mcr-2*, *mcr-3* and $bla_{\text{CTX-M-14}}$ (E.431). The other two *E. coli* isolates carrying both *mcr-1* and *mcr-3* and simultaneously containing $bla_{\text{CTX-M-14}}$/$bla_{\text{CTX-M-55}}$/$bla_{\text{TEM-1B}}$ (ECP.81) and $bla_{\text{CTX-M-14}}$/ $bla_{\text{TEM-1B}}$ (ECP.82) were isolated from clinically sick pigs in 2013.

These three isolates were part of the molecular retrospective study of colistin resistance and ESBL-production in 454 *E. coli* isolates obtained from healthy and clinically sick pigs in Thailand between of 2007-2018. In the study, all *E. coli* strains were examined for their antimicrobial susceptibilities and ESBL production according

to CLSI guidelines (CLSI, 2013). The results were interpreted according to CLSI breakpoints (CLSI, 2013) or EUCAST breakpoints for colistin (EUCAST, 2017). All were screened for *mcr-1* (Liu et al., 2016), *mcr-2* (Xavier et al., 2016), *mcr-3* (Yin et al., 2017) and *mcr-4* (Carattoli et al., 2017). ß-lactamase-encoding genes including bla_{TEM}, bla_{PSE-M}, $bla_{SHV,}$ bla_{CTX-M}, bla_{CMY-1} and bla_{CMY-2} were determined by PCR (Batchelor et al., 2005; Hasman et al., 2005; Li et al., 2013)

5.3.2 Conjugation experiment

Biparental filter mating experiment was conducted to confirm the localization of *mcr* and ESBL genes on conjugative plasmid as previously described (Chen et al., 2004). E. 431, ECP.81 and ECP.82 carrying both ESBL and *mcr* genes served as donors. The plasmid-free SE12Rif^{R}, spontaneous rifampicin-resistant *Salmonella* Enteritidis (rifampicin MIC=256 µg/ml), was used as recipient (Chen et al., 2004). Transconjugants were confirmed to be *Salmonella* on Xylose Lysine Deoxycholate agar (Difco, MD, USA) containing 32 µg/mL rifampicin and an appropriate antibiotic (i.e. 100 µg/mL ampicillin and/or 2 µg/mL colistin). The presence of *mcr* (n = 3) and ESBL (n = 3) genes in transconjugants was determined by PCR using specific primers as described above.

5.3.3 DNA preparation and Whole Genome Sequencing (WGS)

Genomic DNA of E. 431, ECP.81 and ECP.82 were extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Plasmid DNA of two transconjugants obtained from ECP. 81 and ECP.82, namely ECP.81T and ECP.82T, were extracted by using Qiagen Plasmid Maxi Kit (Qiagen). The amount of all DNA samples was quantified according to Illumina sequencing sample requirements as follows. The DNA samples were dissolved in nuclease-free water or 10mM Tris buffer. The DNA concentration required was at

least 10 nM in 10 µl of minimum volume. The purity of DNA was defined using A260/280 and A260/230 ranging from 1.8 to 2.0.

The quantified DNA were subjected to Whole Genome Sequencing (WGS) by using Illumina platform MiSeq or HiSeq PE150 (Illumina, San Diego, CA, US). The libraries were prepared by using Nextera XT sample preparation kit and sequenced with 2×250 or 350 paired-end reads protocol on an Illumina platform (MiSeq or HiSeq PE150) at Omics Sciences and Bioinformatics Center (OSBC), Faculty of Science, Chulalongkorn University and Singapore Joint Venture & Sequencing Center Novogen AIT.

5.3.4 Analysis of DNA sequence data

Timing of raw sequence reads was performed using the CLC Genomics Workbench software version 11.0.0 (CLC bio, Aarhus, Denmark) with default settings. De novo assembly of the trimmed reads was conducted using CLC Genomics Workbench or SPAdes 3.5.0 software (Nurk et al., 2013). Identification of Open Reading Frames (ORFs) and genome annotation of the assembled genetic elements was performed by using Prokka (Seemann, 2014) and/or PATRIC 3.6.5 (Brettin et al., 2015) with default settings.

WGS data sets were analyzed using Open-access bioinformatic webtool available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). *In silico* typing based on WGS of assembled genomes/contigs in FASTA format was carried out by using Serotype Finder 2.0 (Joensen et al., 2015) with selected threshold of 90% identity and 60% total serotype gene length. Multi-Locus sequence typing (MLST 2.0) was applied for molecular typing of *E. coli* (Larsen et al., 2012). The MLST allele sequences and

profile data used in MLST 2.0 were obtained from https://www. PubMLST.org and determined at 90 % identity and 60% minimum length.

Res Finder 4.1 (Bortolaia et al., 2020) and Point Finder (Zankari et al., 2012) were used to predict AMR genes and chromosomal point mutations from genomic sequences based on 90% identity. Plasmid replicon sequence analysis and identification of virulence genes were performed using Plasmid Finder 2.1 (Carattoli et al., 2014) and Virulence Finder 2.0 (Joensen et al., 2014), respectively with the threshold of 90% identity and 60% minimum length.

5.3.5 Plasmid and Phylogenetic analysis

Plasmid reconstruction from WGS data was conducted by using highly homologous complete plasmid sequence references available in NCBI. The alignment and assembly of sequences was performed using CLC Genomics Workbench or PATRIC 3.6.5 (Brettin et al., 2015). Plasmid sequences were annotated by Prokka (Seemann, 2014) or PATRIC 3.6.5 (Brettin et al., 2015) and manually edited. Then, annotated plasmid sequences were analyzed. Circular comparison between IncI2 plasmid carrying *mcr-1* and most similar reference plasmids available at NCBI database was generated by CG view server (Stothard and Wishart, 2005). All reference plasmids used for comparison in this study and obtained from NCBI database are listed in supplementary table (Appendix B).

Phylogenetic analysis of *mcr-1* harboring plasmids was conducted by comparing the assembled *mcr-1* plasmid sequences from two sick pigs (ECR.81 and ECP.82) with 20 complete genome reference sequences of IncI2 plasmids harboring *mcr-1* harboring from GenBank database (supplementary table Appendix B). The data were assessed by using MEGA 10.0 program with the neighbor-joining method (1,000 bootstrap replicates). The phylogenetic relationship of the core genome

segment of *mcr-3* carrying contigs was conducted by MEGA 10.0 program with maximum likelihood method (1,000 bootstrap replicates). All *mcr-3* harboring contig sequences used to produce the phylogenetic trees were generated in this study and the *mcr-3* reference genome sequences were obtained from the GenBank database (supplementary table Appendix B).

5.4 RESULTS

5.4.1 Susceptibilities to antimicrobials and molecular characteristics

The *E. coli* isolates, E.431, ECR.81 and ECP.82 exhibited multidrug resistance (MDR) phenotype (resistance to at least three different antimicrobial classes). The E. 431 from healthy pig was resistant to all antibiotics tested but not to trimethoprim (Table 16). The ECP. 81 from clinically sick pig was resistant to all antimicrobials tested while the other EC.P. 82 remained susceptible to trimethoprim and ceftazidime.

Serotype of all three *E. coli* isolates were *in silico* determined and MLST following the WGS data analysis through the CGE online services. The E. 431 from healthy pig belonged to serotype O142:H32 with sequence type ST29. The other two *E. coli* isolates, ECP.81 and ECP.82, from sick pigs were serotype O9:H9 belonging to ST10.

Plasmid finder showed that all the isolates harbored four plasmid replicons, including IncFIB, IncFII, IncHI2 and Col440I. E. 431 from healthy pig additionally carried Col156, Col440I, and p0111 replicon types, while ECP.81 and ECP.82 from sick pigs additionally contained ColpVC, IncI1-I(**γ**) and IncI2 replicon types (Table 17).

Identification of virulence profiles revealed that E. 431 from healthy pig and ECP.81 and ECP.82 from sick pigs harbored similar virulence genes including heat

stable toxin (*ast*A), fimbrial adhesin (*fed*A and *fed*F), glutamate decarboxylase (*gad*), heat resistance agglutinin (*hra*), tellurium ion resistance protein (*ter*C) and outer membrane protein resistance (*tra*T). The presence of certain virulence genes including *celb*, *cif*, *eae*, *efa*1, *esp*A, *iss*, *iuc*C, *iut*A, *kat*P, *lpf*A, *nle*A, *nle*B, *nel*C, *omp*T, *sep*A, *tox*B and *tsh* varied among the three isolates (Table 17).

5.4.2 Presence of AMR determinants

WGS data analysis using Resfinder revealed the presence of AMR genes that were consistent with their resistance phenotype (Table 17). E.431 caried *mcr-2.1*, *mc-r3.1* and $bla_{\text{CTX-M-14}}$. Both ECP.81 and ECP.82 carried *mcr-1.1* and *mcr-3.1* genes. ECP.81 additionally harbored $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1B}}$, while ECP.82 simultaneously carried $bla_{\text{CTX-M-14}}$ and $bla_{\text{TEM-1B}}$. In addition to ESBL and *mcr* genes, all three isolates concurrently contained various AMR genes conferring (but not limited) resistance to aminoglycoside (*aac(3)-IIa, aadA1, aadA2b, aph(3'')-Ib, aph(3')-IIa* and *aph(6)-Id*), macrolide (*mdf(A)*), phenicol (*cmlA1*), sulphonamide (*sul3*) and tetracycline (*tet(A) and tet(M)*) that were consistent with the AMR phenotypes. The single point mutations in quinolone resistant-determining regions (QRDR) of *gyrA* and *parC* resulting in amino acid substitution in GyrA and ParC were identified including S83L and D87G in GyrA and S80I and E62K in ParC. Plasmid mediated quinolone resistance genes (PMQR) i.e. *oqxA, oqxB and qnrS13* were detected in ECP.81 and ECP.82.

5.4.3 Horizontal transfer of AMR genes

Only ECP.81 and ECP.82 were able to transfer *mcr* and ß-lactamase genes to SE12Rif^{R}. Using ampicillin and rifampicin as selective pressure, ECP.81 conjugally transferred $bla_{\text{CTX-M-55}}$ together with *mcr-3.1* and *aac(3)-IIa*. The NGS analysis of plasmid revealed the presence of Col440I, ColpVC, IncFIB and IncFII plasmid

replicons in the transconjugant ECP.81T. ECP.82 was capable of transfer bla_{TEM-1B}, *mcr 1.1, aac(3)-IIa*, *aadA2b*, *aph(3")-Ib*, *aph(6)-Id*, *qnrS13* and *sul2* at the same time. Several plasmid replicon types were identified in ECP.82T, including IncI2, Col440I, ColpVC, IncFIB, IncFII and IncI1(γ) (Table 18). Under colistin and rifampicin selection pressure, only ECP.82 could transfer *mcr-3* to the recipient strain.

Table 16. Antimicrobial susceptibilities of ESBL-producing *E. coli* carrying *mcr* from pigs in Thailand (n = 3).

Antimicrobial agent	Healthy pig		Clinically ill pig			
	E. 431		ECP. 81		ECP. 82	
	MIC	S/R	MIC	S/R	MIC	S/R
Polymyxin						
Colistin	8	R	8	R	8	R
Beta-lactam						
Ampicillin	>512	R	>512	R	>512	R
Cefotaxime		R[a]		R[a]		R[a]
Ceftazidime		R[a]		R[a]		S[a]
Cefpodoxime		R[a]		R[a]		R[a]
Aminoglycosides						
Streptomycin	32	R	256	R	256	R
Gentamicin	1024	R	256	R	256	R
Fluoroquinolones						
Ciprofloxacin	128	R	32	R	32	R
Phenicol						
Chloramphenicol	64	R	256	R	128	R
Tetracycline						
Tetracycline	1	S	256	R	256	R
Folate pathway inhibitors						
Sulfamethoxazone	256	S	>2048	R	>2048	R
Trimetroprim	64	R	1024	R	1	S

[a] = ESBL screening test by using disk diffusion method.

Table 17. Molecular characteristics of ESBL-producing *E. coli* carrying *mcr* from pigs in Thailand (n = 3)

Strain	Source	Serotype	MLST	Plasmid content	Virulence profile	Resistance genes			Amino acid change in GyrA and/or ParC
						mcr	β-lactamase	Others by WGS	
E. 431	Healthy pig	O142:H31	29	Col156, Col440II, IncFIB, IncFII, IncHI2, p0111	*ast*A, *celb*, *cif*, *eae*, *efa*1, *esp*A, *esp*B, *esp*F, *espJ*, *gad*, *hra*, *iss*, *iuc*C, *iut*A, *kat*P, *lpf*A, *nle*A, *nle*B, *nel*C, *omp*T, *sep*A, *ter*C, *tox*B, *tra*T, *tsh*	*mcr-2.1*, *mcr-3.1*	$bla_{CTX-M-14}$	*aac(3)-IIa, aadA1, aadA2b, aph(3")-Ib, aph(3')-IIa, aph(6)-Id, mdf(A), cmlA1, oqxA, oqxB, sul3, tet(A), tet(M)*	GyrA (S83L, D87G), ParC (S80I)
ECP. 81	Sick pig	O9:H9	10	Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-I(γ), IncI2	*ast*A, *fed*A, *fed*F, *gad*, *hra*, *sta1*, *ter*C, *tra*T	*mcr-1.1*, *mcr-3.1*	$bla_{CTX-M-14}$, $bla_{CTX-M-55}$, bla_{TEM-1B}	*aac(3)-IIa, aadA1, aadA2b, aph(3")-Ib, aph(3')-IIa, aph(6)-Id, cfr, lnu(F), mdf(A), catA2, cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M)*	GyrA (S83L), ParC (S80I)
ECP. 82	Sick pig	O9:H9	10	Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-I(γ), IncI2	*ast*A, *fed*A, *fed*F, *gad*, *hra*, *ter*C	*mcr-1.1*, *mcr-3.1*	$bla_{CTX-M-14}$, bla_{TEM-1B}	*aac(3)-IIa, aadA1, aadA2b, aph(3")-Ib, aph(3')-IIa, aph(6)-Id, mdf(A), cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M)*	GyrA (S83L), ParC (S80I, E62K)

Table 18. Antimicrobial resistance genes and plasmid contents in donors and transconjugants revealed by NGS.

Strain	Role	AMR gene	Plasmid replicon type
EC.P. 81	Donor	*mcr-1.1*, *mcr-3.1*, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}55}$, $bla_{TEM\text{-}1B,}$ *aac(3)-IIa, aadA1, aadA2b, aph(3'')-Ib, aph(3')-IIa, aph(6)-Id, cfr, lnu(F), mdf(A), catA2, cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M)*	Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-I(**γ**), IncI2
EC.P. 81T	Transconjugant	*mcr-3.1*, $bla_{CTX\text{-}M\text{-}55}$ *aac(3)-IIa*	Col440I, ColpVC, IncFIB, IncFII
EC.P. 82	Donor	*mcr-1.1*, *mcr-3.1*, $bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}1B,}$ *aac(3)-IIa, aadA1, aadA2b, aph(3'')-Ib, aph(3')-IIa, aph(6)-Id, mdf(A), cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M)*	Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-I(**γ**), IncI2
EC.P. 82T	Transconjugant	*mcr-1.1*, $bla_{TEM\text{-}1B}$ *aac(3)-IIa, aadA2b, aph(3'')-Ib, aph(6)-Id, qnrS13, sul2*	Col440I, ColpVC, IncFIB, IncFII, IncI1-I(**γ**), IncI2

5.4.4 Characteristics of *mcr*-harboring plasmids

The *mcr 1.1* gene was identified in the *E. coli* isolates from ill pigs, ECP.81 (contig 30, 19,134 bp in length) and ECP.82 (contig 30, 53,048 bp in length) and carried by IncI2 plasmid. The blast alignment of these two contigs showed 97.2% identity across 11,523 lengths of the shortest contig (19,134 bp). The genetic organizations of the regions downstream of these *mcr-1.1* contigs shared the similar genes as follows: haemolysin expression modulating protein, DNA topoisomerase III, PcfJ domain-hypothetical protein and shufflon-specific DNA recombinase. When compared to plasmid pHNSHP45 (Accession number: KP347127), 99% nucleotide homology was found covering the region of the published plasmid from 14,445 to 33,021 bp for ECP. 81 and 34,130 to 64,015 bp for ECP.82.

