GENETIC CHARACTERISTICS OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM PIGS, PORK AND HUMANS IN THAILAND AND LAO PDR BORDER PROVINCES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ลักษณะทางอณูชีววิทยาของการดื้อยาของเชื้อเอสเชอริเชีย โคไลที่แยกได้จากสุกร เนื้อสุกร และคน ในเขตจังหวัดชายแดนประเทศไทยและประเทศลาว

น.ส.ชนิกา พึ่งเพียร

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Thesis Title	GENETIC CHARACTERISTICS OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM PIGS, PORK AND HUMANS IN THAILAND AND LAO PDR BORDER PROVINCES
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Field of Study	Veterinary Public Health
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Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

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ชนิกา พึ่งเพียร : ลักษณะทางอณูชีววิทยาของการดื้อยาของเชื้อเอสเชอริเชีย โคไลที่แยกได้จากสุกร เนื้อสุกร และคนในเขตจังหวัดชายแดนประเทศไทยและประเทศลาว. (GENETIC CHARACTERISTICS OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* ISOLATED FROM PIGS, PORK AND HUMANS IN THAILAND AND LAO PDR BORDER PROVINCES) อ.ที่ปรึกษาหลัก : ศ. สพ.ญ. ดร.รุ่งทิพย์ ชวนชื่น

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความชุกของการดื้อยาและ การศึกษาคุณลักษณะการดื้อยาของเอสเชอริเชีย โคไล ที่แยกได้จากสุกร เนื้อสุกร และคนในเขตพื้นที่ชายแดนประเทศไทยและประเทศลาว และเปรียบเทียบลักษณะการดื้อยาปฏิชีวนะในกลุ่มยาทางเลือกสุดท้ายของเอสเชอริเชีย โคไล และซัลโมเนลลาที่แยกได้จากสุกร และเนื้อสุกร ในเขตพื้นที่จังหวัดชายแดนไทย-ลาว และเขตพื้นที่จังหวัดชายแดนไทยกัมพูชา การศึกษานี้ประกอบด้วย 3 โครงการวิจัย ได้แก่ โครงการวิจัยที่ 1 การศึกษาลักษณะปรากฏของการดื้อยาและการศึกษาลักษณะทางพันธุกรรมของการดื้อยาในเอสเชอริเชีย โคไลที่แยกได้จากสุกร เนื้อสุกร และคนในเขตพื้นที่ชายแดนประเทศไทยและประเทศลาว จากจำนวนเอสเชอริเชีย โคไลทั้งสิ้น 847 เชื้อ เป็นเอสเชอริเชีย โคไลที่แยกได้จากสุกร ซากสุกร เนื้อสุกร และคนในเขตพื้นที่จังหวัดชายแดนประเทศไทยและประเทศลาว ผลการศึกษาพบว่าเชื้อส่วนใหญ่ ร้อยละ 67 มีการดื้อยาปฏิชีวนะหลายชนิดพร้อมกัน Class 1 integrons ที่มี gene cassette array ชนิด *aadA1* (37.2%) พบมากที่สุด การศึกษา ESBL พบว่า เอสเชอริเชีย โคไลที่สามารถผลิตเอนไซม์ ESBL ตรวจพบในอัตราส่วนที่ต่ำทั้งในเชื้อจากประเทศไทย (3.4%) และประเทศลาว (3.2%) ยีน ESBL ที่ถูกตรวจพบ ได้แก่ $bla_{CTX\text{-}M14}$ $bla_{CTX\text{-}M27}$ และ $bla_{CTX\text{-}M55}$ โดยพบว่า $bla_{CTX\text{-}M55}$ เป็นยีนที่ตรวจพบมากที่สุด (58.6%) การเปลี่ยนแปลงของกรดอะมิโนที่ตำแหน่ง Ser-83-Leu และ Asp-87-Asn บนโปรตีน GyrA ถูกพบมากที่สุดในเชื้อที่มีการดื้อต่อยา ciprofloxacin ยีนบนพลาสมิดที่เกี่ยวข้องกับการดื้อยากลุ่ม quinolone ชนิด *qnrA* และ *qnrB* ถูกพบในสัดส่วนที่ต่ำ ร้อยละ 0.1% แต่ยีน *qnrS* ถูกพบในสัดส่วนที่สูง ร้อยละ 23 ของเชื้อทั้งหมด ผลการศึกษา Class 1 integrons ที่มียีน *aadA1* ที่แยกได้จากสุกร (จำนวน 1 เชื้อ) และยีน ESBL ($bla_{CTX\text{-}M55}$ และ $bla_{CTX\text{-}M14}$) จากสุกร (จำนวน 2 เชื้อ) เนื้อสุกร (จำนวน 1 เชื้อ) และคน (จำนวน 7 เชื้อ) อยู่บนพลาสมิดชนิด conjugative plasmids นอกจากนี้พลาสมิดส่วนใหญ่ ร้อยละ 29.3 เป็นพลาสมิดในกลุ่ม IncF โครงการวิจัยที่ 2 ศึกษาการดื้อต่อยา colistin และยีน *mcr* ในเอสเชอริเชีย โคไล และซัลโมเนลลาที่แยกได้จากสุกร ซากสุกร และเนื้อสุกรบริเวณเขตพื้นที่จังหวัดชายแดนไทย-ลาว และเขตพื้นที่จังหวัดชายแดนไทย-กัมพูชา จากจำนวนเอสเชอริเชีย โคไลและและซัลโมเนลลาทั้งสิ้น 1619 เชื้อ ผลการศึกษาพบว่าอัตราการดื้อต่อยา colistin ถูกพบในเอสเชอริเชีย โคไล (8%) มากกว่าซัลโมเนลลา (1%) โดยพบอัตราการดื้อต่อ colistinมากที่สุดในเชื้อจากประเทศกัมพูชา ร้อยละ 10.1 ยีนที่เกี่ยวข้องกับการดื้อยา colistin ที่พบได้แก่ *mcr-1 mcr-3* และ *mcr-5* โดยยีน *mcr-1* และ *mcr-3* พบมากที่สุด พบยีน *mcr-1* หรือ *mcr-3* ในเอสเชอริเชีย โคไล และซัลโมเนลลาจำนวน 7 เชื้อ อยู่บนพลาสมิดชนิด conjugative plasmids ในกลุ่ม IncF และ/หรือ IncI ซัลโมเนลลาที่มียีน *mcr* จากประเทศไทยและประเทศกัมพูชาถูกจัดลักษณะได้ใน 2 กลุ่ม ไม่พบการดื้อต่อยา meropenem ในเอสเชอริเชีย โคไล และซัลโมเนลลาในการศึกษานี้ โครงการวิจัยที่ 3 ศึกษาลักษณะทางอณูชีววิทยาของการดื้อยาในเอสเชอริเชีย โคไล ที่สร้างเอนไซม์ ESBL และมียีน *mcr* จำนวน 7 เชื้อ ประกอบด้วย เอสเชอริเชีย โคไลที่แยกได้จากสุกรจำนวน 2 เชื้อ (TH2 และ TH3) และคนจำนวน 2 เชื้อ (TH8 และ TH9) ในประเทศไทย และจากสุกรในเขตพื้นที่ประเทศลาว จำนวน 3 เชื้อ (LA1 LA2 และ LA3) โดยใช้เทคนิคการหาลำดับเบสของสารพันธุกรรมทั้งหมด (WGS) จากการศึกษาพบ sequence types/serotypes จำนวน 4 ชนิด ได้แก่ ST6833/H20 (TH2 และ TH3) ST48/O160:H40 (TH8 และ TH9) ST5708/H45 (LA1) และ ST10562/O148:H30 (LA2 และ LA3) ทุกเชื้อ มี point mutation ที่ตำแหน่ง Ser-31-Thr บน PmrA และ His-2-Arg บน PmrB (colistin MIC=4-8 µg/mL) นอกจากนี้ LA1 มี point mutation 5 ตำแหน่งบน PmrB (colistin MIC=8 µg/mL) ตรวจพบยีน *mcr-1.1* ทั้งหมดมีโครงสร้างชนิด *ISApl1-mcr-1-pap2* ในขณะที่ *mcr-3.1* ทั้งหมดมี core segment: Δ*TnAs2-mcr-3.1-dgkA-ISKpn40* ตรวจพบ *mcr-3.1* และ $bla_{CTX\text{-}M55}$ อยู่บนพลาสมิดเดียวกันในเชื้อ TH2 TH3 TH8 TH9 และ LA1 และพบการปรากฏร่วมของยีนดื้อยาหลายชนิด (*cml qnrS1* และ *tmrB*) บนพลาสมิดนี้ พบการถ่ายทอดร่วม-ของยีน $bla_{CTX\text{-}M55}$ และ *mcr-3.1* ใน LA1 ภายใต้คัดเลือกด้วยการใช้ยา ampicillin หรือ colistin แต่พบการส่งผ่านยีนดื้อยาเฉพาะ $bla_{CTX\text{-}M55}$ ใน TH8 และ TH9 แม้ว่า $bla_{CTX\text{-}M55}$ อยู่บนพลาสมิดเดียวกันกับยีน *mcr-3.1* ตรวจพบ virulence gene อย่างน้อย 1 ชนิดในเอสเชอริเชีย โคไลทุกตัวอย่าง โดยสรุปจากผลการศึกษานี้พบเอสเชอริเชีย โคไลและซัลโมเนลลาที่มีการดื้อยาปฏิชีวนะหลายชนิดพร้อมกัน ในอัตราสูงและพบการหมุนเวียนของยีนดื้อยาเกิดขึ้นในบริเวณที่ศึกษา พบการดื้อยาในกลุ่มยาทางเลือกสุดท้ายโดยเฉพาะ new generation cephalosporins และ colistin ในเอสเชอริเชีย โคไลและซัลโมเนลลา ที่แยกได้จากสุกร เนื้อสุกร และคนในบริเวณประเทศไทย ประเทศลาว และประเทศกัมพูชา ดังนั้นการควบคุมการดื้อยาจำเป็นต้องมีความร่วมมือจากทุกภาคส่วนภายใต้แนวความคิดสุขภาพเป็นหนึ่งเดียว

สาขาวิชา สัตวแพทยสาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2563 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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The objectives of this study were to monitor the prevalence of antimicrobial resistance (AMR) among *Escherichia coli* isolated from pigs, pork and humans in Thailand-Laos border areas, characterize AMR of *E. coli* isolated from pigs, pork and humans in Thailand-Laos border areas, and compare the resistance to last-line antimicrobials of *E. coli* and *Salmonella* isolated from pigs and pork in Thailand-Laos and Thai-Cambodia border areas. Three study projects were conducted. Project 1 demonstrated the results of phenotypic and genotypic monitoring of AMR in *E. coli* in pigs, pork, and humans in Thailand and Lao PDR border provinces. A total of 847 *E. coli* isolates were obtained from pigs, pig carcasses, pork, and humans in Thailand and Lao PDR border provinces. Most isolates (67%) were multidrug resistance (MDR). Class 1 integrons carrying *aadA1* gene cassette array were most observed (37.2%). The low percentage of ESBL-producing *E. coli* was observed in Thailand (3.4%) and Lao PDR (3.2%). The ESBLs genes found were $bla_{CTX\text{-}M14}$, $bla_{CTX\text{-}M27}$, and $bla_{CTX\text{-}M55}$, of which $bla_{CTX\text{-}M55}$ was the most common (58.6%). Amino acid substitutions, Ser-83-Leu and Asp-87-Asn were predominant in GyrA of ciprofloxacin resistant isolates. Plasmid-mediated quinolone resistance genes, *qnrA* and *qnrB,* was identified at low rate (0.1% each) but at higher rate for *qnrS* (23%). Class 1 integrons carrying *aadA1* from pigs (n = 1) and ESBL genes ($bla_{CTX\text{-}M55}$ and $bla_{CTX\text{-}M14}$) from pigs (n = 2), pork (n = 1), and humans (n = 7) were located on conjugative plasmids. Most plasmid (29.3%) were in IncF group. Project 2 described colistin resistance and plasmid-mediated *mcr* genes in *E. coli* and *Salmonella* isolated from pigs, pig carcass and pork in Thailand, Lao PDR, and Cambodia border provinces. A total of 1,619 *E. coli* and *Salmonella* isolates from pigs, pig carcasses, and pork were obtained. Colistin resistance was more common in *E. coli* (8%) than *Salmonella* (1%) and the highest resistance rate was found in Cambodia (10.1%). Colistin-resistance genes *mcr-1*, *mcr-3* and *mcr-5* were identified, of which *mcr-1* and *mcr-3* were predominant. The *E. coli* and *Salmonella* isolates (n=7) contained *mcr-1* or *mcr-3* associated with IncF and/or IncI conjugative plasmids. The *mcr*-positive *Salmonella* from Thailand and Cambodia were categorized into two clusters. None of meropenem-resistant isolates were detected. Project 3 investigated the genomic characteristics of AMR in seven *mcr*-carrying ESBL producing *E. coli* from two pigs (TH2 and TH3) and two humans (TH8 and TH9) in Thailand, and three pigs from Lao PDR (LA1, LA2, and LA3) by Whole Genome Sequencing analysis. Four different sequence types/serotypes were found, including ST6833/H20 (TH2 and TH3), ST48/O160:H40 (TH8 and TH9), ST5708/H45 (LA1), and ST10562/O148:H30 (LA2 and LA3). The point mutation Ser-31-Thr in PmrA and His-2-Arg in PmrB were identified in all isolates (colistin MIC=4-8 µg/mL). LA1 contained up to five point mutations in PmrB (colistin MIC=8 µg/mL). All *mcr-1.1* resided in *ISApl1-mcr-1-pap2* region, whereas all *mcr-3.1* located in *TnAs2-mcr-3.1-dgkA-ISKpn40* element. Colocalization of *mcr-3.1* and $bla_{CTX\text{-}M55}$ on the same plasmids were detected in TH2, TH3, TH8, TH9, and LA1. All plasmids concurrently carry other resistance genes, including *cml*, *qnrS1*, and *tmrB*. The co-transmission of $bla_{CTX\text{-}M55}$ and *mcr-3.1* genes was found in LA1 under the selective pressure of either ampicillin or colistin. Only $bla_{CTX\text{-}M55}$ could be transferred in TH8 and TH9, although the $bla_{CTX\text{-}M55}$ was co-localized on the same plasmid with *mcr-3.1*. All *E. coli* isolates contained at least one virulence gene. In conclusion, our findings demonstrated the high prevalence of MDR *E. coli* and *Salmonella* and the circulation of their AMR determinants among these areas. Resistance to last line antibiotics, in particular new generation cephalosporins and colistin distributes in *E. coli* and *Salmonella* from pigs, pork and humans along Thailand, Lao PDR, and Cambodia. To contain AMR, the comprehensive collaborations based on One Health at nation, regional and global level is required.

Field of Study:	Veterinary Public Health	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

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LIST OF ABBREVIATIONS

Aad	aminoglycoside adenyltransferase
AMP	ampicillin
AMR	antimicrobial resistance
bp	base pair(s)
bla	ß-lactamase
^{o}C	degree(s) Celsius
CAZ	ceftazidime
CIP	ciprofloxacin
CHC	chloramphenicol
CLSI	Clinical and Laboratory Standards Institute
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 sequence
Kb	kilobase(s)
LB	Luria Bertani
mcr	mobilized colistin resistance
MDR	multidrug resistance
MHA	Mueller Hinton Agar
MHT	modified Hodge test
MIC	minimum inhibitory concentration
mL	milliliter(s)
MLST	multilocus sequence typing
PCR	polymerase chain reaction
STP	streptomycin
SUL	sulfamethoxazole
TET	tetracycline
Tn	transposon
TRI	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. Pungpian C, Sinwat N, Angkititrakul S, Prathan R and Chuanchuen R 2021. Presence and Transfer of Antimicrobial Resistance Determinants in *Escherichia coli* in Pigs, Pork, and Humans in Thailand and Lao PDR Border Provinces. Microbial Drug Resistance. 27:4.
2. Pungpian C, Lee S, Trongjit S, Sinwat N, Angkititrakul S and Chuanchuen R 2021. Colistin resistance and plasmid-mediated *mcr* genes in *Escherichia coli* and *Salmonella* isolated from pigs, pig carcass and pork in Thailand, Lao PDR and Cambodia border provinces. JVS.

CAPTURE I

1.1 Importance and Rationale

Antimicrobial resistance (AMR) in bacterial species has been recognized as a global public health issue that has an impact on human and animal health, society and economy. It is evident that the emergence and spread of AMR is associated with overuse and inappropriate use of antimicrobial agents in humans, animals and environment (Strom et al., 2017). Therefore, AMR is referred to as One Health issue. In food producing animals, antimicrobials are widely used for several reasons, including control and prevention of diseases, treatment of infections and promotion of growth. Such use of antimicrobials may produce antimicrobial residues at sublethal level at downstream, and this may become a selective pressure for AMR in bacteria (Strom et al., 2017).

AMR can distribute by either clonal spread or horizontal gene transfer (HGT). Resistant bacteria can spread via food, water and environment as well as through direct contact. Concurrently, resistance determinants could be horizontally transferred among bacteria intra/inter species and inside/outside animal bodies, resulting in global spread of AMR (Liu et al., 2018). Mobile genetic elements (MGE), particularly plasmids, are thought to be a major factor of HGT. Plasmids are genetic elements that are capable of accumulating many AMR genes simultaneously. The potential of plasmid to disseminate genes encoding adaptive traits, including AMR, throughout bacterial populations, allows bacteria to survive under environmental pressure (Zhang et al., 2019). Resistance plasmids have been reported to spread from food animals to humans through the food chain, and this phenomenon has become a serious public health concern (Liu et al., 2018).

The global situation of AMR is made worse when antimicrobials are not used correctly and appropriately. The increasingly resistant to multiple drugs and resistant to last line antimicrobials (e.g. third generation cephalosporins, carbapenem, and colistin) have been reported (Jin et al., 2018; Zhang et al., 2019). The phenomenon raises particular concerns regarding lack of antimicrobials for future treatment of bacterial infections.

Extended-spectrum ß-lactamases (ESBLs) produced by *Enterobacteriaceae* have been increasingly reported in humans and food animals (Liu et al., 2018). Resistance to oxyimino cephalosporins such as third-generation cephalosporins and aztreonam was revealed by the ESBL producers, resulting in negative outcomes and treatment failure. Carbapenems are crucial antimicrobials used to treat infections caused by MDR bacteria, particularly ESBL-producing *Enterobacteriaceae*. Infections with carbapenem-resistant *Enterobacteriaceae* (CRE) are associated with a high mortality rate that is rapidly increasing in patients in hospitals and has also been found in food animals (Atterby et al., 2019). Colistin is a last resort antibiotic against MDR infections, especially CRE in humans. However, this antibiotic is widely used in pigs to treat *E. coli*-related intestinal illnesses, most commonly postweaning diarrhea. An increasing number of studies describes the global spread of plasmid mediated colistin resistance (*mcr*) gene in *Enterobacteriaceae* in both humans and animals (Atterby et al., 2019). This increases the potential that, when the *mcr* is co-harbored with carbapenemase or ESBL genes on a single bacteria or the same plasmid, the bacteria may become untreatable with most or all antibiotics (Malchione et al., 2019). As a result, the detection of resistance to last line antimicrobials has been suggested in national AMR monitoring and surveillance programs globally.

To address the rising of AMR threat, international organizations (e.g. World Health Organization, WHO; World Organization for Animal Health, OIE; European Food

Safety Authority, EFSA) have lunched guidelines for AMR surveillance intended to apply at national and international levels. As a fundamental agreement, it is suggested that target bacteria for AMR surveillance in food animals and meat products include zoonotic bacteria (*Salmonella enterica* and *Campylobacter* spp.) and commensal bacteria (*Escherichia coli*; *E. coli* and *Enterococcus* spp.) (EFSA, 2014).

E. coli is a commensal bacterium residing in the gastrointestinal tract of humans and animals. This bacterium serves as a reservoir for resistance determinants that are potentially transferred to other bacterial species. The *E. coli* isolates from healthy animals have been approved as an indicator of Gram-negative bacteria in AMR monitoring (EFSA, 2014). Monitoring of the phenotypic and genotypic aspects of AMR, particularly HGT from commensal *E. coli* could highlight the transmission of AMR.

Salmonella is one of the major food borne pathogens with a significant impact on public health worldwide. *Salmonella* infection is usually self-limited and antibiotic therapy may not be always necessary. However, invasive salmonellosis with serious symptoms requires antimicrobial treatment. In the past decades, MDR *Salmonella* has been drastically reported (Sinwat et al., 2016). The emergence of *E. coli* and *Salmonella* resistant to the last resort antimicrobials, including third generation cephalosporins, carbapenem, and colistin has recently been described (Malchione et al., 2019). As the cost and impact of AMR remain largely unclear, AMR data on *E. coli* and *Salmonella* is required and will benefit for the control and prevention strategies of AMR. Thailand is located in the center of Southeast Asia and bordered by four neighboring countries, including Lao PDR and Cambodia to the Northeast, Myanmar to the North and Malaysia to the South. The northeastern region is the largest area of Thailand and shares its 1,800 and 800 kilometers border with Lao PDR and

Cambodia, respectively. Among border trade markets in this region of Thailand, Lao PDR is Thailand's top border trade partner. Thailand and Lao PDR border trade markets have grown rapidly and produced enormous economic benefits, of which trade value was approximately US$ 3,334.46 million in 2011 (Supatn, 2012). The highest trade value sources were from Nong Khai and Mukdahan (Supatn, 2012). Livestock and their products are one of the most extensive market forces. The data from the Ministry of Commerce in Thailand revealed that there is a large movement of swine from Thailand to Lao PDR with a tendency to increase every year (Potapon and Roland-Holst, 2013). In addition to Thailand-Lao PDR border trade, Thailand-Cambodia border markets of livestock and their products are also powerful and dynamic (Potapon and Roland-Holst, 2013). Of all the Thailand-Cambodia trading pointsets, the largest trade value origin is from Sa Kaeo. Despite a massive economic advantage, the border trade markets could generate risk factors to emergence and spread of diseases as well as AMR.

Since AMR can develop and change overtimes, the emergence and spread of AMR via cross border trade has been serious clinical concerns (Potapon and Roland-Holst, 2013). Comparison of AMR data may describe the dynamic of AMR situation in this region as well as bacterial AMR transfer between countries. Therefore, routine AMR monitoring and surveillance is recommended.

Keywords: Antimicrobial resistance, *Escherichia coli*, Lao PDR, Pigs, Thailand

1.2 Literature Review

1.2.1 General characteristics of *E. coli*

E. coli is Gram-negative bacterium as belonging to the family *Enterobacteriaceae*. The *E. coli* is commonly found in large intestine of mammals. Most *E. coli* is harmless and important for healthy intestinal tract. However, some

E. coli is pathogenic with cause intestinal and extraintestinal infections. Pathogenic is differentiated from nonpathogenic strain from the holding of virulence factor genetic elements (Flament-Simon et al., 2020). *E. coli* can contaminate in water and food. Therefore, *E. coli* has been used as an indicator for the presence of fecal contamination in food and water. Particularly, *E. coli* has been used as a sentinel for AMR monitoring (EFSA, 2014).

1.2.2 Antimicrobial resistance in *E. coli*

E. coli is the main reservoir of resistant genes and can transfer to other bacterial species. *E. coli* developed to resist the antimicrobials through mutations or addition of mobile genetic elements. Multidrug resistance (MDR) *E. coli* have been increasing as well as resistance to last line antibiotics, including third generation cephalosporins, carbapenem which is first drug of choice for serious ESBL-producing infections have been reported. Moreover, the emergence of colistin resistance that is a last-resort antibiotic for carbapenem-resistance infection has been detected (Liu et al., 2017).

In food producing animals, antimicrobials are used for several reasons including treatment, disease control and prevention, and growth promotion. Antimicrobials used can induce the progression and circulation of AMR among bacteria that could be transferred to human through food chain. AMR in *E. coli* isolated from pigs and their products have been increasing reported (Liu et al., 2018).

In Thailand, MDR and ESBL producing *E. coli* isolated from humans, pigs and pig products have been reported (Lay et al., 2021; Trongjit et al., 2016). Recently, carbapenem resistance *E. coli* was detected in patients from hospital (Runcharoen et al., 2017).

MDR *E. coli* in Lao PDR that isolated from pigs and their products were resistant to different classes of antimicrobials. The data on ESBL-producing, carbapenem and colistin resistance *E. coli* in this area are restricted.

In Cambodia, MDR *E. coli* isolated from pigs and their products were resistant to various antimicrobials groups. ESBL-producing *Salmonella* has been detected. However, the data on carbapenem and colistin resistance *Salmonella* are limited (Trongjit et al., 2016).

1.2.3 General characteristics of *S. enterica*

Salmonella is bacterium member in family *Enterobacteriaceae,* which is classified into two major species including, *S. enterica* and *S. bongori*. As for *S. enterica* is divided into 6 subspecies, among all subspecies *S. enterica* subspecies *enterica* is mainly found in mammals and generally linked with human and animal infection. Classification of *Salmonella* according to the Kauffmann–White scheme depends on somatic and flagellar antigens. Over 3,000 serovars of *S. enterica* have been recognized (Sinwat et al., 2016).

S. enterica is a food-borne pathogen, which impact on public health. Non-typhoidal *Salmonella* (NTS) serovars, Typhimurium and Enteritidis are broad host range. Food animals play a major role as reservoirs for distribution of *Salmonella* to humans (Sinwat et al., 2016).

1.2.4 Antimicrobial resistance in *S. enterica*

Non-invasive salmonellosis is generally self-limiting, thus antimicrobial treatment may not be required. However, antimicrobial therapy is necessary for invasive S*almonella* infection (Whistler et al., 2018). MDR and resistance rate to the last resort antimicrobials in *Salmonella* isolates has been reported. (Lay et al., 2021; Mulvey et al., 2018). Moreover, AMR in *Salmonella* isolated from pigs and their

products has been described (Lay et al., 2021). The dissemination of AMR from pigs to humans may be driven by cross-contamination in pigs and their carcasses in slaughterhouses (Sinwat et al., 2016).

In Thailand, MDR and ESBL producing *Salmonella* isolated from humans, pigs and their products have been detected (Sinwat et al., 2016; Trongjit et al., 2017). However, the data on carbapenem and colistin resistance *Salmonella* are limited.

In Lao PDR and Cambodia, MDR and ESBL producing *Salmonella* isolated from humans, pigs and their products has been reported (Sinwat et al., 2016; Trongjit et al., 2017). On the other hand, the data on carbapenem and colistin resistance *Salmonella* are restricted.

1.2.5 Genetic of antimicrobial resistance

1.2.5.1 Class 1 integrons

Integrons is mobile genetic element with capability to capture and mobilize AMR genes in bacteria. The increasing of AMR is significantly driven by the possession resistance genes in a variable region. Class 1 integrons is the most ubiquitous in *Enterobacteriaceae*. These genetic elements play an important role in the widespread of AMR in term of MDR Gram negative bacteria. Class 1 integrons is located on plasmid and transferred of resistance genes to other bacteria via horizontal transfer. Several resistance-gene cassettes can be inserted into a variable region within the same integrons. Consequently, the single antimicrobial agent used can choose the co-selection for many AMR genes (Tomova et al., 2018).

Class 1 integrons is composed of two conserve segments, including 5’ and 3’ conserve segments. As for 5’ conserve segment consists of *intI* gene encoding an integrase that responsible for the resistance gene cassettes insertion/excision

and *attI* recombination site where resistance gene cassettes are integrated. The 3' conserve segment contains *qacEΔ1*, *sul1* and *orf5* encoding resistance to quaternarium ammonium compound, sulphonamide, and open reading frame of unknown function, respectively (Tomova et al., 2018). The gene cassettes are integrated between 5' and 3' conserve segments where the region is variable.

1.2.5.2 Quinolone resistance

Quinolone resistance is generally related to the mutations on chromosome in *gyrA* and *parC*, Quinolone Resistance Determining Region (QRDR). Recently, plasmid-mediated quinolone resistance (PMQR) has been played a significant role in fluoroquinolone resistance (Stephenson et al., 2010).

1.2.5.2.1 Mutation in *gyrA* and *parC*

Mutations on Topoisomerase II (DNA gyrase) and topoisomerase IV are common mechanism for quinolones resistance. Topoisomerase II and IV are enzymes for bacterial DNA replication. Topoisomerase II is a tetramer composing four subunits include two GyrA and two GyrB, which encoded by *gyrA* and *gyrB*, respectively. Topoisomerase IV consisted of ParC and ParE subunits, which encoded by *parC* and *parE*, respectively (Johnning et al., 2015). The activity of quinolones is inhibition the bacterial DNA synthesis by forming drug-enzyme-DNA-complex.

In *E. coli* and *Salmonella*, *gyrA* mutation is usually involved in quinolone resistance. The mutations in this region, resulting in reduced drug affinity for enzyme-DNA complex. A study presented that the mutation on *parC* did not affect to quinolone susceptibility level. However, *E. coli* and *Salmonella* strains harboring both of *parC* and *gyrA* mutations exhibited high quinolone resistance (Johnning et al., 2015).