The IncI2 plasmid harboring *mcr-1.1* from ECP. 81 (64,006 bp in length) and ECP.82 (64,023 bp in length) were reconstructed based on the WGS data by comparison to plasmid pHNSHP45. The *mcr-1.1*-IncI2 plasmids had a typical plasmid backbone containing *repA* for plasmid replication, *traL* for maintenance and transfer. Only an IS*Apl1-mcr-1.1* cassette identical to that of pHNSHP45 was found in ECP.81 whereas *mcr-1.1* of ECP.82 was flanked by IS*Apl1* and IS*91* transposase at upstream and downstream regions, respectively (IS*Apl1-mcr-1.1*-orf-IS*91*).

Phylogenetic studies of *mcr-1.1*-harboring IncI2 plasmids revealed 8 distinct clades (Fig 7), of which each clade consisted of sequences from different origins. Both plasmid from ECP.81 and ECP.82 were found on the same clonal linages and clonally related to pHNSHP45. The *mcr-1.1*-harboring IncI2 plasmids identified in ECP. 81 and ECP. 82 were also similar to the *mcr-1* harboring IncI2 plasmids isolated from a cattle in Japan (pMRY16-002, Accession No. AP017622), a wild boar in China

(pMRY15-131, Accession No. AP017614) and chicken in China (pGD16-131, Accession No. MN232187) (Fig 8).

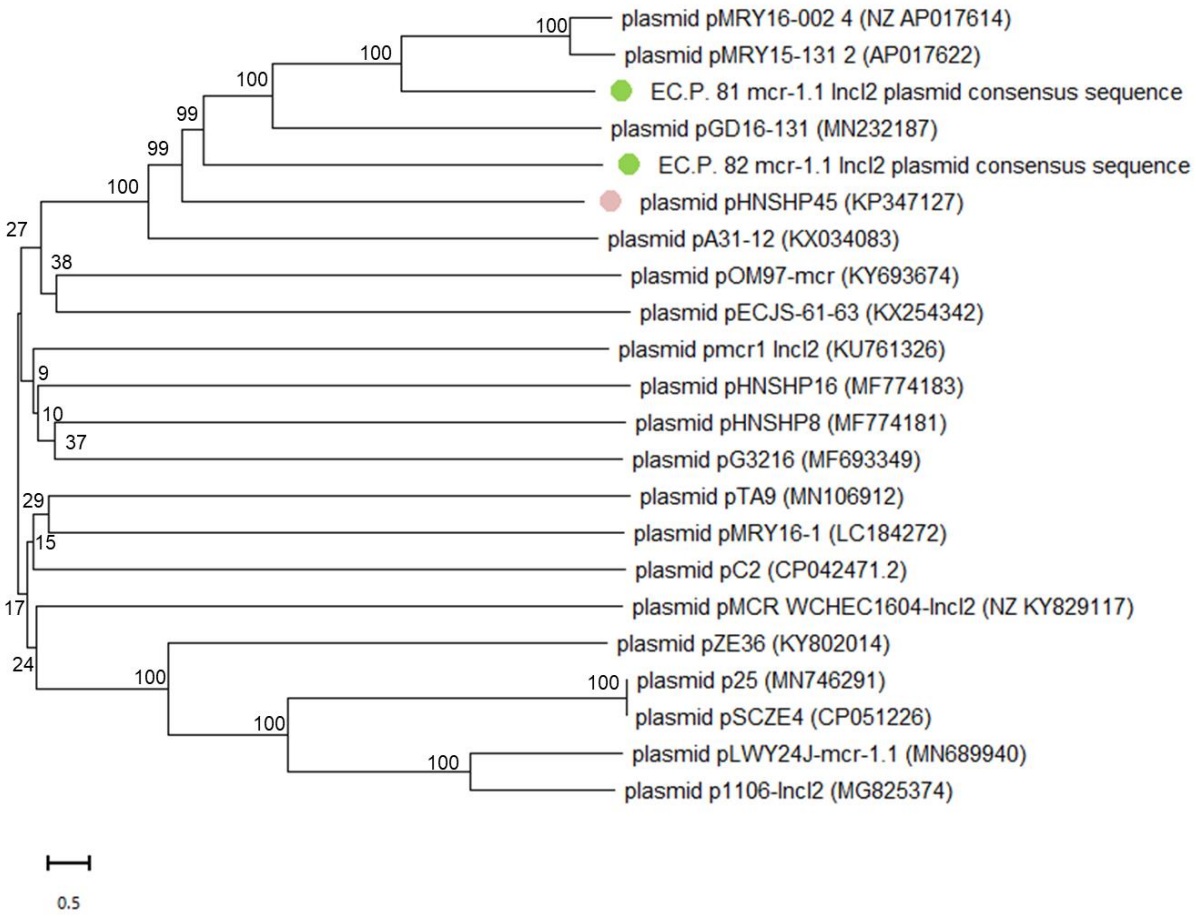


Figure 7. Phylogenetic analysis of the *mcr-1.1*-carrying IncI2 plasmids in ECP.81 and ECP.82 from sick pigs (indicated with green dot) and other *mcr-1*-carrying IncI2 plasmids from *E. coli* deposited in the GenBank database. Sequences were aligned using MUSCLE and phylogenetic interferences were obtained using the neighbor-joining method within the MEGA 10 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis for 1000 times to generate a majority consensus tree.

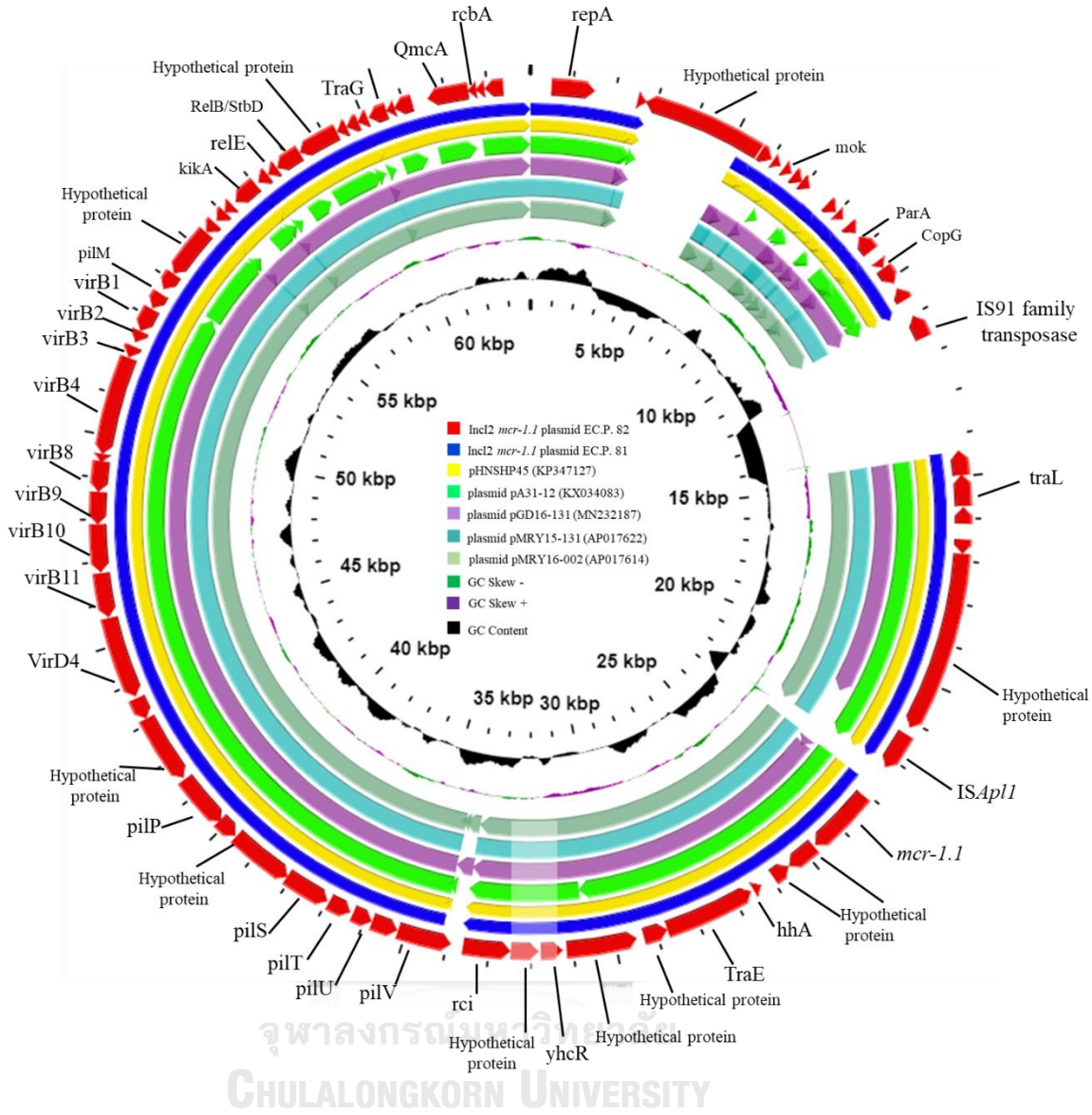


Figure 8. Circular comparison between *mcr-1.1*-carrying IncI2 plasmids from ECP.81 and ECP.82 to five IncI2 type plasmids carrying *mcr-1* with the highest similarity obtained from NCBI database (Accession No. KP347127, KX034083, MN232187, AP017622 and AP017614) generated by the Comparative Genomic tool (CG view) freely available at https://CGView Server.ca. The outer-red circle denotes annotation of *mcr-1.1* plasmids from the present study. The sequencing alignment indicates the high degree similarity of the *mcr-1.1* harboring IncI2 plasmids from ECP.81 and ECP.82 to the *mcr-1*-harboring plasmid, pHNSHP45, isolated in China. The insertions element, IS*Apl1* is conserved in all *mcr-1*-containing plasmids. Gaps indicate regions that were missing in the respective plasmid compared to the reference plasmid.

The *mcr-2.1* gene was identified in E.431 obtained from healthy fattening pig. However, only short assembled contig of 2,142 bp was generated. This *mcr-2.1* cassette contained a hypothetical protein at downstream and its entire length showed 90% identity to the 26687 to 28611 bp region of the published sequence of plasmid pKP37-BE in *E. coli* strain KP37 (Accession No. LT598652). No plasmid replicon and other antimicrobial resistance genes were detected on the same contig.

All three porcine *E. coli* isolates in this study harbored *mcr-3.1*. The size of assembled contigs ranged from 8,412 to 11,172 bp. The WGS data showed that *mcr-3.*1 carrying plasmid in all of them had a similar backbone to *mcr-3.1*-carrying plasmid pWJ1 (Accession No. KY924928) (Fig 9). Five insertion sequences (IS) flanking the *mcr-3.1* cassette were identified. Those located upstream were IS*4321* (ECP.81 and ECP.82) and ΔTn*As2* (E.431, ECP.81 and ECP.82) and those at downstream were IS*Kpn40* and IS*3* family transposase (ECP.81 and ECP.82) and IS*26* (ECP.82). A core segment ΔTn*As2-NimC/NimA-mcr-3.1*-dgkA and conjugative transfer genes (i.e. *trb* and *traO*, and *traG*) were presented in all three *mcr-3.1*-carrying plasmid. The additional insertion sequence, IS*4321*, was localized immediately upstream of the ΔTn*As2-NimC/NimA-mcr-3.1-dgkA* segment in *mcr-3* carrying contigs ECP.81 and ECP.82. However, none of the contigs contained other AMR genes and plasmid replicon type sequence identified in the PlasmidFinder database.

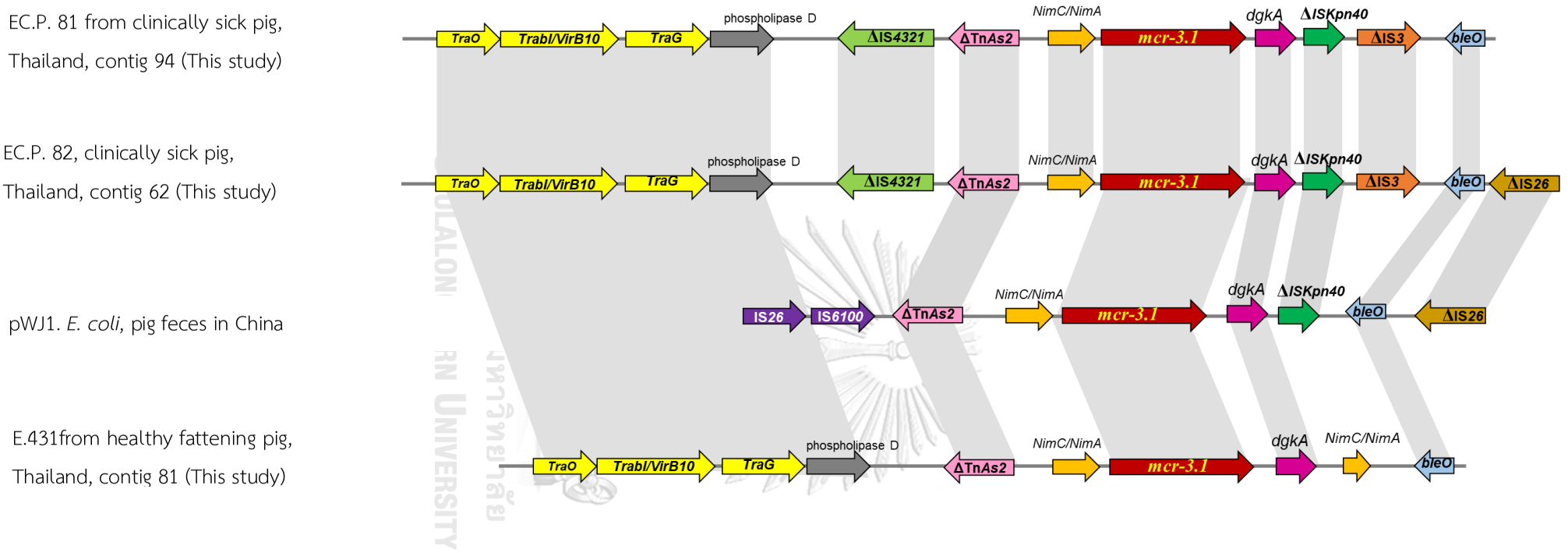


Figure 9. Comparative schematic representation of the flanking regions of the *mcr-3* gene in pWJ1 (Accession no. KY924928) and *mcr-3* carrying contigs in this study. The arrows indicate the positions and directions of the genes. The gray shade indicates homology in the corresponding genetic environment on each contig. AMR genes are indicated in red for *mcr-3.1* and blue for *bleO*, bleomycin resistance gene. The conjugally transferred proteins are indicated in yellow. The green, pink, orange and brown arrows represent transposon-associated genes (ΔIS*4321*, ΔTn*As2*, ΔIS*Kpn40* and ΔIS*26*).

Phylogenetic tree of the core genome sequences was analyzed in all three *mcr-3.1*-carrying isolates and 12 of *mcr-3.1* harboring-plasmids deposited in the GenBank database (Fig 10). The members in this phylogenetic tree can be grouped into three clades. The mcr.3.1 carrying contigs in ECP. 81 and EC.P.82 had a core segment that were similar to pWJ1 (Accession No. KY924928) isolated from in *E. coli* from pigs in China and in *K. pneumoniae* from pigs in Nakhon Pathom, Thailand (Accession No. CO041095 and CP041104). However, the *mcr-3.1* carrying contig from fattening pig (E.431) was different from those in sick pigs and reference plasmids.

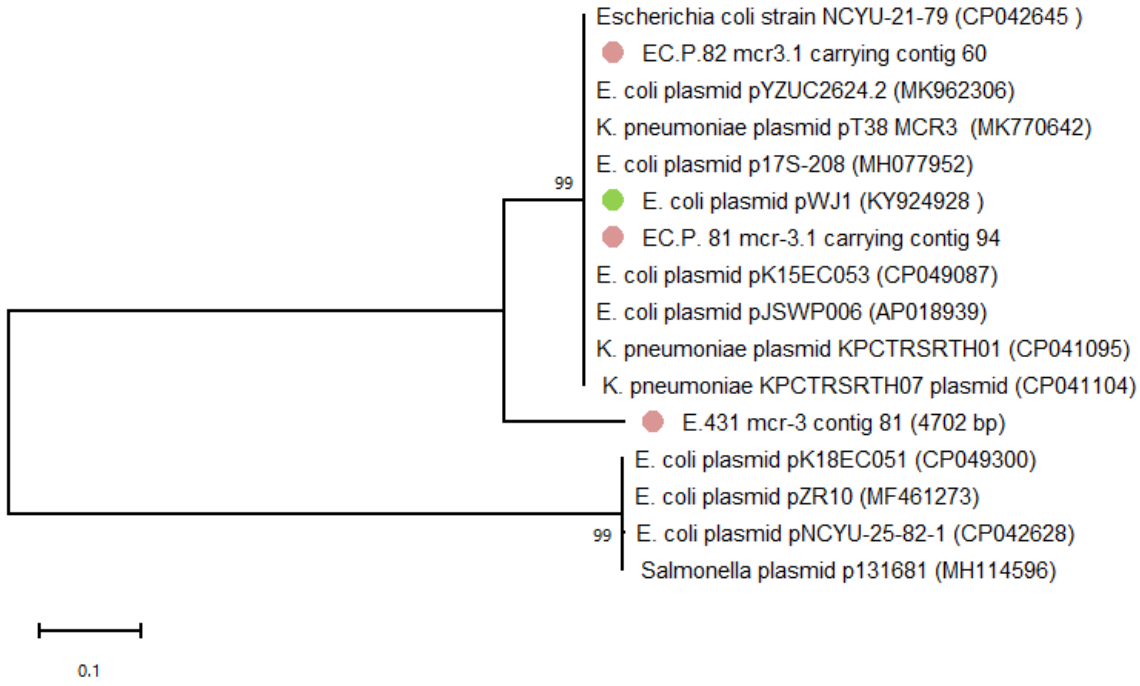


Figure 10. Phylogenetic tree of core genome sequences in all three *mcr-3* carrying isolates (indicated by green dot) and other *mcr-3* plasmids deposited in the GenBank database. Sequences were aligned using MUSCLE and phylogenetic interferences were obtained using the maximum likelihood method within the MEGA 10 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis of 1000 times to generate a majority of consensus tree.

5.5 DISCUSSION

The emergence and spread of colistin resistance mediated by plasmid-borne *mcr* genes has occurred globally, of which the majority were associated with Enterobacteriaceae including *E. coli., Moraxella* spp*., Klebsiella* spp*., Salmonella* spp*., Enterobacter* spp*. and Citrobacter* spp (Flament-Simon et al., 2020). The *mcr*-carrying plasmids frequently co-harbor other resistance determinants (Haenni et al., 2016; Poirel et al., 2016). These bring about a particular challenge for treatment of MDR Gram-negative bacterial infection due to limited availability of effective antibiotics. *E. coli,* particularly from pig, has been a major species among the *mcr*-carrying bacterial strains (Liu et al., 2016; Yin et al., 2017; Garcia-Menino et al., 2019). In the present study, we performed WGS analysis for molecular characterization of three ESBL producing *E. coli* additionally carrying *mcr* originated from pigs.

The *E. coli* isolates from healthy fattening pig, E.431, carrying *mcr-2/mcr-3* belonged to serotype O142:H31 and had sequence type, ST 29. The other two isolates originated from clinically sick pigs, ECP.81 and ECP.82, with coexistence of *mcr*-1 and *mcr-3* appeared to be serotype O9:H9 with sequence type ST10. This is similar to previous studies demonstrating that the ST10 *E. coli* served as an important reservoir of *mcr-1* (Matamoros et al., 2017; Garcia-Menino et al., 2019). The clonal group ST10 of *mcr*-carrying *E. coli* have been reported previously in the clinical isolates from humans (Luo et al., 2017) and food-producing animals (i.e. poultry and swine) in Asia (Bai et al., 2016; Falgenhauer et al., 2016) and some European countries (El Garch et al., 2017; Garcia et al., 2018). It was shown that the ST10 strains harboring *mcr* commonly carried additional-multiple AMR genes e.g. $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$ (Garcia et al., 2018).

E.431, ECP.81 and ECP.82 were MDR, which exhibited resistance to different antimicrobial classes e.g. colistin, ß-lactam, aminoglycosides, fluoroquinolone, tetracycline and sulfa-trimethoprim. The AMR phenotype in all these isolates was consistent with AMR genotype revealed by WGS e.g. resistance to colistin (i.e. *mcr-1.1*, *mcr-2.1* and *mcr-3*), ß-lactams (i.e. $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-1B}}$), aminoglycosides (i.e. *aadA1* and *aadA2*), fluoroquinolones (i.e. *oqxA, oqxB* and *qnrS13*), phenicols (i.e. *catA2* and *cmlA1*), tetracycline (i.e. *tetA* and *tetM*) and sulfamethoxazole (i.e. *sul2* and *sul3*). These results agree with previous studies demonstrating that most colistin resistant bacterial isolates exhibited MDR phenotype and frequently co-harbored multiple AMR genes (Alba et al., 2018; Flament-Simon et al., 2020).

The conjugation experiments and plasmid analysis confirmed that the *mcr-1.1* gene of ECP.81 and ECP. 82 was located on IncI2 plasmid, in agreement with a previous study (Liu et al., 2016). The *mcr-1* gene and its variants have been identified to be associated with four major plasmid incompatibility groups i.e. IncX4, IncI2, IncHI2 and ColE10-like (Matamoros et al., 2017; Garcia-Menino et al., 2019). It was previously shown that IncI2 plasmid can migrate between different bacterial species and *E. coli* serves as a potential carrier of this plasmid replicon (Wong et al., 2015).

The insertion sequence IS*Apl1* has been the main driver of mobilized colistin resistance gene, *mcr-1*, via horizontal gene transfer. Four genetic contexts surrounding *mcr-1* cassette have been reported including the composite transposon Tn6330 (IS*Apl1*–*mcr-1*–orf–*ISApl1*) and a single-IS*Apl1* located upstream (IS*Apl1*–*mcr-1*–orf); a single-IS*Apl1* (*mcr-1*–orf–IS*Apl1*) located downstream and a structure lacking both copies of IS*Apl1* (*mcr-1*–orf) (Snesrud et al., 2018). In present study, the two *mcr-1.1* carrying IncI2 plasmids from ECP.81 and ECP. 82 of sick pigs have the

structure of IS*Apl1*–*mcr-1*–orf. The results from the conjugation experiment and plasmid analysis in both donor (ECP. 82) and transconjugant (ECP. 82T) confirmed that IS*Apl1* is associated with the mobilization of *mcr-1*. This transconjugant, ECP. 82T, also carried *mcr-1.1* with translocation of IS*Apl1* to its upstream or IS*Apl1*–*mcr-1*–orf as observed in its donor strain. A recent study hypothesized that *mcr-1* translocation could be mediated through a circular intermediate that mediates the insertion of the *mcr-1* gene cassette into other bacterial plasmids or genome (Li et al., 2017).

The *mcr-2* gene was first described on IncX4 plasmid in *E. coli* isolated from calves and piglets in Belgium (Xavier et al., 2016). However, the prevalence of *mcr-2* is low and was limited to *E. coli*, *Moraxella* and *K. pneumoniae* from pigs, claves, bird, and chicken in some countries e.g. China (Zhang et al., 2018), Belgium (Xavier et al., 2016), USA and Great Britain (AbuOun et al., 2017). Up to date there are six *mcr-2* variants (Accession no. LT598652, MF176239, NG065452, MT757845, MT757842 and MT757844) were deposited on to GenBank database but only one plasmid sequence, pBK37-BE (Accession no. LT598652), was characterized. In our study, the *mcr-2.1* gene was harbored by E.431 and showed 100% identity to pBK37-BE and IS*1595* is located upstream of the gene. However, analysis of genetic contexts of the genomic location *mcr-2* was limited due to limited size of the contig and non- transferability in the conjugation experiment.

The *mcr-3* gene was first identified on pWJ1, a 261 kb IncHI2-type plasmid, in *E. coli* from pigs in China (Yin et al., 2017). Up to date, the gene was identified on IncP1, IncFII and IncI1 plasmids in many bacteria species e.g. *E. coli*, *K. pneumoniae*, *Salmonella*, *Enterobacter* spp. and *Aeromonas* spp. from pig, chicken, cattle, aquatic environment and human in several countires i.e. China (Ling et al., 2017), Denmark (Litrup et al., 2017) and Spain (Hernandez et al., 2017). In this study, all E.431, ECP.81

and ECP. 82 harbored *mcr-3.1*. Only *mcr-3.1* in ECP. 81 was located on conjugative plasmid. Previous studies described that the dissemination of *mcr-3* is involved in mobile elements that can be horizontally transferred such as ΔTn*As2*, IS*Kpn40,* IS*26 and* IS*15DI* (Wang et al., 2019). The mobile elements ΔTn*As2* and ΔIS*Kpn40* were originated from *Aeromonas* spp and may play an important role in the spread of *mcr-3* between *Aeromonas* spp. and Enterobacteriaceae (Yin et al., 2017). In this study, five mobile elements were identified in the flanking regions of *mcr-3* contigs including ΔIS*4321*, ΔTn*As2*, ΔIS*Kpn40,* IS*26* and IS*3*. ECP.81 and ECP.82 contained a core segment of IS*4321*-ΔTn*pA-NimC/NimA-mcr-3-dgkA*-IS*Kpn40*-IS*3*-Δ*bleO,* while E. 431 from healthy-fattening pig carried ΔTn*pA-nimC/nimA-mcr-3-dgkA-nimC/nimA*-Δ*bleO.* The core segment ΔTn*pA-nimC/nimA-mcr-3-dgkA* in all three isolates was similar to pWJ1. However, the two sick pig isolates, ECP.81 and ECP.82, had IS*4321* instead of IS6100. This IS*4321* originated from *K. aerogenes* and was previously identified in *mcr-3* carrying plasmids, pZR78 (Accession no. MF455226) and pZR12 (Accession no. MF455227) (Wang et al., 2019).

ESBL genes are most commonly on conjugative plasmid and has been previously shown to be associated with several plasmid incompatibility groups e.g. IncF, IncI, IncH and IncA/C plasmids (Rozwandowicz et al., 2018). IncF is the commonly described plasmid type from humans and animals *E. coli* is considered to be a major reservoir of this plasmid. A previous study in Korea demonstrated the dissemination of $bla_{\text{CTX-M-14}}$ gene driven by IncF plasmid (Kim et al., 2011). A study in China reported that $bla_{\text{CTX-M-55}}$ in *E. coli* from pets and food animals were linked to IncI2 plasmid (Lv et al., 2013). Both IncHI1 and IncHI2 plasmids are frequently associated with resistance to multiple drugs e.g. sulfonamides, aminoglycosides, tetracyclines and streptomycin in additions to cephalosporins. Both $bla_{\text{CTX-M-2}}$ and $bla_{\text{TEM-1}}$ were previously reported to be on a IncHI2 plasmid (Egervarn et al., 2014).

In this study, many plasmid replicon types were detected in the three *E. coli* strains, of which the most frequent plasmid replicons were IncFIB, IncFII and IncHI2. The $bla_{CTX\text{-}M\text{-}14}$ was detected in all *E. coli* isolates. The $bla_{CTX\text{-}M\text{-}55}$ gene was found only in ECP.81 and $bla_{TEM\text{-}1B}$ was identified in both isolates from ill pigs. Four mobile elements (i.e. Tn3 transposase, Tn*903*, IS*1* and IS26) were identified in flanking regions of ESBL genes. This supports that, in addition to conjugative plasmid, the dissemination of ESBL genes occurs through transposons.

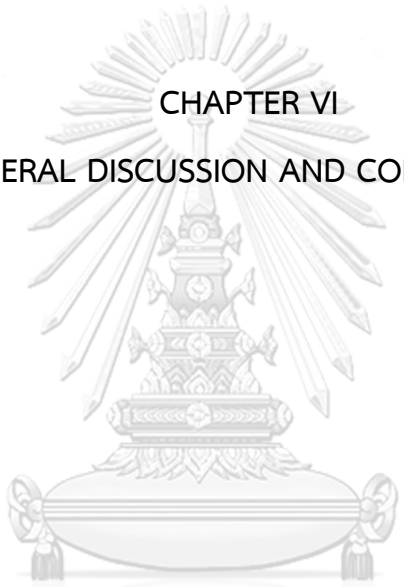
Moreover, the co-transfer of *mcr-1.1* and $bla_{TEM\text{-}1B}$ was observed in ECP. 82 and the co-transfer of *mcr-3.1* and $bla_{CTX\text{-}M\text{-}55}$ was observed in ECP. 81. Using data from WGS analysis of the donor and plasmid analysis of transconjugants, plasmid reconstruction did not show the presence of other AMR genes. This implies that the genes encoding colistin resistance and ESBL production (i.e. *mcr-1.1*/$bla_{TEM\text{-}1B}$ and *mcr-3.*1/$bla_{CTX\text{-}M\text{-}55}$) were not colocalized on the same plasmid with other AMR genes. These observations are in agreement with a previous study in China demonstrating the co-transfer of *mcr-1.1*, *mcr-3.5*, $bla_{NDM\text{-}5}$ and *rmtB* could occurred even they were not located on the same plasmid (Long et al., 2019). The co-transfer of many clinically important AMR at the same time is a big challenge for clinical treatment and disease controlling in both human and veterinary medicine. Such co-transfer of multiple resistance genes could provide the fitness advantage to the host strains (Wu et al., 2018b). Therefore, using colistin might not be the main reason of *mcr* dissemination. This could be supported by the use of ampicillin could select for colistin resistance gene in our study.