1.2.5.2.2 Plasmid-mediated quinolone resistance (PMQR)

PMQR has been shown to contribute in resistance of quinolone and distribution of fluoroquinolone resistance genes by horizontal transfer. The common genes of PMQR include *qnr*, *aac (6')-Ib-cr* and *qepA*. Qnr is a pentapeptide protein that prevents the inhibition from quinolone on DNA gyrase (Ferreira et al., 2018). AAC (6')-Ib-cr is an aminoglycoside acetyltransferase variant which reduce activity of ciprofloxacin. QepA is a plasmid mediated-quinolone efflux pump, and increases resistance level on fluoroquinolone (Yamane et al., 2008).

1.2.5.3 Extended-Spectrum ß Lactamases (ESBL)

ESBL is an enzyme with ability to hydrolyze ß-lactam ring. The results of ESBL-producing organisms are resistance to several types of the ß-lactam antibiotics, including penicillins, 1-, 2-, 3-generation cephalosporins and monobactams. However, ESBL-producing strains are susceptible to cephamycins and carbapenem as well as may inhibited by ß-lactamase inhibitors such as clavulanic acid (Liu et al., 2018).

E. coli are the major of ESBL-producing organisms. ESBL have been found in other bacterial species included *Salmonella* (Batchelor et al., 2005). The spread of ESBL is commonly associated with plasmid-borne mediated ß-lactamase genes, which identified in *E. coli* and *Salmonella* isolated from humans and food-producing animals (Batchelor et al., 2005; Ni et al., 2016). ESBL can be divided into three groups, including Temoniera (TEM), Sulfhydryl variable (SHV), and CTX-M types, of which CTX-M type were predominant.

1.2.5.4 Carbapenem resistance *Enterobacteriaceae* (CPE)

Carbapenemase is an enzyme-mediated resistance to carbapenem and all or almost β-lactam antibiotics. In the functional classification scheme, carbapenemases are divided into two groups. Serine carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC) and Carbapenem-hydrolysing oxacillinase (OXA), which have the capability to hydrolyze a wide variation of β-lactams, including penicillins, cephalosporins, carbapenem, and aztreonam. However, these enzymes are inhibited by β-lactamase inhibitors. Metallo β-lactamases such as Verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM) are categorized by the ability to hydrolyze penicillins, cephalosporins, carbapenems and resistance to the β-lactamase inhibitors, but susceptible to aztreonam and metal ion chelators (Long et al., 2019; Peng et al., 2019).

The prevalence of CPE has been increased. Carbapenem resistance genes usually located on mobile genetic elements, which play a major role of the spread of these genes among bacteria. Carbapenem resistance genes have been detected in *E. coli* and *Salmonella* isolated from humans and animals (Atterby et al., 2019; Long et al., 2019; Peng et al., 2019).

1.2.5.5 Colistin resistance

Colistin belongs to the polymyxins drug group, with broad-spectrum against Gram-negative bacteria. Colistin is considered as a last-resort antibiotic used for carbapenem-resistant *Enterobacteriaceae* infections. Currently, plasmid-encoded colistin resistance, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, *mcr-7*, *mcr-8*, and *mcr-9* genes, has been discovered. These resistance genes are detected on a plasmid with the ability to transfer between animal and human bacterial isolates (Litrup et al., 2017; Long et al., 2019; Wang et al., 2019).

In food producing animal, colistin is mainly used for activity against *E. coli* and *Salmonella enterica*, especially in swine and poultry production. Plasmid mediated colistin resistance genes have been detected in *E. coli* and *Salmonella* isolated from humans and food-producing animals (Garcia-Menino et al., 2019; Litrup et al., 2017; Long et al., 2019; Wang et al., 2019).

1.2.5.6 Plasmid replicon typing

Plasmid is extrachromosomal circular DNA with ability of autonomous replication in bacterial cell. Plasmid can confer resistance to the main antimicrobial classes by contain antimicrobial resistance genes (Carattoli, 2013). Plasmids play an importance role in the spread of AMR via horizontally exchanged among bacteria population (Zhang et al., 2019)..

A formal system to categorization of plasmid is incompatibility (Inc) groups. Inc groups testing is tedious, especially when applied to large number of bacteria. Therefore, PCR based replicon typing has been developed to identify the replicons of the major plasmid families (Carattoli et al., 2005).

1.2.5.7 Multi-Locus Sequence Typing (MLST)

MLST is a procedure for characterization the isolates of bacterial species using the sequencing of internal fragments of well-conserved house-keeping genes within the bacterial genome. The internal fragments of each gene are sequenced. The set of alleles are compared to other isolate profiles in the database. The different of the sequences within bacterial species are assigned as distinct alleles and define the allelic profile or sequence type (ST). The differences of the number of nucleotide sequences are assigned in different allele numbers. Most bacterial species contain variation within house-keeping

genes, which creating the billions of distinct allelic profiles to be distinguished using house-keeping loci (Sukhnanand et al., 2005).

1.2.5.8 Next-generation sequencing (NGS)

NGS is an sequencing innovation that contained a high-throughput and massively parallel DNA sequencing. NGS can generate more than hundreds of millions of nucleotide sequence data in a single instrument run. NGS methods have three common steps, including library preparation, amplification, and sequencing. The libraries are prepared by using random fragmentation of DNA and ligation with the linkers. Then, the libraries are amplified using clonal amplification methods and Polymerase chain reaction (PCR). The DNA is sequenced using one of several different approaches, including reversible terminator sequencing (Illumina). NGS has been used to detect the whole genome sequencing (WGS) in bacteria in many studies. WGS is the procedure of determining the DNA sequence of an bacterial genome. It enables high resolution characterization of bacterial isolates in terms of properties that include antibiotic resistance, molecular epidemiology, and virulence (Flament-Simon et al., 2020).

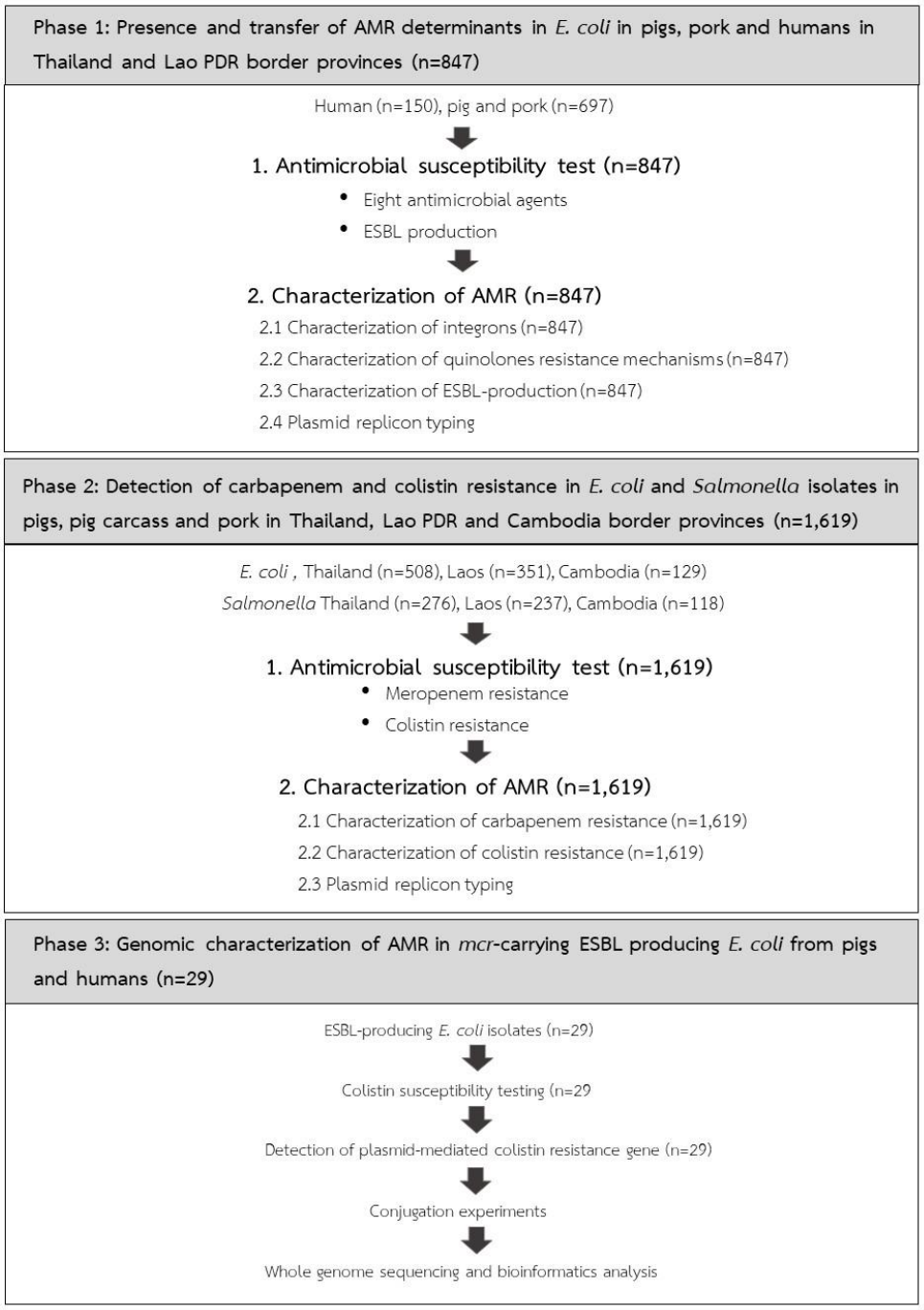
1.3 Research Objectives

1. To monitor the prevalence of AMR among *E. coli* isolated from pigs, pork and humans in Thailand-Laos border areas.

2. To characterize of AMR of *E. coli* isolated from pigs, pork and human in Thailand-Laos border areas.

3. To compare resistance to last-line antimicrobials of *E. coli* and *Salmonella* isolated from pigs and pork in Thailand-Laos and Thai-Cambodia border areas.

1.4 Research outline



1.5 Research Benefits

1.5.1 Novel knowledge

1. Data on rates and genetic characteristics of AMR in *E. coli* isolated from pigs, pork and humans in Thailand and Lao PDR border areas will be obtained.
2. Data on rates and genetic characteristics of resistance to last resort antimicrobials in *E. coli* and *Salmonella* isolated from pigs and pork in Thailand Lao PDR and Cambodia border areas will be obtained.

1.5.2 Application of knowledges

1. The results obtained from this study can be used as part of AMR monitoring in Thailand and neighboring countries.
2. Information on AMR from this project will be used to support the development of guideline for prudent antimicrobial use in food animals in Thailand, Lao PDR and Cambodia countries.
3. The data will support the necessity of protocol to strictly control animals and animal products movement in Thailand and between neighboring areas.
4. Genetic elements and plasmids obtained in this study can be used in future studies.

CHAPTER II

Presence and Transfer of Antimicrobial Resistance Determinants in *Escherichia coli* in Pigs, Pork, and Humans in Thailand and Lao PDR Border Provinces

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Presence and transfer of antimicrobial resistance determinants in *Escherichia coli* in pigs, pork and humans in Thailand and Lao PDR border provinces

2.1 Abstract

This study aimed to investigate antimicrobial resistance (AMR) characteristics of *Escherichia coli* isolates from pig origin (including pigs, pig carcass, and pork) and humans in Thailand and Lao People's Democratic Republic (PDR) border provinces. The majority of the *E. coli* isolates from Thailand (69.7%) and Lao PDR (63.3%) exhibited multidrug resistance. Class 1 integrons with resistance gene cassettes were common (n = 43), of which the most predominant resistance gene cassette was *aadA1*. The percentage of extended-spectrum β-lactamase (ESBL) producers was 3.4 in Thailand and 3.2 in Lao PDR. The ESBL genes found were $bla_{CTX\text{-}M14}$, $bla_{CTX\text{-}M27}$, and $bla_{CTX\text{-}M55}$, of which $bla_{CTX\text{-}M55}$ was the most common (58.6%). Ser-83-Leu and Asp-87-Asn were the predominant amino acid changes in GyrA of ciprofloxacin-resistant isolates. Twenty-two percent of all isolates were positive for *qnrS*. Class 1 integrons carrying *aadA1* from pigs (n = 1) and ESBL genes ($bla_{CTX\text{-}M55}$ and $bla_{CTX\text{-}M14}$) from pigs (n = 2), pork (n = 1), and humans (n = 7) were located on conjugative plasmids. Most plasmids (29.3%) were typed in the IncFrepB group. In conclusion, AMR *E. coli* are common in pig origin and humans in these areas. The findings confirm AMR as One Health issue, and highlight the need for comprehensive and unified collaborations within and between sectors on research and policy.

Keywords: antimicrobial resistance, pigs, human, Thailand, Lao PDR

2.2 Introduction

Antimicrobial resistance (AMR) has become a serious public health problem in most parts of the world, and it is evidently a result of over- and misuse of antimicrobials. AMR is associated with human, veterinary, and environmental health, referred to as One Health. The global AMR situation has been worsened by the emergence and spread of multidrug-resistant (MDR) bacteria, which are resistant to almost all the antibiotics available in the market. Particular concern has been raised on resistance to the last line antibiotics (i.e., new-generation cephalosporins, quinolones, colistin, and meropenem) that could limit choices of antibiotic therapy for bacterial infections in the future.

Commensal *Escherichia coli* serve as indicator bacteria for fecal contamination in foods from animals. *E. coli* are generally harmless but could serve as reservoirs of AMR determinants. These AMR determinants can be shared in a process called horizontal gene transfer that can occur both between bacteria of the same species and between different species. Therefore, commensal *E. coli* are considered a potential AMR hazard (Awosile et al., 2018). AMR monitoring is a priority action to elucidate the root causes and estimate the burden of AMR. It has been justified that resistance phenotype and genotype in commensal bacteria can reflect antimicrobial use in food animals. In addition, commensal *E. coli* are commonly isolated from animal intestinal content and feces. Therefore, commensal *E. coli* are one of the target bacterial species in AMR monitoring (Marc et al., 2019).

To date, extended-spectrum ß-lactamase (ESBL)-producing *E. coli* have been increasingly isolated from humans and food animals (Al-Mayahie et al., 2016). The ESBL producers exhibit resistance to new-generation cephalosporins and multiple antibiotics that are clinically important, and therefore, they have been suggested to be routinely monitored.

Plasmids are transferable genetic elements that are capable of accumulating many resistance genes simultaneously (Rozwandowicz et al., 2018). The genetic elements play an important role in the horizontal transfer of resistance genes, leading to a widespread of AMR. Resistance plasmids have been reported to spread from food animals to humans through the food chain, and this phenomenon has become a serious public health concern (Liu et al., 2018). Therefore, it is essential to monitor and characterize plasmids to follow the emergence and spread of resistant bacteria and their AMR determinants (Rehman et al., 2017).

Plasmid-mediated quinolone resistance (PMQR) genes are increasingly detected worldwide. The presence of PMQR genes (e.g., *qnrA*, *qnrB*, or *qnrS*) may not confer quinolone resistance phenotype based on clinical breakpoints (Liu et al., 2018). However, PMQR genes may coexist with other resistance genes and could lead to the co-selection of resistance to quinolone and other antibiotics, resulting in the emergence of MDR bacteria (Shaheen et al., 2013; Hong et al., 2008). While the majority of ESBL genes are mediated by highly mobilizable plasmids, a transferable plasmid harboring ESBL and PMQR genes was previously reported (Awosile et al., 2018; Liu et al., 2018). In addition, the association between PMQR and class 1 integrons gene cassettes was previously demonstrated (Tomova et al., 2018). Taken together, these observations highlight the role of PMQR genes in the co-selection and spread of resistance to other antibiotics.

Class 1 integrons are one of the most common mobile genetic elements with the capability to capture resistance genes from the environment into their variable regions. The latter has placed class 1 integrons as an indicator of AMR evolution (Velhner et al., 2018) that has been suggested to be included in AMR monitoring.

Thailand is located in the center of Southeast Asia and bordered by four neighboring countries, of which the northeastern region is the largest area and shares

its longest border with Lao People's Democratic Republic (PDR). Thailand and Lao PDR border trade markets have grown rapidly, with pig production being one of the most extensive market forces in these areas. As pigs and their meat products are one of the most important reservoirs of AMR (Lalruatdiki et al., 2018), the increase in the cross border trading of pigs and their products poses a risk of resistant bacterial transfer between humans and food animals in and across the border area, which could spread to other parts of each country (Thu et al., 2019). Monitoring and analysis of AMR in food animals and along the food chain are essential to understand the root causes and estimate the burden of AMR. A One Health approach should be implemented to strengthen the surveillance program and the strategic actions for the control and prevention of AMR. This study is aimed to examine the prevalence and genetics underlying AMR in *E. coli* isolated from pigs, pig carcasses, pork, and humans in Thailand and Lao PDR border provinces.

2.3 Materials and Methods

2.3.1 Sample collection and bacterial isolation

A total of 847 *E. coli* isolates were obtained from pigs, pig carcasses, pork, and humans in Thailand (n = 416) and Lao PDR (n = 431) border provinces during 2013–2018. The isolates were collected as part of our AMR monitoring project in the region and stored in the strain collection of the Department of Veterinary Public Health. The number of collected samples was regularly calculated based on a prevalence of 50% at 95% confidence level and 5% error. The sampling sites were located in four border provinces with crossing points between Thailand and Lao PDR, including Nong Khai-Vientiane and Mukdahan-Savannakhet of Thailand and Lao PDR, respectively. In each province, the sampling sites included one municipal pig slaughterhouse, one municipal fresh market, and one municipal hospital. The slaughterhouses were large-scale abattoirs with 80 and ≥200 pig culling capacity per day in Thailand and Lao

PDR, respectively. The municipal markets selected were the largest in the provinces and located in the same area as the slaughterhouses. The municipal hospitals are the main local government hospitals in the provinces, while private hospitals are not common in these areas.

The fecal samples were obtained by rectal swabbing of dead pigs after bleeding but before the scalding process at the slaughterhouses. This was to minimize interruption of the slaughtering process and avoid heat damage on bacterial cells during the scalding process. The carcass samples were obtained by swabbing pig carcasses after the first wholesale cut that separated the whole carcass into two halves. One of the halves was swabbed from neck to bottom on the inside of red meat. The pork samples were obtained by swabbing ~50 cm^2 area of pork cuts at retail markets (Sinwat et al., 2016). The sample collection at slaughterhouses and retail markets was performed by the sample collection team consisting of laboratory staff trained with the same protocol to ensure the consistency of sampling.

The human samples included self-collected stool samples from workers at slaughterhouses and butchers at retail fresh markets, and stool samples from diarrhea patients collected by on-duty nurses at the local hospitals. The research protocols involving human subjects were approved by the Ethics Committee of the Faculty of Medicine of Khon Kaen University, with the authorization ID: HE592162.

All collected samples were stored in an icebox and transported to shipping carriers immediately. If not possible, the samples were kept in a refrigerator at 4°C until transporting. All samples arrived at the laboratory within 24 hours after sampling.

The *E. coli* strains were isolated and biochemically confirmed using the Indole test according to the Bacteriological Analytical Manual (BAM, 2017). A single colony

from each positive sample was collected (Marc et al., 2019). All *E. coli* isolates were stored in 20% glycerol at -80°C.

2.3.2 Antimicrobial susceptibility test and ESBL-phenotypic detection

Determination of minimum inhibitory concentrations (MICs) was performed in all *E. coli* isolates (n = 847) by using the twofold agar dilution method (CLSI, 2015). The antimicrobial agents and their clinical breakpoints (in parentheses) are as follows: 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). *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 served as quality control strains. All antimicrobial agents were purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO).

ESBL production was examined in all isolates using the standard disk diffusion method (CLSI, 2015). An initial screening test was performed with three indicator cephalosporins, namely ceftazidime (30 μg), cefotaxime (30 μg), and cefpodoxime (10 μg). Isolates resistant to at least one of the indicator cephalosporins were subsequently confirmed for ESBL production using a phenotypic confirmatory test. A difference of ≥5 mm between the inhibition zone of the cephalosporin/clavulanic acid combination and corresponding cephalosporin disks alone was interpreted as a positive ESBL phenotype. All antimicrobial disks were purchased from Oxoid Limited (Oxoid®, Hamshire, England, United Kingdom). *E. coli* ATCC 25922 was used as a quality control strain.

2.3.3 Polymerase chain reaction and DNA sequencing analysis

DNA template for polymerase chain reaction (PCR) was prepared using whole cell boiled lysate from all *E. coli* isolates (n = 847). The oligonucleotide primers used

in this study are listed in Table 1. For nucleotide sequencing analysis, PCR amplicons were purified using Nucleospin® Gel and PCR cleanup (Mccherey-Nagel) and then submitted to First Base Laboratories (Selangor Darul Ehsan, Malaysia), for sequencing. The DNA sequences obtained were analyzed with Chromas programs and compared with the GenBank database using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI) website. The DNA sequences obtained were deposited in the NCBI GenBank database. The genes and accession numbers are as follows: *dfrA25*, MT416084; *aadA1*, MT409422; *aadA2*, MT409423; *dfrA1-aadA1*, MT416086; *aadA2-linF*, MT416087; *dfrA12-aadA2*, MT409424; *dfrA17-aadA5*, MT416083; *dfrA7*, MT416085; $bla_{CTX-M55}$, MT409402- MT409418; $bla_{CTX-M14}$, MT416566-MT416572; and $bla_{CTX-M27}$, MT409419-MT409421.

2.3.4 Characterization of class 1 integrons

All *E. coli* isolates were screened for the presence of *intl1* using PCR with specific primers, Int-F and Int-R (Chuanchuen et al., 2007). The *int1*-positive *E. coli* isolates (Thailand, n = 200 and Lao PDR, n = 194) were characterized for inserted-gene cassettes using PCR and primers in the 5′ conserved segment (5′-CS) and the 3′-CS (Levesque et al., 1995). The CS-PCR amplicons were grouped based on their size, and the representative (s) of each group were submitted for nucleotide sequencing. The CS-PCR amplicons of the same size were further analyzed using restriction digestion with at least two different restriction endonuclease enzymes based on the nucleotide sequencing results, including *Bgl*I, *Bsh*1285I, *Nco*I, *Cla*I, *Dra*I, *Eco*RV, and *Eco*RI (i.e., *aadA1*, *dfrA1-aadA1*, *aadA2-linF*, *dfrA12-aadA2*, and *dfrA17-aadA5*). The amplicons with the same restriction digestion patterns were considered identical. Finally, the CS-PCR amplicons with the same size that yielded different restriction patterns were submitted for DNA sequencing (*dfrA25* and *dfrA7*).

2.3.5 Examination of fluoroquinolone resistance mediating mechanisms

All the ciprofloxacin-resistant *E. coli* (n = 38) were examined for mutations in *gyrA* and *parC* using PCR and DNA sequencing with specific primers, gyrA-F/gyrA-R and parC-F/parC-R, respectively (Chuanchuen et al., 2009). Three ciprofloxacin susceptible isolates were selected and used as controls. The nucleotide sequences were compared with the published *gyrA* (GenBank accession No. X06373) and *parC* (GenBank accession No. M58408) sequences.

The PMQR genes were screened in all the isolates using PCR with specific primers, namely *qnrA*, qnrA-F/qnrA-R; *qnrB*, qnrB-F/qnrB-R; *qnrS*, qnrS-F/qnrS-R; *aac(6´)-Ib-cr*, aac(6´)-Ib-F/aac(6´)-Ib-R; and *qepA*, qepA-F/qepA-R as described previously (Park et al., 2006; Stephenson et al., 2010; Yamane et al., 2008).

2.3.6 Detection of ESBL-encoding genes

The presence of three ß-lactamase gene groups was detected in the ESBL-positive isolates (n = 29) from Thailand (n = 15) and Lao PDR (n = 14) using specific primers, namely bla_{TEM} (blaTEM-F/blaTEM-R), bla_{SHV} (blaSHV-F/blaSHV-R), and bla_{CTX-M} (blaCTX-M-F/blaCTX-M-R) (Hasman et al., 2005; Batchelor et al., 2005). The *E. coli* isolates positive for bla_{CTX-M} were further investigated to determine the specific CTX-M subgroups using specific primers, namely CTX-M1 (MultiCTXMGp1 -F/MultiCTXMGp1 -R), CTX-M2 (MultiCTXMGp2 -F/MultiCTXMGp2 -R), CTX-M9 (CTX-M group 9-F/CTX-M group 9-R), and CTX-M8/25 (CTX-M group 8/25-F/CTX-M group 8/25-F) (Dallenne et al., 2010; Sabate et al., 2000). The *E. coli* isolates positive for CTX-M1 group were tested for the presence of $bla_{CTX-M1\ 5}$ using the primers, *bla*CTX-M1 5 -F/*bla*CTXM1 5 -R (Muzaheed et al., 2008). The PCR amplicons were purified and confirmed through DNA sequencing.

2.3.7 Conjugation experiments

Horizontal transfer of resistance plasmid was examined using the biparental mating technique as described previously (Khemtong et al., 2008). All *E. coli* isolates carrying class 1 integrons with resistance gene cassettes or harboring β-lactamase genes were used as donors. Spontaneous rifampicin-resistant *Salmonella* Enteritidis (SE12) strains (rif^r SE12; MIC = 256 μg/mL) were used as recipients (Khemtong et al., 2008). In brief, the overnight culture of the donor and recipient strains was diluted by adding 80 μL of culture to 4 mL fresh Luria Bertani broth (Difco®) and grown at 37°C to the log phase. The donor and recipient cultures were mixed at a 1:1 ratio in a microcentrifuge. The bacterial cells were collected through centrifugation at 8,000 rpm for 1 minute, placed on a 0.45-μm-pore-size filter (Millipore™, Merck) on LB agar plates, and incubated at 37°C overnight. The conjugation mixture was scraped and washed from the filter into a fresh microcentrifuge with 0.9% NaCl solution. The conjugation cells were collected, resuspended in 200 μL of 0.9% NaCl solution, and spread onto LB agar plates containing a combination of rifampicin (32 μg/mL) and the corresponding antibiotic. They were further confirmed on brilliant green agar and xylose lysine deoxycholate agar (Difco) containing one of the following antibiotics: ampicillin (100 μg/mL), streptomycin (50 μg/mL), and trimethoprim (25 μg/mL). The presence of corresponding class 1 integrons with resistance gene cassettes or β-lactamase genes was detected using PCR as described above. The *Salmonella* transconjugants were examined for plasmid incompatibility (Inc) groups using PCR-based replicon typing (PBRT) as described below.

2.3.8 Plasmids replicon typing

All the *E. coli* isolates were examined for Inc groups using a PBRT (Carattoli et al., 2005). Eighteen genes specific for plasmid Inc groups were screened using five

multiplex PCRs (i.e., HI1/ HI2/I1-I**γ**, X/L-M/N, FIA/FIB/W, Y/P/FIC, and A-C/T/ FIIA) and three simplex PCRs (i.e., F, K, and B/O).