In conclusion, the pandemic spread of *mcr* genes is attributed to several factors e.g. MDR profile in colistin-resistant strains, host fitness adaptation, and co-selection by other antimicrobials. Co-transfer of *mcr* and ESBL genes has generated a challenging for clinical treatment and a serious concern to global health.

Judicious use of antibiotics in livestock and human medicine should be encouraged. Characterization of the coexistence ESBL and *mcr* genes in three *E. coli* isolates by using WGS approach provided large database for rapid analysis of genetic context of resistance determinants and supported better understanding on the dissemination of these resistance markers among bacteria from different sources. However, short read sequences obtained from NGS created some limitations in data analysis. According to the variety of structural variants and repeated sequences in bacterial genomes, these limitations may adversely affect a perfect genome reconstruction. Long-read sequencing (LRS) technologies are suggested for the improvements.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION



6.1 General discussion

The overuse and misuse of antibiotics are highlighted as a major contributor to the development and spread of antimicrobial-resistant pathogens including MDR *E. coli* and *Salmonella* (Byrne et al., 2019). Food animals and their meat products have been considered as one of the most important sources for the distribution of these resistant bacteria to humans via several pathways. Particular concerns have been raised against the rapid increase of ESBL-producing Enterobacteriaceae and colistin resistance in Enterobacteriaceae globally (Rao et al., 2014; Yang et al., 2016; Wang et al., 2018). However, knowledge on the particular resistance is still limited in developing countries including Thailand and other Southeast Asian countries. Thus, determination of AMR phenotype and genotype bacteria isolated from food animals and their associated meat products is urgently needed to explain their spreading and transmission along the food chain. Simultaneously, testing of commensal *E. coli* from food animals and meat is also highly relevant since AMR phenotype in these bacterial species can accurately reflect antimicrobial used in food animals.

The results were discussed in each individual chapter. However, general discussion is made to cover missing or additional messages as follows.

Part 1. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces.

In this present study, one of the main findings was the high-level of *Salmonella* contamination in pigs, broilers and their meat in the Thailand–Cambodia border provinces. The observations could be resulted from many factors example transportation and lairage stress that may induce the more-frequent *Salmonella* shedding, poor hygiene practices and biosecurity measures in both slaughterhouses and fresh markets in the study area (Mannion et al., 2012). The overall prevalence of

Salmonella in these areas was 34.2% that was higher than the previous studies from food animals and meat products in other countries e.g. South Korea (11.8%) (Lee et al., 2016), China (13.7%) (Zeng et al., 2019) and Germany (8.9%) (Schwaiger et al., 2012). However, the prevalence was lower than the other reports in Vietnam (68.4%) (Thai et al., 2012), Georgia, U.S. (42.8%) (Guran et al., 2017) and Thailand-Laos border provinces (39.5%) (Sinwat et al., 2016). The total prevalence of *Salmonella* was significantly higher in the samples collected from Cambodia than those in Thailand ($P < 0.05$), especially among those samples of pig origin. This may reflect the differences in farm biosecurity and management in the two countries. Livestock production in Thailand mainly aims to support domestic demands with the potential to exportation (SPEA, 2013). Thailand has introduced Good Manufacturing Practices (GMP) for swine (ACSF, 2006a) and poultry abattoirs issued by Thai Agricultural Standard (TAS) (ACSF, 2006b). However, traditional small slaughterhouses with poor hygienic practices still present in remote area. Cambodian households traditionally raise poultry and pigs in their backyards for consumption and selling. Although, there are law and regulations concerning the Cambodian slaughterhouse system introduced in 2008, Cambodian farmers still tend to use a small killing point for slaughter (Tornimbene and Drew, 2012). Poor sanitation and hygienic manners potentially underline the high prevalence *Salmonella* contamination in Cambodian samples.

The prevalence of *Salmonella* was much higher in the samples collected at fresh markets than those from slaughterhouses in both Thailand and Cambodia provinces ($P < 0.05$), which is discordant with a previous study in food animals in northern Thailand (Padungtod and Kaneene, 2006). In contrast, these results are in agreement with a previous report in Thailand-Laos border provinces (Sinwat et al., 2016) and in Germany (Schwaiger et al., 2012). There were several *Salmonella*

serovars identified in the retail samples and some serovars were consistent to those in slaughtering samples. This may indicate a cross contamination along the processing chain such as unclean shipping container, improper packaging, decomposition, dirty equipment or via butchers and uncontrolled environmental conditions (Carrasco et al., 2012). This highlights that the enforcement of hygiene regulations serves as a key element in verifying food safety as well as controlling the transmission of resistant bacteria from food to humans. In addition, genetic relatedness of *Salmonella* should be determined for better clarification of the relationship along the food chain.

Salmonella Typhimurium, Rissen and Corvallis were prominent serovars identified in Thailand-Cambodia border provinces, in agreement with previous studies demonstrating that *S*. Typhimurium and *S*. Rissen were common serotypes distributing in food-producing animals and meat products in Thailand (Lertworapreecha et al., 2013; Sinwat et al., 2016), South Korea (Lee et al., 2016), Vietnam (Thai et al., 2012), China (Zeng et al., 2019) and the US (Foley et al., 2008). *S*. Corvallis has been particularly reported from poultry in specific geographic areas e.g. Brazil (Yamatogi et al., 2015), Japan (Murakami et al., 2017), Turkey (Erol et al., 2013) and Bulgaria (Archambault et al., 2006). *S*. Corvallis were found in a few studies in Thailand mostly from poultry origin (Chotinun et al., 2014; Lampang et al., 2014).

Approximately 45% of the *Salmonella* isolates from Thailand-Cambodia provinces were MDR, which is much lower than previous studies in Thailand (64%) (Wannaprasat et al., 2011) and Thailand-Laos border provinces (98%) (Sinwat et al., 2016). However, it was similar to previous reports in China (Zeng et al., 2019) and Germany (Schwaiger et al., 2012). In contrast, it was higher than the reports in Vietnam (27%) (Thai et al., 2012) and Denmark (19%) (Arguello et al., 2013). These

data confirmed the role of pigs, broilers and their associated meat products as reservoir of MDR *Salmonella* strains and highlighted the importance of implementation legislation on prudent antimicrobial use in humans and food animals. In fact, the epidemiological data of AMR and resistance mechanisms is still limited in most Southeast Asian countries. As the data is invaluable, more investigation should be carried out by the relevant. The obtained findings may provide useful information for further public health projects in Thailand and Southeast Asia region.

The highest proportions of resistance were to ampicillin (70.7%), sulfamethoxazole (69.5%) and trimethoprim (29.6%) in the *Salmonella* isolates from both countries, in agreement with earlier studies (Wannaprasat et al., 2011; Sinwat et al., 2016). These results were also consistent with the *E. coli* isolates from the same samples. It is not surprising because these antimicrobials have been used for maintaining animal health and productivity in food animals for a quite long time in many countries in Asia (Vergne et al., 2014). Some antimicrobial agents (i.e. penicillin and sulfonamides) have been used at subtherapeutic doses for long periods of time in order to reduce bacterial competition in the gut and improve feed conversion (growth promotion) (Vergne et al., 2014). Such common practice is considered the improper use of antimicrobials in food-animal productions and may be the cause of the high rates of ampicillin, sulfamethoxazole and trimethoprim resistance were observed.

All ciprofloxacin-resistant *Salmonella* isolates (0.6%) in this study were also resistant to multiple drugs, in agreement with previous studies (Vo et al., 2006; Thai et al., 2012). Even though resistance to quinolones was at low prevalence, it is still a particular concern since fluoroquinolones are an antibiotic of choice for treatment of

multidrug resistant enteric fever and invasive *Salmonella* infection in human (WHO, 2019).

Resistance to 3rd-generation cephalosporins (ceftazidime, cefotaxime and cefpodoxime) was still low in both Thai (2–4.1%) and Cambodian (4.5–6%) isolates, in agreement with previous studies (Padungtod and Kaneene, 2006; Sinwat et al., 2016). These results are similar to those for *E. coli* isolates from the same sources in our recent study (Trongjit et al., 2016). Cephalosporins are among the most expensive antibiotics for feed medication in food-producing animals. Presently, the Thai DLD has banned the use of cephalosporins as medicated feed in food animals for any indications (MOAC, 2018; Lekagul et al., 2020). In Cambodia, use of cephalosporins in food animals is not prohibited. However, cephalosporins are not antibiotics for first-choice empiric treatment in animals due to its high cost. This may partly explain for low-level cephalosporin resistance rate among *Salmonella* in the areas.

The *dfrA12-aadA2* cassette array was predominate class 1 integrons-gene cassette in both Thai and Cambodian isolates, especially in pig samples, in agreement with previous reports (Padungtod et al., 2011; Sinwat et al., 2016). This *dfrA12-aadA2* gene cassette was previously reported in *Salmonella* strains from different sources and different geographical regions e.g. poultry in Korea (Dessie et al., 2013), farm animals in China (Zhao et al., 2017) and swine and human in Italy (Beutlich et al., 2013) (Fig. 11). The reports of class 1 integrons in several bacterial pathogens from different sources and different world region indicate the widespread of *dfrA12-aadA2* and highlight the important role of horizontal gene transfer across the world region. This was supported by our finding that class 1 integrons containing *dfrA12-aadA2* were conjugally transferred. Approximately 50% of *intI1* positive isolates from each country had no resistance gene cassette in variable region (i.e. empty integrons). This could be because resistance gene cassettes were cured in the

absence of antibiotic selective pressures or exchanged with other bacterial cells. Nevertheless, these empty integrons are available for capturing of new resistance gene cassettes and contribute to the spread of resistance genes among the bacterial pathogen (Bissonnette and Roy, 1992)

Low prevalence of ESBL-producing *Salmonella* detected in this study (1.7%) was in accordance to a previous study in Thailand-Laos border areas (Sinwat et al., 2016) and in the *E. coli* isolates from the same sources (Trongjit et al., 2016). All ESBL-producing *Salmonella* strains carrying $bla_{\text{CTX-M-55}}$ and simultaneously carried broad spectrum ß-lactamase, $bla_{\text{TEM-1}}$ gene. This finding indicated that ESBL-producing *Salmonella* frequently co-harbored more than one ß-lactamase gene (Nadimpalli et al., 2019; Dong et al., 2020). The dissemination of $bla_{\text{CTX-M-55}}$ has been demonstrated in many *Salmonella* serotypes from different origins, for example, *S.* Typhimurium from human in China (Dong et al., 2020), *S.* Indiana, *S*. Typhimurium and *S*. Chester from food animal and raw meat in China (Zhang et al., 2019a). Recently, the gene was found in *S.* Rissen, *S*. Typhimurium and *S.* Newport from retail meat and fish in Phnom Penh, Cambodia (Nadimpalli et al., 2019). The widespread of $bla_{\text{CTX-M-55}}$ in several *Salmonella* serotypes from different sources and different regions indicates that horizontal transfer serves as a significant key in the spread of ESBL genes (Fig. 12). This was additionally supported by our study that $bla_{\text{CTX-M-55}}$ was located on conjugative plasmids, confirming the potential spread of these ß-lactamase genes to other bacteria via horizontal transfer.

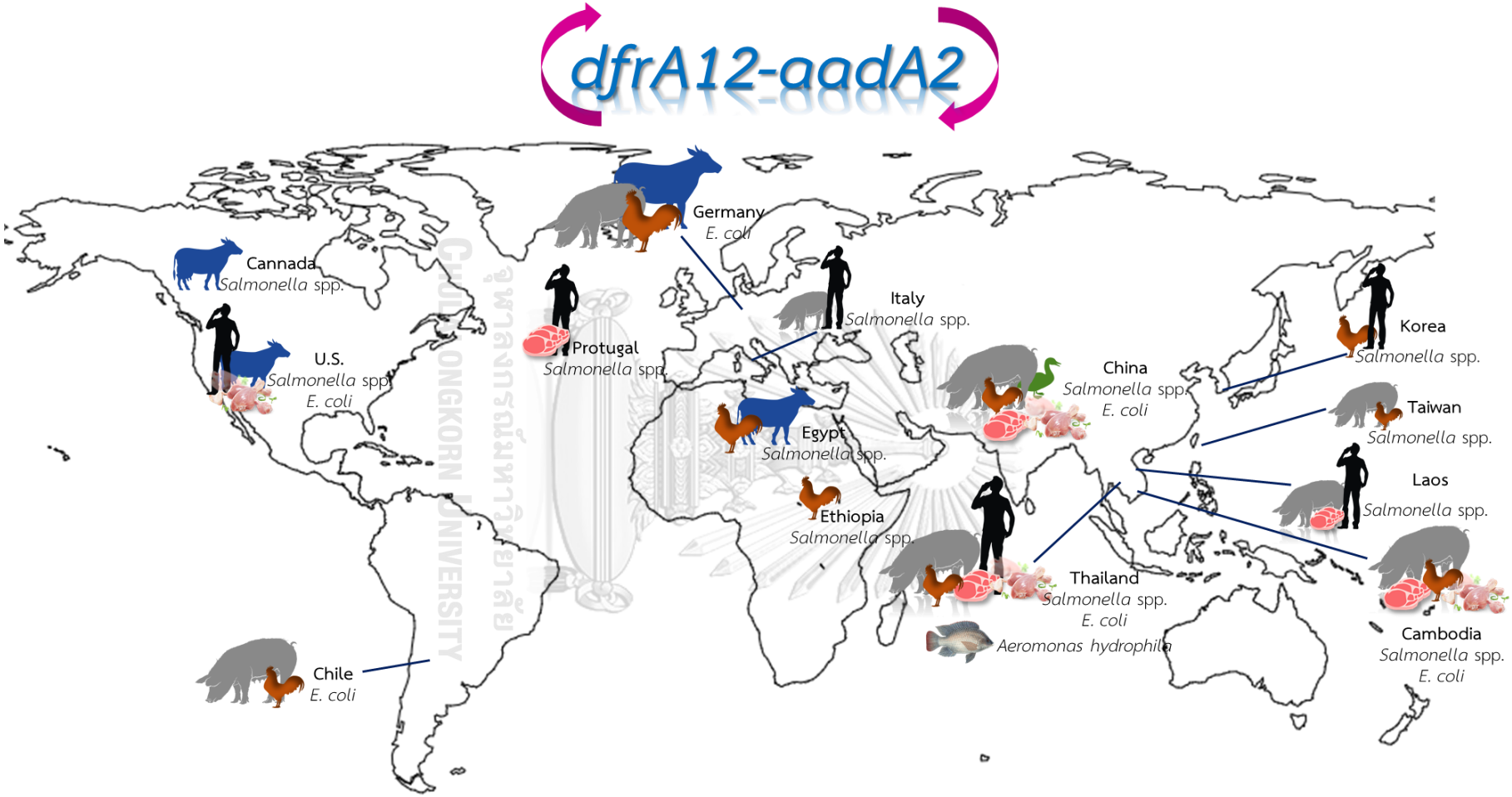


Figure 11. Global distribution of class 1 integrons harboring *dfrA12-aadA2* gene cassette array in bacteria isolates obtained from food-producing animals, meat products and human.

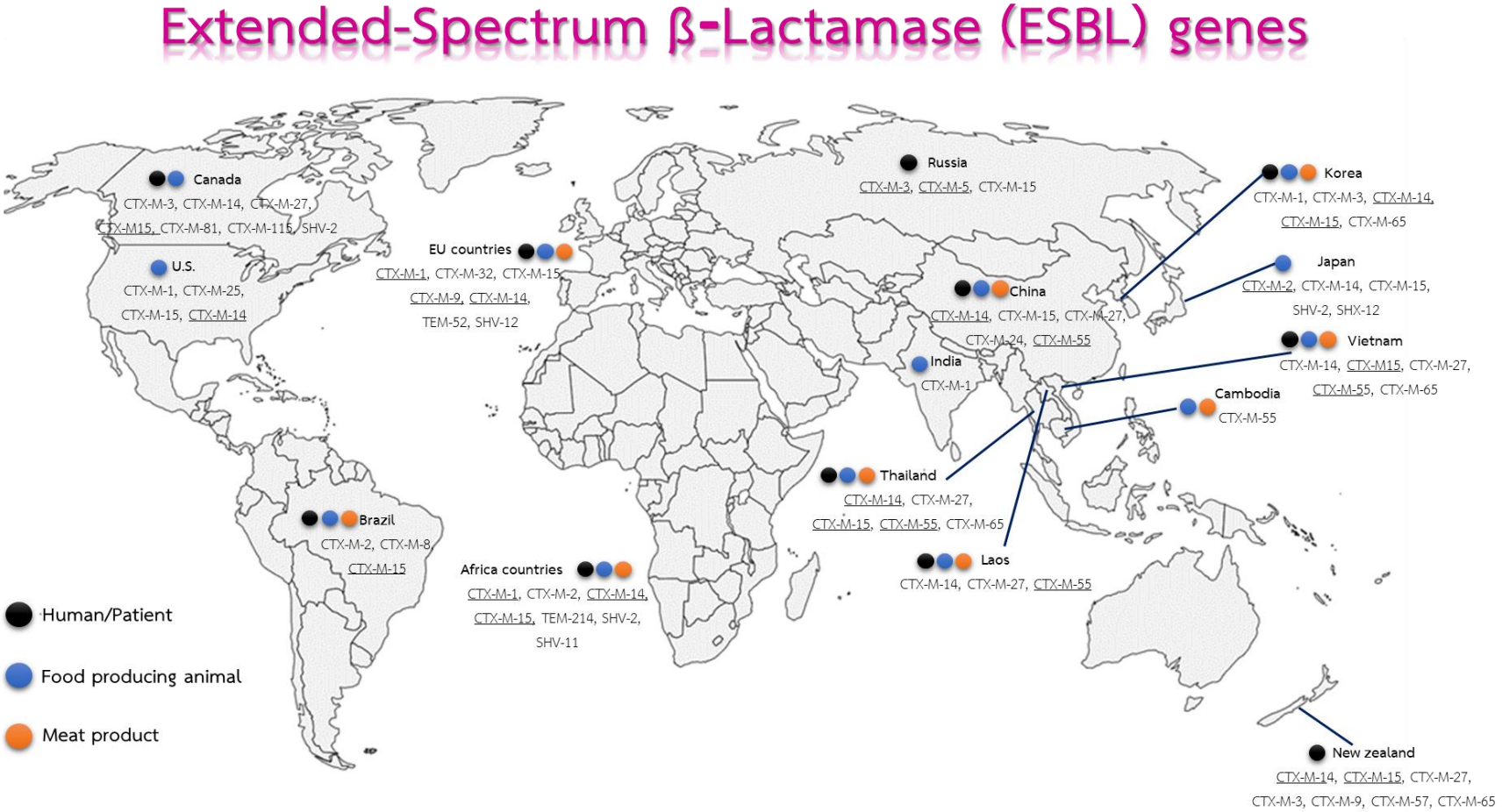


Figure 12. Global dissemination of ESBL genes frequently (underlined) reported in *Salmonella* and *E. coli* obtained from food producing animals, meat products and humans (isolated from 1983 to 2020).

Part 2. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces

In this present study, the overall prevalence of *E. coli* isolated from pigs, broilers and their meat products in Sa Kaeo, Thailand and Banteay Meanchey and Siem Reap, Cambodia were 57.1% and 58.8%, respectively. This prevalence was higher than previous studies in swine and chicken farming communities in Northern Thailand (Hanson et al., 2002), animals and their meat products in the US (21.7%) (Zhao et al., 2001) and Korea (14.9%) (Lee et al., 2009)

The prevalence of *E. coli* in the pig and pork samples in Thailand was significantly higher than that in Cambodia ($P < 0.05$). In contrast, the higher prevalence was observed in the broiler samples in Cambodia. The observation may be a result of poor sanitation and manufacturing practices during slaughtering, carcass cutting and meat handling processes, leading to excessively contaminated pork and broiler carcasses. Pig production in Thailand has been changed from small scale farming to intensive with medium- to large-scale production (Robinson et al., 2011). The modern technologies and farm management were introduced to pig farming to increase the productivity of animals on the farm (Thanapongtharm et al., 2016). Slaughtering process is regularly performed under GMP for pig abattoirs issued by ACSF (ACSF, 2006a) and animal welfare protocol (ACSF, 2010). However, inadequate hygienic practice can be observed at provincial slaughterhouses. In Cambodia, swine and poultry are mostly raised by semi commercial farm or backyard system. There are three commercial pig farms in the country financially supported by foreign-private companies (Tornimbene and Drew, 2012). Biosecurity in farms is generally inadequate especially in the backyard farm. It was pointed out that hygienic

practices during slaughter and dressing are common problems in Cambodia (Tornimbene and Drew, 2012).

One of the main finding was that the *E. coli* prevalence in broiler carcasses from fresh markets in Cambodia was significantly higher than that in Thailand ($P < 0.05$). In general, pork and chicken meat in fresh markets in Thailand and Cambodia are sold in the open air at ambient temperature. Thailand has specific regulations on fresh food markets' sanitary conditions, food safety and consumer protection authorized by the Thai Ministry of Public Health (Luong, 2016). The butchers are generally trained and awareness of hygiene and sanitation practice has been raised. Meat processing hygiene is given a priority and used of standard practice is regularly monitored. In Cambodia, where the rules and regulations on food hygiene and sanitation practice has got less attention. Raw meat is frequently delivered to fresh markets by open buckets on mopeds or small trailers without cooling system (Tornimbene and Drew, 2012). However, it should be noted that slaughterhouse and one fresh market from each province were included in this study. Thus, further research should be conducted with a larger population and in different areas in these countries.

The majority of the *E. coli* isolates from Thailand (77.4%) and Cambodia (72.4%) were MDR strains. The high resistance rates to ampicillin (88.8%), trimethoprim (75.9%) and sulfamethoxazole (68.6%) were observed in both countries. The data indicate that food animals and meat products are potential sources of AMR *E. coli* and pose a risk for the transfer of resistant bacteria to the food chain, environment and humans. Our findings appear to be well supported by a recent research showing that approximately 50% of Thai farmers have been using antibiotics for disease prevention depending on farmers' experience and farm income without veterinarian prescription (Lekagul et al., 2020). In Cambodia,

antimicrobial use in urban and peri-urban pig farming was commonly based on farmers' experiences and there was limited awareness of the risks and consequences related to AMU and AMR (Strom et al., 2018). Therefore, the restrictive policies on the use of antimicrobials in food animals are necessary to be enforced in both countries. Besides, improving of the farmer's knowledge on animal health and potential alternative interventions to antimicrobial use such as farm management, vaccination, disease prevention and control should be encouraged.

Low-resistance rate to fluoroquinolones (Thailand, 5% and Cambodia, 7%) and 3rd generation cephalosporins (Thailand, 9-10% and Cambodia, 7-8.4%) was observed in the areas of study. These resistance rates were quite lower than the recent studies in swine (Seenama et al., 2019) and poultry (Tansawai et al., 2019) from Northern Thailand, but higher than a previous study in Cambodia (Strom et al., 2018). Nevertheless, this should be again a particular concern because fluoroquinolones and 3rd generation cephalosporins are the first choice for treatment of *E. coli* infections in human (Terahara and Nishiura, 2019).

Approximately 53% of the *E. coli* isolates were positive to *intl1.* The prevalence was lower than previous studies in swine from central part of Thailand (71%-73%) (Lay et al., 2012; Changkaew et al., 2015) and other countries e.g. swine and broilers from China (59%) and broilers from the Netherlands (76%) (Van Essen-Zandbergen et al., 2007). There were 77% of Thai isolates and 86% of Cambodia isolates harbored empty integrons. These empty integrons might lost their gene cassettes in the absence of antibiotic selective pressure or due to the weak promotor that could not express resistance genes cassettes in the variable region. The empty integrons have been reported by other studies (Lapierre et al., 2008; Lay et al., 2012), indicating that these bacterial strains have the potential to capture

resistance gene cassettes and contribute to multidrug resistance phenotype (Lapierre et al., 2008).

The most predominate gene cassette in the Thai isolates was *dfrA12-aadA2* (41.5%), whereas the *dfrA1-aadA1* cassette array (70.8%) was most frequently found in the isolates from Cambodia. The different combination of *dfr* and *aadA* genes could be associated with the different type of trimethoprim and/or aminoglycoside usage or the different treatment protocol used in different geographic regions. These resistance gene cassette arrays were formerly identified in *E. coli* isolates from poultry and swine in Chile (Lapierre et al., 2008), *Salmonella* from pigs and human in Thailand-Laos border provinces (Sinwat et al., 2016), *Salmonella* from pigs, ducks and chickens in China (Li et al., 2013) and *Aeromonas hydrophila* from Nile tilapia in Thailand (Lukkana et al., 2012). Once again, it emphasized that horizontal gene transfer play an important role in the dissemination and the circulation of the resistance genes globally (Fig. 11).

Three *E. coli* isolates from Thailand (two isolates from chicken carcass and one from chicken meat) harbored two class 1 integrons, *dfrA12-aadA2* and *aadA2-linF*. The latter gene cassette array encoded resistance to spectinomycin and streptomycin -lincosamide. The *aadA2-linF* cassette array was individually frequently detected among Enterobacteriaceae isolates (Kor et al., 2013). However, the *aadA2-linF* cassette array is a rare combination. In Thailand, lincosamide has been commonly used in combination with spectinomycin in medicated feed for treatment of swine respiratory diseases and mucohemorrhagic diarrheal diseases (swine dysentery). In chicken production, it has been commonly used in form of injectable antibiotic combined with spectinomycin to increase the potency for treatment Infectious Coryza (*Avibacterium paragallinarum* infection). The combined use of lincosamide and spectinomycin in livestock could cause the high resistance rate of

drugs in these group and promote the spread of resistance gene cassette array. This could explain the coexistence of *aadA2-linF* gene cassette in *E. coli* isolates from Thai chicken in this study.

Sixteen *E. coli* isolates were confirmed to be ESBL producers (2.4%). This prevalence was lower than a previous study in healthy poultry (40 %) and swine (76.7%) farms from Northern and Eastern provinces of Thailand (Boonyasiri et al., 2014). All ESBL-producing *E. coli* isolates in this study were MDR, in agreement with previous studies (Boonyasiri et al., 2014; Seenama et al., 2019). Of all ESBL genes tested, only $bla_{\text{CTX-M-15}}$ was identified in four *E. coli* isolates from Sa Kaeo. The gene was previously reported in poultry, farmers and environment in Northern Thailand (Tansawai et al., 2019). It was shown that $bla_{\text{CTX-M-15}}$ has increasingly distributed across the world and is common in many countries (Figure 12). This was different from China, South-East Asia, South Korea, Japan and Spain, where CTX-M group 9 variants (especially $bla_{\text{CTX-M-14}}$) were predominant (Bevan et al., 2017). This discrepancy may be associated with different geographical locations, different types of cephalosporins used, and different antimicrobial selective pressure. The *ampC* gene, $bla_{\text{CMY-2}}$ was detected at low frequency in this present study. It has been previously described worldwide in *E. coli* from humans, animals and meat product in many countries i.e. Denmark (Noguchi et al., 2019), Norway (Berg et al., 2017), the Netherlands (De Been et al., 2014) and Korea (Seo et al., 2019).

Part 3. Plasmid-mediated colistin resistance and ESBL production in *E. coli* isolated from clinically healthy and sick pigs in Thailand, 2007-2018

The main finding in this study was high MDR rates in *E. coli* from healthy (97.5%) and sick (99%) pigs. The high rate of resistance to tetracycline (healthy pigs, 96.6% and sick pigs, 100%), ampicillin (healthy pigs, 90.1% and sick pigs, 97%) and

chloramphenicol (healthy pigs, 74.6% and sick pigs, 91%) were observed. These results were similar to a previous study in *E. coli* from pigs in Thailand (Ström et al., 2017). Sick pigs are expected to receive antimicrobial treatments for curing infection. In healthy pigs, it is more likely due to the long-term use of these antimicrobial classes for disease prevention in pig farms in Thailand.

Tetracycline is considered one of the most versatile antibiotics for therapeutic and prophylaxis purposes in pigs since it has a broad spectrum of activity. The common tetracyclines used in pig production are chlortetracycline and oxytetracycline (Lekagul et al., 2020). Chlortetracycline is recommended for fatteners, while oxytetracycline are frequently used in medicated feed for sows and piglets (Lekagul et al., 2020). Ampicillin is regularly administered for disease prevention and treatment swine respiratory infections and swine colibacillosis by injection route. The high chloramphenicol resistance rates were observed in all pig groups, although it has been banned for using in food producing animals in Thailand since 1988 (MOPH, 1988). This observation could be a result of co-selection and/or cross-resistance created by other antimicrobials. The phenomenon indicates that withdrawn a single antibiotic from livestock production could not completely eradicate AMR.

The prevalence of colistin resistance was high among healthy pigs (41%) and sick pigs (73%). It was much higher than previous studies in *E. coli* from diseased and healthy pigs in China (Li et al., 2018) and Japan (Fukuda et al., 2018). The majority of colistin resistance rate was found in the isolates from piglet (95.2%) and sick pig (73%). Colistin has been frequently recommended for addition to medicated feed for treatment of swine gastrointestinal tract infection. However most of colistin were intended for suckling and nursery pigs to treat post-waning diarrhea (PWD) and colibacillosis (Lekagul et al., 2020). Such excessive colistin consumption in pig

production may underly the presence of high colistin resistance in piglets and sick pigs in this study.

Of all *E. coli* isolates, 142 healthy pig isolates (40.1%) and 77 sick pig isolates (77%) were *mcr* carriers, of which the majority (45%) carried *mcr-3* (healthy pigs, 37.8% and sick pig, 70%). The worldwide distributed *mcr-1* was found at low frequency (healthy pig, 7.6% and sick pigs, 20%), while *mcr-2* was detected in five isolates from fattening pigs (1.4%). This is contradicted to previous studies reporting that *mcr-1* was the major colistin resistance gene in *E. coli* isolated from healthy and diseased pigs (Delannoy et al., 2017; Fukuda et al., 2018; Zhang et al., 2018). While *mcr-3* was very common among the isolates in this collection, especially in piglets (89%) and sick pigs (70%), a recent study in South Korea showed the prevalence of *mcr-3* in *E. coli* from diseased pigs has a tendency to increase during 2014-2018. It suggests that *mcr-3* and other *mcr* variants in livestock should be carefully monitored (Mechesso et al., 2020). The observation of different *mcr*-variants might be related to geographical location, antibiotic pressure, sample sources and sampling period. Co-occurrence of *mcr-1/mcr-3* (23% of piglets and 13% of sick pigs) and *mcr-2/mcr-3* (3.5% of fattening pigs) was detected in this study. Up to date, the co-occurrence of *mcr* genes has been reported in very few studies e.g. co-existence of *mcr-1/mcr-3* in *E. coli* from pigs, poultry and dogs in China (Zhang et al., 2018; Du et al., 2020) and cattle in Spain (Hernandez et al., 2017) and co-harboring of *mcr-2/mcr-3* in *E. coli* from pigs and poultry in China (Zhang et al., 2018) and pigs in Canada (Rhouma et al., 2019) (Fig. 13).