Table 1. Primers used in this study

Target	Primer	Amplicon size (bp)	Primer sequenc (5′-3′)	Reference
Class 1 integrons *intI1*	Int-F	497	CCTGCACGGTTCGAATG	Chuanchuen et al. (2007)
	Int-R		TCGTTTGTTCGCCCAGC	
QRDR				
gyrA	gyrA-F	436	GCTGAAGAGCTCCTATCTGG	Chuanchuen & Padungtod
	gyrA-R		GGTCGGCATGACGTCCGG	(2009)
parC	parC-F	390	GTACGTGATCATGGATCGTG	Chuanchuen & Padungtod
	parC-R		TTCCTGCATGGTGCCGTCG	(2009)
PMQR				
qnrA	qnrA-F	516	ATTTCTCACGCCAGGATTTG	Stephenson et al. (2010)
	qnrA-R		GATCGGCAAAGGTTAGGTCA	
qnrB	qnrB-F	469	GATCGTGAAAGCCAGAAAGG	Stephenson et al. (2010)
	qnrB-R		ACGATGCCTGGTAGTTGTCC	
qnrS	qnrS-F	417	ACGACATTCGTCAACTGCAA	Stephenson et al. (2010)
	qnrS-R		TAAATTGGCACCCTGTAGGC	
qepA	qepA-F	199	GCAGGTCCAGCAGCGGGTAG	Yamane et al. (2008)
	qepA-R		CTTCCTGCCCGAGTATCGTG	
aac(6')-Ib-cr	aac(6')-Ib-F	482	TTGCGATGCTCTATGAGTGGCTA	Park et al. (2006)
	aac(6')-Ib-R		CTCGAATGCCTGGCGTGTTT	
ESBLs				
bla_{CTX-M}	blaCTX-M-F	585	CGATGTGCAGTACCAGTAA	Batchelor et al. (2005)
	blaCTX-M-R		AGTGACCAGAATCAGCGG	
bla_{TEM}	blaTEM-F	964	GCGGAACCCCTATTT	Hasman et al. (2005)
	blaTEM-R		TCTAAAGTATATATGAGTAAACTTGGTCT	
bla_{SHV}	blaSHV-F	854	TTCGCCTGTGTATTATCTCCCTG	Hasman et al. (2005)
	blaSHV-R		TTAGCGTTGCCAGTGYTG	
[b]CTXM subgroup 1	MultiCTXMGp1-F	688	TTAGGAARTGTGCCGCTGYA[a]	Dallenne et al. (2010)
	MultiCTXMGp1-R		CGATATCGTTGGTGGTRCCAT[a]	
[c]CTXM subgroup 2	MultiCTXMGp2-F	404	CGTTAACGGCACGATGAC	Dallenne et al. (2010)
	MultiCTXMGp2-R		CGATATCGTTGGTGGTRCCAT[a]	
[d]CTXM subgroup 8/25	CTX-M group 8/25-F	326	AACRCRCAGACGCTCTAC[a]	Dallenne et al. (2010)
	CTX-M group 8/25-R		TCGAGCCGGAASGTGTYAT[a]	
[e]CTXM subgroup 9	CTX-M group 9-F	850	GTGACAAAGAGAGTGCAACGG	Sabate et al. (2000)
	CTX-M group 9-R		ATGATTCTCGCCGCTGAAGCC	
$bla_{CTX-M15}$	blaCTX-M15-F	996	CACACGTGGAATTTAGGGACT	Muzaheed et al. (2008)
	blaCTX-M15-R		GCCGTCTAAGGCGATAAACA	
PBRT				
Multiplex 1	HI1-F	471	GGAGCGATGGATTACTTCAGTAC	Carattoli et al. (2005)
	HI1-R		TGCCGTTTCACCTCGTGAGTA	
	HI2-F	644	TTTCTCCTGAGTCACCTGTTAACAC	
	HI2-R		GGCTCACTACCGTTGTCATCCT	
	I1-F	139	CGAAAGCCGGACGGCAGAA	
	I1-V		TCGTCGTTCCGCCAAGTTCGT	

Target	Primer	Amplicon size (bp)	Primer sequenc (5′-3′)	Reference
Multiplex 2	X-F	376	AACCTTAGAGGCTATTTAAGTTGCTGAT	Carattoli et al. (2005)
	X-R		TGAGAGTCAATTTTTATCTCATGTTTTAGC	
	L/M-F	785	GGATGAAAACTATCAGCATCTGAAG	
	L/M-R		CTGCAGGGGCGATTCTTTAGG	
	N-F	559	GTCTAACGAGCTTACCGAAG	
	N-R		GTTTCAACTCTGCCAAGTTC	
Multiplex 3	FIA-F	462	CCATGCTGGTTCTAGAGAAGGTG	Carattoli et al. (2005)
	FIA-R		GTATATCCTTACTGGCTTCCGCAG	
	FIB-F	702	GGAGTTCTGACACACGATTTTCTG	
	FIB-R		CTCCCGTCGCTTCAGGGCATT	
	W-F	242	CCTAAGAACAACAAAGCCCCCG	
	W-R		GGTGCGCGGCATAGAACCGT	
Multiplex 4	Y-F	765	AATTCAAACAACACTGTGCAGCCTG	Carattoli et al. (2005)
	Y-R		GCGAGAATGGACGATTACAAAACTTT	
	P-F	534	CTATGGCCCTGCAAACGCGCCAGAAA	
	P-R		TCACGCGCCAGGGCGCAGCC	
	FIC-F	262	GTGAACTGGCAGATGAGGAAGG	
	FIC-R		TTCTCCTCGTCGCCAAACTAGAT	
Multiplex 5	A/C-F	465	GAGAACCAAAGACAAAGACCTGGA	Carattoli et al. (2005)
	A/C-R		ACGACAAACCTGAATTGCCTCCTT	
	T-F	750	TTGGCCTGTTTGTGCCTAAACCAT	
	T-R		CGTTGATTACACTTAGCTTTGGAC	
	FIIS-F	270	CTGTCGTAAGCTGATGGC	
	FIIS-R		CTCTGCCACAAACTTCAGC	
Simplex 1	FrepB-F	270	TGATCGTTTAAGGAATTTTG	Carattoli et al. (2005)
	FrepB-R		GAAGATCAGTCACACCATCC	
Simplex 2	K-F	160	GCGGTCCGGAAAGCCAGAAAAC	Carattoli et al. (2005)
	K-R		TCTTTCACGAGCCCGCCAAA	
Simplex 3	B/O-F	159	GCGGTCCGGAAAGCCAGAAAAC	Carattoli et al. (2005)
	B/O-R		TCTGCGTTCCGCCAAGTTCGA	

[a]Group 1 includes CTX-M-1, -3, -10 to -12, -15, -22, -23, -28, -29, and -30.

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

[c]Group 2 includes CTX-M-2, -4 to -7, and -20.

[d]Group 8/25 includes CTX-M-8, CTX-M-25, CTX-M-26, and CTX-M-39 to CTX-M-41.

[e]Group 9 includes CTX-M-9, -14, -16 to -19, -21, and -27.

ESBLs, extended-spectrum ß-lactamases; PBRT, polymerase chain reaction-based replicon typing; PMQR, plasmid-mediated quinolone resistance

2.3.9 Statistical analysis

The chi-squared test with SPSS version 22.0 (IBM Corporation) program was used to compare the AMR phenotype and genotype. A p-value of <0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 AMR of the *E. coli* isolates (n = 847)

Overall, the majority of *E. coli* isolates (76.15%) were resistant to at least one antimicrobial agent. Sixty-seven percent of the isolates were MDR (being resistant to at least three different antimicrobial classes).

In Thailand, most *E. coli* isolates were resistant to at least one antimicrobial agent (88.34%) and MDR (69.7%). High resistance rates were observed for ampicillin (80.8%), tetracycline (68.3%), sulfamethoxazole (57.2%), and trimethoprim (54.8%) (Table 2). When considering sample sources, the *E. coli* isolates of pig origin (i.e., pigs, pig carcasses, and pork) were commonly resistant to ampicillin (84.1%), tetracycline (71.7%), sulfamethoxazole (61.3%), and trimethoprim (58.7%). The human isolates were frequently resistant to ampicillin (64.3%) and tetracycline (51.4%) (Table 2). The resistance rates to all antimicrobials, except ciprofloxacin, in the *E. coli* of pig origin were higher than those in the human isolates. MDR was more common in the isolates of pig origin than in the human isolates, of which the highest proportion was identified in the pig isolates (84.6%).

In Lao PDR, the majority of the *E. coli* isolates were resistant to at least one antimicrobial agent (80.1%) and MDR (63.3%). High resistance rates were observed for tetracycline (74%), ampicillin (68.4%), sulfamethoxazole (59.6%), and trimethoprim (55.9%) (Table 2). The *E. coli* isolates of pig origin were frequently resistant to tetracycline (77.2%), ampicillin (71.5%), sulfamethoxazole (62.4%), and trimethoprim

(59.3%), while the human isolates were frequently resistant to tetracycline (60%) and ampicillin (55%) (Table 2). Resistance rates to all antimicrobials, except ciprofloxacin, were significantly higher in the pig isolates than those from pig carcasses, pork, and humans. The MDR phenotype was most common in the isolates from pigs (76.5%).

Table 2. Resistance rates of *Escherichia coli* from pigs, pig carcasses, pork and human in Thailand and Lao PDR (n = 847)

Antimicrobial agent†	No. of isolates (%)										
	Thailand					Lao PDR					Grand total
	Pig (n=123)	Carcass (n=111)	Pork (n=112)	Human (n=70)	Total (n=416)	Pig (n=115)	Carcass (n=132)	Pork (n=104)	Human (n=80)	Total (n=431)	(n=847)
MDR	104 (84.6)[a]	81 (72.9)[b]	77 (68.8)[b]	32 (45.7)[c]	290 (69.7)*	88 (76.5)[e]	87 (65.9)[e, f]	59 (56.7)[f, g]	39 (48.8)[g]	273 (63.3)**	563 (66.5)
Ampicillin	107 (86.9)[a]	100 (90.1)[a]	84 (75)[b]	45 (64.3)[c]	336 (80.8)*	90 (78.3)[e]	95 (72)[e, f]	66 (63.5)[f, g]	44 (55)[g]	295 (68.4)**	631 (74.5)
Chloramphenicol	61 (49.6)[a]	37 (33.3)[b]	41 (36.6)[a, b]	14 (20)[c]	153 (36.8)	73 (63.5)[e]	71 (53.8)[e, f]	48 (46.2)[f]	23 (29)[g]	215 (49.9)	368 (43.4)
Ciprofloxacin	6 (4.9)[a]	3 (2.7)[a]	5 (4.5)[a]	3 (4.3)[a]	17 (4.1)	5 (4.3)[e, f]	2 (1.5)[e]	2 (1.9)[e]	9 (11.3)[f]	18 (4.2)	35 (4.1)
Gentamicin	12 (9.8)[a]	9 (8.1)[a]	18 (16.1)[a]	11 (15.7)[a]	50 (12)	19 (16.5)[e]	17 (12.9)[e]	9 (8.6)[e]	7 (8.8)[e]	52 (12.1)	102 (12)
Streptomycin	80 (65)[a]	61 (55)[a]	45 (40.2)[b]	21 (30)	207 (49.8)*	40 (34.8)[e]	37 (28)[e]	26 (25)[e]	21 (26.3)[e]	124 (28.8)**	331 (39)
Sulfamethoxazole	89 (72.4)[a]	65 (58.6)[b]	58 (51.8)[b, c]	26 (37.1)[d]	238 (57.2)	79 (68.7)[e]	84 (63.6)[e, f]	56 (53.8)[f, g]	38 (47.5)[g]	257 (59.6)	495 (58.4)
Trimethoprim	81 (65.9)[a]	62 (55.9)[a]	60 (53.6)[a]	25 (35.7)[b]	228 (54.8)	78 (67.8)[e]	76 (57.6)[e, f]	54 (51.9)[f, g]	33 (41.3)[g]	241 (55.9)	469 (55.3)
Tetracyclines	92 (74.8)[a]	79 (71.2)[a]	77 (68.8)[a]	36 (51.4)[b]	284 (68.3)*	99 (86.1)[e]	97 (73.5)[f, g]	75 (72.1)[g, h]	48 (60)[h]	319 (74)**	603 (71.2)
Ceftazidime	4 (3.3)[a]	1 (0.9)[a]	5 (4.5)[a, b]	10 (14.3)[b]	20 (4.9)	10 (8.7)[e]	7 (5.3)[e]	4 (3.8)[e]	9 (11.3)[e]	30 (7)	50 (5.9)
Cefotaxime	7 (5.7)[a]	4 (3.6)[a]	6 (5.4)[a]	26 (37.1)[b]	43 (10.3)*	15 (13)[e]	19 (14.4)[e]	11 (10.6)[e]	22 (27.5)[f]	67 (15.5)**	110 (13)
Cefpodoxime	5 (4.1)[a, b]	2 (1.8)[a]	5 (4.5)[a, b]	11 (15.7)[b]	23 (5.5)	13 (11.3)[e]	7 (5.3)[e]	7 (6.7)[e]	9 (11.3)[e]	36 (8.4)	59 (7)

[a-d] Values with different superscripts in the same row (for *E. coli* from Thailand) are statistically different ($p \leq 0.05$).

[e-h] Values with different superscripts in the same row (for *E. coli* from Lao PDR) are statistically different ($p \leq 0.05$).

*, ** Indicate statistical difference ($p \leq 0.05$) of total within the same row between *E. coli* from Thailand and Lao PDR.

†Antimicrobial susceptibility of all antimicrobials were tested by agar dilution method, except ceftazidime, cefotaxime and cefpodoxime that were tested by disk diffusion method

2.4.2 Class 1 integrons and resistance-gene cassettes

Overall, class 1 integrons were found in 48.4% of the *E. coli* isolates, including 23.6% from Thailand and 22.9% from Lao PDR. Forty-seven percent of the Thai isolates were positive for *intl1*, including the isolates from pigs (54.5%), pig carcasses (46.9%), pork (45.5%), and humans (29.1%). The *intl1* gene was more common among the isolates from pig origin than those from humans (p = 0). Seven percent of the *intl1*-positive isolates carried class 1 integrons with variable region inserts of size ranging from 750 to 1,700 bp. Nucleotide sequencing analyses revealed seven gene cassettes (i.e., *dfrA25*, *aadA1*, *aadA2*, *dfrA1-aadA1*, *aadA2-linF*, *dfrA12-aadA2*, and *dfrA17-aadA5*). The most common gene cassette array was *aadA1* (33.3%) (Table 3).

Among the isolates from Lao PDR, 44% were positive for *intl1*, including 56.5% from pigs, 45.5% from pig carcasses, 40.4% from pork, and 33.8% from humans. The prevalence of *intl1*-positive *E. coli* from pigs was significantly higher than that from pork and humans (p = 0.017 and 0.002, respectively). Three percent of the *intl1*-positive isolates harbored class 1 integrons with inserted-gene carcasses 750–1,700 bp in size. Four resistance gene cassette arrays (i.e., *dfrA7*, *aadA1*, *dfrA12-aadA2*, and *dfrA17-aadA5*) were identified, of which *aadA1* was the most common (55.6%) (Table 3).

Eighty-nine percent of all *intl1*-positive *E. coli* isolates (n = 394) yielded a 150 bp variable region without inserted gene cassettes, which corresponded to empty integrons. These included isolates from Thailand (84%) and Lao PDR (94.4%). The majority of the *E. coli* isolates harboring *intl1* (87.6%) and those carrying empty integrons (87.7%, n = 351) were resistant to sulfamethoxazole.

Three *E. coli* isolates from Thailand harbored class 1 integrons on a transferable plasmid, including one pig isolate carrying class 1 integrons with *dfrA1-*

aadA1, one pork isolate carrying class 1 integrons with *dfrA1-aadA1*, and one isolate from pig carrying class 1 integrons with *aadA1*.

2.4.3 Mutations in quinolone resistance-determining regions and the presence of PMQR genes

All ciprofloxacin-resistant isolates from Thailand (MIC range = 8–64 µg/mL) and Lao PDR (MIC range = 4–128 µg/mL) carried at least one point mutation C-248-T in QRDR of *gyrA*, leading to Ser-83-Leu amino acid substitute in GyrA (Table 4). The most common point mutations identified in *gyrA* were C-248-T (100%) leading to amino acid substitution Ser-83-Leu, followed by G-259-A leading to Asp-87-Asn (94.3%), and G-259-T leading to Asp-87-Tyr (2.8%). The most common point mutations in *parC* were A-298-G leading to amino acid changes in ParC, Thr-100-Ala (54.3%), and G-191-A, leading to Cys-64-Tyr (2.8%). No mutations were found in GyrA and ParC of the ciprofloxacin susceptible isolates. Double mutations in GyrA, Ser-83-Leu, and Asp-87-Asn were common in the isolates from Thailand (100%) and Lao PDR (88.9%). Most isolates with double mutations in GyrA (54.3%) additionally harbored the amino acid substitution, Thr-100-Ala, in ParC. Only one Thai isolate with a double mutation in GyrA additionally had Cys-64-Tyr substitution in ParC. The isolates with amino acid substitution in both GyrA and ParC had varied ciprofloxacin MICs ranging from 8 to 64 µg/mL. The isolates with amino acid changes in only GyrA exhibited ciprofloxacin MICs ranging from 4 to 128 µg/mL.

Of all the PMQR genes tested, *qnrA* (0.1%), *qnrB* (0.1%), and *qnrS* (23%) were found. Twenty-six percent of the Thai isolates (ciprofloxacin MIC range = 0.0625–16 µg/mL) carried *qnrS*, including 31.7% (39/123) from pigs, 29.7% (33/111) from pig carcasses, 24.1% (27/112) from pork, and 25.7% (18/70) from humans. The *qnrS* positive *E. coli* isolates from pigs ($p = 0.014$) and pig carcasses ($p = 0.036$) were significantly higher than those from humans. The *qnrA* and *qnrB* genes were found in

only two *E. coli* isolates in Thailand: one from human (ciprofloxacin MIC, 0.125 μg/mL) and the other from pig carcass (ciprofloxacin MIC, 0.125 μg/mL).

In Lao PDR, the *E. coli* isolates (18%) from pigs (17.4%, 20/115), pig carcasses (18.2%, 24/132), pork (18.3%, 19/104), and humans (18.8%, 15/80) harbored *qnrS*. The ciprofloxacin MICs ranged between 0.0625 and 8 μg/mL. No significant difference in *qnrS* was observed between the isolates of pig origin and humans.

Table 3. Characteristics of class 1 integrons carrying *Escherichia coli* from pigs, pig carcasses, pork, and humans in Thailand and Lao PDR provinces (n = 847)

IP	Gene cassette	bp	Thailand (n=416)			Lao PDR (n=431)		
			Isolate (No.)	PMQR	Replicon typing	Isolate (No.)	PMQR	Replicon typing
1	*dfrA25*	750 bp	Pig carcass (1)	*qnrB*	-	-	-	-
2	*dfrA7*	750 bp	-	-	-	Pig (1)	*qnrS* (1)	FIB (1), P (1), F_{repB} (1)
3	*aadA1*	1000 bp	Pig (6),	*qnrS* (4)	I1 (2), FIA (1), FIB (2), P (1), Y (2), A/C (1), FIIS (1), F_{repB} (4)	Pig (3)	-	Y (1), F_{repB} (3)
			Pig carcass (1)	-	P (1)	Pig carcass (2)	-	F_{repB} (1)
			Pork (3)	*qnrS* (1)	A/C (2), F_{repB} (2)	Human (1)	-	P (1), F_{repB} (1)
4	*aadA2*	1000 bp	Human (1)	-	-	-	-	-
5	*dfrA1-aadA1*	1500 bp	Pig (2)	-	FIC (1), F_{repB} (2)	Human (1)	-	FIB (1), FIC (1), F_{repB} (1)
			Pig carcass (1)	-	FIC (1)			
			Pork (2)	-	I1 (1), FIC (2), F_{repB} (2)			
			Human (2)	-	HI1 (1), FIC (2)			
6	*aadA2-linF*	1700 bp	Pig (2)	*qnrS* (1)	HI1 (1), N (1), FIB (1), F_{repB} (1)	-	-	-
			Pork (1)	-	-			
7	*dfrA12-aadA2*	1700 bp	Pig carcass (3)	*qnrS* (1)	FIIS (1)	Pig (1)	-	-
			Human (1)	*qnrS* (1)	F_{repB} (1)			
8	*dfrA17-aadA5*	1700 bp	Pig (1)	*qnrS* (1)	F_{repB} (1)	Pig (1)	-	F_{repB} (1)
			Pig carcass (1)	*qnrS* (1)	F_{repB} (1)	Human (1)	-	F_{repB} (1)
			Pork (4)	*qnrS* (1)	Y (1), F_{repB} (1)			

IP, integron profile; PMQR, plasmid-mediated quinolone resistance.

Table 4. Amino acid substitutions in the QRDR of GyrA and/or ParC in ciprofloxacin resistance *Escherichia coli* (n = 38)

Mutation		PMQR	Ciproflxacin MIC (µg/mL)	No. Isolates		
gyr A	*parC*			Thailand (n=21)	Lao PDR (n=18)	Total
Ser-83-Leu, Asp-87-Asn	Thr-100-Ala	-	8-64	8	7	15
Ser-83-Leu, Asp-87-Asn	Thr-100-Ala	*qnrS*	8-16	2	1	3
Ser-83-Leu, Asp-87-Asn	Cys-64-Tyr	-	32	1	0	1
Ser-83-Leu, Asp-87-Asn	None	-	4-128	8	7	15
Ser-83-Leu, Asp-87-Asn	None	*qnrS*	16	2	0	2
Ser-83-Leu, Asp-87-Tyr	None	-	32	0	1	1
Ser-83-Leu	None	*qnrS*	4	0	1	1

2.4.4 ESBL phenotype and ß-lactamase-encoding genes

Twenty-nine *E. coli* isolates (3.4%) in this study produced ESBL (Table 5). Four percent (15/416) of the Thai isolates (n = 15) including pigs (n = 4), pig carcasses (n = 1), pork (n = 1), and humans (n = 8) were confirmed to be ESBL producing strains. The prevalence of ESBL-producing *E. coli* in Thailand was not significantly different from that in Lao PDR. Only ESBL genes in the CTX-M group 1 (i.e., $bla_{\text{CTX-M55}}$) and group 9 (i.e., $bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M27}}$) were found. The CTX-M group 1, $bla_{\text{CTX-M55}}$, was predominant and found in the isolates from pigs (n = 4), pork (n = 1), and humans (n = 4). The CTX-M group 9 genes, $bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M27}}$, were additionally found in the isolates from pig carcasses ($bla_{\text{CTX-M14}}$, n = 1) and humans ($bla_{\text{CTX-M14}}$, n = 4 and $bla_{\text{CTX-M27}}$, n = 1). The $bla_{\text{TEM-1}}$ gene encoding broad-spectrum ß-lactamase was also detected in 12 *E. coli* isolates positive for ESBL (pigs, n = 4; pig carcasses, n = 1; pork, n = 1; and humans, n = 6). The *E. coli* isolates carrying $bla_{\text{CTX-M55}}$ (n = 9) and those carrying $bla_{\text{CTX-M14}}$ (n = 2) additionally carried $bla_{\text{TEM-1}}$.

In Lao PDR, 3.2% (14/431) of the *E. coli* isolates were ESBL-producing strains, including the isolates from pigs (n = 5), pig carcasses (n = 1), pork (n = 4), and humans (n = 4). The ESBL genes found were $bla_{\text{CTX-M55}}$ of the CTX-M group 1 and $bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M27}}$ of the CTX-M group 9. The $bla_{\text{CTX-M55}}$ gene was found in eight isolates, including pigs (n = 4), pig carcasses (n = 1), pork (n = 1), and humans (n = 2). Six isolates carried the CTX-M group 9 genes, including $bla_{\text{CTX-M14}}$ (pig, n = 1 and pork, n = 3) and $bla_{\text{CTX-M27}}$ (human, n = 2). The $bla_{\text{TEM-1}}$ gene was detected (n = 15) and found in pigs (n = 4), pig carcasses (n = 1), pork (n = 5), and humans (n = 5).

Almost all ESBL-producing *E. coli* isolates in both countries additionally harbored both $bla_{\text{TEM-1}}$ and *qnr* (Table 5). Twelve ESBL-producing *E. coli* isolates of Thailand carried *qnrS* (80%), including the isolates from pigs (n = 4), pig carcasses (n = 1), pork (n = 1), and humans (n = 6). Nine ESBL-producing *E. coli* from Lao PDR harbored *qnrS* (64.3%) (9/14), including the isolates from pigs (n = 4), pig carcasses (n = 1), and pork (n = 4). One *E. coli* isolate from humans in Thailand coharbored *qnrA* and $bla_{\text{TEM-1}}$ (Table 5).

Ten *E. coli* isolates horizontally transferred ESBL genes. These included seven isolates carrying $bla_{\text{CTX-M55}}$ from pork (n = 1) and humans (n = 4) from Thailand and pigs (n = 2) from Lao PDR, and $bla_{\text{CTX-M14}}$ from humans in Thailand (n = 3). Based on the conjugation experiment, five *E. coli* isolates harbored $bla_{\text{CTX-M55}}$ and *qnrS* on Inc F_{repB} type plasmids that were cotransferred to the *Salmonella* recipient when ampicillin was used as a selective agent.

Table 5. Characteristic of Extended-Spectrum ß-Lactamase-Producing *Escherichia coli* (n = 34)

Country	Sample source[a]	ß-lactamase	PMQR	Inc group	Transferability of ß-lactamase gene[b]
Thailand	Pig (n=2)	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}, K	-
(n=16)	Pig	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	-
	Human (n=2)	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	+
	Human	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	-	FIA	+
	Human	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	I1	+
	Pig	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	HI, Y, A/C, K	-
	Pork	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	HI	+
	Human	*bla* $_{CTX-M\ 14}$	*qnrS*	F_{repB}, FIB, A/C	+
	Human	*bla* $_{CTX-M\ 14}$	*qnrS*	F_{repB}, FIB	+
	Pig carcass	*bla* $_{CTX-M\ 14}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}, FIB	-
	Human	*bla* $_{CTX-M\ 14}$	-	F_{repB}	+
	Human	*bla* $_{CTX-M\ 14}$, *bla* $_{TEM-1}$	*qnrS*	HI	-
	Human	*bla* $_{CTX-M\ 27}$	-	F_{repB}, FIB	-
	Human	*bla* $_{TEM-1}$	*qnrA*	-	-
Lao PDR	Pig	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	+
(n=18)	Pig	*bla* $_{CTX-M\ 55}$	*qnrS*	F_{repB}	+
	Pig	*bla* $_{CTX-M\ 55}$	*qnrS*	Y	-
	Pig carcass	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	-
	Pork	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	-
	Pig	*bla* $_{CTX-M\ 55}$	-	Y	-
	Human	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	-	K	-
	Human	*bla* $_{CTX-M\ 55}$, *bla* $_{TEM-1}$	-	F_{repB}, FIB	-
	Pork (n=2)	*bla* $_{CTX-M\ 14}$, *bla* $_{TEM-1}$	*qnrS*	N	-
	Pork	*bla* $_{CTX-M\ 14}$, *bla* $_{TEM-1}$	*qnrS*	F_{repB}	-
	Human	*bla* $_{CTX-M\ 27}$, *bla* $_{TEM-1}$	-	F_{repB}, FIB	-
	Human	*bla* $_{CTX-M\ 27}$, *bla* $_{TEM-1}$	-	F_{repB}	-
	Pig	*bla* $_{CTX-M\ 14}$, *bla* $_{TEM-1}$	*qnrS*	HI	-
	Pig (n=2)	*bla* $_{TEM-1}$	-	F_{repB}	-
	Pork	*bla* $_{TEM-1}$	-	F_{repB}, K	-
	Human	*bla* $_{TEM-1}$	-	F_{repB}	-

[a]Only n > 1 is indicated in parentheses.

+, Transfer; -, nontransfer; Inc, incompatibility; PMQR, plasmid-mediated quinolone resistance.

2.4.5 Plasmid replicon typing

Of all 847 isolates tested, 18 plasmid replicon types were identified (Table 6). The most common plasmid replicon type was $IncF_{repB}$ (29.3%), followed by IncFIB (13%) and IncY (11.2%). Among the Thai isolates, 12 different plasmid replicons were identified, of which the most common replicon type was $IncF_{repB}$ (33.7%). $IncF_{repB}$ were mostly found among the isolates from humans (48.6%), followed by pigs (36.6%), pig carcasses (31.3%), and pork (23.4%). In addition, $IncF_{repB}$ was predominantly detected in the *E. coli* isolates carrying resistance genes, including *intI1* (39.5%), *qnrS* (39.3%), and ESBL genes (71.4%) (Table 7).

Eleven different replicon types were identified among the Laos isolates. The most predominant Inc group was $IncF_{repB}$ (25.1%). The most commonly found Inc group among the isolates of pig origin and humans was $IncF_{repB}$ (17.4% and 17.5%, respectively). The $IncF_{repB}$ group was common among the *E. coli* isolates positive for *intI1* (36.6%), *qnrS* (29.5%), and ESBL (57.1%) (Table 7)

Table 6. Distribution of plasmid replicon type of *Escherichia coli* isolates from pigs, pig carcasses, pork, and human in Thailand and Lao PDR border area (n = 847)

Plasmid replicon type	No. of isolates (%)										Total
	Thailand (n=416)					Lao PDR (n=431)					
	Pigs	Pig carcass	pork	Human	Total	Pigs	Pig carcass	pork	Human	Total	
HI 1	17 (4)	6 (1.4)	4 (1)	6 (1.4)	33 (7.9)	9 (2.1)	8 (1.9)	3 (0.7)	4 (0.9)	24 (5.6)	57 (6.7)
I 1	8 (1.9)	8 (1.9)	6 (1.4)	7 (1.7)	29 (7)	1 (0.2)	3 (0.7)	1 (0.2)	1 (0.2)	6 (1.4)	35 (4.1)
N	16 (3.8)	10 (2.4)	3 (0.7)	3 (0.7)	32 (7.7)	5 (1.2)	5 (1.2)	2 (0.5)	3 (0.7)	15 (3.5)	47 (5.5)
FIA	6 (1.4)	2 (0.5)	3 (0.7)	5 (1.2)	16 (3.8)	0	2 (0.5)	2 (0.5)	2 (0.5)	6 (1.4)	22 (2.6)
FIB	25 (6)	16 (3.8)	11 (2.6)	18 (4.3)	70 (16.8)	6 (1.4)	13 (3)	11 (2.6)	10 (2.3)	40 (9.3)	110 (13)
FIC	9 (2.2)	9 (2.2)	7 (1.7)	2 (0.5)	27 (6.5)	0	3 (0.7)	1 (0.2)	1 (0.2)	5 (1.2)	32 (3.8)
P	4 (1)	6 (1.4)	3 (0.7)	7 (1.7)	20 (4.8)	5 (1.2)	9 (2.1)	5 (1.2)	10 (2.3)	29 (6.7)	49 (5.8)
IncY	18 (4.3)	8 (1.9)	12 (2.9)	6 (1.4)	44 (10.6)	23 (5.3)	10 (2.3)	12 (2.8)	6 (1.4)	51 (11.8)	95 (11.2)
A/C	3 (0.7)	2 (0.5)	4 (1)	1 (0.2)	10 (2.4)	0	0	0	0	0	10 (1.2)
FIIS	6 (1.4)	10 (2.4)	7 (1.7)	1 (0.2)	24 (5.8)	0	0	1 (0.2)	0	1 (0.2)	25 (3)
F_{repB}	45 (10.8) [a]	26 (6.3) [b]	35 (8.4) [a, b]	34 (8.2) [a, b]	140 (33.7)*	26 (6) [c, d]	27 (6.3) [d]	34 (7.9) [c]	21 (4.9) [c, d]	108 (25.1)**	248 (29.3)
B/O	0	0	0	1 (0.2)	1 (0.2)	1 (0.2)	0	1 (0.2)	0	2 (0.5)	3 (0.4)

[a, b] Values with different superscripts in the same row (for E. coli from Thailand) are statistically different ($p \leq 0.05$).