Interestingly, some *mcr-3* harboring *E. coli* were colistin-susceptible, while all *mcr-1* and *mcr*-2 carrying *E. coli* isolates exhibited resistance to colistin. This was in agreement with a previous study in Japan (Fukuda et al., 2018). These data suggest that colistin susceptibility and presence of *mcr* may not be consistently associated

and there are other factors affecting the resistance phenotype. Further study to examine the effect of individual *mcr* on colistin susceptibility is suggested. Besides, testing MIC of colistin and molecular screening of *mcr*-variants should be performed simultaneously in both colistin susceptible or resistant strains as the prevalence of *mcr-3* gene or other *mcr* genes may be underestimated.

The prevalence of ESBL-producing *E. coli* isolates was significantly higher in sick pigs (44%) than healthy pigs (19.2%) ($P < 0.05$). Among the healthy isolates, the piglet isolates (45.8%) presented the highest proportion of ESBL producers ($P < 0.05$) that was much lower than a previous study in *E. coli* from healthy pigs in Thailand (Boonyasiri et al., 2014). The 3rd generation cephalosporins have been frequently used for treatment of respiratory disease and diarrhea in clinically ill pigs. They were also considered prophylactic antibiotics for piglets after birth, during castration and prevention of diarrhea (Callens et al., 2012). Such extensive use of cephalosporins as therapeutic and prophylaxis treatment could explain the high prevalence of ESBL producers in sick pigs and piglet isolates. The $bla_{\text{CTX-M-14}}$ gene (54.5%) was dominant among healthy and sick pig isolates in this study, followed by $bla_{\text{CTX-M-55}}$ (42.9%). This is in agreement with previous reports conducted in ESBL-producing *E.coli* from livestock in other countries (i.e. chicken in Japan (Hayashi et al., 2018), swine and cattle in Korea (Tamang et al., 2013) and swine in China (Liao et al., 2015) (Fig. 12). In Thailand, the $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$ genes were the two major ESBL encoding gene in *E. coli* isolates from humans (Niumsup et al., 2008), animals (Seenama et al., 2019) and environment samples (Runcharoen et al., 2017), however the prevalence of each gene may vary depending on source of samples, study location and types of cephalosporins.

Co-existence of ESBL and *mcr* gene in ESBL-producing strains was commonly observed in this study (80.4%). The combination of $bla_{\text{CTX-M-55}}$ and *mcr-3* genes was

prevalent, in consistence with a previous study that reported the raising trend of co-existence ESBL and *mcr-1* in ESBL-producing *E. coli* from broiler in China. It was proposed that ESBL-producing strains are more likely to carry *mcr*-gene than non-ESBL producing strains (Wu et al., 2018a). Besides, all ESBL-*E. coli* harboring *mcr* strains in the present study were MDR, in agreement with a previous study (Wu et al., 2018a). By using ampicillin as a selectable marker, the co-transfer of ß-lactamase genes (bla_{TEM-1} or $bla_{CTX-M-55}$) and *mcr-3* was detected. This suggests that the global spread of *mcr*-genes can be a result of the co-selection by using other antimicrobial classes or vice versa. A study in China proposed that IncI2 harboring *mcr-1* plasmids conferred fitness advantage for its host (Wu et al., 2018b). Thus, the ban of colistin use in pig production may not effectively prevent the spread of colistin resistance.

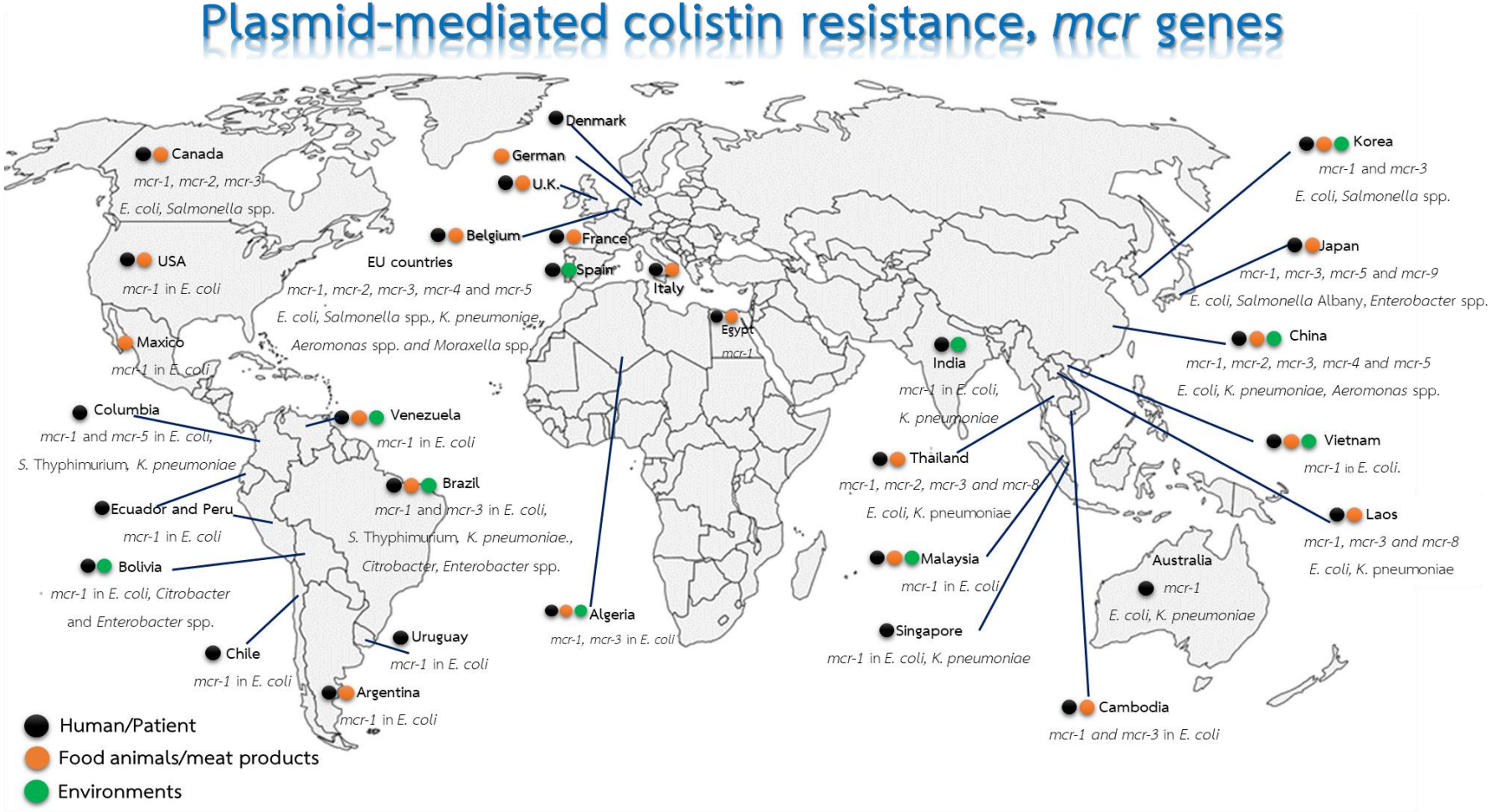


Figure 13. Global distribution of plasmid-mediated colistin resistance, *mcr* gene carrying bacteria isolates obtained from human, food animals, meat products and environment (isolated from 1980 to 2020).

Part 4. Whole Genome Sequencing and Characteristics of three *Escherichia coli* isolates with co-existence of ESBL and *mcr* genes from pigs in Thailand

The WGS analysis was performed in three porcine *E. coli* with *mcr* and ESBL co-expressing strains. One fattening pig isolate harboring *mcr-2.1/mcr-3.1* belonged to serotype O142:H31 and had ST 29 sequence type. The other two isolates from sick pigs with co-existence of *mcr-1.1/mcr-3.1* had serotype O9:H9 with ST10 sequence type. Several AMR genes were explored by using WGS, which is consistent to their AMR phenotype. This finding is in accordance with previous studies reporting most colistin-resistant strains were also multidrug resistance (Garcia-Menino et al., 2019; Flament-Simon et al., 2020), especially resistance to β-lactams or fluoroquinolones (Alba et al., 2018; Garcia et al., 2018).

The *mcr-1.1* gene from two sick pig isolates was located on IncI2 plasmid. This is consistent with previous reports demonstrating that the worldwide dissemination of *mcr-1* is mainly mediated by IncI2, IncX4 and IncHI2-type plasmid (Li et al., 2017; Matamoros et al., 2017; Poirel et al., 2017). Approximately 75% of *mcr-1* positive strains carrying an IncHI2 plasmid originated from Europe, while IncI2 plasmids have been prevalent replicon type among *mcr-1* carrying Enterobacteriaceae in Asia (Matamoros et al., 2017). The prevalence of particular plasmids carrying *mcr-1* may vary depending on geographical regions, antibiotic pressure and sample sources. Blast comparison of the *mcr-1.1*-IncI2 harboring-plasmids from two sick pig isolates with the referent plasmids sequence in NCBI database found that the *mcr-1.1*-IncI2 harboring plasmids of porcine *E. coli* had a typical plasmid backbone and high degree of similarity to the original plasmid pHNSHP45 in China (Liu et al., 2016). The IS*Apl1* transposon element serves as the main driver in horizontal gene transfer of *mcr-1* (Li et al., 2017; Snesrud et al., 2018).

In this study, the genetic context of *mcr-1.1* from two sick pig isolates contained a single-IS*Apl1* at upstream (IS*Apl1*–*mcr-1*–orf). This is in agreement with previous studies reporting that the IS*Apl1* at upstream structure was frequently observed in IncI2 and IncHI2 plasmids, while the lacking IS*Apl1* structure (*mcr-1*–orf) was commonly associated with IncX4 plasmids (Li et al., 2017; Matamoros et al., 2017; Sun et al., 2017). These differences in the genetic context of *mcr-1* gene could be a result of plasmid evolution after integrating the transposon. Losing the IS*Apl1* elements possibly makes plasmid become more stable.

The WGS data analysis performed in one *E. coli* isolate from fattening pig provided limited results on genetic context surrounding *mcr-2*. It was found that this *mcr-2.1* gene (1,617 bp) showed 100% similarity to the original plasmid pKP37-BE (LT598652) and it was directly flanked by IS*1595* at upstream region. This IS*1595* transposon was proposed to be an important key in the dissemination of *mcr-2* gene by the formation of a circular intermediate. Presently, the IS*1595* was renamed to ISE*c69* (Partridge, 2017).

Genetic context analysis of the genomic location plasmid harboring *mcr-3.1* were limited by the size of the contigs in this study. The obtained data revealed a core segment ΔTn*As2-mcr-3.1-dgkA* in all three *E. coli* isolates in addition to the assembly scaffolds containing *mcr-3.1* region had similar plasmid backbone to the originally published *mcr-3.1*-carrying plasmid pWJ1 (KY924928). The ΔTn*As2-mcr-3* element was previously identified in *E. coli* from pigs in Malaysia, *K. pneumoniae* from humans in Thailand, and *S.* Typhimurium from humans in the US (Yin et al., 2017). The truncated transposon element, Tn*As2*, was characterized only in *A. salmonicida* (Siguier et al., 2006). These findings indicated that the *mcr-3* gene in Enterobacteriaceae might have originated from *Aeromonas* species, and already widely disseminated in humans, animals, and the environment (Yin et al., 2017).

Moreover, several transposases and IS elements, including ΔIS*4321*, ΔTn*As2*, ΔIS*Kpn40*, IS*26* and IS*3* were identified in the flanking regions of *mcr-3.1* in three *E. coli* isolates in the present study. It is well recognized that the transposases and insertion sequences (IS) play an importance role in spreading of resistance determinants. The presence of several transposases and IS elements implies that the *mcr-3* may have a stronger potential in the dissemination than *mcr-1*. However, the contributive effect of individual IS elements and transposons on *mcr-3* mobility among different bacterial species remains to be clarified.

This study demonstrates the benefits of applying an advanced innovation of WGS by using Illumina sequencing technology to study the basic information on genetic variation and dynamics of plasmid transfer. The results obtained from WGS provide a comprehensive information to predict resistance genes from genomic data and the highest potential resolution for pathogen subtyping. However, a limitation of short reads obtained by WGS may adversely affect a perfect genome reconstruction due to the high number of repeated sequences in the bacterial genomes. Long read sequencing technologies may probably be a valuable tool bring new perspectives in future studies.

6.2 Conclusion and suggestions

In conclusion, the objectives of this study were accomplished. The results from this research project are invaluable as AMR data either phenotypic or genotypic levels is in demand. It can magnify the understanding of AMR related to animal sector in the region. Due to limitation of sample numbers, time from resistance genes detected, the results may not be able to repeat the whole Southeast Asia. Suggestions for data usage and future investigation is made.

The summary according to the research objectives are as follows:

Objective 1. To determine the occurrence of antimicrobial resistance among *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces.

The present study revealed the AMR situation of *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Thailand-Cambodia border provinces as well as *E. coli* from clinically healthy and sick pigs in Thailand. The main finding of this study was high contamination rate of MDR *E. coli* and *Salmonella enterica* in pigs, broilers and their associated meat products along Thailand-Cambodia border provinces. High prevalence of MDR in both commensal and pathogenic *E. coli* isolates from clinically healthy and sick pigs in Thailand was highlighted. The resistance rate to ampicillin, trimethoprim, sulfamethoxazole, and tetracycline was common among MDR *E. coli* and MDR *Salmonella*. The data highlighted the significant roles of *Salmonella* as zoonotic bacteria, which can potentially contaminated food animal and meat products to human by the food-borne route. These findings confirmed the important role of commensal *E. coli* as indicator bacteria for examination the effects of antimicrobial usage (AMU) and the spread of AMR in animal population in this area. Overall, the data in this study strongly suspected that AMR is a global problem and can affect to multi-health sectors (humans, animals and agricultural) as well as the global environment. Thus, the effective addressing on the raising threat of AMR requires a holistic and multisectoral approach. The necessary actions consist of regulation and policy on judicious antimicrobial use, AMR surveillance and monitoring program, disease prevention and infection control and alternative ways for antimicrobial treatments in all health sectors should be encouraged. In addition, appropriate hygiene protocols and biosecurity on farm, effective cleaning and disinfecting in slaughterhouses and

fresh markets should be implemented to minimize the dissemination of MDR organisms.

Objective 2. To characterize antimicrobial resistance of *Salmonella* and commensal *E. coli* from broilers, pigs and their meat products in Sa Kaeo, Banteay Meanchey and Siem Reap provinces.

Evidently, these is the widespread of class 1 integrons among *Salmonella* and *E. coli* obtained from broilers, pigs and their meat products in Thailand-Cambodia border provinces. These findings confirmed that class 1 integrons are important mobilized genetic elements that play a significant role in the dissemination of multidrug-resistance phenotype and AMR genes among pathogenic and commensal bacteria. The gene cassette array, *dfrA12-aadA2* was the dominant resistance gene cassettes. The results supported that horizontal transfer of resistance determinants contributes significantly to the spread of AMR bacteria. ESBL-producing *Salmonella* and *E. coli* were rather limited in this study area. Nevertheless, it should be a particular attention because the rapid increase in of ESBL-producing *E. coli* and *Salmonella* have been observed not only in clinical medicine, but also widely in food animal production. The latter finally affects human and veterinary medicine, society and economy.

The study of colistin resistance and ESBL producing *E. coli* in clinically healthy and sick pigs in Thailand highlighted that commensal and pathogenic *E. coli* serve as an important reservoir of ESBL and *mcr* genes encoding resistance to the highest priority critically important antimicrobials (HP-CIAs). This can pose a significant global health risk as these resistance genes can spread rapidly through horizontal transfer. The use of ampicillin could select for colistin resistance, highlighting the pandemic spread of *mcr*-genes can be a result of co-selection by other antimicrobial classes.

Particular concern is the coexistence of multiple clinically important antimicrobials (i.e. *mcr* and ESBL genes) in commensal *E. coli*, indicating a particular challenge for future treatment options in health care setting.

Taken together, these findings highlight the role of food animals and their meat products as a major reservoir of AMR determinants and horizontal gene transfer is a major route in their dissemination. Our research suggests that the AMR monitoring and surveillance programs by using standardized methods for sampling and assessing antimicrobial susceptibility should be regularly performed in both pathogenic and commensal bacteria at national and international levels. Besides, AMR surveillance program of ESBL production and colistin resistance at phenotypic and genotypic levels should be implemented at local national, regional and global levels. This will facilitate the understanding of the current trend and impact of AMR. Applying an innovative whole genome sequencing (WGS) in AMR surveillance can provide the rapid channel to obtain the vast data of AMR related to emerging public health threats. Integrating this advance technology along with comprehensive analysis within one health concept will help to predict the current trend and early emergence of AMR. The obtained sequencing data is also useful for describing the underlying mechanisms of AMR dissemination and provide key information to design strategies and action plans for minimizing the emergence and spread of AMR.

Additional suggestions are as follows:

1. The obtained data from this study could be applied in development of guidelines for prudent antimicrobial use in food animals in Thailand and neighboring countries.
2. These data can be utilized for veterinary education and animal health personnel and raising awareness and understanding on antimicrobial use in livestock.
3. The achieved data ca be used for controlling of cross border movements and facilitation of trade in animals and animal products.
4. These data can be used to monitor the newly emerging AMR determinants that could emerge at the border of Thailand and neighboring countries.
5. The results from WGS and plasmid mapping can be used to describe the co-existence of multiple drug resistance and can be applied for further study.
6. The national antimicrobial resistance database should be established, which can be used for surveillance of antimicrobial consumption in food animals, monitoring newly emerging AMR determinants, updating the current AMR situation in food animals and human and provide necessary data for risk assessment.
7. The national database to provide accessible bioinformatic resources should be founded to allow all countries, institutions and individuals to take advantage of the novel sequencing technologies.

6.3 Further studies are suggested, but not limited to, as follows:

1. The occurrence and characterization of antimicrobial resistance of *Salmonella* and commensal *E. coli* from food animals, their meat products along the food chain and human should be investigated in a larger population and in different areas in the region.
2. Genetic relatedness or clonal relationship in *Salmonella* and *E. coli* isolates from humans, livestock animals and foodstuffs along the food chain should be determined.
3. Since congregation of resistance and virulence plasmid in one isolate could lead to more resistance and pathogenic *Salmonella* and *E. coli* strains. The study on virulence factors in *Salmonella* and *E. coli* should be performed simultaneously with resistance profiles.
4. Plasmid characterization and plasmid mapping by using advanced technology in *Salmonella* and *E. coli* isolates in a larger number will provide comprehensive useful data for explanation of underlying resistance mechanism, evolution of resistance and spreading.
5. Risk assessment of AMR in food borne pathogens will be helpful to descript the potential impact on public health of the use of antimicrobials in animals.

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VITA

NAME	Suthathip Trongjit
DATE OF BIRTH	11 March 1988
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Doctor of Veterinary Medicine from Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2011
HOME ADDRESS	27 Soi Ekachai43/2, Ekachai Rd. Bangbon district, Bangkok, Thailand 10150
PUBLICATION	1. Occurrence and molecular characteristics of antimicrobial resistance of Escherichia coli from broilers, pigs and meat products in Thailand and Cambodia provinces (Microbiol Immunol 2016; 60: 575–585, doi: 10.1111/1348-0421.12407)
	2. Prevalence and antimicrobial resistance in Salmonella enterica isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces (Microbiol Immunol 2017; 61: 23–33, doi: 10.1111/1348-0421.12462)

APPENDICS

APPENDIX A

Parts of this dissertation have been published or presented in the conferences as follow:

List of international publication

1. Trongjit, S., Angkittitrakul, S. and Chuanchuen, R., 2016. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. Microbiology and immunology, 60(9), pp.575-585. doi: 10.1111/1348-0421.12407

2. Trongjit, S., Angkititrakul, S., Tuttle, R.E., Poungseree, J., Padungtod, P. and Chuanchuen, R., 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces. Microbiology and immunology, 61(1), pp.23-33. doi: 10.1111/1348-0421.12462

3. Trongjit, S., Assavacheep P., Samngamnim, S., Tran Hoang, M., Vo Thi Tra, An., Simjee, S., and Chuanchuen, R., 2021. Plasmid-mediated colistin resistance and ESBL production in *Escherichia coli* from clinically healthy and sick pigs in Thailand, 2007-2018 (submitted to Frontiers in Microbiology Journal)

List of international conferences

1. Trongjit, S, Angkittitrakul, S, Chuanchuen, R. Presence and phenotypic-genotypic antimicrobial resistance of *Escherichia coli* in broilers, pigs and meat products in the Thailand and Cambodia border provinces. Research

links on “Antimicrobial resistance in bacteria associated with livestock and animal products for Southeast Asian countries”, 16th-19thAugust 2016, Faculty of Vet Sci., Chulalongkorn University, Bangkok, Thailand.

2. Trongjit, S, Angkittitrakul, S, Chuanchuen, R. Molecular characteristics of antimicrobial resistances in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand-Cambodia border provinces. Research links on “Antimicrobial resistance in bacteria associated with livestock and animal products for Southeast Asian countries”, 16th-19th August 2016, Faculty of Vet Sci., Chulalongkorn University, Bangkok, Thailand

3. Trongjit, S, Assavacheep, P, Chuanchuen, R. Characterization of extended-spectrum beta-lactamase and colistin resistance mechanism in *Escherichia coli* isolated from pigs in Thailand. The 3rd International Conference on One Medicine One Science, 29th April – 2nd May 2018, University of Minnesota, USA.

List of local conferences

Trongjit, S., Angkititrakul, S., Tuttle, R.E., Padungtod, P. and Chuanchuen, R. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* and *Salmonella* from broilers, pigs and meat products in Thailand and Cambodia border provinces. RGJ - Ph.D. Congress 19 “INNOVATION CHALLENGES TOWARD THAILAND 4.0: Research Inspiration, Connectivity and Transformation”, 7th-9thJune, 2018, Jomtien Palm Beach Hotel & Resort, Chon Buri, Thailand.

APPENDIX B

Antimicrobial resistance pattern of *Salmonella* from Thailand and Cambodia (n=345)

Resistance patterns	No. of isolates(%)	
	Thailand (n=145)	Cambodia (n=200)
AMP	8 (5.5)	10 (5)
CHC	1 (0.7)	3 (1.5)
SUL	13 (8.9)	11 (5.5)
TRI	1 (0.7)	0
TET	1 (0.7)	0
CAZ	0	1 (0.5)
SUL-TET	2 (1.4)	4 (2)
SUL-TRI	2 (1.4)	2 (1)
CHC-SUL	1 (0.7)	0
AMP-CHC	0	1 (0.5)
AMP-TRI	0	3 (1.5)
AMP-TET	8 (5.5)	1 (0.5)
AMP-SUL	40 (27.6)	19 (9.5)
AMP-STP	2 (1.4)	11 (5.5)
TET-CAZ	0	1 (0.5)
SUL-CPD	0	1 (0.5)
STP-SUL	0	10 (5)
AMP-SUL-TET	8 (5.5)	3 (1.5)
AMP-SUL-TRI	1 (0.7)	20 (10)
AMP-SUL-CPD	1 (0.7)	0
AMP-STP-TET	5 (3.4)	5 (2.5)
AMP-STP-SUL	9 (6.2)	2 (1)
AMP-GEN-TET	1 (0.7)	0
AMP-GEN-SUL	1 (0.7)	1 (0.5)
AMP-CHC-TET	1 (0.7)	0
AMP-CIP-TET	1 (0.7)	0
AMP-CIP-STP	1 (0.7)	0
STP-SUL-TRI	0	1 (0.5)
STP-SUL-TET	0	3 (1.5)
CAZ-CTX-CPD	0	1 (0.5)

Antimicrobial resistance pattern of *Salmonella* from Thailand and Cambodia (n=345) (continued)

Resistance patterns	No. of isolates(%)	
	Thailand (n=145)	Cambodia (n=200)
CHC-GEN-SUL-CPD	1 (0.7)	0
GEN-CAZ-CTX-CPD	1 (0.7)	0
GEN-STP-SUL-TET	1 (0.7)	0
AMP-SUL-TRI-TET	3 (2.1)	12 (6)
AMP-STP-SUL-TET	6 (4)	1 (0.5)
AMP-STP-SUL-TRI	0	19 (9.5)
AMP-CHC-SUL-TRI	3 (2.1)	7 (3.5)
CHC-STP-SUL-TRI	0	1 (0.5)
STP-SUL-TRI-TET	0	1 (0.5)
TRI-CAZ-CTX-CPD	0	1 (0.5)
TET-CAZ-CTX-CPD	0	1 (0.5)
AMP-CHC-GEN-SUL-TET	0	1 (0.5)
AMP-CHC-STP-SUL-TRI	0	4 (2)
AMP-CHC-SUL-TRI-TET	0	6 (3)
AMP-GEN-SUL-TRI-TET	0	1 (0.5)
AMP-STP-SUL-TRI-TET	0	4 (2)
AMP-SUL-TET-CTX-CPD	1 (0.7)	0
AMP-STP-CAZ-CTX-CPD	0	1 (0.5)
STP-SUL-CAZ-CTX-CPD	0	1 (0.5)
AMP-CHC-STP-SUL-TRI-TET	3 (2.1)	1 (0.5)
AMP-CHC-GEN-SUL-TRI-TET	0	1 (0.5)
AMP-SUL-TRI-CAZ-CTX-CPD	0	1 (0.5)
AMP-STP-SUL-TRI-CAZ-CTX-CPD	0	3 (1.5)
AMP-SUL-TRI-TET-CAZ-CTX-CPD	0	1 (0.5)
AMP-CIP-CHC-STP-SUL-TET-CAZ-CPD	1 (0.7)	0
AMP-CHC-GEN-STP-SUL-TET-CAZ-CTX-CPD	1 (0.7)	0
Susceptible to all	16 (11)	18 (9)

AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim.

Antimicrobial resistance pattern of *Escherichia coli* from Thailand and Cambodia (n=667)

Resistance patterns	No. of isolates (%)	
	Thailand (n=381)	Cambodia (n=286)
AMP	21 (5.5)	16 (5.6)
SUL	1 (0.3)	1 (0.3)
TET	1 (0.3)	0
TRI	3 (0.8)	4 (1.4)
CPD	0	1 (0.3)
AMP-GEN	1 (0.3)	1 (0.3)
AMP-CHC	0	2 (0.7)
AMP-SUL	2 (0.5)	8 (2.8)
AMP-STP	3 (0.8)	2 (0.7)
AMP-TRI	15 (3.9)	12 (4.2)
AMP-TET	22 (5.8)	1 (0.3)
CHC-TET	0	1 (0.3)
STP-SUL	1 (0.3)	0
STP-TRI	5 (1.3)	0
SUL-TRI	2 (0.5)	10 (3.5)
TRI-TET	2 (0.5)	0
GEN-STP	1 (0.3)	0
AMP-GEN-STP	3 (0.8)	1 (0.3)
AMP-GEN-TET	1 (0.3)	0
AMP-GEN-SUL	1 (0.3)	0
AMP-TRI-TET	12 (3.1)	2 (0.7)
AMP-SUL-TET	2 (0.5)	2 (0.7)
AMP-SUL-TRI	29 (7.6)	48 (16.8)
AMP-STP-SUL	1 (0.3)	3 (1)
AMP-STP-TET	3 (0.8)	1 (0.3)
AMP-STP-TRI	1 (0.3)	0
AMP-CHC-TET	2 (0.5)	0
AMP-CHC-TRI	1 (0.3)	1 (0.3)
AMP-CHC-CTX	1 (0.3)	0
AMP-TRI-CAZ	1 (0.3)	0
CAZ-CTX-CPD	1 (0.3)	0

Antimicrobial resistance pattern of *Escherichia coli* from Thailand and Cambodia (n=667) (continued).

Resistance patterns	No. of isolates (%)	
	Thailand (n=381)	Cambodia (n=286)
STP-TRI-CAZ	1 (0.3)	0
TRI-TET-CTX	1 (0.3)	0
CHC-SUL-TRI	0	2 (0.7)
STP-SUL-TRI	0	1 (0.3)
SUL-TRI-TET	1 (0.3)	0
GEN-TRI-TET	2 (0.5)	0
AMP-CIP-SUL-TRI	0	2 (0.7)
AMP-CAZ-CTX-CPD	1 (0.3)	3 (1)
AMP-TRI-TET-CTX	1 (0.3)	0
AMP-CHC-SUL-TRI	1 (0.3)	21 (7.3)
AMP-CHC-SUL-TET	0	1 (0.3)
AMP-SUL-TRI-TET	47 (12.3)	10 (3.5)
AMP-SUL-TRI-CPD	0	1 (0.3)
AMP-STP-TRI-TET	4 (1)	0
AMP-STP-SUL-TET	1 (0.3)	1 (0.3)
AMP-STP-SUL-TRI	7 (1.8)	46 (16.1)
AMP-GEN-SUL-TET	1 (0.3)	0
AMP-GEN-SUL-TRI	0	3 (1)
AMP-GEN-STP-TET	3 (0.8)	1 (0.3)
AMP-CHC-STP-SUL	0	2 (0.7)
AMP-CHC-GEN-TRI	1 (0.3)	0
AMP-CHC-GEN-STP	2 (0.5)	0
AMP-CHC-GEN-SUL	0	1 (0.3)
AMP-CIP-SUL-TET	1 (0.3)	0
STP-TRI-TET-CPD	1 (0.3)	0
STP-SUL-TRI-CPD	1 (0.3)	0
GEN-STP-SUL-TRI	1 (0.3)	0
AMP-TRI-CAZ-CTX-CPD	2 (0.5)	1 (0.3)
AMP-TET-CAZ-CTX-CPD	2 (0.5)	0
AMP-SUL-TRI-TET-CTX	1 (0.3)	0
AMP-SUL-TRI-CAZ-CTX	0	2 (0.7)

Antimicrobial resistance pattern of *Escherichia coli* from Thailand and Cambodia (n=667) (continued).