[c, d] Values with different superscripts in the same row (for E. coli from Lao PDR) are statistically different ($p \leq 0.05$).

*, ** Indicate significant difference ($p \leq 0.05$) of values within the same row between E. coli from Thailand and Lao PDR.

Table 7. Plasmid replicon type in *Escherichia coli* carrying different resistance determinants (n = 491)

Resistance genes	Country	Sample source	Plasmid replicon type (%)											
			HI 1	I 1	N	FIA	FIB	FIC	P	IncY	A/C	FIIs	FrepB	B/O
intI1	Thailand	Pig	14.9	9	13.4	3	22.4	9	1.5	19.4	4.5	1.5	38.8	0
(n=394)		Pig carcass	7.7	7.7	9.6	1.9	19.2	7.7	3.8	9.6	1.9	1.9	21.2	0
		Pork	5.9	7.8	3.9	3.9	11.8	9.8	0	13.7	7.8	2	52.9	0
		Human	20	3.3	6.7	13.3	20	6.7	13.3	20	0	0	50	0
		Subtotal	11.5	7.5	9	4.5	18.5	8.5	3.5	15.5	4	1.5	39.5	0
	Lao PDR	Pig	12.3	0	4.6	0	6.2	0	1.5	30.8	0	0	32.3	0
		Pig carcass	6.7	1.7	3.3	0	10	0	0	15	0	0	26.7	0
		Pork	7.1	0	2.4	0	9.5	0	0	23.8	0	0	50	0
		Human	7.4	0	7.4	3.7	14.8	3.7	7.4	14.8	0	0	48.1	0
		Subtotal	8.8	0.5	4.1	0.5	9.3	0.5	1.5	22.2	0	0	36.6	0
	Total		10.2	4.1	6.6	2.5	14	4.6	2.5	18.8	2	0.8	38.1	0
qnrS	Thailand	Pig	15.4	2.6	7.7	0	7.7	10.3	2.6	15.4	2.6	10.3	33.3	0
(n=195)		Pig carcass	3	9.1	3	3	21.2	6.1	0	6.1	3	6.1	36.4	0
		Pork	3.7	0	0	7.4	14.8	7.4	0	18.5	0	7.4	37	0
		Human	27.8	11.1	11.1	0	22.2	0	5.6	5.6	5.6	0	61.1	0
		Subtotal	11.1	5.1	5.1	2.6	15.4	6.8	1.7	12	2.6	6.8	39.3	0
	Lao PDR	Pig	15	0	5	0	5	0	5	30	0	0	25	0
		Pig carcass	0	0	0	0	0	0	0	8.3	0	0	29.2	0
		Pork	5.3	0	10.5	0	0	5.3	0	15.8	0	5.3	52.6	5.3
		Human	26.7	6.7	13.3	0	0	0	0	13.3	0	0	6.7	0
		Subtotal	10.3	1.3	6.4	0	1.3	1.3	1.3	16.7	0	1.3	29.5	1.3
	Total		10.8	3.6	5.6	1.5	9.7	4.6	1.5	13.8	1.5	4.6	35.4	0.5
ESBLs	Thailand	Pig	25	0	0	0	0	0	0	25	25	0	75	0
(n=30)		Pig carcass	0	0	0	0	100	0	0	0	0	0	100	0
		Pork	100	0	0	0	0	0	0	0	0	0	0	0
		Human	12.5	12.5	0	0	37.5	0	0	0	12.5	0	75	0
		Subtotal	21.4	7.1	0	0	28.6	0	0	7.1	14.3	0	71.4	0
	Lao PDR	Pig	20	0	0	0	0	0	0	20	0	0	40	0
		Pig carcass	0	0	0	0	0	0	0	0	0	0	100	0
		Pork	0	0	50	0	0	0	0	0	0	0	50	0
		Human	0	0	0	25	50	0	0	25	0	0	75	0
		Subtotal	7.1	0	14.3	7.1	14.3	0	0	14.3	0	0	57.1	0
	Total		14.3	3.6	7.1	3.6	21.4	0	0	10.7	7.1	0	64.3	0
Grand total			10.5	3.9	6.3	2.3	13	4.4	2.1	16.9	2.1	1.9	38.4	0.2

2.5 DISCUSSION

The high prevalence of MDR *E. coli* in pigs (80.7%), pig carcasses (69.1%), pork (63%), and humans (47.3%) in Thai–Lao PDR border provinces was highlighted in this study. The *E. coli* isolates were commonly resistant to ampicillin (74.5%), tetracycline (71.2%), sulfamethoxazole (58.4%), and trimethoprim (55.3%), in agreement with a previous study in the same area (Sinwat et al., 2016). This could be related to the types of antimicrobials used in pig production, which is influenced by the relatively low cost of the antibiotics (Strom et al., 2017). It is noted that almost all AMR rates in the isolates from pig origin were higher than those from humans, in agreement with a previous study (Tseng et al., 2015). This suggests that there may be more frequent use of antimicrobial agents in pig farming than in humans. However, the exception was resistant to ciprofloxacin. Ciprofloxacin resistance rates in the pig (4%) and human (4.3%) isolates were not different in Thailand, but the rate was higher in the human isolates (11.3%) in Lao PDR. The results may reflect the frequent use of ciprofloxacin in humans in Lao PDR. The ciprofloxacin resistance rate in this study is higher than that in a previous study on *Salmonella* in the same area (Sinwat et al., 2016; Whistler et al., 2018). The possible explanation could be that *E. coli* are commensal bacteria and reside in the intestinal tract of the host pigs. Therefore, they have longer antimicrobial contact duration than *Salmonella*. It could also be because *E. coli* develop resistance to antimicrobials more easily and rapidly than *Salmonella* (Varga et al., 2008).

Almost half of the *E. coli* isolates from Thailand (47%) and Lao PDR (44%) harbored *intI1*, of which most were empty integrons (84% and 94.4% in Thailand and Lao PDR, respectively). All the *E. coli* isolates carrying empty integrons yielded a 150 bp amplicon of the variable region, indicating the absence of gene cassettes inserted between the conserved segments of the integron gene cassettes. These could be a

result of losing gene cassettes in the absence of selection pressure (Oliveira-Pinto et al., 2017), or it could represent ancestral elements that have never acquired gene cassette inserts (Clarisse et al., 2017). It was previously demonstrated that the lack of a 3´-conserved region can result in the existence of empty integrons and can be confirmed by the susceptibility to sulfamethoxazole (Mujeeb et al., 2017). However, this is not the case for the presence of empty integrons in this study. Some of the isolates positive for *intl-1* or carrying empty integrons were susceptible to sulfamethoxazole. The possible explanation could be that the relevant integrons were carrying atypical conserved regions or nonfunctional *sul* genes in their conserved regions. However, the characterization of the 3´ conserved segments was not pursued in this study. Regardless, the empty integrons are available for the acquisition of new resistance genes to adapt to new selective pressure in a different environment. Thus, the limited use of antimicrobials could decrease the spread of resistance genes mediated by class 1 integrons among bacteria. In this study, the prevalence of *intI1* in the isolates from pig origin was higher than that from humans, which is in agreement with a previous study (Phongpaichit et al., 2007), indicating that appropriate antimicrobial use must be encouraged in pig production.

The most common resistance gene cassette among the isolates from both countries was *aadA1*, which was found to be located on a conjugative plasmid. In some *E. coli* isolates carrying class 1 integrons with *dfrA1-aadA1*, only *intI1* was transferred to the *Salmonella* recipient. It is unusual that *intI1* is located on more than one plasmid in the same bacterial cell. Therefore, the results are likely due to losing resistance gene cassettes when antimicrobial selective pressure is diluted in vitro.

The *aadA2-linG* gene cassette array was detected in the *E. coli* isolates from pigs and pork in Thailand (n = 3), similar to a previous study in *Salmonella* (Sinwat et

al., 2016). Lincomycin is often used in combination with spectinomycin to treat swine dysentery and respiratory infection in pigs in Thailand. This possibly poses selective pressure on lincomycin-resistant bacteria including *E. coli*. Taken together, the observations suggest the possible sharing of the gene cassettes across bacterial species in this area.

The *dfrA17-aadA5* and *dfrA12-aadA2* cassette arrays were commonly found among the isolates from humans, pigs, and pork in this study. These gene cassette arrays have been previously reported in many countries (e.g., China, Taiwan, Korea, India, and Australia) (Oliveira-Pinto et al., 2017). The *dfrA12-aadA2* gene cassettes were previously identified in *Salmonella* from companion animals in Thailand (Srisanga et al., 2017). These findings support the proposition that the resistance gene cassettes are ubiquitous and AMR is a borderless threat.

The association between class 1 integrons and *qnrS* has been previously reported (Tomova et al., 2018). This is in agreement with this study, where almost half of the class 1 integrons carrying isolates additionally contained *qnrS* (26.1%) and exhibited MDR phenotype (98.1%). It is possible that class 1 integrons, *qnrS*, and nonintegrons borne resistance genes coexist on the same plasmid that could be coselected by a single antimicrobial agent (Ferreira et al., 2018). However, plasmid characterization was not pursued in this study.

All ciprofloxacin-resistant isolates (n = 38) in this study carried the Ser-83-Leu mutation in GyrA, in agreement with previous studies (Johnning et al., 2015; Ni et al., 2016). Previous studies showed that the isolates containing many mutation points or additionally carrying the PMQR gene had a higher MIC level of quinolone drugs (Ni et al., 2016; Rezazadeh et al, 2016), which is inconsistent with this study. For example, one isolate with only C-248-T in *gyrA* leading to Ser-83-Leu in GyrA and one isolate with C-248-T in *gyrA* leading to Ser-83-Leu, and G-259-A leading to Asp-87-Asn had

ciprofloxacin MIC of 4 µg/mL. The isolates with amino acid changes in both GyrA and ParC without *qnrS* had ciprofloxacin MIC of 8 µg/mL, while those with *qnrS* had ciprofloxacin MIC of 8–16 µg/mL. Therefore, the contribution of mutations in GyrA and ParC and the presence of PMQR genes to a ciprofloxacin resistance level are dynamic and different from cell to cell.

The *qnrS* gene was the predominant PMQR gene, which is in agreement with previous studies (Liu et al., 2018). The *qnrS* gene is usually located on small mobilizable plasmids (Carattoli, 2009), and this may explain the current observation. It was previously shown that the presence of the *qnr* gene does not always confer fluoroquinolone resistance (Ferreira et al., 2018), which is in agreement with this study where a human isolate with *qnrA* and a pig isolate with *qnrB* exhibited low ciprofloxacin MIC (0.125 µg/mL). In contrast, the presence of *qnr* genes was associated with the reduction in ciprofloxacin susceptibility in the *E. coli* clinical isolates (Rezazadeh et al., 2016). The latter may be a combination effect with other ciprofloxacin-resistance mechanisms, for example, the presence of porins, multidrug efflux systems, and DNA gyrase or topoisomerase mutations that were not investigated. Regardless, the dissemination of *qnr* genes plays a role in coselection for the MDR phenotype in bacteria.

The strong association between PMQR and ESBL genes has been previously reported due to their colocalization on the same plasmid (Liu et al., 2018), in agreement with this study. As most ESBL-producing isolates carried *qnrS* (19/30), all ESBL producers carrying *qnrS* exhibited the MDR phenotype. The results support the notion that ESBL genes are frequently located on large plasmids containing many resistance genes (Paterson, 2000), even though plasmids were not characterized in this study.

In this study, the ESBL-producing *E. coli* were more commonly found in humans (8.7%) than in the pig origin (2.3%), which is consistent with a previous study in China (Hu et al., 2013). This could be a result of the limited use of cephalosporins in pig production in this area. The latter is mainly due to the high price of the antibiotics, which will affect the investment cost. In addition, bla_{TEM-1}, a ß-lactamase gene, was commonly found, which is in agreement with a previous study in Thailand (Trongjit et al., 2017).

The $bla_{CTX-M55}$ gene was the most common ESBL gene detected in the isolates from pig origin and humans (58.6%), which is consistent with studies in other Asian countries, including Taiwan, Japan, and China (Norizuki et al., 2018). However, these results are inconsistent with previous reports in European countries, where CTX-M1 was predominant among livestock (Hammerum et al., 2014). In our study, all the *E. coli* isolates carrying $bla_{CTX-M-55}$ in Thailand harbored a non-ESBL TEM1 , in agreement with a previous study in humans in Thailand (Kiratisin et al., 2007). The $bla_{CTX-M-14}$ gene was found at a lower rate (31%), which differs from a previous study reporting that $bla_{CTX-M-14}$ was most frequently identified in pigs (Liu et al., 2018). The $bla_{CTX-M-55}$, $bla_{CTX-M-14}$, and bla_{TEM-1} genes were found to be located on transferable plasmids, supporting the dissemination of the genes in humans, pigs, pig carcasses, and pork in this area.

It is important to note that $bla_{CTX-M-55}$ and $bla_{CTX-M-14}$ were horizontally transferred by using ampicillin as a selective pressure in the conjugation experiment. A similar phenomenon was observed for the ESBL-producing isolates carrying both $bla_{CTX-M-55}$ and *qnrS* on the same plasmid (Table 5). This highlights the role of the old-generation antibiotic that has been widely used for a long time as a selective pressure for the new generation of clinically important antibiotics. The strategic actions in antimicrobial distribution and usage as a whole are essential.

In this study, $IncF_{repB}$ plasmid was commonly identified in the isolates with resistance determinants, which is consistent with a study in China (Yang et al., 2015). Replicon $IncF_{repB}$ belonging to the IncF class has been previously identified as the most common Inc group in *Enterobacteriaceae* from different sources (Odetoyin et al., 2017). These plasmids have been associated with MDR bacteria, and shown to commonly confer resistance to ß-lactams and quinolones (Yang et al., 2015). In contrast, $bla_{CTX\text{-}M\text{-}1}$ that was the most common ESBL gene in European countries was usually located on IncI1 (Zurfluh et al., 2014). Therefore, it is likely that different variants of $bla_{CTX\text{-}M}$ are related to different replicon type plasmids in different geographic regions (Carattoli, 2009).

In addition, the *qnrS*-positive isolates mostly carried $IncF_{repB}$ plasmid (35.4%), in agreement with a previous study (Rezazadeh et al., 2016). As the *E. coli* isolates carrying both $bla_{CTX\text{-}M}$ and *qnrS* were positive for only the $IncF_{repB}$ class of plasmid replicon, it suggests that colocalization on the same plasmid and cotransfer of the genes contribute to the emergence and spread of MDR *E. coli* in this area. The results in this study revealed the high prevalence of MDR *E. coli* isolates in pigs, pig carcasses, pork, and humans. These isolates harbored a variety of AMR determinants, including class 1 integrons with resistance gene cassettes, PMQR genes, and ESBL genes. $IncF_{repB}$ plasmid was commonly identified in *intI*, $bla_{CTX\text{-}M}$, and/or *qnrS*-carrying isolates. Cotransfer of the genes on the same transmissible plasmid was observed and contributed to the spread of MDR *E. coli*. The monitoring of AMR at the phenotype and genotype levels needs to be encouraged to allow epidemiological tracing of resistance patterns. The One Health approach to national AMR surveillance tracking in human and animal populations is required to strengthen the understanding and support control and prevention strategic actions of AMR.

CHAPTER III

Colistin resistance and plasmid-mediated *mcr* genes in *Escherichia coli* and *Salmonella* isolated from pigs, pig carcass and pork in Thailand, Lao PDR and Cambodia border provinces

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Detection of Carbapenem and Colistin resistance in *Escherichia coli* and *Salmonella* isolates in pigs, pig carcass and pork in Thailand, Lao PDR and Cambodia border provinces

3.1 Abstract

Background: Colistin and carbapenem-resistant bacteria have emerged and become a serious public health concern. However, their epidemiological data is still limited.

Objectives: This study aimed to examine colistin and carbapenem resistance in *Escherichia coli* and *Salmonella* from pigs, pig carcasses and pork in Thailand, Lao PDR, and Cambodia border provinces.

Methods: Phenotypic and genotypic resistance to colistin and meropenem was determined in *E. coli* and *Salmonella* from pigs, pig carcasses and pork (n=1,619). Conjugative experiment was performed in all isolates carrying *mcr* gene (s) (n=68). Plasmid replicon type was determined in the isolates carrying conjugative plasmid with *mcr* by PCR-based replicon typing (n=7). Genetic relatedness of *mcr*-positive *Salmonella* (n=11) was investigated by multi-locus sequence typing.

Results: Colistin resistance was more common in *E. coli* (8%) than *Salmonella* (1%), of which the highest resistance rate was found in *E. coli* (17.8%) and *Salmonella* (1.7%) from Cambodia. Colistin-resistance genes, *mcr-1*, *mcr-3* and *mcr-5*, were identified, of which *mcr-1* and *mcr-3* were predominant in *E. coli* (5.8%) and *Salmonella* (1.7%), respectively. The *mcr-5* gene was found in *E. coli* from pork in Cambodia. Two colistin-susceptible pig isolates from Thailand carried both *mcr-1* and *mcr-3*. Seven *E. coli* and *Salmonella* isolates contained *mcr-1* or *mcr-3* associated with IncF and IncI plasmids. The *mcr*-positive *Salmonella* from Thailand and Cambodia were categorized into two clusters with 94%-97% similarity. None were meropenem resistant.

Conclusions: Colistin-resistant *E. coli* and *Salmonella* distributed in pigs, pig carcass and pork in the border areas. Undivided-One Health collaboration is needed to address the issue.

Keywords: Antimicrobial resistance, Pigs, Thailand, Lao PDR, Cambodia

3.2 Introduction

Antimicrobial resistance (AMR) is an ongoing One Health problem of global dimension. Emergence and rapid spread of multidrug resistance (MDR) has complicated the AMR situation. Last-line antibiotics e.g. polymyxins and carbapenems have been used for treatment of infections involved in MDR Gram negative bacterial pathogens. However, resistance to last-resort treatment has been increasingly reported (Peng et al., 2019) and raised alarm over limiting treatment option in the near future.

Carbapenems are critically important antimicrobials and used for treatment of infections caused by MDR, including ESBL-producing, *Enterobacteriaceae* (Peng et al., 2019). Carbapenem-resistant *Enterobacteriaceae* (CRE) infections with high mortality have rapidly increased in hospitals (Atterby et al., 2019) and CRE were additionally reported in food animals (Peng et al., 2019). Carbapenem resistance is commonly mediated by carbapenemase genes (e.g. bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA}) that are located on large conjugative plasmids carrying other resistance genes (Long et al., 2019). Co-existence of carbapenem and colistin resistance genes was previously reported in *E. coli* from patients and animals (Peng et al., 2019; Liu et al., 2017).

Colistin or polymyxin B is a last-resort antibiotic against MDR infections, especially CRE in humans (Liu et al., 2017). It is widely used in pigs for treatment of intestinal infections caused by *Escherichia coli*, particularly post weaning diarrhea (Wang et al., 2019). Colistin resistance has been identified in pathogens from various

sources e.g. *E. coli* from pigs in China (Wang et al., 2019); poultry in Argentina (Dominguez et al., 2018); patients, pigs and poultry in Cambodia (Atterby et al., 2019) and *Salmonella* from patients in Denmark (Litrup et al., 2009). Since the report of plasmid-mediated *mcr-1* in *E. coli* and *Klebsiella pneumoniae* from animals and patients in China in 2016 (Liu et al., 2016), several *mcr* variants have been discovered [9]. Currently, detection of plasmid-mediated colistin resistance is included in national AMR monitoring and surveillance programs globally (ESFA, 2017).

Coexistence of $bla_{\text{NDM-1}}$ and *mcr-1* was detected in *E. coli* from patients in China and Taiwan and CRE from broilers and pigs in China (Peng et al., 2019). Co-transfer of carbapenem and colistin resistance genes located on different plasmids was demonstrated (Garcia-Menino et al., 2019). These could contribute to emergence and spread of superbugs no longer susceptible to any antibiotics commercially available.

Pig and pork production is one of the largest market forces worldwide and several antimicrobials have been used to create more quantity and quality of products (Olaitan et al., 2014). This phenomenon also occurs in Southeast Asia border trade areas where imports and exports of live animals and products represent an important contributor to increase cross-border trade value. As a borderless One Health threat, AMR in one region may pose a risk to public health, animal health, and environmental health within or outside the region. AMR monitoring and surveillance is the foundation for assessing the burden of AMR and providing the information for strategic action to control and prevent the threat and evaluate the strategic interventions. The fact is that many developing countries still lack national AMR surveillance. Epidemiological data on AMR is still insufficient and that of last-line antibiotics is much less, particularly in these areas. Therefore, this study aimed to examine the prevalence and genetics underlying resistance to carbapenems and

colistin in E. coli and Salmonella isolated from pigs, pig carcasses and pork in Thailand, Lao PDR and Cambodia border provinces.

3.3 Materials and Methods

3.3.1 Sample collection and bacterial isolation

A total of 988 *E. coli* and 631 *Salmonella* isolates from pigs (n = 377 and 139), pig carcasses (n = 328 and 174), and pork (n = 283 and 318) were included. They were previously isolated as part of AMR epidemiological studies in food animals and products in Southeast Asia during 2014-2018 (Sinwat et al., 2017; Trongjit et al., 2016; Trongjit et al., 2017; Pungpian et al., 2021). The *E. coli* and *Salmonella* isolates were obtained from Thailand (n= 508 and 276), Lao PDR (n= 351 and 237), and Cambodia (n= 129 and 118) (Table 8). The sampling sites were located in provinces with two largest border-crossing points between Thailand-Lao PDR (i.e. Nong Khai-Vientiane and Mukdahan-Savannakhet) and Thailand-Cambodia (i.e. Sa Kaeo-Banteay Meanchey).

In Thailand-Lao PDR border areas, the sampling sites included one municipal pig slaughterhouse and one municipal fresh market in each province. The slaughterhouses have large-scale facilities with a daily throughput of 50-200 or more pigs, and the municipal markets were located in the same area. In Thailand-Cambodia border provinces, the samples were collected from 3 private pig slaughterhouses and one municipal fresh market in Sa Kaeo. The pig slaughterhouses are small-scale facilities producing pork for local consumption only. For the Cambodia sampling sites, the samples were obtained from one municipal pig slaughterhouse and one municipal fresh market in Banteay Meanchey.

The rectal swabs were collected from dead pigs immediately after bleeding but before the scalding process at slaughterhouses. The pig carcass samples were obtained by swabbing the inner side of a half pig carcass from neck to bottom after the first whole carcass was cut lengthwise and divided into two halves. The pork samples were collected by swabbing at least 50 cm^2 of raw meat at retail fresh markets. The sample collection at slaughterhouses and retail markets was performed by our sample collection team consisting of laboratory staff and veterinarians trained with the same sampling protocol (FAO. 2019). All collected samples were stored in an icebox and transported to the laboratory within 24 h after sampling.

The *E. coli* strains were isolated and biochemically confirmed (BAM, 2017). The *E. coli* isolates were selected on MacConkey agar and Eosin Methylene Blue agar and further confirmed by indole test. One *E. coli* colony was collected from each positive sample. The *Salmonella* strains were isolated according to ISO6579:2002 (E) (ISO, 2002) and subjected to serotyping by slide agglutination (Popoff and LeMinor, 1992). A single colony of each serotype was collected from each positive sample. Forty-five serovars were included, of which serovars Typhimurium and Rissen were most common (Table 9).

Table 8. Source and number of *E. coli* and *Salmonella* isolates (n = 1,619)

Bacteria	Country	Provinces	Sampling location	Sample type	No. of isolates	Total
E. coli (n=988)	Thailand (n=508)	Nong Khai, Mukdahan, Sa Kaeo	Slaughterhouse	Pig rectal swab	180	508
			Slaughterhouse	Pig carcass swab	185	
			Retail meat market	Pork	143	
	Lao PDR (n=351)	Vientiane, Savannakhet	Slaughterhouse	Pig rectal swab	115	351
			Slaughterhouse	Pig carcass swab	132	
			Retail meat market	Pork	104	
	Cambodia (n=129)	Banteay Meanchey, Siem Reap	Slaughterhouse	Pig rectal swab	82	129
			Slaughterhouse	Pig carcass swab	11	
			Retail meat market	Pork	36	
Salmonella (n=631)	Thailand (n=276)	Nong Khai, Mukdahan, Sa Kaeo	Slaughterhouse	Pig rectal swab	58	276
			Slaughterhouse	Pig carcass swab	63	
			Retail meat market	Pork	155	
	Lao PDR (n=237)	Vientiane, Savannakhet	Slaughterhouse	Pig rectal swab	59	237
			Slaughterhouse	Pig carcass swab	82	
			Retail meat market	Pork	96	
	Cambodia (n=118)	Banteay Meanchey, Siem Reap	Slaughterhouse	Pig rectal swab	22	118
			Slaughterhouse	Pig carcass swab	29	
			Retail meat market	Pork	67	
Grand total						1,619

Table 9. *Salmonella* serovars from pig, pig carcass, and pork samples in Thailand, Lao PDR, and Cambodia border provinces in this study (n = 631)

Salmonella serovars	No. of isolates												Total
	Thailand				Lao PDR				Cambodia				
	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	
Afula					2			2					2
Agona							1	1					1
Anatum	3		15	18	9	**25**	16	50	1	1		2	70
Braenderup											1	1	1
Chincol											1	1	1
Corvallis			21	21									21
Derby			4	4	4	**3**	10	17	3	6		9	30
Dessau					2			2					2
Doncaster										1		1	1
Duesseldort			4	4									4
Eindegi					1	**6**	2	9					9
Enteritidis	1			1						1		1	2
Fufu									1			1	1
Give	1	2	1	4	1		1	2					6
Havana							2	2					2
Hvittingfoss			2	2		**1**		1					3
Indians											1	1	1
Kedougou	11	4	11	26									26
Kentucky		1		1									1
Lexington									3		2	5	5
London						**1**		1					1
Mbandaka											1	1	1

Salmonella serovars	No. of isolates												Total
	Thailand				Lao PDR				Cambodia				
	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	
Menden	1			1									1
Monschaui			2	2									2
Mowanjum											1	1	1
Newmexico									2	1		3	3
Norwish										1		1	1
Panama		1	2	3	1			1					4
Parathyphi		1	1	2					3	3	13	19	21
Preston							2	2					2
Readind					1			1					1
Rissen	11	20	16	47	7	**11**	17	35	4		42	46	128
Saintpaul		6	7	13									13
Schleissheim										1	1	2	2
Schwarzengrund			1	1									1
Singapore										2	1	3	3
Stanley	1	6	9	16	11	**10**	13	34		1	1	2	52
Stuttgart			1	1									1
Tsevie							**2**	2					2
Typhimurium	27	16	52	95	15	**22**	**19**	56	3	8		11	162
Urbana			2	2									2
Wandsworth										1		1	1
Warragul									1	1		2	2
Welterreden	1	6	4	11	5	**3**	**1**	9	1			1	21
Worthington	1			1			**10**	10		1	2	3	14
Total	58	63	154	276	59	**82**	**96**	237	22	29	67	118	631

3.3.2 Determination of colistin and meropenem susceptibility (n = 1,619)

Minimum inhibitory concentrations (MICs) of colistin was determined using agar dilution method (CLSI, 2015). The joint CLSI-EUCAST Polymyxin Breakpoints Working Group has recommended broth microdilution method for determining colistin MIC. However, plastic binding feature of colistin and a synergistic effect of polysorbate 80 with colistin has been observed (Dafopoulou et al., 2015). Agar dilution method generates colistin MIC value comparable to broth microdilution method (Dafopoulou et al., 2015) and is superior in reproducibility, robustness, and user-friendly. Therefore, agar dilution method was used to determine colistin MIC in this study. Colistin concentration range was 0.125-64 µg/mL with MIC breakpoint of 2 µg/mL (EUCAST, 2017). *E. coli* ATCC25922, *Staphylococcus aureus* ATCC29213 and *Pseudomonas aeruginosa* ATCC27853 served as quality control strains.

Carbapenemase production was screened by disk diffusion method using meropenem disks (10 µg) (CLSI, 2015) and confirmed by modified Hodge test (MHT) with meropenem (10 µg). *E. coli* ATCC25922 was control strain.

3.3.3 PCR and DNA sequencing analysis

Total DNA prepared by whole cell boiling were used as PCR template (Levesque et al., 1995). All primers are listed in Table 10. PCR products were purified using Nucleospin® Gel and PCR clean up (Macherey-Nagel, Düren, Germany) and submitted for DNA sequencing using PCR primers (First Base Laboratories, Selangor Darul Ehsan, Malaysia). The nucleotide sequences were analyzed by comparison to published sequences available at NCBI website.

All bacterial isolates (n=1619) were screened for colistin-resistance encoding genes, including *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* by using multiplex PCR with specific primers (Rebelo et al., 2018). Representatives of PCR amplicons were purified

and submitted for DNA sequencing to confirm the specificity of the PCR amplification. The DNA sequences obtained were deposited in GenBank database, of which accession numbers are as follows: *mcr-1*, MW314264; *mcr-3*, MW314265 and *mcr-5*, MW314266.

The presence of carbapenem-resistance encoding genes was detected in all bacterial isolates using multiplex PCR with specific primers (Poirel et al., 2011), including bla_{IMP}, bla_{VIM}, bla_{OXA}, bla_{NDM-1} and bla_{KPC}.

Table 10. Primers used in this study

Target	Primer	Primer sequenc (5′-3′)	Product size (bp)	Reference
MCR				
Multiplex	*mcr1*-F	AGTCCGTTTGTTCTTGTGGC	320	(Rebelo et al., 2018)
	mcr1-R	AGATCCTTGGTCTCGGCTTG		
	mcr2-F	CAAGTGTGTTGGTCGCAGTT	715	(Rebelo et al., 2018)
	mcr2-R	TCTAGCCCGACAAGCATACC		
	mcr3-F	AAATAAAAATTGTTCCGCTTATG	929	(Rebelo et al., 2018)
	mcr3-R	AATGGAGATCCCCGTTTTT		
	mcr4-F	TCACTTTCATCACTGCGTTG	1116	(Rebelo et al., 2018)
	mcr4-R	TTGGTCCATGACTACCAATG		
	mcr5-F	ATGCGGTTGTCTGCATTTATC	1644	
	mcr5-R	TCATTGTGGTTGTCCTTTTCTG		
Carbapenemase genes				
Multiplex	bla_{IMP} -F	GGAATAGAGTGGCTTAAYTCTC	232	(Poirel et al., 2011)
	bla_{IMP}-R	GGTTTAAYAAAACAACCACC		
	bla_{VIM} -F	GATGGTGTTTGGTCGCATA	390	(Poirel et al., 2011)
	bla_{VIM} -R	CGAATGCGCAGCACCAG		
	bla_{OXA} -F	GCGTGGTTAAGGATGAACAC	477	(Poirel et al., 2011)
	bla_{OXA}-R	CATCAAGTTCAACCCAACCG		
	bla_{NDM}-F	GGTTTGGCGATCTGGTTTTC	621	(Poirel et al., 2011)
	bla_{NDM}-R	CGGAATGGCTCATCACGATC		
	bla_{KPC}-F	CGTCTAGTTCTGCTGTCTTG	798	(Poirel et al., 2011)
	bla_{KPC}-R	CTTGTCATCCTTGTTAGGCG		
PBRT				
Multiplex 1	HI1-F	GGAGCGATGGATTACTTCAGTAC	471	(Carattoli et al., 2005)
	HI1-R	TGCCGTTTCACCTCGTGAGTA		
	HI2-F	TTTCTCCTGAGTCACCTGTTAACAC	644	(Carattoli et al., 2005)
	HI2-R	GGCTCACTACCGTTGTCATCCT		
	I1-F	CGAAAGCCGGACGGCAGAA	139	(Carattoli et al., 2005)
	I1-V	TCGTCGTTCCGCCAAGTTCGT		

Target	Primer	Primer sequenc (5′-3′)	Product size (bp)	Reference
Multiplex 2	X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	(Carattoli et al.,
	X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC		2005)
	L/M-F	GGATGAAAACTATCAGCATCTGAAG	785	(Carattoli et al.,
	L/M-R	CTGCAGGGGCGATTCTTTAGG		2005)
	N-F	GTCTAACGAGCTTACCGAAG	559	(Carattoli et al.,
	N-R	GTTTCAACTCTGCCAAGTTC		2005)
Multiplex 3	FIA-F	CCATGCTGGTTCTAGAGAAGGTG	462	(Carattoli et al.,
	FIA-R	GTATATCCTTACTGGCTTCCGCAG		2005)
	FIB-F	GGAGTTCTGACACACGATTTTCTG	702	(Carattoli et al.,
	FIB-R	CTCCCGTCGCTTCAGGGCATT		2005)
	W-F	CCTAAGAACAACAAAGCCCCCG	242	(Carattoli et al.,
	W-R	GGTGCGCGGCATAGAACCGT		2005)
Multiplex 4	Y-F	AATTCAAACAACACTGTGCAGCCTG	765	(Carattoli et al.,
	Y-R	GCGAGAATGGACGATTACAAAACTTT		2005)
	P-F	CTATGGCCCTGCAAACGCGCCAGAAA	534	(Carattoli et al.,
	P-R	TCACGCGCCAGGGCGCAGCC		2005)
	FIC-F	GTGAACTGGCAGATGAGGAAGG	262	(Carattoli et al.,
	FIC-R	TTCTCCTCGTCGCCAAACTAGAT		2005)
Multiplex 5	A/C-F	GAGAACCAAAGACAAAGACCTGGA	465	(Carattoli et al.,
	A/C-R	ACGACAAACCTGAATTGCCTCCTT		2005)
	T-F	TTGGCCTGTTTGTGCCTAAACCAT	750	(Carattoli et al.,
	T-R	CGTTGATTACACTTAGCTTTGGAC		2005)
	FIIS-F	CTGTCGTAAGCTGATGGC	270	(Carattoli et al.,
	FIIS-R	CTCTGCCACAAACTTCAGC		2005)
Simplex 1	FrepB-F	TGATCGTTTAAGGAATTTTG	270	(Carattoli et al.,
	FrepB-R	GAAGATCAGTCACACCATCC		2005)
Simplex 2	K-F	GCGGTCCGGAAAGCCAGAAAAC	160	(Carattoli et al.,
	K-R	TCTTTCACGAGCCCGCCAAA		2005)
Simplex 3	B/O-F	GCGGTCCGGAAAGCCAGAAAAC	159	(Carattoli et al.,
	B/O-R	TCTGCGTTCCGCCAAGTTCGA		2005)
MLST	*fimA*-F	TCAGGGGAGAAACAGAAAACTAAT	760	(Sukhnanand et
	fimA-R	TCCCCGATAGCCTCTTCC		al., 2005)
	manB-F	CCGGCACCGAAGAGA	893	(Sukhnanand et
	manB-R	CGCCGCCATCCGGTC		al., 2005)
	mdh-F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	849	(Sukhnanand et
	mdh-R	ATATCTTTYTTCAGCGTATCCAGCAT		al., 2005)

3.3.4 Conjugation experiments and plasmid replicon typing

Biparental mating experiment was performed in all *mcr*-positive *E. coli* and *Salmonella* isolates. All *mcr*-carrying *E. coli* (n=57) were used as donors and spontaneous rifampicin-resistant *Salmonella* Enteritidis SE12 (SE12rifr, MIC = 256 μg/mL) was used as recipient (Khemtong and Chuanchuen, 2008). Transconjugants were selected on LB agar containing rifampicin (32 μg/mL) with colistin (2 μg/mL) and confirmed to be *Salmonella* on brilliant green agar and xylose lysine deoxycholate agar (Difco®, MD, USA) containing 2 μg/mL colistin. For all *mcr*-positive *Salmonella* isolates (n=11), the spontaneous rifampicin-resistant *E. coli* K12 strain MG1655 (MG1655rifr) was used as recipient (Khemtong and Chuanchuen, 2008). Transconjugants were selected on LB agar containing rifampicin (32 μg/mL) with colistin (2 μg/mL) and confirmed to be *E. coli* on MacConkey agar and Eosin Methylene Blue agar (Difco®, MD, USA) containing 2 μg/mL colistin. The presence of the corresponding genes in all transconjugants was confirmed by PCR with specific primers as described above.

Plasmid Inc groups were determined in all the isolates contained conjugative plasmid by a PCR-based replicon typing (PBRT) (Carattoli et al., 2005). Eighteen plasmid Inc groups were detected by using 5 multiplex PCR (i.e., HI1/HI2/I1-Iγ; X/L-M/N; FIA/FIB/W; Y/P/FIC and A-C/T/FIIA) and 3 simplex PCR (i.e. F, K and B/O).

3.3.5 *Salmonella* phylogenetic analysis

All *mcr*-positive *Salmonella* (n=11) were characterized for phylogenetic groups using multilocus sequence typing (MLST). Three housekeeping genes, *fimA*, *manB* and *mdh* were PCR amplified as previous described (Sukhnanand et al., 2005). All PCR products were purified and submitted for nucleotide sequencing. The obtained sequences were assembled and proofreaded using the DNASTAR program

(Burland, 2000) and aligned using the MUSCLE options in MEGA-X software (Kumar et al., 2018). A phylogenetic tree with 1000 bootstrap replicates was constructed based on a concatenated alignment of *fimA*, *manB*, and *mdh* sequences using the Neighbor-Joining (NJ) method (Sukhnanand et al., 2005). The Maximum Composite Likelihood method was used to calculates the evolutionary distances (Kumar et al., 2018). All positions containing gaps and missing data were removed for each sequence pair. A total of 18,129 positions was present in the final data set. Evolutionary analyses were conducted using MEGA X (Kumar et al., 2018).

3.3.6 Statistical analysis

Statistical analysis was performed by using SPSS version 22.0 (IBM Corporation) program. The chi-squared test was used to compare AMR phenotype and genotype. The statistical significance ($p<0.05$) of differences between AMR phenotypic and genotypic percentage was determined.

3.4 Results

3.4.1 Phenotypic resistance to colistin and meropenem (n = 1,619)

Colistin resistance rates of *E. coli* and *Salmonella* from Thailand, Lao PDR and Cambodia were presented in Table 11. The prevalence of colistin-resistant *E. coli* was higher than that of *Salmonella* in all countries ($p<0.05$). Colistin resistance was commonly observed in either *E. coli* (17.8%; 23/129) or *Salmonella* (1.7%; 2/118) from Cambodia ($p<0.05$). When considering sample sources, colistin-resistant *E. coli* were most common in pigs in Thailand (9.4%; 17/180), Lao PDR (7.8%; 9/115) and Cambodia (22%; 18/82). Colistin-resistant *Salmonella* was most frequently identified in pigs in Thailand (3.4%; 2/58) and Cambodia (4.5%; 1/22) (Table 11). None of the isolates in this collection were meropenem resistant.

3.4.2 Distribution of *mcr* genes (n = 1,619)

Of all the *E. coli* isolates (n = 988), 57 isolates (5.8%; 57/988) carried at least one *mcr* gene including the isolates from Thailand (3.5%; 18/508), Lao PDR (6%; 21/351) and Cambodia (14%; 18/129) (Table 12). The percentage of *mcr*-carrying *E. coli* isolates in Cambodia was significantly higher than Thailand and Lao PDR ($p<0.05$). The *mcr* genes were most common among the pig isolates (8.8%; 33/377), followed by the pig carcasses (4.3%; 14/328) and pork (3.5%; 10/283) isolates and corresponded to their colistin resistance phenotype. The percentage of *mcr*-carrying *E. coli* from pigs was significantly higher than that from pork ($p<0.05$). The *mcr-1* gene was predominant (56.1%; 32/57), followed by *mcr-3* (38.6%; 22/57), *mcr-1/mcr-3* (3.5%; 2/57), and *mcr-5* (1.8%; 1/57). The *mcr* genes were more commonly identified among *E. coli* (5.8%; 57/988) than *Salmonella* (1.7%; 11/631) isolates ($p<0.05$). No *mcr-2* and *mcr-4* were detected.

The co-occurrence of *mcr-1/mcr-3* was detected in two colistin-susceptible isolates (colistin MIC=1 µg/mL) from pigs in Thailand. Based on our best knowledge, this is the first report of *mcr-5* in a pork isolate in Cambodia (colistin MIC=2 µg/mL).

In *Salmonella* (n=631), 11 isolates (1.7%; 11/631) were positive to at least one *mcr* gene tested, including 6 Thai (2.2%; 6/276) and 5 Cambodian (4.2%; 5/118) isolates. The *mcr* genes were more common in the pig isolates than the pork isolates ($p<0.05$). The *mcr-3* gene (90.9%; 10/11) was most common, followed by *mcr-1* (9.1%; 1/11). None were positive to *mcr-2*, *mcr-4*, or *mcr-5*.

None of the *E. coli* and *Salmonella* isolates were positive to carbapenem-resistance genes tested.

Table 11. Colistin resistance rates of *E. coli* and *Salmonella* from pigs, pig carcasses and pork in Thailand, Lao PDR and Cambodia (n = 1,619)

Bacteria	Number of bacterial isolates												
	Thailand [a]				Lao PDR [a]				Cambodia [a]				Grand total
	Pig	Carcass	Pork	Total	Pig	Carcass	Pork	Total	Pig	Carcass	Pork	Total	
E. coli	9.4% (17/180)	5.4% (10/185)	7.7% (11/143)	7.5% (38/508)	7.8% (9/115)	6.1% (8/132)	1.9% (2/104)	5.4% (19/351)	22% (18/82)	9.1% (1/11)	11.1% (4/36)	17.8% (23/129)	8% (80/988)
Salmonella	3.4% (2/58)	0 (0)	0.6% (1/155)	1.1% (3/276)	0 (0)	1.2% (1/83)	0 (0)	0.5% (1/218)	4.5% (1/22)	0 (0)	1.5% (1/67)	1.7% (2/118)	1% (6/631)

[a] Number of colistin resistant isolates/ Number of bacterial isolates from each source

Table 12. Presence and transfer of *mcr* genes in *E. coli* and *Salmonella* (n = 1,619)

Bacteria	Country	***mcr*-gene**	**Sample source**	**MIC (µg/mL)**	**No. of isolates (%)**	**Transferability** [a]
E. coli	Thailand	*mcr-1*	Pig	8	4	-
(n=988)	(n=508)		Pig carcass	0.5, 8	3	+ (1)
			Pork	4	1	+ (1)
		mcr-3	Pig	4-8	6	-
			Pig carcass	0.5	1	-
			Pork	8	1	+ (1)
		mcr-1/mcr-3	Pig	1	2	-
		Subtotal				18 (4%)
	Lao PDR	*mcr-1*	Pig	8	3	-
	(n=351)		Pig carcass	4-8	6	-
			Pork	4-8	2	-
		mcr-3	Pig	4-8	5	+ (1)
			Pig carcass	2-8	3	-
			Pork	0.5-4	2	-
		Subtotal				21 (6%)
	Cambodia	*mcr-1*	Pig	8-16	10	-
	(n=129)		Pig carcass	8	1	-
			Pork	4-8	2	-
		mcr-3	Pig	4	3	-
			Pork	0.5	1	-
		mcr-5	Pork	2	1	-
		Subtotal				18 (14%)
	Grand Total					57 (6%)
Salmonella	Thailand	*mcr-1*	Pig	1	1	-
(n=631)	(n=276)	*mcr-3*	Pig	1-8	3	+ (2)
			Pork	1-2	2	-
		Subtotal				6 (2.2%)
	Cambodia	*mcr-3*	Pig	0.5-4	4	+ (1)
	(n=118)		Pork	4	1	-
		Subtotal				5 (4.2%)
	Grand total					11 (2.8%)

[a]The number indicates the colistin-resisant isolate that can transfer *mcr*

3.4.3 Horizontal transfer and plasmid replicon typing of *mcr* gene carrying isolates

Two *E. coli* isolates from pig carcass and pork in Thailand conjugally transferred *mcr-1* to *Salmonella* recipients. The *mcr-3* gene in 2 *E. coli* isolates (one from Thai pork and the others from Laos pig) and 3 *Salmonella* (2 from Thai pigs and one from Cambodian pig) was horizontally transferred. All the isolates were resistant to colistin (colistin MIC = 4-8 μg/mL) and harbored IncF plasmid. The *mcr*-conjugative plasmids were in IncF and IncI groups (Table 13).

Table 13. Characteristics of *E. coli* and *Salmonella* transconjugant (n = 7)

Bacteria	Country	Sample	MIC (μg/mL)	Bacterial donor		Bacterial recipient	
				mcr gene	Inc group	*mcr* gene	Inc group
E. coli	Thailand	Carcass	8	*mcr-1*	IncI, IncFIB, IncFrepB	*mcr-1*	IncI
E. coli	Thailand	Pork	4	*mcr-1*	IncFrepB	*mcr-1*	IncFrepB
E. coli	Thailand	Pork	8	*mcr-3*	IncY, IncFrepB	*mcr-3*	IncFrepB
E. coli	Lao PDR	Pig	8	*mcr-3*	IncFrepB	*mcr-3*	IncFrepB
Salmonella	Thailand	Pig	8	*mcr-3*	IncI, IncFIB	*mcr-3*	IncI
Salmonella	Thailand	Pig	8	*mcr-3*	IncI, IncFIB	*mcr-3*	IncI
Salmonella	Cambodia	Pig	4	*mcr-3*	IncFrepB	*mcr-3*	IncFrepB

3.4.4 Evolutionary relationship among *mcr*-carrying *Salmonella*

NJ phylogenetic tree showed that *mcr*-carrying *Salmonella* isolates (n=11) were classified into two clusters, Clusters A and B (Table 14). The percentage of similarity within each cluster ranged from 94% to 97% and the average branch lengths of the *Salmonella* isolates within the same cluster ranged from 0.00 to 0.01 (Figure 1). The phylogenetic cluster A (n= 7) was predominant, including 4 isolates from Thailand [*S.* Typhimurium from pigs (n=2), *S.* Kedougou from pork (n=1) and *S.*

Rissen from a pig (n=1)] and 3 isolates from Cambodia [*S.* Parathyphi from pigs (n=2) and *S.* Kentucky from a pig (n=1)]. Cluster B consists of 4 *Salmonella* isolates, including 3 isolates from Thailand [*S*. Typhimurium from a pig (n=1), *S*. Huettwillen from a pig (n=1), and *S*. Rissen from pork (n=1)] and one isolate from Cambodia [*S*. Typhimurium from pork (n=1)].

Table 14. Cluster and characteristics of selected *Salmonella* in MLST (n = 11)

Cluster	*Salmonella* Serovars (n=11)	Country	Source	*mcr* gene
A	Parathyphi (n=2)	Cambodia	Pig	*mcr 3*
	Kentucky (n=1)	Cambodia	Pig	*mcr 1*
	Typhimurium (n=2)	Thailand	Pig	*mcr 3*
	Kedougou (n=1)	Thailand	Pork	*mcr 3*
	Rissen (n=1)	Thailand	Pig	*mcr 1*
B	Typhimurium (n=1)	Cambodia	Pig	*mcr 3*
	Typhimurium (n=1)	Cambodia	Pork	*mcr 3*
	Huettwillen (n=1)	Thailand	Pig	*mcr 3*
	Rissen (n=1)	Thailand	Pork	*mcr 3*

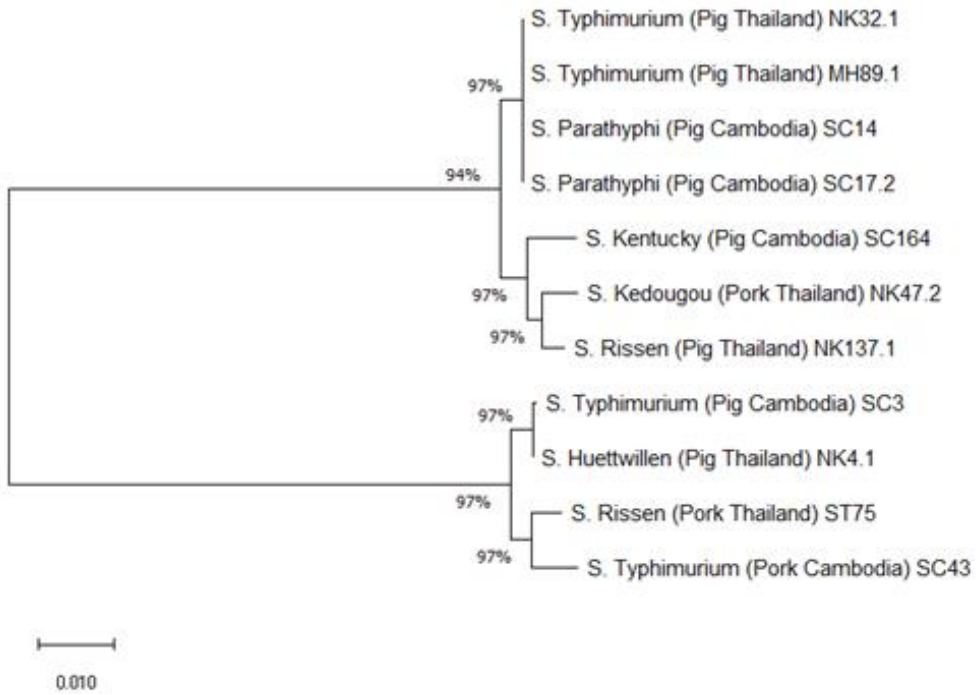

Figure 1. Phylogenetic tree of *Salmonella* isolates based on the concatenated *manB*, *mdh* and *fimA* sequence (n = 11)

3.5 Discussion

One of the major findings of this study was the presence of colistin-resistant *E. coli* and *Salmonella* harboring *mcr* in pigs, pig carcass, and pork, indicating the circulation of colistin-resistant bacteria and genes in pig production chain in the region. A previous study demonstrated that the flattening pigs entering slaughterhouses acted as a vehicle of colistin-resistant bacteria distributed to slaughterhouse environment (Zhang et al., 2019). This is in agreement with the observation of high percentage of colistin-resistant *E. coli* in pigs in this study. Pig carcasses might be contaminated with resistant bacteria during slaughtering process and transfer to pork at retail meat markets (Huang et al., 2017). The resistant bacteria may spread to workers at pig farms or slaughterhouses and butchers at the retail markets by direct contact and food chain. These results support that the circulation of colistin-resistant bacteria in pig and pork production chain serve as a potential route for introducing resistant bacteria to humans and environments (Huang et al., 2017).

For Thailand, colistin-resistant *E. coli* (7.5%) in this study was more common than a previous study reporting the absence of colistin-resistant *E. coli* in pig farms in other provinces of Northeastern Thailand (Olaitan et al., 2014). Even though the extent of antimicrobial use remains largely unclear, the results may reflect the increase of colistin consumption in pig production in the area (Zhang et al., 2019). This discrepancy may be associated with diverse sources and different geographical origins of the samples as well. Additionally, colistin resistance could be a result of co-selection caused by other antimicrobials. A previous study in *E. coli* showed that host cationic antimicrobial peptides (e.g. LL-37 and lysozyme) can promote cross-resistance to polymyxins, which possibly explain the spread of colistin resistance in the limited colistin use (Xu et al., 2018). In contrast, the percentage of colistin

resistance in Cambodia (17.8%) was significantly higher than that in Thailand (7.5%) and Lao PDR (5.4%), but it was slightly lower than a previous study in Cambodia (Strom et al., 2017). This may be due to the higher use of colistin and other antimicrobial drugs in the country, in agreement with a previous study revealing that higher and longer application of antimicrobials in pig feed on-farm may cause the increase AMR prevalence in Cambodia (Strom et al., 2017).

The phenotypic colistin resistance and *mcr* in *E. coli* is more common than that in *Salmonella* ($P<0.05$). Previous study showed that resistance rates to antimicrobials in different classes in *E. coli* were frequently higher than those in *Salmonella* (Langata et al., 2019). *E. coli* are commensals existing in the intestinal tract of all animals and have longer contact duration to antimicrobials administered to animals than *Salmonella*. Therefore, they have greater effectiveness in development of resistance to antimicrobials than *Salmonella* (Varga et al., 2008). In addition, AMR development in *E. coli* is more affected by antimicrobials used than that in *Salmonella* in the same condition (Varga et al., 2008).

The *mcr-1* and *mcr-3* genes were predominant *mcr* variants in this study. The *mcr-1* gene was most common among colistin-resistant *E. coli* (59.6%), in agreement with previous study in pigs in China (Zhang et al., 2019). This gene has been detected in *E. coli* and *Salmonella* from humans and livestock in most parts of the world, including Asia (e.g. China) (Huang et al., 2017), Europe (e.g. Spain) (Garcia-Menino et al., 2019) America (e.g. Argentina) (Dominguez et al., 2018) and South Africa (e.g. Algeria) (Touati et al., 2019). The *mcr-1* gene was located on conjugative plasmid, supporting its wide presence in *E. coli* and *Salmonella* observed. However, it was previously noted that *mcr-1* is stable and can be transferred in the absence of selective pressure from colistin (Xu et al., 2018). The *mcr-3* gene was found in *E. coli* and *Salmonella* isolates from pigs, pig carcasses, and pork in Thailand and Cambodia

but predominant among colistin-resistant *Salmonella* (10/11). Previous studies reported *mcr-3* in *Salmonella* from patients in Canada (Mulvey et al., 2018) and Denmark (Litrup et al., 2009) who traveled to Thailand and *E. coli* and *Klebsiella pneumoniae* from patients in Thailand (Wise et al., 2018). Plasmid carrying *mcr-3* was previously observed in *E. coli* from pigs in China and Malaysia, of which its sequence was similar to that in the *K. pneumoniae* patient isolates in Thailand (Yin et al. 2017). These data indicate the worldwide spread of *mcr-3* in humans and animals. An *E. coli* isolate from pork in Cambodia was found to carry *mcr-5*. The *mcr-5* gene was previously detected in *E. coli* from pigs and poultry in China, and Spain (Garcia-Menino et al., 2019), suggesting the wide distribution of *mcr-5*. To our knowledge, this is the first report of *mcr-5* in *E. coli* from pork in Cambodia.

Coexistence of *mcr-1* and *mcr-3* was observed in *E. coli* from pigs in Thailand, in agreement with previous studies in Taiwan and China (Liu et al., 2017). Almost all the *E. coli* and *Salmonella* isolates carrying individual *mcr-1* or *mcr-3* were resistant to colistin (MIC≥2 µg/mL), suggesting that the presence of *mcr* in these isolates was well corresponded to colistin resistance phenotype. In contrast, the *E. coli* isolates carrying *mcr-1/mcr-3* were susceptible to colistin (MIC = 1 µg/mL), in agreement with the study in China (Liu et al., 2017) and supporting that coexistence of *mcr* in a single *E. coli* isolate does not generate cumulative effect on colistin susceptibility (Liu et al., 2017). These results indicate that the contribution of *mcr* to colistin resistance phenotype varies and suggest that *mcr* genes should be screened in either colistin-susceptible or resistant isolates.

In this study, transconjugants (n=7) harbored *mcr* gene on IncF (n=4) or IncI (n=3) plasmids, in agreement with a previous study demonstrating IncF, IncI, IncHI2, and IncX4 plasmids carrying *mcr-1* and *mcr-3* (Khine et al., 2020). IncF plasmid has been shown to be associated with several resistance genes worldwide. Taken

together, these observations highlight the potential distribution of *mcr* genes globally.

The prevalence of *mcr* genes in *E. coli* and *Salmonella* from Cambodia was significantly higher than the isolates from Thailand and Lao PDR, in agreement with the colistin resistance phenotype observed and suggesting that the colistin resistance phenotype was being driven by the presence of the *mcr* genes (Peng et al., 2019). Such higher prevalence may be associated with the use of colistin and/or antimicrobial drugs in other classes on-farm, resulting in the preserving and transferring of the genes (Olaitan et al. 2014).

Infection with *mcr*-carrying *Salmonella* may be difficult to treat and result in treatment failure. Knowledge of the genetic relatedness of *mcr*-harboring *Salmonella* will increase the understanding of their transmission mode (Sukhnanand et al., 2005). In this study, all *mcr*-positive *Salmonella* were categorized into two clusters by MLST. Each cluster contained the pig and pork isolates from Thailand and Cambodia. The close similarity within the same cluster indicates the clonal expansion of *mcr*-positive *Salmonella* between the two countries. The *mcr*-positive *Salmonella* in Thailand and Cambodia were categorized into two clusters and these could be due to the multiple and independent acquisitions of *mcr* in bacteria in the same geographical area. This is similar to a study in China where *mcr-1* was harbored by multiple clones of *E. coli* isolates (e.g. ST10, ST101, and ST410) from hospital sewage water (Jin et al., 2018). *E. coli* ST624 with *mcr-1* isolates from a patient in South Africa was previously identified in *E. coli* from poultry in Spain and Japan (Poirel et al., 2016). Taken together, these observations indicate that *mcr-1* has circulated among different bacterial clones within and across countries and these may occur by diverse independent acquisitions of *mcr*-carrying plasmids (Jin et al., 2018).

CRE has spread globally and was previously reported in patients in the study areas (Atterby et al., 2019). In contrast, all *E. coli* isolates in this study were susceptible to meropenem and not positive to any carbapenem resistance genes tested. Carbapenems are not approved for use in livestock in any countries, resulting in minimized selective pressure conditions (Long et al., 2019). Simultaneously, carbapenem use is uncommon in livestock production in the region, mainly due to their high cost.

In summary, colistin resistance was detected in *E. coli* and *Salmonella* isolated from pigs, pig carcasses, and pork in Thailand, Lao PDR, and Cambodia border areas. The *mcr-1*, *mcr-3*, and *mcr-5* genes were found and the co-occurrence of *mcr-1* and *mcr-3* in the same *E. coli* isolates from pigs was observed. The *mcr-1* and *mcr-3* genes were horizontally transferred, while resistance to meropenem was absent. Monitoring and surveillance of resistance to last line antibiotics at phenotypic and genotypic level should be encouraged as a collaboration between countries. Further study to characterize genetic structure of *mcr*-carrying plasmids is in progress.

CHAPTER IV

Genomic characterization of antimicrobial resistance in *mcr*-carrying ESBL producing *Escherichia coli* from pigs and humans

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Genomic characterization of antimicrobial resistance in *mcr*-carrying ESBL producing *Escherichia coli* from pigs and humans

4.1 Abstract

Whole genome sequencing (WGS) was conducted to characterize *mcr*-carrying ESBL-producing *E. coli* (n=7). These *E. coli* isolates originated from two pigs (TH2 and TH3) and two humans (TH8 and TH9) of Thailand, and three pigs of Lao PDR (LA1, LA2, and LA3). Four *E. coli* sequence types/serotypes, including ST6833/H20 (TH2 and TH3), ST48/O160:H40 (TH8 and TH9), ST5708/H45 (LA1), and ST10562/O148:H30 (LA2 and LA3) were identified. The plasmid replicon type IncF was identified in all isolates. The point mutations Ser31Thr in PmrA and His2Arg in PmrB were concurrently found in all isolates (colistin MIC=4-8 µg/mL). LA1 contained up to 5 point mutations in PmrB, of which colistin MIC was not significant different with other isolates. All *mcr-1.1* was located in *ISApl1-mcr-1-pap2* element, while all *mcr-3.1* was located in *TnAs2-mcr-3.1-dgkA-ISKpn40* element. The *mcr-3.1* and $bla_{\text{CTX-M55}}$ genes were co-localized on the same plasmid, which concurrently contained *cml*, *qnrS1*, and *tmrB*. The $bla_{\text{CTX-M55}}$ and *mcr-3.1* genes were located on conjugative plasmid and could be horizontally transferred under selective pressure of ampicillin or colistin. In conclusion, the comprehensive insights into the genomic information of ESBL-producing *E. coli* harboring *mcr* were obtained As *mcr*-carrying ESBL-producing *E. coli* were detected in pigs and humans, a holistic and multisectoral One Health approach is required to contain AMR.

Keywords: Antimicrobial resistance, Pigs, Humans, Thailand, Lao PDR

4.1 Introduction

Third and new generation cephalosporins and polymyxins (e.g. colistin) are WHO-highest priority critically important antimicrobials (HPCIA) and reserved as last resort antibiotics for human patients. These antibiotics should not be used as first-line treatment in food animals with the exception that there is no other effective treatment option. Third and new generation cephalosporins are used for treatment of respiratory infections in pigs, while colistin is used for treatment of intestinal infections, particularly post weaning diarrhea and colibacillosis in pigs (Wang et al. 2019; Urumova 2015). Up to date, resistance to 3rd and newer generation cephalosporins and colistin has been increasingly reported in food animals (Zhang et al. 2019).

ESBL-producing Enterobacteriaceae have increased in prevalence and currently become a critical antimicrobial-resistant pathogen posing a major threat to human an animal health (Liu et al. 2018). ESBL production is usually plasmid mediated and frequently associated with plasmid replicons of IncFII, IncFIB, IncI1, and IncN group (Kim et al. 2018). The CTX-M type ESBLs have become the most dominant genotype globally and are usually associated with co-resistance to fluoroquinolones and aminoglycosides (Velhner et al. 2018). Several CTXM-type have been reported among bacteria from human and food animal populations, of which *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-55}}$ are the most common variants in Asian countries (Kim et al. 2018). *Escherichia coli* plays a role as one of the most important reservoirs of ESBL genes. This has become a major public health concern due to the discovery of plasmid-mediated colistin resistance *mcr* genes co-localized on the same conjugative plasmid with ESBL genes with its potential for global spread (Zhang et al. 2019).

The first plasmid-mediated *mcr-1* gene was discovered in members of Enterobacteriaceae from humans and food-producing animals in China in 2015. Since

then, many *mcr* variants have been reported (Liu et al. 2016). Up to date, *mcr-1* to *mcr-10* have been identified in Enterobacteriaceae, of which *mcr-1* and *mcr-3* have disseminated globally (Li et al. 2021). Prior to the discovery, all focused on chromosomally-mediated colistin resistance mechanisms, the PhoPQ component and the pmrCAB operon. Up to date, many plasmid replicon types have been shown to be related to horizontal transfer of *mcr*, such as IncI2, IncHI1, IncHI2, IncP, IncFIB, and IncX4 (Zhang et al. 2019).

The genetics underlying AMR are complicated and dynamic and the inherent adaptability of plasmids happens all the time (Orlek et al. 2017). The study of individual resistance mechanism has been shown inadequate. Whole genome sequencing (WGS) analysis for AMR bacteria is an effective method providing a comprehensive characterization of the bacteria to elucidate the genetic relatedness, the full picture of resistance genes and mutations conferring resistance. Together with the development of new bioinformatics tools, novel or uncharacterized AMR genes can be discovered, without time and labor-intensive procedure as required in phenotypic testing.

Recently, ESBL production and colistin resistance in *E. coli* isolated from food-producing animals and humans in Thailand and Lao PDR have been reported (Lay et al. 2021; Khine et al. 2020; Eiamphungporn et al. 2018). However, comprehensive knowledge regarding AMR by WGS is rather limited. Therefore, this study aimed to characterize the genomic information of *mcr* carrying-ESBL producing *E. coli* isolated from pigs and humans in Thailand and Lao PDR border area by WGS analysis.

4.2 Materials and Methods

4.2.1 Source and identification of bacterial isolates

During 2013-2018, 847 *E. coli* isolates were collected from pigs, pig carcasses, pork, and humans in Thailand and Lao PDR border provinces as part of our AMR epidemiological study (Pungpian et al., 2021). The samples included rectal swab from dead pigs and carcass swab from pig carcasses at the slaughterhouses; pork swab at retail markets; self-collected stool samples from workers at slaughterhouses and butchers at retail markets and stool samples from diarrhea patients collected by on-duty nurses at local hospitals. The sampling sites were border provinces with crossing points between Thailand and Lao PDR, including Nong Khai-Vientiane and Mukdahan-Savannakhet, respectively. Antimicrobial susceptibility, ESBL production and ß-lactamase genes were detected in all isolates (Batchelor et al. 2005; CLSI, 2015; Hasman et al. 2005).

All ESBL-producing *E. coli* isolates (n=29) were included in this study (Table 15). These ESBL-producing *E. coli* were resistant to at least three antimicrobial groups or multidrug resistance (MDR). All most all ESBL-producing *E. coli* were extensive multidrug resistance or resistant to at least five classes of antimicrobials (n=25). The ESBL genes detected were $bla_{\text{CTX-M14}}$, $bla_{\text{CTX-M27}}$, and $bla_{\text{CTX-M55}}$, of which $bla_{\text{CTX-M55}}$ were predominant among ESBL-producing *E. coli*.

Table 15. Characteristics of ESBL-producing *E. coli* isolates included in this study (n = 29)

Country	*E. coli* isolate	Sample source	AMR pattern	ESBL gene
Thailand (n=15)	TH1	pig rectal swab	AMP-CHP-SUL-TET-TRI	$bla_{CTX-M55}$
	TH2	pig rectal swab	AMP-CHP-COL-GEN-STR-SUL-TET-TRI	$bla_{CTX-M55}$
	TH3	pig rectal swab	AMP-CHP-COL-GEN-STR-SUL-TET-TRI	$bla_{CTX-M55}$
	TH4	pig rectal swab	AMP-CHL-GEN-STR-SUL-TET-TRI	$bla_{CTX-M55}$
	TH5	carcass	AMP-GEN-STR-SUL-TET-TRI	$bla_{CTX-M14}$
	TH6	pork	AMP-CHL-GEN-STR-SUL-TET-TRI	$bla_{CTX-M55}$
	TH7	stool swab	AMP-GEN-STR-SUL-TET	$bla_{CTX-M14}$
	TH8	stool swab	AMP-CHP-COL-GEN-SUL-TET	$bla_{CTX-M55}$
	TH9	stool swab	AMP-CHP-COL-GEN-STR-SUL-TET	$bla_{CTX-M55}$
	TH10	stool swab	AMP-CHP-COL-GEN-SUL-TET	$bla_{CTX-M55}$
	TH11	stool swab	AMP-STR-SUL	$bla_{CTX-M55}$
	TH12	stool swab	AMP-CHP-GEN-SUL-TET	$bla_{CTX-M14}$
	TH13	stool swab	AMP-CIP-SUL-TRI	$bla_{CTX-M14}$
	TH14	stool swab	AMP-CHP-GEN-SUL-TET-TRI	$bla_{CTX-M14}$
	TH15	stool swab	AMP-SUL-TET	$bla_{CTX-M27}$
Lao PDR (n=14)	LA1	Pig rectal swab	AMR-CHP-COL-GEN-TET-TRI	$bla_{CTX-M55}$
	LA2	Pig rectal swab	AMR-CHP-STR-SUL-TET-TRI	$bla_{CTX-M55}$
	LA3	Pig rectal swab	AMR-CHP-COL-STR-SUL-TET-TRI	$bla_{CTX-M14}$
	LA4	Pig rectal swab	AMR-CHP-STR-SUL-TET	$bla_{CTX-M55}$
	LA5	Pig rectal swab	AMR-COL-STR-TET-TRI	$bla_{CTX-M55}$
	LA6	carcass	AMR-CHP-COL-STR-TET	$bla_{CTX-M55}$
	LA7	pork	AMP-CHP-SUL-TET-TRI	$bla_{CTX-M14}$
	LA8	pork	AMP-CHP-SUL-TET-TRI	$bla_{CTX-M14}$
	LA9	pork	AMP-CHP-GEN-STR-SUL-TET-TRI	$bla_{CTX-M14}$
	LA10	pork	AMR-CHP-COL-STR-TET	$bla_{CTX-M55}$
	LA11	Stool swab	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	$bla_{CTX-M27}$
	LA12	Stool swab	AMP-CHP-CIP-GEN-STR-SUL-TET-TRI	$bla_{CTX-M27}$
	LA13	Stool swab	AMP-GEN-TET	$bla_{CTX-M55}$
	LA14	Stool swab	AMP-CIP-CHP-STR-SUL-TET-TRI	$bla_{CTX-M55}$

4.2.2 Colistin susceptibility testing

The minimum inhibitory concentration (MIC) of colistin was determined in all ESBL-producing *E. coli* (n=29) using broth microdilution (CLSI, 2015). The 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.

4.2.3 Detection of plasmid-mediated colistin resistance gene

The presence of *mcr-1* to *mcr-5* genes was determined by Multiplex PCR using the following primers, *mcr-1* (*mcr1*-F, 5′-AGTCCGTTTGTTCTTGTGGC-3′ and *mcr1*-R, 5′-AGATCCTTGGTCTCGGCTTG-3′), *mcr-2* (*mcr2*-F, 5′-CAAGTGTGTTGGTCGCAGTT-3′ and *mcr2*-R, 5′-TCTAGCCCGACAAGCATACC-3′), *mcr-3* (*mcr3*-F, 5′-AAATAAAAATTGTTCCGCTTATG-3′ and *mcr3*-R, 5′- AATGGAGATCCCCGTTTTT-3′), *mcr-4* (*mcr4*-F, 5′- TCACTTTCATCACTGCGTTG-3′ and *mcr4*-R, 5′-TTGGTCCATGACTACCAATG-3′), and *mcr-5* (*mcr5*-F, 5′-ATGCGGTTGTCTGCATTTATC-3′ and *mcr5*-R, 5′-TCATTGTGGTTGTCCTTTTCTG-3′) (Rebelo et al. 2018). All *mcr*-positive isolates were subjected to whole-genome sequencing.

4.2.4 Conjugation experiment

Horizontal transfer of ESBL and *mcr* genes was performed in all *mcr*-positive ESBL-producing *E. coli* (n=7) by using biparental mating experiment. The spontaneous rifampicin-resistant *Salmonella* Enteritidis SE12 (SE12rif^r, MIC = 256 µg/mL) was used as a recipient (Khemtong and Chuanchuen 2008). LB agar containing either rifampicin (32 µg/mL)/colistin (2 µg/mL) or rifampicin (32 µg/mL)/ ampicillin (200 µg/mL) was used to select transconjugants. The transconjugants were confirmed to be

Salmonella by growing on Brilliant Green Agar and Xylose Lysine Deoxycholate agar (Difco®, MD, USA) containing corresponding antibiotics. All transconjugants were confirmed to contain *mcr* genes as described above and ESBL genes as previously described (Batchelor et al. 2005; Hasman et al. 2005).

4.2.5 Whole genome sequencing (WGS) and bioinformatics analysis

WGS was used to determine the genetic background of all seven *mcr*-carrying ESBL producing *E. coli* isolates. DNA extraction were performed by using the DNeasy Blood & Tissue kits (Qiagen, Hilden, Germany) as manufacturer's instructions. The DNA concentration was quantified by using Spectrophotometer Nanodrop (Thermo Scientific, Massachusetts, USA). Libraries were constructed using the QIAGEN FX kit (Qiagen) and submitted for sequencing using an Illumina HiSeq X ten according to a 2x150 paired-end protocol (Illumina, San Diego, CA, US) at the Omics Science and Bioinformatics Center, Chulalongkorn University.

The quality of raw read was determined using FASTQC software (Andrews, 2010). Fastp was used to remove adaptors and low-quality reads. The filtered reads were used as an input for genome assembly using the Unicycler program (Wick et al., 2017). Annotation of the assembled genome was performed using the PATRIC RASTtk-enabled Genome Annotation Service and CLC Genomics Workbench software version 11.0.0 (CLC bio, Aarhus, Denmark).

Multi-Locus Sequence Typing (MLST) based on 7 housekeeping genes i.e. *adk, fumC, gyrB, icd, mdh, purA,* and *recA* was carried out to associate all assembled genomes by using MLST 2.0 (Larsen et al. 2012). Serotyping of the *E. coli* isolates was performed using SerotypeFinder 2.0 (Joensen et al. 2015). The assembled genomes were uploaded to the ResFinder v4.1 web server (Bortolaia et al. 2020) and VirulenceFinder (Joensen et al. 2014) to identify resistance genes and virulence

genes, respectively. Plasmid replicons were identified using PlasmidFinder v2.1 (Carattoli et al. 2014).

Point mutations was determined in chromosomally-encoded genes related to colistin resistance, including *pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *pmrD* by using ResFinder v4.1 web server (Bortolaia et al. 2020). The genes containing point mutation (s) were further analyzed for the presence of amino acid substitutions using the MEGA-X program and the published sequence of *E. coli* K-12 MG1655 available at the NCBI GenBank was used as a reference.

The genetic environment of the *mcr-1.1* genes detected was compared to plasmid pHNSHP45 (GenBank accession number KP347127) using CLC Sequence Viewer version 8.9 and Easyfig version 2.1. The structure of plasmids carrying *mcr-3.1* were compared and mapped to the plasmid reference pCHL5009T-102k IncFII (CP032937) using CLC Sequence Viewer version 8.9 and CGView Server.

4.3 Results

4.3.1 Selected mcr-carrying ESBL producing *E. coli* for WGS analysis

All ESBL producers were MDR. Ten ESBL-producing *E. coli* exhibited resistance to colistin (MIC = 4 and 8 µg/mL), of which seven isolates carried *mcr* genes tested and were subjected to WGS analysis (Table 16). Of the seven *mcr*-carrying ESBL producing *E. coli*, two pig isolates (TH2 and TH3) were obtained from two individual pigs from the same farm and slaughtered at the same slaughterhouse in Nong Khai, Thailand. Two human isolates (TH8 and TH9) were collected from 2 different patients receiving treatment for the same local hospital in Nong Khai. Three pig isolates from Lao PDR (LA1, LA3 and LA5) were originated from 3 individual pigs slaughtered on different days in the same local slaughterhouses in Savannakhet province.

Two pig isolates from Lao PDR i.e. LA3 and LA5 carried only *mcr-1.1*, while two human isolates from Thailand i.e. TH8 and TH9 and one pig isolate from Lao PDR i.e. LA1 contained a single *mcr-3.1*. TH2 and TH3 from Thai pigs coharbored *mcr-1.1* and *mcr-3.1*. None of the isolates were positive to *mcr-2*, *mcr-4*, and *mcr-5*.

4.3.2 Horizontal transfer of ESBL and *mcr* genes in mcr-carrying ESBL producing *E. coli*

All ESBL-producing *E. coli* carrying *mcr* gene (n=7) were examined for their transferability of ESBL and *mcr* genes by using ampicillin and colistin as a selective pressure. TH8 and TH9, the human isolates from Thailand, carrying $bla_{\text{CTX-M55}}$ and *mcr-3.1* could transfer only $bla_{\text{CTX-M55}}$ when ampicillin was used as the selection agent. LA1 could transfer both $bla_{\text{CTX-M55}}$ and *mcr-3.1* when either ampicillin or colistin were used as selective agents.

4.3.3 Sequence type and subtype of *mcr*-carrying ESBL producing *E. coli*

Based on WGS data, 4 different STs and 4 serotypes were identified in all the *E. coli* isolates (Table 16). Two ST/serotypes combinations were found in the isolates from Thailand, including ST6833/ H20 in TH2 and TH3 from pigs and ST48/O160:H40 TH8 and TH9 from humans. The pig isolates from Lao PDR displayed ST10562/O148:H30 (LA3 and LA5) and ST5708/ H45 (LA1).

Table 16. Characteristics of *mcr*-carrying ESBL-producing *E. coli* subjected to WGS analysis (n = 7)

E. coli isolate	Sample source	Sample location	Colistin MIC (μg/mL)	Serotype	MLST	Plasmid type	Mutation on *pmrA*	Mutation on *pmrB*	*mcr*-gene	ESBL gene	Transferable gene (Ampicillin)[1]	Transferable gene (Colistin)[1]
TH2	Pig	Slaughterhouse at Nong Khai	4	H20	ST6833	IncFIA, IncFIB, IncFII, IncQ1, IncX1	Ser-31-Thr	His-2-Arg	*mcr-1.1*, *mcr-3.1*	$bla_{CTX-M55}$	-	-
TH3	Pig	Slaughterhouse at Nong Khai	4	H20	ST6833	IncFIA, IncFIB, IncFII, IncQ1, IncX1	Ser-31-Thr	His-2-Arg	*mcr-1.1*, *mcr-3.1*	$bla_{CTX-M55}$	-	-
TH8	Human	Hospital at Nong Khai	4	O160:H40	ST48	IncFII, p0111	Ser-31-Thr	His-2-Arg	*mcr-3.1*	$bla_{CTX-M55}$	*bla* $_{CTX-M\ 55}$	-
TH9	Human	Hospital at Nong Khai	4	O160:H40	ST48	IncFII, p0111	Ser-31-Thr	His-2-Arg	*mcr-3.1*	$bla_{CTX-M55}$	*bla* $_{CTX-M\ 55}$	-
LA1	Pig	Slaughterhouse at Savannakhet	8	H45	ST5708	IncFII, IncR, IncX1	Ser-31-Thr	His-2-Arg, Thr-235-Asn, Asp-283-Gly, Asn-358-Tyr, Ala-360-Thr	*mcr-3.1*	$bla_{CTX-M55}$	*bla* $_{CTX-M\ 55}$, *mcr-3.1*	*bla* $_{CTX-M\ 55}$, *mcr-3.1*
LA3	Pig	Slaughterhouse at Savannakhet	4	O148:H30	ST10562	ColE10, IncFIA, IncHI1A, IncHI1B	Ser-31-Thr	His-2-Arg	*mcr-1.1*	$bla_{CTX-M14}$	-	-
LA5	Pig	Slaughterhouse at Savannakhet	4	O148:H30	ST10562	ColE10, IncFIA, IncHI1A, IncHI1B	Ser-31-Thr	His-2-Arg	*mcr-1.1*	$bla_{CTX-M14}$	-	-

[1] The antibiotic used as a selective pressure condition in conjugation experiment

4.3.4 Resistance and amino acids alterations in two-component systems in *mcr*-carrying ESBL producing *E. coli*

The WGS data revealed that all the *mcr*-carrying ESBL producing *E. coli* contained both *mcr* and bla_{CTX-M}, of which $bla_{CTX-M55}$ and *mcr-3.1* were most common. *E. coli* of the ST10562 i.e. LA3 and LA5 harbored $bla_{CTX-M14}$ gene, while ST6833 (TH2 and TH3), ST48 (TH8 and TH9), and ST5708 (LA1) carried $bla_{CTX-M55}$ (Figure 2). For *mcr* genes, ST48 (TH8 and TH9) and ST5708 (LA1) carried a single *mcr3.1* while ST10562 (LA3 and LA5) carried a single *mcr1.1*. ST6833 (TH2 and TH3) contained both *mcr-3.1* and *mcr-1.1*. Several AMR genes conferred resistance to aminoglycoside, macrolide, phenicol, sulfonamide, fluoroquinolones, fosfomycin, tetracycline, and trimethoprim were present in all isolates (Figure 2).

Based on mutation analysis in chromosomal-mediated colistin resistance gene, amino acid substitutions in PmrAB were found in all *mcr*-harboring ESBL isolates. No mutations were detected in *phoPQ*, *mgrB*, and *pmrD*. Amino acid substitution Ser31Thr in PmrA and His2Arg in PmrB were observed in all *E. coli* isolates. LA1with colistin MIC of 8 µg/mL contained up to 5 amino acid substitution in PmrB including His2Arg, Thr235Asn, Asp283Gly, Asn358Tyr, and Ala360Thr were identified (Table 16). The other isolates (colistin MIC = 4 µg/mL) carried a single amino substitution in PmrA and PmrB individually.

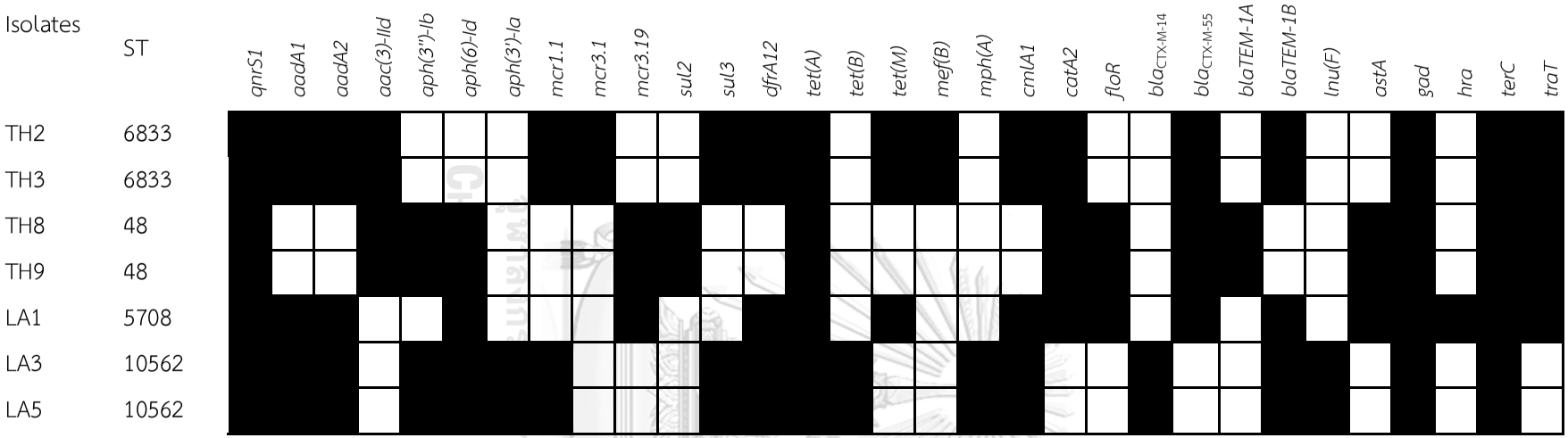


Figure 2. Antimicrobial-resistance and virulence genes of *mcr*-carrying ESBL producing *E. coli* (n=7). Black box indicates the presence of the genes.

4.3.5 Comparative analysis of the ESBL and *mcr* genes in *mcr*-carrying ESBL producing *E. coli*

Two isolates from pigs in Lao PDR (i.e. LA3 and LA5) contained $bla_{\text{CTX-M14}}$, whereas five isolates including TH2, TH3, TH8, TH9 and LA1 contained $bla_{\text{CTX-M55}}$. According to WGS data, the 4,694 bp-insertion sequence IS*Ecp1* was found to be located upstream $bla_{\text{CTX-M14}}$ cassettes (IS*Ecp1*-$bla_{\text{CTX-M14}}$) with 100% identity to the region of the published sequence (Accession number AF252622). Assembled contigs carrying $bla_{\text{CTX-M55}}$ were obtained from WGS analysis of TH2, TH3, TH8, TH9, and LA1. The genetic structural comparison to plasmid pCHL5009T (Accession number: CP032937) showed 100% similarity to IS*26*-$bla_{\text{CTX-M55}}$-Tn*2* with 4,607 bp in range in all contigs.

From WGS analysis, 4 isolates, including TH2, TH3, LA3, and LA5 carried *mcr-1.1*. Genetic environment surrounding the *mcr-1.1* gene was identified by comparison with plasmid pHNSHP45 (Accession number: KP347127). The *mcr-1.1* assembled contigs from all the isolates shared a 3,629-bp DNA segment of *ISApl1-mcr-1.1-pap2* with 100% identity. This homologous segment contained a single *ISApl1* and PAP2 encoding type-2 phosphatidic acid phosphatase enzyme (Figure 3).

Five isolates contained *mcr-3.1*, including TH2, TH3, TH8 and TH9 from Thailand and one LA1 from Lao PDR. The genetic content covering *mcr-3.1* was compared with plasmid pCHL5009T (Accession number: CP032937). The homologous regions between plasmids in the five isolates and pCHL5009T shared 100% identity of *TnAs2-mcr-3.1-dgkA-ISKpn40* with 4,478 bp in length (Figure 4).

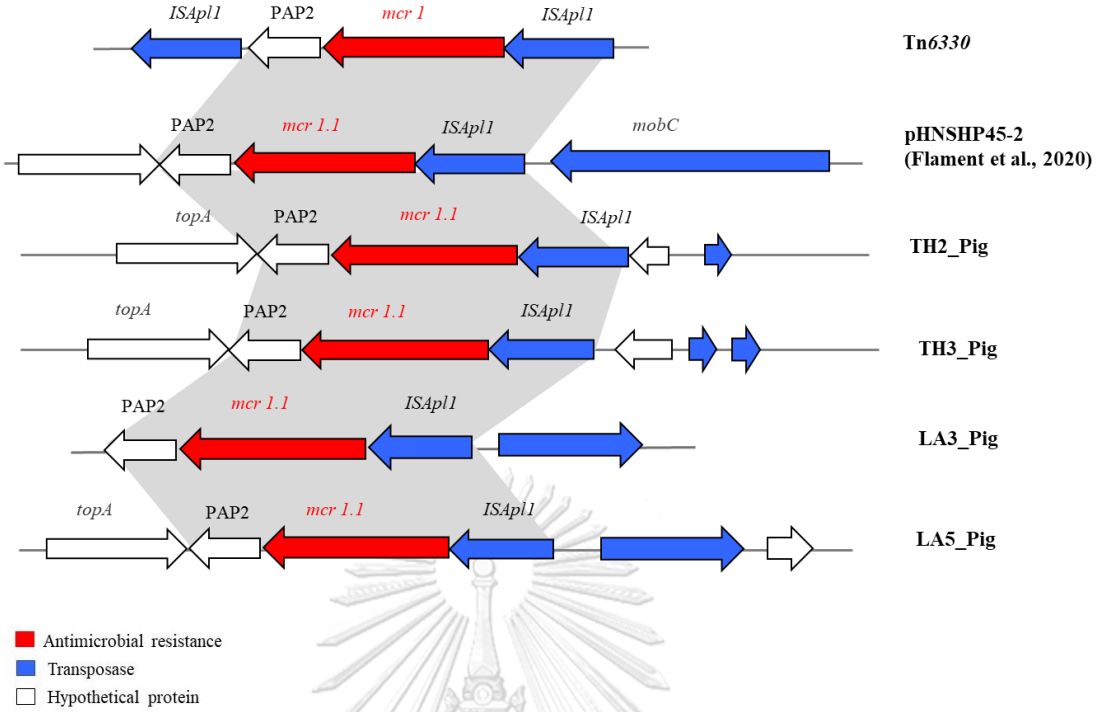


Figure 3. The schematic structure of *mcr-1* flanking regions. ORFs are represented by boxes or arrows. Gray highlighted area, homologous segments of the associated genetic environment; Red arrows, antibiotic resistance genes; Blue arrows, transposon-associated genes and mobile element proteins; White arrows, hypothetical proteins.

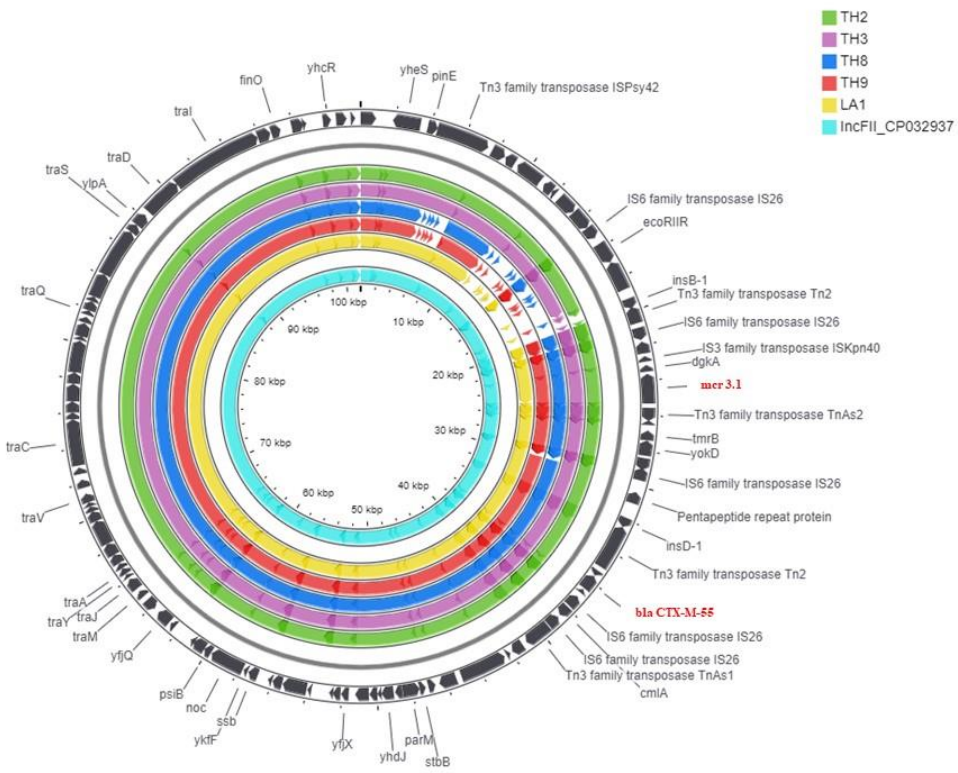


Figure 4. Structural comparison between *mcr-3.1* plasmids and IncFII reference plasmid pCHL5009T (Accession number: CP032937). The outer circle with black arrows denotes the annotation of reference sequence pCHL5009T. The alignment was made for five *mcr-3.1*–carrying plasmids from TH2, TH3, TH1, LA1, LA3 and LA5. The image was generated using CGview server.

4.3.6 Plasmid analysis in *mcr*-carrying ESBL producing *E. coli*

Overall, 10 plasmid replicon types were detected, of which IncFII replicon was the most predominant type. All *mcr-carrying ESBL producing E. coli* contained at least two plasmid replicons. Each ST contained different plasmid type, including ST6833 (i.e. TH2, TH3) carried IncFIA (HI1), IncFIB (K), IncFII, IncQ1, and IncX1. ST48 (i.e. TH8 and TH9) harbored IncFII and p0111. ST5708 (i.e. LA1) contained IncFII, IncR, and IncX1, while ST10562 (i.e. LA3 and LA5) carried ColE10, IncFIA (HI1), IncHI1A, and IncHI1B (Table 2).

The plasmid harboring *mcr-3.1* from TH2, TH3, TH8, TH9, and LA1 were reconstructed by mapping the WGS data to pCHL5009T-102k IncFII plasmid (Accession number: CP032937). The blast alignment showed 99.85-100% identity with the reference plasmid pCHL5009T. All the reconstructed plasmids were IncFII replicon with a size of approximately 101,854-101,860 bp. All plasmids contained the MDR region comprising six different mobile elements, including IS*Psy42*, IS*26*, Tn*2*, IS*Kpn40*, Tn*As2*, and Tn*As1* (Figure 3).

The $bla_{\text{CTX-M55}}$ gene was found to co-localize on the same plasmid with *mcr-3.1* and concurrently harbored other AMR genes, including *cml, tmrB*, and *qnrS1* (Figure 3). However, $bla_{\text{CTX-M14}}$ and *mcr-1* carrying plasmids could not be reconstructed due to the limited length of sequence ontained.

4.3.7 Virulence genes in *mcr*-carrying ESBL producing *E. coli*

All the isolates carried genes encoding glutamate decarboxylase *(gad)* and tellurium ion resistance protein *(terC)* (Figure 2). The virulence genes detected were different among the strains with different STs (i.e. ST6833 and ST10562, *gad, terC*, and *traT*; ST48, *astA*, *gad*, *terC*, and *traT* and ST5708, *astA*, *gad*, *hra*, *terC*, and *traT*).

4.4. Discussion

In the present study, 7 ESBL-producing *E. coli* harboring *mcr* gene (s) was analyzed using WGS. Based on MLST analysis, all the isolates were categorized into four different sequence types/serotypes, including ST6833/ H20 (i.e. TH2 and TH3 from 2 pigs in Thailand), ST48/ O160:H40 (i.e. TH8 and TH9 from 2 patients in Thailand), ST5708/ H45 (i.e. LA1 from a pig in Lao PDR), and ST10562/ O148:H30 (i.e. LA3 and LA5 from 2 pigs in Lao PDR. It should be noted that the isolates originated from different sources but had the similar genetic characteristics, TH2 and TH3 from different pigs at the same slaughterhouse, TH8 and TH9 from different patients at the same hospital and LA1, LA3 and LA5 from different pigs slaughtered on different days in the same local slaughterhouses. This suggests the distribution of the same clones in the same environment.

All the *E. coli* isolates carried at least one $bla_{\text{CTX-M}}$ and one *mcr* gene. This supports that the presence of ESBL and *mcr* genes was not sequence types- and serotypes-specific. The presence of *mcr* genes in *E. coli* from different STs in humans and animals demonstrated that their spread is more likely due to effective transfer of plasmids and mobile elements rather than spread of single *E. coli* clones (Zhang et al. 2019).

All isolates carried the IncFII and IncFIA plasmids, supporting that IncF plasmids are the plasmid replicons associated with the epidemic diversity of resistance genes in *Enterobacteriaceae* (Zhang et al. 2019). This is also consistent with a previous study conducted in *E. coli* isolated from pigs in Thailand, where IncF plasmids were the most common plasmid type that carried *mcr* (Khine et al. 2020). The latter also showed that *mcr-1* and *mcr-3* resided in IncI, IncHI2, and IncX4 plasmids and suggested that *mcr* genes can locate and/or transfer to a range of distinct plasmid types, facilitating their global widespread (Khine et al. 2020).

Intrinsic colistin resistance is mediated by mutations in PhoP/PhoQ and PmrA/PmrB that modify lipid A moiety and it was previously shown that *pmrA* and *pmrB* mutations could result in the extremely-high colistin MIC (Sato et al. 2018). In the present study, all the isolates contained at least one point mutation in *pmrA* and *pmrB* genes but their colistin MIC was not high (4-8 µg/mL). In addition, regulatory mutations in *mgrB* and *pmrD* upregulates the expression of the PhoP/PhoQ and PmrAB system and confer colistin resistance (Sato et al. 2018). However, these regulatory mutations were not observed in this study.

A previous genomic comparison study demonstrated that the MCR-negative group contained more amino acid substitutions in PmrAB than the MCR-positive group (Luo et al. 2019). Also, PmrAB was overexpressed in all MCR-negative group, but this was not always the case in the MCR-positive strains. The expression of MCR in the MCR-positive group could affect the expression of *pmrAB* operon. All isolates in this study harbored the point mutations on *pmrA* and *pmrB* genes and contained *mcr*. However, the effect of MCR on the regulation mechanism of colistin resistance was not pursued in this study and is a topic of future study.

All the *mcr-1* genetic contexts in TH2, TH3, LA3 and LA5 contained a single-ended structure of *ISApl1* (Figure 2), in agreement with previous studies (Zhang et al. 2019; Flament-Simon et al. 2020). Recently, three structures of mobile elements carrying *mcr-1* have been identified, including *ISApl1–mcr-1–orf–ISApl1*, *ISApl1–mcr-1–orf* (or *mcr-1–orf–ISApl1*), and *mcr-1–orf* (sequences lacking *ISApl1*) (Flament-Simon et al. 2020). Previous studies showed that *mcr-1* gene can be translocated as an *ISApl1*-flanked composite transposon, Tn*6330*, to plasmid or chromosome (Flament-Simon et al. 2020; Snesrud, McGann, and Chandler 2018). An engineered variant of Tn*6330*, Tn*6330.2*, was previously constructed and able to mobilize the *mcr-1* fragment, confirming that Tn*6330* is a main carrier for *mcr-1* transposition (Poirel, Kieffer, and

Nordmann 2017). The *mcr-1* gene is all isolates in this study had *pap2* located downstream, in concordant with a recent study revealing that most *mcr-1* segment contained *mcr-1* and a putative open reading frame (ORF) gene encoding a PAP2 superfamily protein (Snesrud, McGann, and Chandler 2018). Our observations support that *ISApl1* and *pap2* usually flank *mcr-1* and *ISApl1* plays role in spreading *mcr-1*cassette among bacterial strains from different sources.

The *mcr-3.1* gene in all isolates was located in the Tn*As2-mcr-3.1-dgkA*-IS*Kpn40* element on reference plasmid, pCHL5009T that contained other AMR genes, including $bla_{\text{CTX-M55}}$, *cml*, *qnrS1*, and *tmrB*. This element was previously reported in *E. coli* from pigs in China (Wang et al. 2019; Li et al. 2021). It was suggested that Tn*As2* and IS*Kpn40* may possibly originate from *Aeromonas* spp. and contribute to the transmission of *mcr-3.1* gene between *Aeromonas* and *Enterobacteriaceae* (Wang et al. 2019). Various transposases and IS elements were previously identified in the flanking areas of *mcr-3* including IS*4321*, *IS26*, *IS15*, Tn*As2* and IS*Kpn40* (Wang et al. 2018; Hadjadj et al. 2019). The presence of different types of transposases and IS elements suggest that *mcr-3* might have a greater capacity for transmission than *mcr-1*, of which only the IS*Apl1* element is important its dissemination (Wang et al. 2019).

Coexistence of *mcr-1.1* and *mcr-3.1* was observed in TH2 and TH3 from pigs in Thailand, in agreement with previous studies (Liu et al. 2017; Long et al. 2019; Li et al. 2021). The *mcr-1* and *mcr-3* genes were previously found to be located on two distinct plasmids in a single *E. coli* isolate (Liu et al. 2017; Long et al. 2019). While *mcr-3* are mostly localized on plasmid, *mcr-1.1* could be either chromosomally located or plasmid mediated. The latter was supported by a previous study showing that *mcr-1* was initially discovered on Tn*6330* that could mobilize between chromosome and plasmid (Shen et al. 2020; Sun et al. 2018). Therefore, the localization and route of transmission of *mcr-1.1* in this study remained unclear.

The coexistence of two distinct *mcr* genes in a single *E. coli* isolate could be explained by the presence of multiple antibiotic resistance genes on plasmids-containing *mcr-1* or *mcr-3.1.* Under exposure to antibiotic selective pressure during serial passages, *mcr-1* was more stably maintained than *mcr-3* in bacterial cells carrying both genes (Li et al. 2021). It was suggested that *mcr-3.1* could have been co-selected by other resistance genes located on the same plasmid (Hadjadj et al. 2019). In this study, the colistin MIC of the isolates carrying both *mcr-1.1* and *mcr-3.1* (i.e. TH2 and TH3) was not significantly different from those with a single *mcr-1* or *mcr-3* (i.e. TH8, TH9, LA1, LA3 and LA5), in agreement with a study in China (Liu et al. 2017). This result suggests that the co-occurrence of *mcr* in a single *E. coli* cell does not always potentiate colistin MIC level.

The co-transfer of $bla_{\text{CTX-M55}}$ and *mcr-3.1* was observed in LA1 an *E. coli* isolate from a pig in Lao PDR under the selective pressure of either ampicillin or colistin. This result suggests the co-localization of $bla_{\text{CTX-M55}}$ and *mcr-3.1* on the same plasmid and therefore, a single antimicrobial used could result in the co-selection of both resistance genes. This agrees with the previous study in *E. coli* from China, where *mcr-1* and $bla_{\text{CTX–M55}}$ on the same plasmid were co-transferred to other bacterial species (Zhang et al. 2019). However, transmission of *mcr-3.1* and $bla_{\text{CTX-M55}}$ may individually occur and mediated by the transposases and IS elements surrounding both genes (Wang et al. 2018).

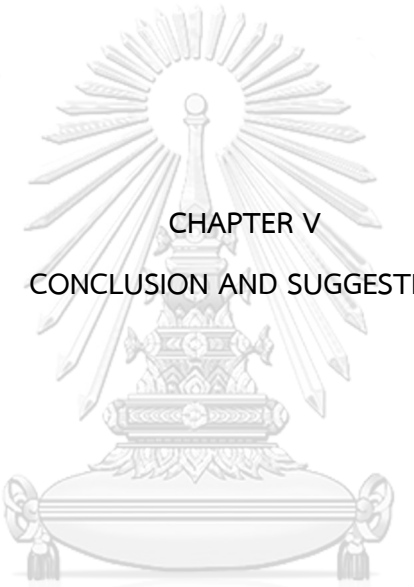
In TH2 and TH3, *mcr-3.1* and $bla_{\text{CTX-M55}}$ were located on the same plasmid but the transmission was observed only for $bla_{\text{CTX-M55}}$ under the selective pressure of ampicillin. The $bla_{\text{CTX-M55}}$ gene was included in the *IS26-Tn2-*$bla_{\text{CTX-M55}}$*-IS26* element. The transposition of $bla_{\text{CTX-M55}}$ might be mediated by *IS26*. *IS26* has been frequently found to mobilize adjacent DNA segments by intramolecular replicative transposition, particularly AMR genes (Wang et al. 2018). *IS26* has also been reported to be

associated with bla_{CTX-M} genes with or without the association with other IS (Shropshire et al. 2021).

All the ESBL-producing *E. coli* carrying *mcr* gene isolates contained at least one virulence gene tested. The *gad* and *terC* genes were found in all isolates. The *gad* gene allows bacteria to survive in severe acidic condition in the gastric environment (Capitani et al. 2003), while *terC* encodes resistance to infection with several bacteriophages (Anantharaman, Iyer, and Aravind 2012). In addition, the toxin *astA* gene associated with acute diarrhea was found in TH8, TH9 and LA1. The *E. coli* isolates were obtained from healthy pigs, therefore, pigs could be an important reservoir of ESBL-producing *E. coli* carrying *mcr* that can cause a serious infection in humans (Khine et al. 2020). These results raise the particular public health concern that the *E. coli* isolates had the potential to cause severe infection and survive longer in the last line antibiotic therapies.

Conclusion

The comprehensive insights into the genomic information of ESBL-producing *E. coli* harboring *mcr* were obtained. ESBL-producing *E. coli* harboring *mcr* were isolated from pigs and humans. This confirms the transmission of the genes among *E. coli* from humans and animals. The findings highlight the requirement of cross-sector collaborations “One Health approach” to facilitate understanding and implementing control and prevention program. The effective strategic actions are required to contain AMR, for example, implantation of national active surveillance on AMR and antimicrobial usage, encouraging guideline and policies on prudent use of antimicrobials in animals and humans and promoting disease control and prevention program.



CHAPTER V

CONCLUSION AND SUGGESTION

5.1 General discussion

AMR has been recognized as a major threat to public health and has a significant impact on humans, animals, and environment. Currently, at least 700,000 people die each year from AMR infections globally. It is estimated that approximately 10 million people will die annually as a result of drug-resistant bacteria by 2050, with up to 90% mortality occurring in low- and middle-income countries, in particular Africa and Asia (O'Neill J, 2014). Southeast Asia is one of the fastest-growing hotspots for emerging infectious diseases and AMR (Walther et al., 2016). Additionally, the ASEAN Economic Community (AEC) has a goal of creating a unified market with free movement of products, services, capital, labor, and other elements. Increased international trade and large cross-border movement of people, food animals and their products are a major driving factor behind emergence and spread of AMR among bacteria, particularly *E. coli* and *Salmonella*. However, data on the AMR is still limited in the region. Therefore, monitoring the AMR phenotype and genotype of bacteria from humans, food producing animals, and their meat products in the area is necessary to understand their dissemination and transmission mode, root causes and burden, with the expectation to implement effective control and prevention strategic action plan.

Our study project started with the monitoring and surveillance of AMR in *E. coli* in Thailand and Lao PDR. Then, the investigation was focused to resistance to highest priority critically important antimicrobials, quinolone, 3rd and newer generation cephalosporin, carbapenems, and colistin. Finally, we characterized the co-existence ESBL and *mcr* isolates by WGS to give more comprehensive details for deep understanding on the dissemination and diversity of resistant bacteria (Figure 5).

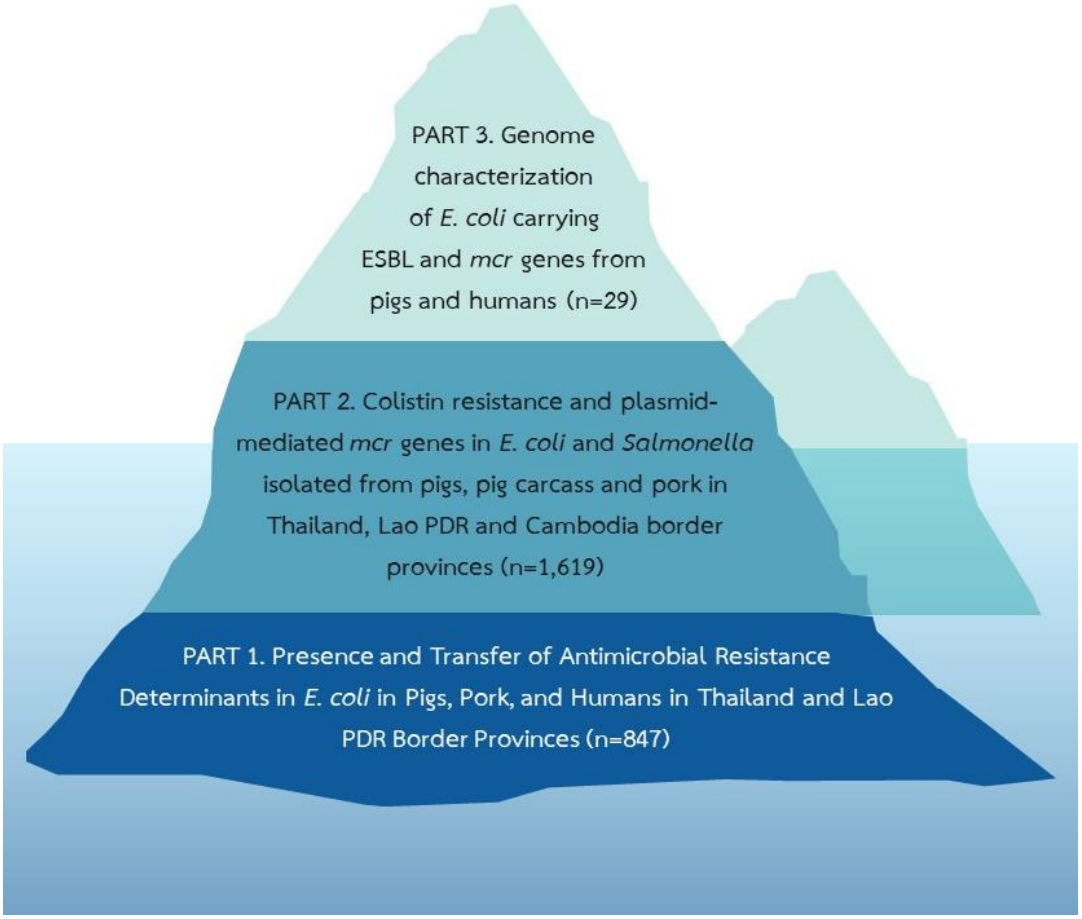


Figure 5. The surveillance and characterization on AMR among *E. coli* isolates from pigs, pig carcass, pork, and humans

The total findings from this project were complicated and diverse. They were discussed in detail in each chapter. The following general discussion is provided to cover the missing and additional messages.

Part 1. Presence and Transfer of Antimicrobial Resistance Determinants in *E. coli* in Pigs, Pork, and Humans in Thailand and Lao PDR Border Provinces

This study highlighted the high prevalence of MDR *E. coli* in pigs (80.7%), pig carcasses (69.1%), pork (63%), and humans (47.3%) in Thailand and Lao PDR border areas. The high resistance rate to ampicillin (74.5%), tetracycline (71.2%), sulfamethoxazole (58.4%), and trimethoprim (55.3%) were observed in the *E. coli* isolates in these countries. The AMR pattern observed was in agreement with a previous study conducted in *Salmonella* in the same area (Sinwat et al., 2016). This may be due to the common use of these antimicrobials in the region. These antimicrobials are relatively inexpensive and widely available in the markets. They may be easy to reach and available without a prescription from veterinarian (Lay et al., 2021). In addition, these antimicrobials are in old generation that have been used for a quite long time. Such prolonged use could generate long-term selection pressure for bacteria (Piccirillo et al., 2014). Still, the resistance rates in this study were lower than those in a previous study of *Salmonella*. Regardless, the results point out that MDR *Salmonella* causes infection that is more difficult to treat. Simultaneously, *E. coli* that are usually considered harmless can act as important reservoir for AMR genes that can be transmitted to pathogenic and non-pathogenic bacterial strains.

This study described a low proportion of ciprofloxacin resistance (4.1%), which is in accordance with a previous report of *Salmonella* in the same area (Sinwat et al., 2016). This indicates that ciprofloxacin, the antibiotic of choice for treating *Salmonella* infection and frequently used to treat human invasive salmonellosis,

remain an effective antibiotic therapy. However, the AST diagnostic result is required, prior to decision making if the antibiotics of this class should be used. The ciprofloxacin resistance rate in *E. coli* was higher when compared to *Salmonella* in the study, supporting the important role of *E. coli* as an indicator for antimicrobial usage in food animals. In this study, a low rate of ciprofloxacin resistance was determined. However, AMR is a dynamic process and can change overtime. Therefore, the monitoring of quinolone resistance should be continued as national, regional, and global level. In contrast, the result was contradicted to a previous study in *E. coli* from pigs in China (Wu et al. 2019). The resistance rate to ciprofloxacin was high since it has been widely used for treatment and prevent diseases in pig farms in China (Wu et al. 2019). Taken together, the different resistance patterns could be caused by the different of antimicrobials used in each area (or country).

In this study, the resistance to 3rd cephalosporins or ESBLs in Thai isolates (4.9-10.3%) was less than that was observed in Lao PDR (7-15.5%). The difference could be resulted from different antimicrobial use and stewardship practices in the countries. However, the use of cephalosporins may not always directly result in the spread of ESBL producers. ESBL genes are commonly plasmid-mediated and usually co-localize with other AMR genes on the same plasmid. Therefore, the presence of ESBL producing bacteria could be due to co-selection of ESBL genes by other antibiotics such as ampicillins, aminoglycosides, and tetracyclines, which were widely used in pig industry in these areas (Lay et al., 2021). In addition, the percentage of ESBL-producing *E. coli* in this study was higher than that of *Salmonella* in previous study at the same locations (Sinwat et al., 2016). This result was in agreement with a previous study in *Salmonella* and *E. coli* conducted along Thailand, Cambodia, Lao PDR, and Myanmar border areas (Lay et al., 2021). The observations highlight that ESBL-producing *E. coli* was more prevalent than ESBL-producing *Salmonella* (Lay et al., 2021). This could be explained by the fact that *E. coli* is a commensal bacterium

residing in intestinal tract of humans and animals. Therefore, *E. coli* has more opportunity and longer period to contact with antimicrobials than *Salmonella* where antimicrobials is regularly applied to the animal host. Therefore, *E. coli* could develop AMR easier and more efficient than *Salmonella* (Varga et al., 2019).

Almost haft of the *E. coli* isolates in this study harbored class 1 integrons, which was consistent with a former study (Sinwat et al., 2016). Class 1 integrons have been reported in a variety of bacteria from diverse sources worldwide, including *E. coli* isolated from humans and animals in Norway (Sunde et al., 2015), *Salmonella* isolated from animals in Thailand (Srisanga et al., 2017), *Acinetobacter baumannii* isolated from patients in China (Huang et al., 2015), *E. coli* isolated from patients in Brazil (Clarisse et al., 2017), and *E. coli* isolated from farm environment in Argentina (Colello et al., 2018). This could be explained by the typical nature of class 1 integrons that are generally associated with transposons. The association allows class 1 integrons to migrate either between different plasmids or between plasmids and bacterial chromosome. Therefore, the transposition or mobilization into various locations promote the wide dissemination of class 1 integrons among bacteria (Sunde et al., 2015).

Many *intI1*-positive isolates contained empty integrons in this study. The presence of the empty integrons have been frequently reported, e.g. *Salmonella* from food animals in Thailand and Lao PDR (Sinwat et al., 2016), *E. coli* from food animals in China (Zou et al., 2018), and *E. coli* from patients in Nigeria (Odetoyin et al., 2017). These empty integrons are available for capturing new resistance genes in response to diverse selective pressure conditions (Sinwat et al., 2015). In addition, it was formerly demonstrated that the resistance determinants in the resistant isolates with class 1 integrons were transferred more efficiently and frequently than those lacking class 1 integrons. It is suggested that class 1 integrons are highly associated

with many transmissible plasmids, with enables them to spread throughout diverse bacterial populations (Sunde et al., 2015).

In this study, the resistance gene cassettes *dfrA17-aadA5*, *dfrA1-aadA1*, and *dfrA12-aadA2* combinations were identified within class 1 integrons in bacterial strains from both Thailand and Lao PDR. This is in agreement with our previous study where various combinations of class 1 integrons- resistance gene cassettes, including *dfrA17-aadA5*, *dfrA1-aadA1*, and *dfrA12-aadA2* were described (Sinwat et al., 2015). The diverse combinations of *dfr* and *aadA* genes are associated with the type of trimethoprim and/or aminoglycosides administered to animals and different treatment procedure applied in different geographic regions (Sinwat et al., 2015). In addition, these resistance gene cassettes might be a part of integrons borne on certain transposons, plasmids, or antibiotic resistance islands, resulting in a widely dissemination in bacteria (Sunde et al., 2015). Moreover, the present study revealed up to eight different types of class 1 integrons gene cassette arrays, supporting the diversity of the resistance gene cassettes in class 1 integrons among the member of bacteria in the *Enterobacteriaceae* family (Li et al., 2013).

Chromosomal mutations in *gyrA* and/or *parC* is one of the most important mechanisms conferring resistance to fluoroquinolones. The mutations alter the antibiotic target site and halt drug-target binging affinity. This eventually results in lowering treatment efficacy (Ferreira et al., 2018). This agreed with the observation that all ciprofloxacin resistant isolates in this study contained at least one point mutation in the *gyrA* and/or *parC* genes. In contrast, the susceptible isolates carried none of point mutations.

The PMQR determinants including *qnr* (i.e. *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*), *aac(6′)-Ib–cr*, *qepA* and *oqxAB* genes were identified. The *qnrS* gene was the most prevalent PMQR gene in this study, in consistent with a previous report of

Salmonella in the same area (Sinwat et al., 2016). However, the prevalence of *qnrS* in this study (23%) was higher than the previous report (7.1%) (Sinwat et al., 2016). In contrast, a previous study in Brazil reported that *qnrB* was more common than other *qnr* genes in *Enterobacteriaceae* (Ferreira et al., 2018). According to the study in Morocco, *aac(6)* was the most common PMQR gene among the *E. coli* and *Klebsiella* isolates (Benaicha et al., 2017). These results indicated that the distinct PMQR genes could be found in distinct geographical regions. Almost all *qnr* positive isolates in this study were susceptible to ciprofloxacin. This finding is consistent with the previous study demonstrating that 93.3% of *qnr* positive isolates were susceptible to ciprofloxacin, whereas the highest ciprofloxacin MIC were related to QRDR mutations (Benaicha et al., 2017). Therefore, the existence of PMQR determinants does not always confer quinolone resistance phenotype.

In this study, all ESBL genes detected were in the group of bla_{CTXM}. As seen in many studies, the CTX-M type was more frequently identified in ESBL-producing *E. coli* than other types of ESBLs. For example, $bla_{CTX\text{-}M}$ was detected as the most ESBL type in *E. coli* from pigs in China (Liu et al., 2018), *E. coli* from pigs in Japan (Norizuki et al., 2018), and *E. coli* from meat in South Korea (Kim et al., 2018). These results indicated that the CTX-M type have emerged and replaced TEM and SHV and become the most prevalent type of ESBL. In this study, the $bla_{CTX\text{-}M55}$ gene was detected in the majority of ESBL producers isolated from pigs, pig carcasses, pork, and humans (58.6%). This is contradicted to previous reports from *Salmonella* in the same area (Sinwat et al., 2016) and *E. coli* in China (Liu et al., 2018). In those previous studies, the most CTX-M type was $bla_{CTX\text{-}M14}$. Moreover, $bla_{CTX\text{-}M1}$ was widespread in livestock in Europe, where it was clonally related to ESBL-producing *E. coli* (Hammerum et al., 2014). Taken together, the subtype of CTX-M gene was diverse depending on the bacterial species, bacterial clonal type, and geographic region.

The association of *qnrS* and ESBL genes, $bla_{\text{CTX-M 55}}$ and $bla_{\text{CTX-M 14}}$ was observed in this study. Many previous studies revealed a relationship between ESBL-producing *E. coli* isolates and PMQR. A study in *E. coli* from dogs in China reported that 84% of ESBL producers carried at least one *qnr* gene, of which *qnrS* was most commonly identified (Liu et al., 2018). Moreover, a study in *E. coli* isolated from pigs in China showed a significant association between $bla_{\text{CTX-M14}}$ and *oqxAB* genes, which were frequently located on the same plasmid (Xu et al., 2015). Therefore, the co-selection of ESBL and PMQR genes by a single antibiotic might occur and faciloitate the spread of MDR determinants.

Part 2. Detection of Carbapenem and Colistin resistance in *E. coli* and *Salmonella* isolates in pigs, pig carcass and pork in Thailand, Lao PDR and Cambodia border provinces

One of the main results of this study was the presence of colistin-resistant in *E. coli* and *Salmonella* isolates from pigs, pig carcasses, and pork along Thailand-Lao PDR and Thailand-Cambodia border areas. Colistin resistance rate in Cambodia (17.8%) was higher than those in Thailand (7.5%) and Lao PDR (5.4%). This is in contrast to a previous study conducted in *E. coli* and *Salmonella* from pigs and pork along Thailand, Cambodia, Lao PDR, and Myanmar border areas, where colistin resistance was highest in Lao PDR (17.8 %), followed by Cambodia (8.6%), Thailand (4.7%), and Myanmar (2%), respectively (Lay et al., 2021). The discrepancy could be associated with sample collecting methods, sampling locations, and sampling timing. In Thailand, polymyxins cannot be added to animal feed for preventive purposes by law (Lay et al., 2021). However, colistin can be used in swine production for a short-term therapy for treatment of post-waning diarrhea (PWD) and colibacillosis. The occurrence of colistin-resistant bacteria may contaminate the food chain and cause a

severe problematic issue to public health. Therefore, monitoring and surveillance of colistin resistance is needed to keep track of the current situation globally.

Since the first plasmid-mediated *mcr-1* gene was identified in China in 2015, many *mcr* variants have been reported (Liu et al. 2016) (Figure 6; Figure 7). Among the current *mcr* genes (*mcr-1* to *mcr-10*), *mcr-1* and *mcr-3* have spread globally. This agrees with the result of this study where *mcr-1* and *mcr-3* genes were discovered as the predominant variants of *mcr* in *E. coli* and *Salmonella*. It is also in agreement with many previous studies conducted in *E. coli* and *Salmonella* isolates obtained from pigs and pork in Thailand, Cambodia, Lao PDR, and Myanmar border areas (Lay et al., 2021), *E. coli* isolates from pigs of Thailand (Khine et al., 2020), pigs in China (Wang et al., 2019), pigs in Cambodia (Strom Hallenberg et al., 2019) and agricultural environments in Algeria (Touati et al., 2019).

Twelve *mcr-1*- or *mcr-3*-carrying *E. coli* (n=6) or *Salmonella* (n=6) isolates in this study were susceptible to colistin (n=12). This included one *E. coli* isolate containing *mcr-1*, four *E. coli* isolates carrying *mcr-3*, one *E. coli* isolate containing *mcr-5*, one *Salmonella* isolate carrying *mcr-1*, and five *Salmonella* isolates harboring *mcr-3*. This is in agreement with a previous study conducted in Japan (Fukuda et al., 2018). Among these colistin susceptible isolates (n=12), *mcr-3* carrying isolates (n=9) were higher than *mcr-1* carrying isolates (n=2). It is supported by the results from the previous study demonstrating that the effect of *mcr-3* on colistin resistance tended to be weaker than *mcr-1* genes (Fukuda et al., 2018). These findings imply that the presence of *mcr* is not be always related to the colistin resistance phenotype. However, further study is required to characterize the function of individual *mcr* gene.

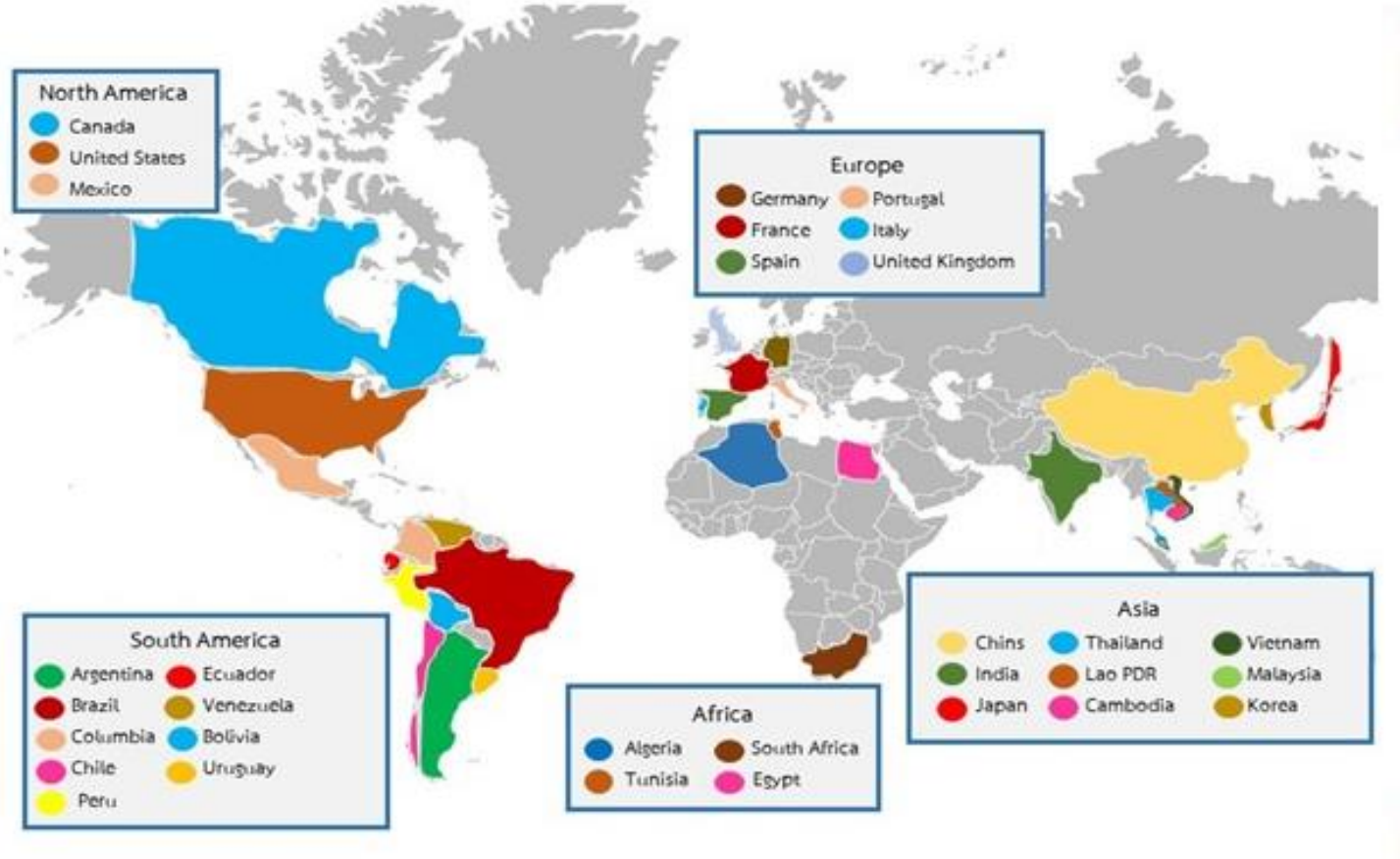


Figure 6. The distribution of plasmid-mediated colistin resistance, *mcr* gene carrying bacteria on a global scale.

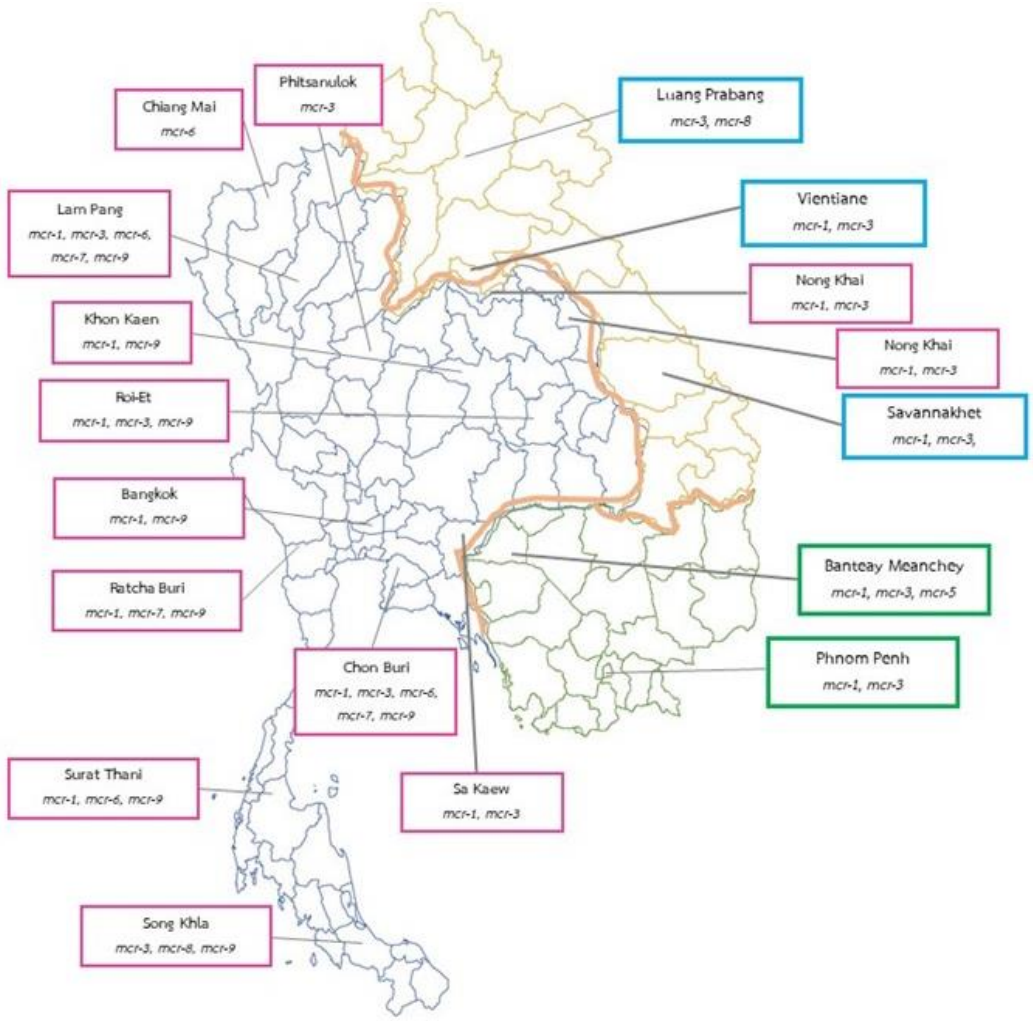


Figure 7. The distribution of plasmid-mediated colistin resistance, *mcr* gene carrying bacteria in Thailand, Lao PDR, and Cambodia.

In this study, the *mcr* genes were located on the plasmid IncI and IncF replicon types. These results were consistent with a previous study of *E. coli* in Thailand, of which IncF replicon types were the most prevalent in *mcr*-carrying *E. coli* (Khine et al., 2020). This plasmid type was associated with various AMR genes and frequently detected in *Enterobacteriaceae* members. Many replicon types were shown to be associated to *mcr*- carrying plasmid. A previous study in *E. coli* from pigs in China demonstrated that IncX1, IncX4, and IncF plasmids carried *mcr-1.1*, whereas IncP-1 plasmids carried *mcr-3.5* (Wang et al., 2019). This indicate that the *mcr* genes could locate and/or transfer to various plasmid types (Khine et al., 2020).

Part 3. Genome characterization of *Escherichia coli* carrying ESBL and *mcr* genes from pigs and humans.

In this study, 10 ESBL-producing *E. coli* isolates from pigs, pig carcasses, pork, and humans exhibited colistin resistance. This is in agreement with previous studies reporting that ESBL-producing *E. coli* from different sources displayed colistin resistance, e.g. *E. coli* from pigs in China (Zhang et al., 2019), water samples in Brazil (Sacramento et al., 2018), and a patient in The US (McGann et al., 2016). These data suggest that *E. coli* co-expressing ESBLs and colistin resistance has distributed in all sections worldwide, referred to as of One Health. This is a critical threat to human and animal health because new generation cephalosporins and colistin are classified as WHO-highest priority critically important antimicrobials for humans and considered last resort antimicrobials for treatment of MDR infection. Therefore, the united collaborations among different sectors on research and control and prevention of AMR is required.

Three *mcr*-negative isolates exhibited resistant to colistin, in agreement with the previous study (Luo et al., 2019). Colistin resistance in these isolates may be due to the chromosomal mutation in *phoP, phoQ, pmrA, pmrB, mgrB*, and *pmrD* that are

involved in the modification of lipid A and is one of the key mechanisms of colistin resistance (Liu et al., 2016). Other colistin resistance mechanisms, such as the presence of porins, multidrug efflux systems, and cross-resistance by host cationic antimicrobial peptides have been characterized (Xu et al., 2018). These could be explained the dissemination of colistin resistance phenotype in the absence of the *mcr* gene (Xu et al., 2018). However, such chromosomal encoded mechanisms were not investigated in these three isolates. Also, *mcr-6* to *mcr-10* genes, a new variant of *mcr* genes, were not examined in this study.

This study revealed the distribution of *mcr* gene (s) among the different ST of *E. coli*. Many studies previously demonstrated that the various types of ST harbored *mcr* genes (Figure 7). For example, *E. coli* carrying *mcr-1* in China were identified to be ST88, ST93, ST609, ST162, and ST457 (Zhang et al., 2019). *E. coli* carrying *mcr-1* in Spain was assigned to ST131 (Flament-Simon et al., 2020). STs, ST10, ST405, and ST345 were identified among *mcr-1*-positive *E. coli* isolates from Algeria, whereas the isolates carrying *mcr-3* were assigned to ST155 (Touati et al., 2019). Taken together, the *mcr* gene could exist among the diversity of *E. coli* clones, promoting the global dissemination.

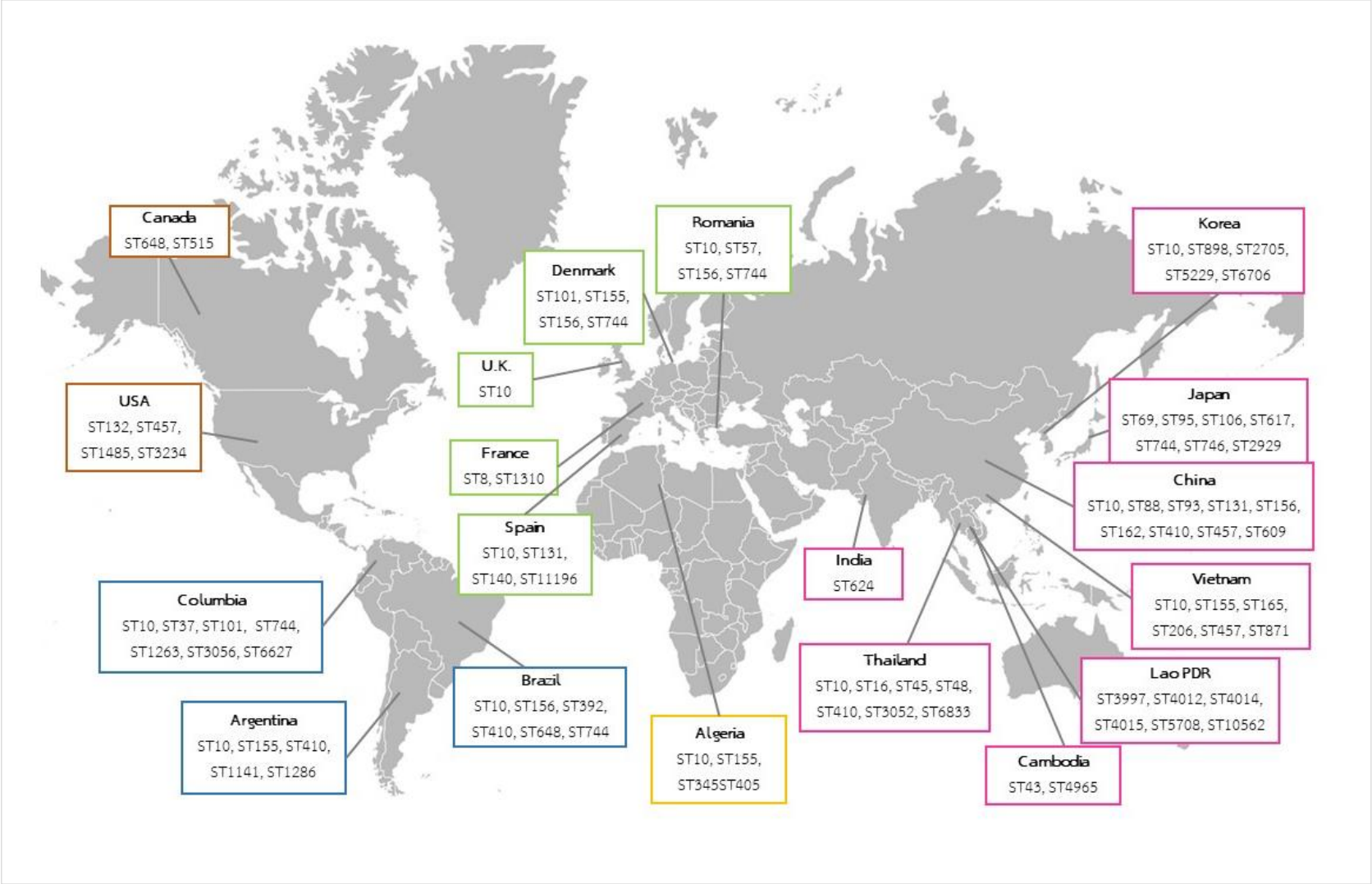


Figure 8. The diversity of ST type among *E. coli* carrying plasmid-mediated colistin resistance, *mcr* gene.

All *mcr-1.1* flanking regions in this study shared the homologous region, *ISApl1-mcr-1-pap2*. The *ISApl1* is a member of *IS30* family, which most likely mediated via composite transposon Tn*6330* (Snesrud et al., 2018). The *ISApl1* gene was initially found in *Actinobacillus pleuropneumoniae*, a Gram-negative rod belonging to the *Pasteurellaceae* family that causes necrotic pleuropneumonia in pigs. Currently, three genetic structures surrounding *mcr-1* gene were identified, which included *ISApl1–mcr-1–*orf*–ISApl1* structure (11%), *ISApl1–mcr-1–*orf or *mcr-1–*orf*–ISApl1* structure (23%), and *mcr-1–*orf structure (66%) (Snesrud et al., 2018). The *ISApl1–mcr-1–*orf*–ISApl1* structure could result in the mobilization of the *mcr-1* gene (Poirel et al., 2017). However, a previous study demonstrated that the single-ended versions of *ISApl1*, *ISApl1–mcr-1–*orf or *mcr-1–*orf*–ISApl1* structure, can mobilize *mcr-1* gene (Snesrud et al., 2018).

In this study, the *TnAs2-mcr-3.1-dgkA-ISKpn40* element was the homologous segments in all the *mcr-3.1*-carrying isolates. *ISKpn40* is a 1213-bp insertion sequence that is identical to transposon from *Aeromonas* spp.. This could imply that *ISKpn40* was originated from *Aeromonas* spp. and mediated transmission of *mcr-3.1* gene between *Aeromonas* and *Enterobacteriaceae* (Wang et al., 2019). *TnAs2* was originated from *Aeromonas* spp. (Wang et al., 2019). Many transposons and IS were associated to *mcr-3* gene reported in many studies. These included, *ISKpn40*, *TnAs2* and *IS4321* in *E. coli* from pigs from China (Wang et al., 2019) and *TnAs2*, *IS26*, and *ISKpn40* were detected in *E. coli* isolate from pigs in China (Wang et al., 2018). Therefore, many transposases and IS elements could mediate the translocation of *mcr-3* (Wang et al., 2019).

Overall from 3 parts, 10 AMR genes were identified in both humans and animals, including *aadA1***,** *dfrA1-aadA1, dfrA12-aadA2, dfrA17-aadA5, qnrS, mcr-1, mcr-3,* $bla_{\text{CTX-M 55}}$, $bla_{\text{CTX-M 14}}$, and $bla_{\text{TEM-1}}$ (Figure 8). This result highlights the same

AMR gene dissemination in human and animal sectors. Co-localization of *bla*$_{CTX-M55}$ and *mcr-3.1* on the same plasmid were detected in humans and pigs isolates from Thailand and Lao PDR, which might result from the plasmid transmission between *E. coli* isolates from humans and pigs between countries.

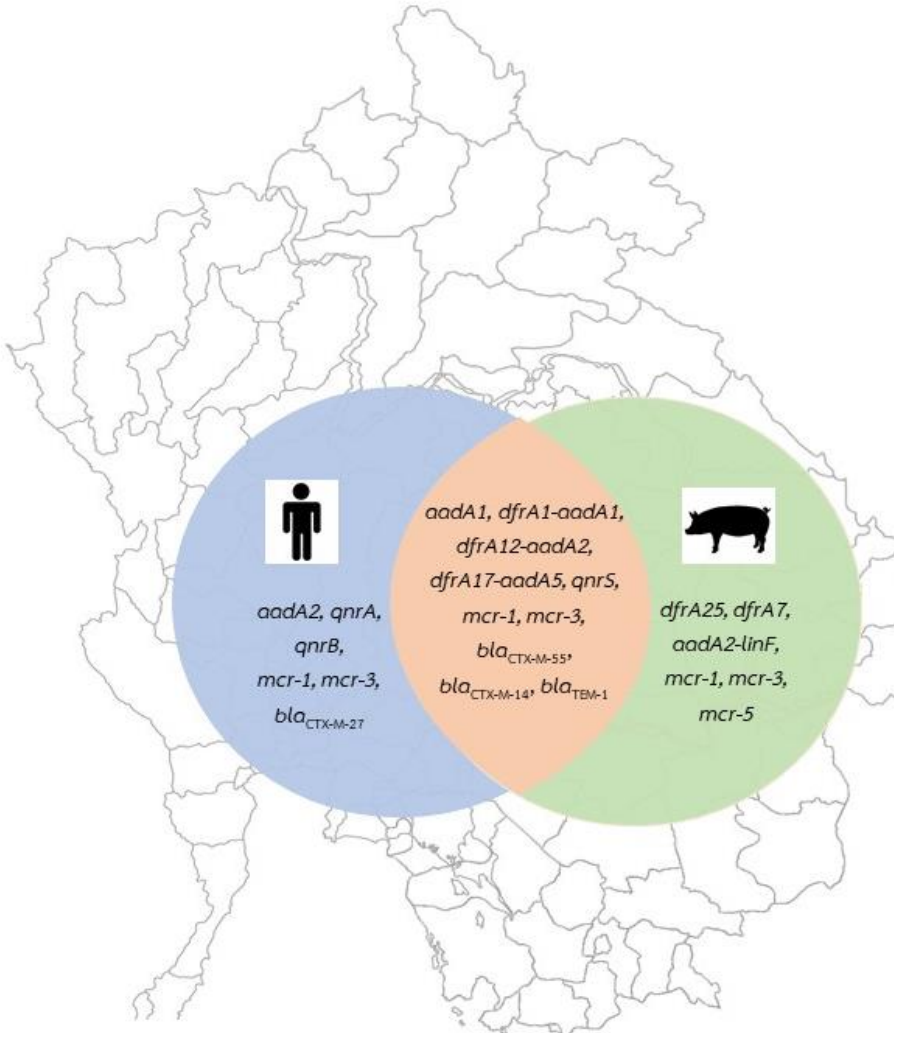


Figure 9. The distribution of AMR genes among *E. coli* isolates from humans and animals in this project.

5.2 Conclusion and suggestion

The objectives of this study project were accomplished. The research was carried out successfully as planed and the findings were published in international journals. The results from this study have expanded the understanding of AMR among bacteria isolated from humans and pigs (food animals). Suggestions are offered for data usage and further study.

The summary of this study could be described for each objective as follows:

Objective 1: To monitor the prevalence of antimicrobial resistance among *E. coli* isolated from pigs, pork and humans in Thailand-Laos border areas.

Our study examined the AMR situation in *E. coli* in the border areas between Thailand and Lao PDR. The results revealed the high prevalence of MDR *E. coli* isolates in pigs, pig carcasses, pork, and humans. Resistance to ampicillin, tetracycline, sulfamethoxazole, and trimethoprim was reported at high prevalence of *E. coli* isolates. The data suggested that resistant *E. coli* may contaminate food animals and meat products and transfer to humans via the food chain. This data obtained supports that AMR is a global concern and affects multiple sectors, including humans, animals, and the environment. In this region, resistance to fluoroquinolones, cephalosporins, and colistin is still uncommon. However, this is also a concern because the antimicrobials are all regarded as clinically significant drugs for treating MDR *E. coli* infection and last resort antimicrobials. As a result, it is necessary to raise awareness to limit the use of fluoroquinolones, cephalosporins, and colistin in food animals.

Objective 2: To characterize of antimicrobial resistance of *E. coli* isolated from pigs, pork and human in Thailand-Laos border areas.

The genotypic characteristics of AMR were examined in *E. coli* isolates from Thailand and Lao PDR border areas. Class 1 integrons was a key genetic factor

contributing to the spread of AMR. The *aadA1* gene array was commonly found and this phenomenon was supported by the horizontal transferability of this gene. Three ESBL genes were identified, including $bla_{CTX\text{-}M14}$, $bla_{CTX\text{-}M27}$, and $bla_{CTX\text{-}M55}$, of which $bla_{CTX\text{-}M55}$ was the most common. The plasmid mediated colistin resistance genes, *mcr-1* and *mcr-3*, were detected in *E. coli* isolates from humans and animals. Importantly, the co-localization of $bla_{CTX\text{-}M55}$ and *mcr-3.1* genes on the same plasmid was identified in the isolates from humans and pigs in Thailand and Lao PDR, where one isolate could transfer both $bla_{CTX\text{-}M55}$ and *mcr-3* genes to recipient. These highlight the co-transmission of both resistance genes under a single antimicrobial selective pressure.

Horizontal transfer of resistance determinants is a significant mode of AMR spread. As a result, AMR phenotypes and genotypes, including ESBL production and colistin resistance, should be monitored and surveilled at the national and worldwide level. WGS provides a comprehensive data on AMR-related emergent public health issues. With the advance of this technology, we can identify the current trend and emergence of AMR. Additionally, the acquired data is important for elucidating the underlying mechanisms of AMR dissemination and developing strategies or action plans aimed at preventing the spread of AMR.

Objective 3: To compare resistance to last-line antimicrobials of *E. coli* and *Salmonella* isolated from pigs and pork in Thailand-Laos and Thai-Cambodia border areas.

Colistin resistance was observed in *E. coli* and *Salmonella* in the provinces bordering Thailand, Lao PDR, and Cambodia. The colistin-resistance genes, *mcr-1*, *mcr-3*, and *mcr-5* were found. The colistin-resistant *E. coli* were more common than colistin-resistant *Salmonella,* as well as the presence of *mcr*. These observations highlight the important role of commensal *E. coli* as an important reservoir of *mcr* genes. Additionally, *Salmonella*, a pathogenic bacterium, harbored *mcr* gene. This

could cause a serious public health problem. The *E. coli* and *Salmonella* isolates from pigs and pork could carry *mcr-1* or *mcr-3* on IncF or IncI conjugative plasmids. These indicated the diversity of *mcr*-carrying plasmids, which transmission among *Enterobacteriaceae*.

The findings emphasize that colistin resistance could be detected among the bacterial strains originated from food-producing animals along Thailand, Lao PDR, and Cambodia border area. This may cause contamination to humans via the food chain. The surveillance and monitoring of the resistance to colistin should be continued. The data will increase the awareness of antimicrobial use in livestock animals and support the development of public health policies, and responsible antimicrobial use guidelines.

5.3 Suggestion and further studies

1. To address the growing threat of AMR, the effectiveness of AMR surveillance and continuous monitoring programs at the local, national, and global levels is required. The implementation of the program needs to be encouraged to allow epidemiological tracing of resistance patterns. One Health approach to national AMR surveillance in human and animal populations is required to strengthen the understanding and support control and prevention strategic actions.
2. The prevalence and genetic characteristics of AMR in *E. coli* from pigs and other food-producing animals should be studied in a larger population across the region.
3. A genetic and clonal relationship between *E. coli* and other bacteria from humans and food-producing animals should be investigated.
4. Characterization of plasmid-mediated AMR in *E. coli* and other bacteria from humans and food animals will offer valuable information about

the evolution, circulation, and spread of plasmid-mediated resistance genes in the region.

5. National monitoring and surveillance of antimicrobial use in humans and food-producing animals should be performed. Together national AMR data, this will support the development and implementation of control and prevention strategic action plan to contain AMR.
6. The coexistence of resistance and virulence genes in a single isolate may result in a severe infection and difficulty of failure of treatment. The study of virulence profile should be conducted concurrently with the study of resistance profiles.

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