Resistance patterns	No. of isolates (%)	
	Thailand (n=381)	Cambodia (n=286)
AMP-STP-TRI-TET-CPD	1 (0.3)	0
AMP-GEN-STP-SUL-CTX	1 (0.3)	0
AMP-STP-SUL-TRI-TET	24 (6.3)	2 (0.7)
AMP-STP-SUL-TRI-CAZ	0	1 (0.3)
AMP-STP-SUL-TRI-CTX	0	1 (0.3)
AMP-GEN-SUL-TRI-TET	6 (1.6)	1 (0.3)
AMP-GEN-STP-TRI-TET	4 (1)	0
AMP-GEN-STP-SUL-TRI	11 (2.9)	1 (0.3)
AMP-CHC-SUL-TRI-TET	3 (0.8)	6 (2.1)
AMP-CHC-STP-SUL-TRI	3 (0.8)	11 (3.8)
AMP-CHC-GEN-SUL-TRI	0	2 (0.7)
AMP-CIP-SUL-TRI-TET	2 (0.5)	0
AMP-CIP-STP-SUL-TET	1 (0.3)	0
AMP-CIP-STP-SUL-TET	1 (0.3)	0
AMP-CIP-STP-SUL-TRI	1 (0.3)	0
AMP-CIP-GEN-SUL-TRI	1 (0.3)	0
AMP-CIP-GEN-STP-TET	1 (0.3)	0
TRI-TET-CAZ-CTX-CPD	1 (0.3)	0
GEN-STP-TRI-CAZ-CPD	1 (0.3)	0
CHC-GEN-STP-SUL-TRI	1 (0.3)	0
AMP-SUL-TRI-CAZ-CTX-CPD	5 (1.3)	2 (0.7)
AMP-TRI-TET-CAZ-CTX-CPD	0	1 (0.3)
AMP-STP-SUL-TRI-TET-CTX	1 (0.3)	0
AMP-STP-SUL-TRI-TET-CPD	1 (0.3)	1 (0.3)
AMP-STP-TRI-CAZ-CTX-CPD	0	1 (0.3)
AMP-STP-SUL-CAZ-CTX-CPD	0	1 (0.3)
AMP-STP-SUL-TRI-CAZ-CTX	0	1 (0.3)
AMP-GEN-SUL-TRI-TET-CTX	2 (0.5)	0
AMP-CIP-GEN-SUL-TRI-CPD	1 (0.3)	0
AMP-GEN-STP-SUL-TRI-TET	20 (5.2)	1 (0.3)
AMP-CHC-STP-SUL-TRI-TET	7 (1.8)	2 (0.7)

Antimicrobial resistance pattern of *Escherichia coli* from Thailand and Cambodia (n=667) (continued).

Resistance patterns	No. of isolates (%)	
	Thailand (n=381)	Cambodia (n=286)
AMP-CHC-STP-SUL-TRI-CAZ	0	2 (0.7)
AMP-CHC-GEN-SUL-TRI-TET	4 (1)	0
AMP-CHC-GEN-STP-SUL-TET	1 (0.3)	0
AMP-CHC-GEN-STP-SUL-TRI	2 (0.5)	5 (1.7)
AMP-CIP-STP-SUL-TRI-TET	1 (0.3)	0
AMP-CIP-GEN-SUL-TRI-TET	1 (0.3)	0
GEN-TRI-TET-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-SUL-TRI-CAZ-CTX-CPD	1 (0.3)	1 (0.3)
AMP-SUL-TRI-TET-CAZ-CTX-CPD	4 (1)	1 (0.3)
AMP-STP-SUL-TRI-CAZ-CTX-CPD	2 (0.5)	2
AMP-GEN-STP-SUL-TRI-TET-CPD	1 (0.3)	0
AMP-GEN-STP-SUL-TRI-TET-CTX	1 (0.3)	0
AMP-CHC-STP-SUL-TRI-TET-CPD	1 (0.3)	0
AMP-CHC-STP-SUL-TRI-CAZ-CPD	0	1 (0.3)
AMP-CIP-GEN-STP-SUL-TET-CTX	1(0.3)	0
AMP-CIP-GEN-STP-SUL-TRI-TET	2(0.5)	0
AMP-CIP-CHC-STP-SUL-TRI-TET	2(0.5)	0
AMP-CHC-GEN-STP-SUL-TRI-TET	15(3.9)	0
AMP-CHC-GEN-STP-SUL-TRI-CAZ	0	1 (0.3)
AMP-STP-SUL-TRI-TET-CAZ-CTX-CPD	3(0.8)	0
AMP-GEN-STP-SUL-TET-CAZ-CTX-CPD	1(0.3)	0
AMP-GEN-SUL-TRI-TET-CAZ-CTX-CPD	0	1 (0.3)
AMP-CHC-SUL-TRI-TET-CAZ-CTX-CPD	2(0.5)	0
AMP-CHC-STP-SUL-TRI-CAZ-CTX-CPD	1(0.3)	1 (0.3)
AMP-CIP-CHC-GEN-STP-SUL-TRI-TET	2(0.5)	0
AMP-CHC-STP-SUL-TRI-TET-CAZ-CTX-CPD	1(0.3)	1 (0.3)
AMP-CHC-GEN-SUL-TRI-TET-CAZ-CTX-CPD	2(0.5)	0
AMP-CIP-CHC-STP-SUL-TRI-TET-CAZ-CTX-CPD	1(0.3)	0
Susceptible all	6(1.6)	20 (7)

AMP, ampicillin; CAZ, ceftazidime; CHC, chloramphenicol; CIP, ciprofloxacin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim.

Antimicrobial resistance patterns of *E. coli* from healthy pigs (n=354) and clinically sick pigs (n=100) in Thailand from 2007-2018

Resistance pattern	No. of isolates (%)	
	Healthy pigs (N=354)	Sick pigs (N=100)
CIP	1 (0.3)	0
CIP-GEN	2 (0.6)	0
CIP-TET	0	1 (1)
TET-TMP	2 (0.6)	0
AMP-CHC-TET	8 (2.3)	1 (1)
AMP-CIP-STR	1 (0.3)	0
AMP-COL-TET	1 (0.3)	0
AMP-STR-TET	2 (0.6)	0
AMP-SUL-TET	2 (0.6)	0
CHC-TET-TMP	11 (3.1)	0
CIP-SUL-TET	0	1 (1)
SUL-TET-TMP	1 (0.3)	0
AMP-CHC-CIP-TET	2 (0.6)	0
AMP-CHC-SUL-TET	1 (0.3)	0
AMP-CHC-TET-TMP	6 (1.7)	0
AMP-GEN-STR-TET	2 (0.6)	0
AMP-STR-TET-TMP	1 (0.3)	0
AMP-STR-SUL-TET	17 (4.8)	0
AMP-SUL-TET-TMP	4 (1.1)	1 (1)
CHC-CIP-TET-TMP	1 (0.3)	0
CHC-SUL-TET-TMP	1 (0.3)	0
CHC-STR-SUL-TET	1 (0.3)	0
CHC-STR-TET-TMP	3 (0.8)	0
CIP-COL-GEN-TET	0	1 (1)
STR-SUL-TET-TMP	2 (0.6)	0
AMP-CHC-COL-STR-TET	1 (0.3)	0
AMP-CHC-SUL-TET-TMP	9 (2.5)	0
AMP-CHC-STR-TET-TMP	1(0.3)	0
AMP-CHC-STR-SUL-TET	1 (0.3)	0
AMP-COL-STR-SUL-TMP	2 (0.6)	0
AMP-COL-SUL-TET-TMP	1 (0.3)	0

Antimicrobial resistance patterns of *E. coli* from healthy pigs (n=354) and clinically sick pigs (n=100) in Thailand from 2007-2018. (continued)

Resistance pattern	No. of isolates (%)	
	Healthy pigs (N=354)	Sick pigs (N=100)
AMP-GEN-SUL-TET-TMP	1 (0.3)	0
AMP-GEN-STR-SUL-TET	1 (0.3)	1 (1)
AMP-STR-SUL-TET-TMP	21 (5.9)	0
CHC-CIP-COL-SUL-TET	1 (0.3)	0
CHC-COL-STR-TET-TMP	4 (1.1)	0
AMP-CHC-COL-STR-SUL-TET	1 (0.3)	0
AMP-CHC-COL-SUL-TET-TMP	2 (0.6)	0
AMP-CHC-STR-SUL-TET-TMP	44 (12.4)	0
AMP-CHC-GEN-STR-TET-TMP	3 (0.8)	0
AMP-CHC-GEN-SUL-TET-TMP	3 (0.8)	0
AMP-CHC-CIP-GEN-STR-TET	0	1 (1)
AMP-CIP-COL-SUL-TET-TMP	1 (0.3)	1 (1)
AMP-CIP-STR-SUL-TET-TMP	1 (0.3)	0
AMP-COL-STR-SUL-TET-TMP	1 (0.3)	0
AMP-COL-GEN-SUL-TET-TMP	1 (0.3)	0
AMP-GEN-STR-SUL-TET-TMP	3 (0.8)	1 (1)
AMP-CHC-CIP-STR-SUL-TET-TMP	5 (1.4)	0
AMP-CHC-COL-STR-SUL-TET-TMP	2 (0.6)	0
AMP-CIP-GEN-STR-SUL-TET-TMP	1 (0.3)	0
AMP-COL-GEN-STR-SUL-TET-TMP	3 (0.8)	0
AMP-COL-STR-TET-CAZ-CTX-CPD	1 (0.3)	0
AMP-COL-GEN-STR-SUL-TET-TMP	4 (1.1)	0
AMP-CHC-CIP-GEN-SUL-TET-TMP	1 (0.3)	0
AMP-CIP-COL-GEN-STR-TET-TMP	1 (0.3)	0
AMP-CHC-COL-STR-SUL-TMP-TET	0	1 (1)
AMP-CHC-GEN-STR-SUL-TET-TMP	24 (6.8)	0
AMP-CHC-CIP-COL-GEN-SUL-TET	1 (0.3)	1 (1)
CHC-CIP-COL-GEN-STR-SUL-TET	1 (0.3)	0
AMP-CHC-CIP-COL-SUL-TET-TMP	5 (1.4)	0
AMP-CHC-CIP-COL-GEN-TET-TMP	1 (0.3)	0
AMP-CHC-CIP-GEN-SUL-TET-TMP	0	1 (1)

Antimicrobial resistance patterns of *E. coli* from healthy pigs (n=354) and clinically sick pigs (n=100) in Thailand from 2007-2018. (continued)

Resistance pattern	No. of isolates (%)	
	Healthy pigs (N=354)	Sick pigs (N=100)
AMP-CIP-COLGEN-SUL-TET-TMP	0	1(1)
AMP-CHC-COL-GEN-STR-SUL-TET-TMP	4 (1.1)	1 (1)
AMP-CHC-CIP-COL-GEN-SUL-TET-TMP	6 (1.7)	1 (1)
AMP-CHC-CIP-COL-GEN-STR-TET-TMP	0	3 (3)
AMP-CHC-CIP-COL-STR-SUL-TET-TMP	13 (3.7)	4 (4)
AMP-CHC-CIP-STR-SUL-TET-TMP-CPD	1 (0.3)	0
AMP-CHC-CIP-GEN-STR-SUL-TET-TMP	5 (1.4)	7 (7)
AMP-CHC-CIP-COL-GEN-TET-CTX-CPD	0	1 (1)
AMP-CHC-STR-SUL-TET-TMP-CTX-CPD	0	1 (1)
AMP-CHC-GEN-SUL-TET-TMP-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-STR-TET-TMP	2 (0.6)	0
AMP-COL-GEN-SUL-TET-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP	0	16 (16)
AMP-CHC-COL-GEN-STR-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-COL-GEN-STR-SUL-TET-CTX-CPD	1 (0.3)	0
AMP-CHC-COL-STR-SUL-TET-TMP-CTX-CPD	0	1 (1)
AMP-CHC-GEN-STR-SUL-TET-TMP-CTX-CPD	4 (1.1)	1 (1)
AMP-CHC-GEN-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-CIP-COL-GEN-SUL-TET-TMP-CAZ	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP	19 (5.4)	2 (2)
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-CTX	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-SUL-TET-CTX-CPD	0	1 (1)
AMP-CHC-CIP-GEN-SUL-TET-TMP-CTX-CPD	0	1 (1)
AMP-CHC-STR-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CIP-GEN-STR-SUL-TET-TMP-CTX-CPD	2 (0.6)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-CTX-CPD	0	3 (3)
AMP-CHC-COL-GEN-STR-SUL-TET-TMP-CTX-CPD	3 (0.8)	0
AMP-CHC-COL-GEN-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CIP-COL-GEN-STR-TET-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CIP-COL-STR-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-CIP-COL-GEN-SUL-TET-TMP-CTX-CPD	2 (0.6)	1 (1)

Antimicrobial resistance patterns of *E. coli* from healthy pigs (n=354) and clinically sick pigs (n=100) in Thailand from 2007-2018. (continued)

Resistance pattern	No. of isolates (%)	
	Healthy pigs (N=354)	Sick pigs (N=100)
AMP-CHC-GEN-STR-SUL-TET-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-TET-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-GEN-STR-SUL-TMP-TMP-CTX-CPD	1 (0.3)	3 (3)
AMP-CHC-CIP-COL-GEN-STR-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-STR-TET-TMP-CTX-CPD	4 (1.1)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP-CAZ	2 (0.6)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-CTX-CPD	6 (1.7)	0
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP-CPD	1 (0.3)	0
AMP-CHC-CIP-COL-GEN-SUL-TET-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-CIP-COL-STR-SUL-TET-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-GEN-STR-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-CIP-COL-GEN-SUL-TET-TMP-CAZ-CTX-CPD	6 (1.7)	0
AMP-CHC-COL-GEN-STR-SUL-TET-TMP-CAZ-CTX-CPD	1 (0.3)	0
AMP-CHC-CIP-GEN-STR-SUL-TET-TMP-CAZ-CTX-CPD	1 (0.3)	3 (3)
AMP-CHC-CIP-COL-GEN-SUL-TET-TMP-CAZ-CTX-CPD	4 (1.1)	2 (2)
AMP-CHC-CIP-COL-GEN-STR-TET-TMP-CAZ-CTX-CPD	7 (2)	1 (1)
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-CAZ-CTX-CPD	1 (0.3)	4 (4)
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP-CTX-CPD	10 (2.8)	3 (3)
AMP-CHC-CIP-COL-STR-SUL-TET-TMP-CAZ-CTX-CPD	0	1 (1)
AMP-CHC-CIP-COL-GEN-STR-SUL-TET-TMP-CAZ-CTX-CPD	8 (2.3)	19 (19)
Susceptible to all	5 (1.4)	0

AMP, ampicillin; CAZ, ceftazidime; CIP, ciprofloxacin; CHC, chloramphenicol; COL, colistin; CPD, cefpodoxime; CTX, cefotaxime; GEN, gentamicin; STP, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim