Phenotypic and Genotypic Characteristics of Extended-spectrum β -Lactamase (ESBL) Production and Colistin-resistance in *Salmonella enterica* and *Escherichia coli* Isolated from Pigs and their Meat Products in the Border Provinces between Thailand and Cambodia, Lao PDR and Myanmar

Mr. Kyaw Phyoe Sunn -

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Veterinary Science and technology
Common Course
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2018

ลักษณะการดื้อยาและลักษณะทางอณูชีววิทยาของการดื้อยาต่อ Extended-spectrum β-lactamases (ESBL) และยา Colistin-ในเชื้อซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไล ที่แยกได้จากสุกรและเนื้อสุกรในเขตจังหวัดชายแดนประเทศไทยและประเทศกัมพูชา ลาวและพม่า

นายเคาว์ พโย ซุนน์ -

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	Phenotypic and Genotypic Characteristics of Extended-spectrum β -Lactamase (ESBL) Production and Colistin-resistance in *Salmonella enterica* and *Escherichia coli* Isolated from Pigs and their Meat Products in the Border Provinces between Thailand and Cambodia, Lao PDR and Myanmar
By	Mr. Kyaw Phyoe Sunn -
Field of Study	Veterinary Science and technology
Thesis Advisor	Associate Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the Faculty of Veterinary Science
(Professor ROONGROJE THANAWONGNUWECH, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor CHANNARONG RODKHUM, D.V.M., Ph.D.)

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

..... Examiner
(Dr. TARADON LUANGTONGKUM, D.V.M., Ph.D.)

..... Examiner
(Dr. Saharuetai Jeamsripong, D.V.M., M.P.V.M., Ph.D.)

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

เคาว์ พโย ซุนน์ - : ลักษณะการดื้อยาและลักษณะทางอณูชีววิทยาของการดื้อยาต่อ Extended-spectrum β -lactamases (ESBL) และยา Colistin-ในเชื้อซัลโมเนลลา เอนเทอริกา และเอสเชอริเชีย โคไล ที่แยกได้จากสุกรและเนื้อสุกรในเขตจังหวัดชายแดนประเทศไทยและประเทศกัมพูชา ลาวและพม่า. (Phenotypic and Genotypic Characteristics of Extended-spectrum β -Lactamase (ESBL) Production and Colistin-resistance in *Salmonella enterica* and *Escherichia coli* Isolated from Pigs and their Meat Products in the Border Provinces between Thailand and Cambodia, Lao PDR and Myanmar) อ.ที่ปรึกษาหลัก : รศ.รุ่งทิพย์ ชวนชื่น

ตัวอย่างทั้งหมดจำนวน 809 ตัวอย่าง มาจาก rectal swab ของสุกรในโรงฆ่าสัตว์ จำนวน 441 ตัวอย่าง และจากเนื้อสุกรจำนวน 368 ตัวอย่างถูกเก็บจากจังหวัดที่อยู่ในเขตชายแดนระหว่างประเทศไทยกับกัมพูชา ลาว และพม่า ระหว่างเดือนตุลาคมปี 2559 จนถึงเดือนมีนาคม 2560 จุดประสงค์ของการศึกษาในครั้งนี้เพื่อตรวจติดตามการดื้อยาในกลุ่ม extended-spectrum β- lactams (ESBLs) และ ยาโคลิสติน ในซัลโมเนลลา เอนเทอริกาและเอสเชอริเชีย โคไล จากตัวอย่างดังกล่าวสามารถแยกเชื้อซัลโมเนลลาได้จำนวน 463 เชื้อและอีโคไลจำนวน 767 เชื้อ แบคทีเรียทั้งหมดถูกนำมาตรวจหาการผลิตเอนไซม์ ESBLs และความไวรับต่อยา ยาโคลิสติน รวมถึงศึกษาการปรากฏของยีน ESBLs และยีน *mcr* ผลการศึกษาพบว่า ความชุกของซัลโมเนลลาที่แยกได้จากตัวอย่างของประเทศกัมพูชาพบมากที่สุด (65.8%) และ ตัวอย่างของประเทศพม่ามีความชุกของซัลโมเนลลาที่แยกได้น้อยที่สุด (13.6%) และ serovar ของซัลโมเนลลา ที่พบมากที่สุด คือ Rissen และพบอัตราการปนเปื้อนอีโคไลในเนื้อสุกรมากกว่า 87% ในตัวอย่างจากทุกประเทศ พบซัลโมเนลลา (1.9%) และอีโคไล (6.3%) ที่ผลิตเอนไซม์ ESBLs ในระดับต่ำ พบแบคทีเรียจำนวน 5 isolates ประกอบด้วยซัลโมเนลลา (n=1) และอีโคไล (n=4) ที่ดื้อต่อยา ยาโคลิสติน และผลิตเอนไซม์ ESBLs ด้วย ตามที่ตรวจหายีนที่ควบคุมการสร้างเอนไซม์ ESBLs พบยีน bla_{CTX-M} และ bla_{TEM} ในตัวอย่างที่แยกได้จากทุกประเทศที่ศึกษา ซัลโมเนลลาจำนวน 12 isolates และอีโคไลจำนวน 68 isolates ให้ผลบวกต่อยีน *mcr-1* และ ซัลโมเนลลาจำนวน 1 isolate และอีโคไลจำนวน 31 isolates ให้ผลบวกต่อยีน *mcr-3* นอกจากนั้นซัลโมเนลลาจำนวน 1 isolate ที่แยกได้จากตัวอย่างเนื้อสุกรที่เก็บจากประเทศลาวพบทั้งยีน *mcr-1* และยีน bla_{CTX-M} พร้อมกัน นอกจากนั้น อีโคไลจำนวน 1 isolate ที่แยกได้จากตัวอย่างสุกรที่เก็บจากประเทศไทยและอีโคไลจำนวน 1 isolate ที่แยกได้จากทั้งตัวอย่างสุกรและเนื้อสุกรในประเทศกัมพูชา พบว่ามีทั้งยีน *mcr-3* และยีน bla_{CTX-M} ด้วยกัน จากผลการศึกษาทั้งหมดพบว่าสุกรและเนื้อสุกรเป็นพาหะของซัลโมเนลลาและอีโคไลที่ดื้อต่อยาในกลุ่ม next-generation cephalosporins และยา ยาโคลิสติน ดังนั้นควรมีการเฝ้าระวังและติดตามการดื้อต่อยาปฏิชีวนะ ในกลุ่มนี้ในตัวอย่างจากสัตว์ที่เลี้ยงเพื่อการบริโภค.

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5975401631 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY

KEYWORD: Colistin / Escherichia coli / Extended-spectrum β-lactams / Salmonella enterica

Kyaw Phyoe Sunn - : Phenotypic and Genotypic Characteristics of Extended-spectrum β-Lactamase (ESBL) Production and Colistin-resistance in *Salmonella enterica* and *Escherichia coli* Isolated from Pigs and their Meat Products in the Border Provinces between Thailand and Cambodia, Lao PDR and Myanmar. Advisor: Assoc. Prof. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

A total of 809 samples of pig rectal swab from slaughterhouses (n=441) and pork from retail market (n=368) were collected in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar between October 2016 and March 2017. The objective of this study was to determine resistance to extended-spectrum β-lactams (ESBLs) and colistin in *Salmonella enterica* and *Escherichia coli*. A total of *Salmonella* (n=463) and *E. coli* (n=767) were collected and determined for the ESBL-production and for the Minimum Inhibitory Concentrations (MICs) of colistin and; the presence of ESBL gene and *mcr* gene. The results showed that the prevalence of *Salmonella* was highest in Cambodia (65.8%) and lowest in Myanmar (13.6%). Serovar Rissen was mostly observed. The prevalence of *E. coli* in pork was above 87% in all countries. ESBL-producing *Salmonella* (1.9%) and *E. coli* (6.3%) were detected at low level. Five bacterial isolates (1 *Salmonella* and 4 *E. coli*) were simultaneously resistant to colistin produced by ESBL enzymes. Among the ESBL genes tested, bla_{CTX-M} and bla_{TEM} genes were found in all countries. Twelve *Salmonella* and 68 *E. coli* isolates were positive to *mcr-1* gene. One *Salmonella* and 31 *E. coli* isolates harbored *mcr-3* gene. In addition, one *Salmonella* isolate from pork in Lao PDR carried both *mcr-1* and bla_{CTX-M}. One *E. coli* isolate from pigs in Thailand and one *E. coli* isolate from pig and pork in Cambodia belonged to *mcr-3* and bla_{CTX-M}. These findings demonstrated that pigs and pork serve as reservoirs for the next-generation cephalosporins and colistin-resistant *Salmonella* and *E. coli*. Monitoring of resistance to these antibiotics in food animals is needed.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my academic advisor, Associate Professor Dr. Rungtip Chuanchuen, for her valuable advice and enthusiastic kind encouragement throughout my study. I believe that this thesis would not have been completed in time without her kind guidance and appreciation.

I would like to give a special thanks to my committee members, Assistant Professor Dr. Channarong Rodkhum, the chairman of the thesis committee (Director of Veterinary Science and Technology Program), Dr.Taradon Luangtongkum, Dr. Saharuetai Jeamsripong and Associate Professor Dr. Sunpetch Angkititrakul for their constructive criticisms and fruitful suggestions.

I appreciate the staffs and friends especially my lab-mates from the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University for their help during my study and their kind friendship.

I would also like to extend my sincere thanks to H.E. Dr. Aung Thu (Union Minister), Dr. Khin Zaw (Permanent Secretary) and Dr. Ye Tun Win (Director General of Livestock Breeding and Veterinary Department), Ministry of Agriculture, Livestock and Irrigation, Myanmar for their permission to study the postgraduate program in Chulalongkorn University, Thailand.

I wish to thank Dr. Khin Khin Lay, University of Veterinary Science, Myanmar, for her providing laboratory training and kind support in my research work. I would express a deep sense of gratitude to my family, for their continuous encouragement, moral support, and blessings.

Finally, I would like to thank “Scholarship Program for ASEAN/ Neighbouring Countries”, Chulalongkorn University for providing a scholarship on Veterinary Science and Technology program, Chulalongkorn University. I also would like to thank the contract of NRU 59_015_HER from the National Research University Project, Office of Higher Education Commission, Thailand for their grant of my research.

Kyaw Phyoe Sunn -

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AEC	ASEAN Economic Community
AMR	Antimicrobial resistance
AMU	Antimicrobial use
bp	base pair (s)
BPW	buffer peptone water
CTX-M	Cefotaximase-munich
CFU	colony-forming unit
CMY	Cephamycinase
℃	degree Celsius
CLSI	Clinical and Laboratory Standards Institute
DNA	deoxyribonucleic acid
DW	distilled water
E.	*Escherichia*
EC	*Escherichai coli* medium
ECDC	European Center for Disease Control
EDTA	ethylene diamine teteraacetic acid
e.g.	exampla gratia, for example
EMB	Eosin Methylene Blue medium
ESBL	Extended-Spectrum β-lactamase
et al.	et alii, and others
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organizations of the United Nations
g	gram (s)

h	hour (s)
ISO	International Organization for Standardization
LB	Luria-Bertani medium
M	molar
mcr	Mobilized colistin-resistant
MDR	Multidrug-resistant
mg	milligram (s)
min	minute (s)
ml	milliliter (s)
mm	millimeter
MHA	Muller Hilton agar
MIC	minimal inhibitory concentrations
mPCR	multiplex Polymerase Chain Reaction
NA	Nutrient agar
No.	number
NSS	Normal saline solution
OIE	World Organization for Animal Health
PCR	Polymerase Chain Reaction
pH	The negative logarithm of hydrogen ion concentration
PSE	*Pesudomonas*-specific enzymes
rpm	round per minutes
S.	*Salmonella*
sec	second (s)
SHV	sulfhydryl variable
spp.	species
TAE	Tris-Acetate-EDTA
TEM	temoniera

TSI	Triple Sugar Iron
UN	United Nations
UTI	urinary tract infection
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate
%	Percentage
µg	microgram (s)
µl	microliter (s)
µm	micrometer

CHAPTER I

INTRODUCTION

Emergence and spread of antimicrobial resistance (AMR) among bacterial pathogens has created a significant impact on public health, animal health, economy, society and international trade worldwide. Acquired AMR has threatened the efficacy of antimicrobial drugs for the treatment of bacterial infections and has been placed as one of the greatest problematic issues of human beings (Collignon et al., 2016). Besides usage in humans, antimicrobial has been used in food-animal production for a quite long time. The agents are mainly delivered for therapy, disease prevention and growth promotion in food-producing animals. However, any uses of antimicrobial drugs have created ideal selective pressure for the emergence of AMR bacteria and also has accelerated spread of either resistant bacteria or resistant determinants (ECDC, 2015).

Currently, the situation of AMR has become worse due to the emergence and spread of multidrug-resistant (MDR) bacteria. The rise of MDR bacteria have resulted in increased severity of infections, increased the frequency of therapeutic failures and elevated costs associated with more expensive antibiotics (Prestinaci et al., 2015). The World Health Organization (WHO) has recognized that resistance to all first-line and last resort antimicrobial drugs has been continuously raised in many regions. This has raised particular concern that there may be a lack of antibiotics that can efficiently treat bacterial infection in the future.

Antimicrobial resistant bacteria have arisen among humans, animals and the environment and may spread from one to another, and from country to the others. The resistant bacteria and/or resistance determinants do not memorize geographic

borders or human-animal borders. Therefore, AMR is referred to as a One Health concept. The Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and the WHO have worked closely with the United Nations (UN) agencies to take part in national and international level of combating AMR (Robinson et al., 2016).

Livestock production, particularly pig production, contributes significantly to the world's trading economy over the last couple of decades while antimicrobial substances are widely delivered in pig production for several purposes (Thacker, 2013). The major problem in developing countries, leading to inappropriate use of antimicrobials, is that most pig farmers use antimicrobial drugs imprudently and without prescription of veterinarians. It is evident that any usage of antimicrobials can result in emergence and spread of AMR in bacteria. This raises particular concern that pigs and their meat products could consider a potential reservoir of AMR bacteria and resistance determinants that could enter through the food chain (Barton, 2014) .

Extended-spectrum cephalosporins are new generation ß-lactam antibiotics and one of the last-line antibiotics for the treatment of multidrug-resistant *Enterobacteriaceae* infections in humans. The antibiotics are considered critically important antimicrobial drugs by WHO (Angulo et al., 2009) and the use of antimicrobials in animals should be restricted. Broad-spectrum third-generation and fourth-generation cephalosporins have also been used for the treatment of serious infections (e.g. urinary and respiratory tract infection) caused by *Enterobacteriaceae* family in both humans and animals. These antimicrobials have been categorized as high priority critically important antimicrobials in human medicine (WHO, 2012) and also listed as veterinary importance (OIE, 2007). As the major cause of bacterial resistance to ß-lactams is ß-lactamase enzymes production, resistance to extended-spectrum cephalosporins associated Extended-spectrum ß-lactamase (ESBL) enzymes

has been increasingly reported worldwide (Canton et al., 2008). ESBL-producing *Enterobacteriaceae* were previously reported to be associated with hospitals and are now increasingly found in communities (Le et al., 2015).

ESBLs are mainly encoded by plasmid-borne genes. The ESBLs-carrying plasmids may be transferred horizontally and harbor genes encoding resistance to multiple classes of antimicrobials. This raises a particular concern of wide distribution of pathogenic bacteria resistant to new generation cephalosporins with multidrug resistance phenotype. ESBL enzymes are commonly produced by *Escherichia coli* and *Klebsiella pneumonia*. Recently, the resistance rates of ESBL-producing *Salmonella* and *E. coli* are gradually endemic in livestock productions from different parts of the world (Nguyen et al., 2016). Therefore, role of food-producing animals and food-animal origins as potential reservoirs of ESBL-producing bacteria has been suggested (Geser et al., 2012).

Colistin (polymyxin-E) is a cationic polypeptide antimicrobial drug commercialized in human and veterinary medicine. It is a narrow-spectrum bactericidal against Gram-negative bacteria. For several years, colistin is considered the last resort option in human medicine for treatment of MDR Gram-negative bacterial infections (Catry et al., 2015). Colistin has been widely used in food-animals in Asia, Europe and North America countries, particularly in pigs for prevention and treatment of *Enterobacteriaceae* infections. According to field studies, colistin is generally mixed into feed or drinking water and dispensed to pigs at a group level to treat gastrointestinal tract infections caused by *Salmonella* and *E. coli*.

Recently, the emergence of associated with transferable plasmids *mcr-1* gene in *E. coli* has been reported in food-producing animals and has now been identified in other bacterial strains from animals and humans (Liu et al., 2016a). The report of

plasmid-borne *mcr-1* in early November in China has alarmed the public about the widespread of colistin-resistant pathogens that will adversely affect both human and animal medicine (Newton-Foot et al., 2017). Up to date, the emergence and global spread of *mcr* have now been commonly identified in livestock in many parts of the world including countries with zero to marginal use of colistin (Butaye and Wang, 2018b). Therefore, the hope of novel antimicrobial discovery is uncertain in the future, especially in Gram-negative spectrum (Chaudhary, 2016).

Thailand, Cambodia, Lao PDR and Myanmar are located in the Mekong region and share a common land border. Cross-border trade has been focused on improve their commerce, trade, tourism, and transportation (Manarungsan, 2010). Moreover, the ASEAN Economic Community (AEC) has been already established since 2015 and ASEAN cooperation has touched the significance of food safety, in which AMR is one of the major challenges for developing countries. As a consequence, it led to routine movement of live animals and meat products, particularly pigs and pork that imported and/or exported within and across the bordering partner countries. Sometimes, formal quarantine approaches are generally not supported by traders or not available. Therefore, the emergence and spread of AMR in those countries could be a reflection of the movement of animals and their meat products.

Moreover, more than millions of people travel across the borders per year. These include also tourists, merchants, villagers and migrant workers. Overseas travel has faced as a risk factor for the international emergence and wide-spread dissemination of AMR bacteria from one country to another (Senok et al., 2012). The emergence and wide-spread dissemination of AMR bacteria are carried in connection of large movements of people in the border provinces among Thailand, Cambodia, Lao PDR, and Myanmar. Thus, regional cooperation on standardize and harmonize for AMR monitoring and surveillance programs are essential for the future development

and to implement ASEAN export and import markets for all livestock and livestock products among those bordering partner countries (Archawakulathep et al., 2014).

Salmonella enterica remain one of the most common zoonotic food-borne pathogens that can impact on public health worldwide. According to the European surveillance data, food-producing animals are main vectors of bacteria and contaminated foods are potential transmission of AMR *Salmonella* (Humphrey, 2000). *Salmonella* serovars are commonly found in swine production and humans may get infected through direct contact, contaminated food and water and the environment (DuPont and Steele, 1987). Salmonellosis is usually self-limited, and the infected people may recover within a week without antimicrobial treatments. However, patients with invasive *Salmonella* infections and enteric fever are more likely to require suitable antimicrobial drugs. Recently, the occurrence of AMR in *Salmonella* spp. has been increasingly reported in many parts of the world (Skov et al., 2007).

E. coli is a commensal bacterium and commonly found in the gastrointestinal tract of humans and animals. It has been used as a good indicator bacterium for selection pressure imposed by antimicrobial use (Li et al., 2014). The bacterium could serve as a major reservoir of resistance determinants that transfer to other bacterial species including *Salmonella*. It has been suggested that commensal *E. coli* has an exceptional capability for the spreading and acquiring of resistance genes from one to other bacterial spp. (Smith et al., 2007). Recently, the prevalence of MDR *E. coli* has been increasing found. Therefore, commensal *E. coli* of food-producing animals are considered as a key reservoir for the transfer of AMR bacteria and AMR genes to humans (Dyar et al., 2012).

Homologous relationships between AMR determinants in humans and food-producing animals have been commonly recorded for food-borne bacteria such as *Salmonella* and *E. coli* (Marshall and Levy, 2011). These bacteria may enter to the food chain and cause infections in humans that are difficult to treat. Due to these particular concerns, special attention is needed to reduce the development of these bacteria on food products and to minimize the emergence of AMR genes and determinants in *Salmonella* and *E. coli* in developing countries. However, the knowledge of ESBL-producing and colistin-resistant *Salmonella* spp. and *E. coli* from pigs and pork is still limited. Therefore, detection of ESBL production and colistin resistance in *Enterobacteriaceae* isolated from pigs and pork were performed among Thailand, Cambodia, Lao PDR, and Myanmar based on the understanding the reported experiences of AMR studies.

Objectives of study

1. To determine the prevalence of ESBL-producing and colistin-resistant *Salmonella* and *E. coli* in pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar.
2. To characterize genetic under ESBL-production and colistin-resistance in *S. enterica* and *E. coli* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar.

Questions of study

1. What is the percentage of ESBL-producing and colistin-resistant *S. enterica* and *E. coli* in pigs and pork in the border provinces among Thailand, Cambodia, Laos PDR and Myanmar?

2. What is the genetic characteristics of ESBL-production and colistin-resistance in *S. enterica* and *E. coli* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar?

CHAPTER II

LITERATURE REVIEW

1. General characteristics of *Salmonella* spp. and *E. coli*

1.1. General characteristics of *S. enterica*

Salmonella was first found by Karl Eberth in 1880s, but *Salmonella* pathogen was discovered by Salmon's group. *Salmonella* is a rod-shaped and facultatively anaerobic Gram-negative bacterium belonging to the *Enterobacteriaceae* family. Cell is approximately 2-5 μm length and 0.7-1.5 μm width. It is mesophile, and the optimal temperature for growth of *Salmonella* is at 37°C. However, it survives well at least seven years under the freezing environment (-23 to 18°C). *Salmonella* grows on ferrous sulphate containing media (Triple Sugar Iron Test). It includes two genus: *Salmonella bongori* and *Salmonella enterica* (Agbaje et al., 2011).

At present, the taxonomic group of *Salmonella* spp. comprises more than 2,600 serotypes and are identified by the somatic O (lipopolysaccharide) and flagella H antigens according to the Kauffman-White classification (Gal-Mor et al., 2014). Moreover, they remain one of the most common food-borne illness in humans. *Salmonella* spp. are more prevalent in the environment and are detected not only in domestic animals but also in wild animals as pathogens or commensals. The routes of bacterial transmission from food-animals to humans can take place through the food-chain.

The common clinical signs are fever, vomiting, abdominal pain and diarrhea, but sometimes severe infections depending on *Salmonella* strains and hosts. Moreover, non-typhoidal *Salmonella* infections are more severe than in immuno-

compromised people, children and older people. The typhoidal *Salmonella* strains infections are usually acquired to humans through contaminated food and water. Among them, *S. enterica* sub-species are commonly related with Salmonellosis in humans and animals cased, gastroenteritis (Callaway et al., 2008).

Antimicrobial drugs are critically used for the treatment of patients with invasive *Salmonella* infections. Unfortunately, the emergence of AMR *Salmonella* strains has been reported to improper use of antimicrobial drugs. This may lead to prolonged hospitalization in patients due to the lack of effective treatments.

1.2. General characteristics of *E. coli*

E. coli was first discovered in 1885s by Theodor Escherich. It is a rod-shaped and facultatively anaerobic Gram-negative bacterium belonging to the *Enterobacteriaceae* family. The diameter of the cell is approximately 0.5 μm and 2.0 μm long. Cell volume are 0.46-0.7 μm3 (Kubitschek, 1990). The optimal temperature for growth of *E. coli* is at 37°C, however, it grows well up to 49°C with optimum pH 6-7. It can grow in all common laboratory media, including MacConkey Agar or Eosin Methylene-Blue Agar, which differentiate bacteria that ferment lactose with nucleated colonies.

E. coli serotypes are identified by surface antigens such as the somatic (O), flagella (H), and capsular (K) based on the modified Kauffmann-White classification. According to the Robins-Browne and Hartland, 2002, there are over 180 various O-antigens and at least 60 H-antigens have also been observed in their study (Robins-Browne and Hartland, 2002). Commensal *E. coli* exist in the gastrointestinal tract of humans and warm-blooded animals gut to support digestion as well as defend

against enteric pathogens and usually do not cause disease to their hosts (Wieler et al., 2001).

Some *E. coli* strains is the major pathogen causing severe diarrhea in piglets and also impact on economic losses in pig rearing. It can be transmitted to humans through consumption of contaminated foods, such as raw or undercooked ground meat products. Sometimes it causes lethal infections such as meningitis, gastroenteritis, urinary tract infections, septicemia and epidemic diarrhea of adults and children (Schierack et al., 2006).

Since commensal *E. coli* are originally susceptible to several antimicrobial drugs, it has been used as an indicator organism not only for detection fecal contamination food, but also for AMR monitoring among Gram-negative bacteria. The incidence of AMR *E. coli* has also been reported (Barber et al., 2013). Particularly, *E. coli* have the ability to accept and transfer AMR genes and therefore, serve as a model for studying the emergence and spread of AMR and the health risks posed by antimicrobial use (AMU). In order to contribute to this knowledge, determination of AMR commensal *E. coli* has become an international topic of both human and veterinary concerns globally as well.

2. ESBL-production in *S. enterica* and *E. coli*

2.1 Description and mechanisms of action and resistance

Beta-lactams are one of the broad-spectrum antimicrobial drugs and are characterized by possession of ß-lactam ring. Beta-lactam antimicrobial agents are commonly used as a first-line therapy to treat a wide range of bacterial infections caused by susceptible organisms in human and veterinary medicine. There are many classes for ß-lactam antibiotics according to their bacterial spectrum (broad versus

narrow) or their type of activity (bactericidal vs. bacteriostatic). Among ß-lactam antibiotics, cephalosporins are also divided due to their antibacterial activities and properties depending on their side chain configurations. In the third and fourth generation broad-spectrum cephalosporins, they can inactivate for Gram-positive and Gram-negative bacteria.

Currently, one of the important resistance mechanisms to ß-lactam antimicrobial agents in *Enterobacteriaceae* is caused by production of plasmid-encoded ß-lactamases that inactivate those drugs by hydrolyzing of their rings, and these enzymes are called ESBLs (Dierikx et al., 2010). The *Enterobacteriaceae* need a certain resistance gene to produce ESBL enzymes. During propagation period, this genetic property can be moved from one drug resistant bacteria to another through cell division. However, ESBL is a new group of enzymes that has the ability to break down or hydrolyze penicillin, extended-spectrum cephalosprins and monobactams, while they are generally susceptible to cephamycins, carbapenems and ß-lactamase inhibitors (Coque et al., 2008).

ESBL is class A ß-lactamase and the predominant ESBL types are temoniera (*TEM*), sulfhydryl variable (*SHV*), and cefotaximase-munich (*CTX-M*). ESBL-producing *Enterobacteriaceae*, especially plasmid-borne *SHV* and *TEM* types were mostly observed in hospital-acquired infections since 1980s and 1990s, respectively. Most ESBLs are descended from plasmid-mediated penicillinases, such as *TEM* or *SHV* types through the process of mutations that change near the activated site of these ß-lactamases. However, a typical ESBL phenotype is a new class of ß-lactamases and it is not close related to *TEM* or *SHV* types (Bonnet, 2004).

After a few years ago, *CTX-M* types with a typical ESBL resistance phenotype has become predominantly in *Salmonella, E. coli* and *Klebsiella pneumoniae* than

any other β-lactamases (Chong et al., 2013). *CTX-M* enzymes are greater active against cefotaxime than ceftazidime, ceftriaxone or cefepime. Indeed, ESBL-producing organisms frequently carry co (or) multi-resistant encoding genes to other antimicrobial classes by chromosomal or plasmid-borne (Jacoby and Munoz-Price, 2005).

2.2 Epidemiology of ESBL-production in *S. enterica* and *E. coli*

ESBL-producing *Enterobacteriaceae* has been published in food-animals and humans (Blanc et al., 2006). Although the resistance rate of ESBL family were almost detected under 10% before 2008, but the prevalence varies in geographical distribution and is increasingly forwards (Woerther et al., 2013). Several studies from European countries have been published that ESBL-producing *Salmonella* and *E. coli* have been detected in animal origins (Brinas et al., 2005; Carattoli, 2008). Different studies performed in Belgium, Denmark, France, Greece, the Netherlands, Spain and the UK, ESBL-producing *Salmonella* and *E. coli* isolates have also been found in farm animals, pets and beef meat sources (Hasman et al., 2005; Cloeckaert et al., 2007).

Moreover, ESBL-producing *Salmonella* spp. and *E. coli* strains have been found in patients suffering from urinary tract infections (UTI) since 2000 (Livermore et al., 2007). The recent study in Turkey demonstrated a prevalence of ESBL-producing *E. coli* have also been detected 21% in community acquired UTI between 2004 and 2005 (Yumuk et al., 2008). This UTI prevalence was higher than Spanish survey in 2006 (Andreu and Planells, 2008). The prevalence of ESBLs was higher than 10% in Hungary, Poland, Romania, Russia and Turkey (Korten et al., 2007; Damjanova et al., 2008; Empel et al., 2008).

The first report from the Eastern Mediterranean region, showed a prevalence of 2.4% ESBL producers in young healthy students in 2005 (Moubareck et al., 2005). During the past decade, a typical *CTX-M* group with ESBL resistance phenotype have widely distributed and it is important for nosocomial infections in Japan (Hiroi et al., 2012). Previous report in Asian countries have been stated that ESBL-producing bacteria has gradually increased up to 70% among the Asian community (Nakayama et al., 2015).

Moreover, ESBL *Salmonella* isolates have also been identified in humans, pigs and pork in the border crossings among Thailand-Lao PDR and Thailand-Cambodia (Sinwat et al., 2016; Trongjit et al., 2017). However, *E. coli* is the major bacteria associated to ESBL-production. Reports concerning ESBL *E. coli* isolated from food-animals have been published in many regions (Smet et al., 2010). The major reservoirs and sources of ESBL- producing *E. coli* transmission have not been known, but food-producing animals are the primary sources of ESBL-production (European Food Safety Authority, 2011b).

3. Colistin-resistant *S. enterica* and *E. coli*

3.1 Description and mechanisms of action and resistance

Colistin is one of the cationic polypeptide antimicrobial drugs produced by *Paenibacillus polymyxa*. Polymyxin groups consist of five different classes, including polymyxins A, B, C, D and E. Among them, Colistin (polymyxin-E) and polymyxin-B are being reconsidered as last-resort antimicrobial drug in human medicine. Colistin or polymyxin-E was first manufactured by *Bacillus colistinus* in 1947s (Poirel et al., 2017). It has been widely operated to treat serious bacterial infections since 1959s. The use of colistin was gradually reduced between 1970 and 1990s in both humans

and animals because of the high incidence of its systemic toxicity. There are two forms of colistins are available in the market, such as colistin sulfate for topical use and sodium colistin methanesulphonate for parenteral use. However, parenteral use of colistin has been related to its toxicity (Beringer, 2001).

Colistin consists of a cyclic decapeptide bound to fatty acid chain and its molecular weight is 1750 Da. Colistin is a bactericidal in action by binding to lipopolysaccharide (LPS) and phospholipids in the outer membrane of Gram-negative bacteria. Colistin displaces divalent cations from the phosphate groups of membrane lipids, leading to a local disturbance of the outer cell membrane, resulting in leakage of cell contents and bacterial death (Landman et al., 2008). Polymyxins also inhibit vital respiratory enzymes (type II NADH-quinone oxidoreductases (NDH-2) in the inner membrane of bacteria (Deris et al., 2014).

Colistin is commonly used to treat and prevent bacterial infections caused by *Salmonella* and *E. coli* in food-producing animals. Particularly, colistin is administered with food during or post weaning in swine production and other uses have also been found in all areas. Although colistin has not been administered in the US, it is largely used for the treatment in animal health in Europe, and also promotion of animals growth in several Asian countries (Butaye and Wang, 2018a). Colistin is also used for growth promotion in some non-European countries (Livermore, 2002).

More recently, the reintroduction of colistin for treatment has been followed by the development of AMR among Gram-negative bacteria. At present, colistin has been acted as a last line antibiotic in human medicine for treating carbapenem-resistance in Gram-negative bacterial infections. Unfortunately, Gram-negative bacteria can develop resistance to colistin through mutation or adaption

mechanisms. High percentages of colistin-resistance may be considered with suboptimal dosage (Kempf et al., 2013).

At present, five different transferable plasmid-encoded colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* have been reported and *mcr*-resistance genes possess multiple variants (Rebelo et al., 2018). Among five different emerging *mcr*-genes, *mcr-1, mcr-2*, and *mcr-3* were originated on plasmids in *Enterobacteriaceae* family (Liu et al., 2016b; Zhang et al., 2018). They contribute to the emergence and transmission of colistin-resistance mediated by plasmid-borne *mcr*-genes.

3.2 Epidemiology of colistin-resistant *S. enterica* and *E. coli*

The epidemiology of colistin resistance was poorly known and few researchers were interested regarding with colistin resistance in general. After the first finding of *mcr-1* encoding genes in China has been published, the prevalence of *mcr-1* resistant *Salmonella* and *E. coli* have also been identified in food-animals, meat products and humans, including countries with zero to marginal use of colistin (Anjum et al., 2016). After the first discovery of *mcr-1* gene, additional new type of plasmid-borne colistin resistance encoding genes were detected mcr-2 genes in *E. coli* isolated from porcine and bovine origin in Belgium (Xavier et al., 2016), followed by *mcr-3* genes in *E. coli* isolated from pigs in China (Yin et al., 2017), *mcr-4* genes in *Salmonella* and *E. coli* isolated from pigs in Italy, Spain and Belgium (Carattoli et al., 2017) and *mcr-5* genes in *Salmonella* isolated from poultry in Germany (Borowiak et al., 2017), respectively.

In Latin America and the Asia Pacific region, colistin resistant *E. coli* was detected from distinct geographical regions, but a greater resistance rate of colistin

was found in *Klebsiella* spps. Moreover, colistin-resistance in *K. pnemoniae* and *E. coli* have also been detected from Chinese patients in China hospital (Paterson and Harris, 2016). A recent report in Cambodia, colistin-resistant Salmonella has been detected from chicken carcasses (Lay et al., 2011). In Lao PDR, colistin-resistance in *E. coli* has been reported from a pig and a person (Olaitan et al., 2014). In Thailand, colistin-resistance has also been found in chicken isolates (Angkititrakul et al., 2005). Moreover, colistin-resistance has also been identified in *Salmonella* and *E. coli* from pigs and pork in the border between Thailand - Cambodia (Trongjit et al., 2016b; Trongjit et al., 2017). However, AMR data including colistin resistance is limited in Southeast Asia countries.

4. Co-occurrence of ESBL-producing and colistin-resistant *S. enterica* and *E. coli*

Beta-lactam drugs (carbapenems) are first used as a last line therapy against ESBL-producing organisms, which are increasing resistant to other classes of antimicrobials in communities and hospitals. However, the emergence carbapenems-resistant isolates are more common in Gram-negative bacteria due to the introduction of carbapenems treatment (McKenna, 2013). At present, colistin has been reintroduced in human medicine as a last line antibiotic to treat in MDR patients due to carbapenem-resistant *Enterobacteriaceae.* Resistance to colistin by Gram-negative bacteria that are normally susceptible to carbapenem has also been detected (Johansen et al., 2008).

After the discovery of *mcr-1* gene, the higher prevalence of plasmid-borne *mcr-1* gene has been identified in animal origins and livestock production has been singled out as a major reservoir of colistin resistance amplification and spread (Rhouma et al., 2016). The most significant development in the last few years is *mcr-1* positive isolates carried in several resistance genes that were located on the

mcr-1 carrying plasmids. The previous study from China indicated that the co-occurrence of $bla_{CTX\text{-}M\text{-}55}$ and *mcr-1* encoding genes located on same plasmid in *S. enterica* (Yang et al., 2016).

The co-localization of *mcr-1* gene and ESBL genes has been detected in many isolates from poultry and porcine origins (Falgenhauer et al., 2016; Yao et al., 2016). The prevalence of *mcr-1* harboring ESBL-producing *Enterobacteriaceae* has been found in renal deficiency patient in Switzerland (Poirel et al., 2016) and also in patient with fungal meningitis in France hospital (Caspar et al., 2017).

The resistance to colistin has been observed in Italy for treating carbapenemase-producing *Enterobacteriaceae* infections (Monaco et al., 2014). In Denmark, the prevalence of *mcr-1* harboring ESBL-producing *E. coli* from veal calves was much reported than that found in ESBL-producing *E. coli* from human and chicken meat (Hasman et al., 2015). Therefore, the increasing use of colistin may lead to co-occurrence of colistin-resistance with carbapenems and is of great concern to public health services globally (Timofte et al., 2015).

CHAPTER III

MATERIALS AND METHODS

This experiment was divided into three phases, including Phase 1: Sample collection, isolation and identification of *Salmonella* and *E. coli* isolated from pigs and their products, Phase 2: Determination of ESBL-production and colistin-resistance in *Salmonella* and *E. coli*, and Phase 3: Detection of resistance determinants underlying ESBL-production and colistin-resistance of *Salmonella* and *E. coli* (Figure 1).

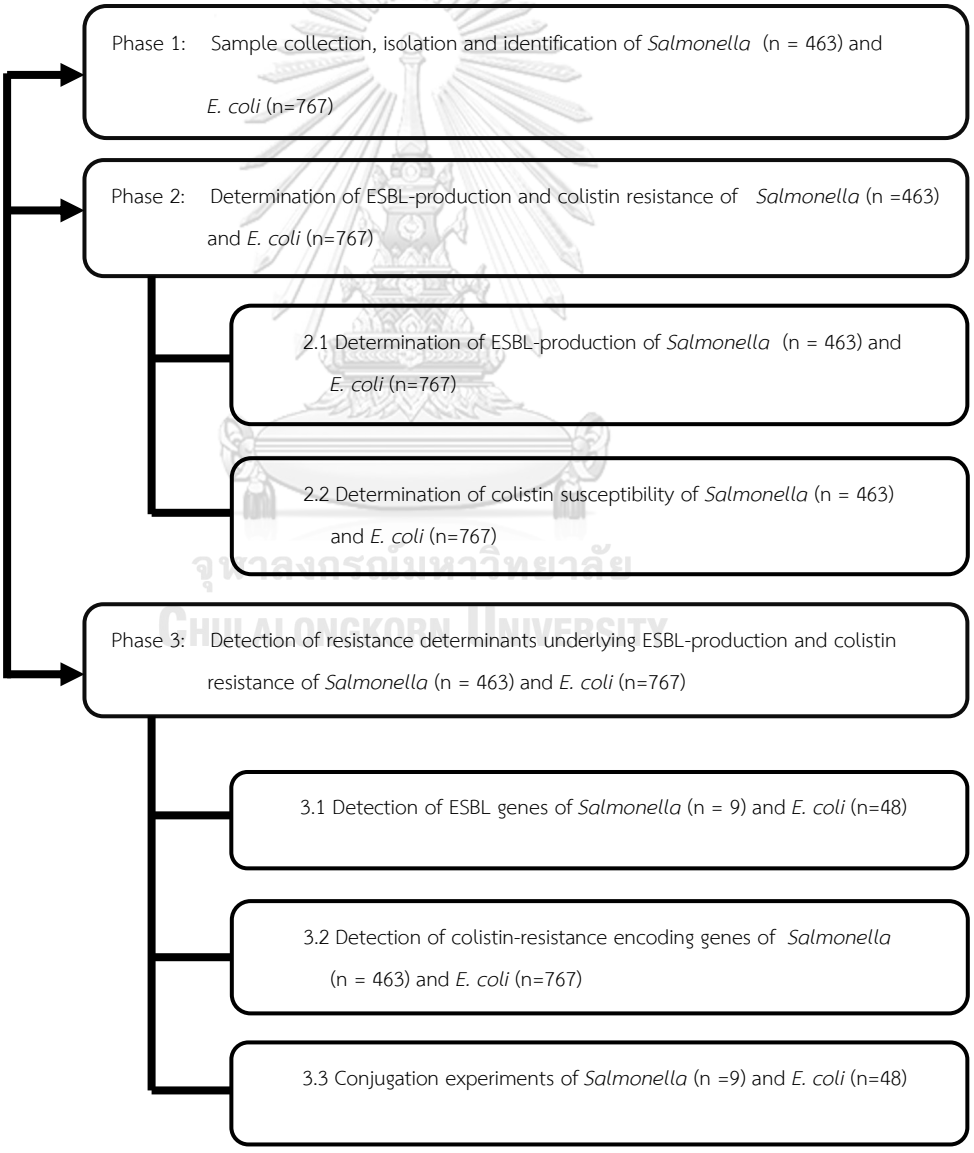

Figure 1: Flow chart of the experiments

Phase I: Sample collection, isolation and identification of *Salmonella* and *E. coli*

1. Sampling location and sample collection

1.1 Sampling location

All samples were collected in the border provinces between Thailand and Cambodia (Sa Kaeo - Banteay Meanchey), Thailand and Lao PDR (Nong Khai - Vientiane) and Thailand - Myanmar (Chiang Rai - Tachileik). The crossing points were chosen due to their flourishing owing to their geographical advantages situated along the Mekong River and high movement of live animals and humans (Manarungsan, 2010). Sampling location is shown in Figure 2.

Figure 2: Map of the border provinces among Thailand, Cambodia, Lao PDR and Myanmar. The provinces where were the sample collection sites are shown with location vector (and).

1.2 Sample collection

A total of 809 samples were collected by rectal swab (n=441) from municipal pig slaughterhouses and carcass swab (n=368) from municipal retail markets (Table 1). Sample collection was jointly performed with the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University, Thailand.

The samples were collected by purposive sampling in three separate occasions in six months from each province between October 2016 and March 2017. The samples were obtained from municipal slaughterhouses and provincial retail markets of each province are described in Figure 3-5.

In Thailand, the pig slaughterhouse in Nong Khai province was large-scale facilities and eighty or more pigs were processed per day, while the slaughterhouses from Sakaeo and Chaing Rai provinces were small-scale modern facilities with a throughout of fifty or fewer pigs per day.

The pig slaughterhouses in Banteay Meanchey province in Cambodia and from Tachileik province in Myanmar were traditionally small slaughterhouses with a throughput of thirty or fewer pigs per day. Pigs were mostly processed in simple and traditional slaughtering in both provinces from Cambodia and Myanmar.

In Lao PDR, the pig slaughterhouse from Vientaine was large-scale modern facilities with a throughput of two hundred or more pigs per day. All pigs in all provinces were from commercial production farms that provide meat for domestic consumption. The slaughterhouses and retail markets were selected so that pigs and pork could be tracked and sample at each point in the food supply chain.

Table 1: Source and number of samples obtained from each border province among Thailand, Cambodia, Lao PDR and Myanmar (n=809)

Location	Origin	Sample Type	No. of collected samples									
			Thailand				Cambodia		Lao PDR		Myanmar	
			Nong Khai	Sa Kaeo	Chiang Rai	**Total**	Banteay Meanchey	**Total**	Vientiane	**Total**	Tachileik	**Total**
Slaughter-house	Pig	Rectal swab	80	60	60	**200**	84	**84**	82	**82**	75	**75**
Retail market	Pork	Carcass swab	60	60	60	**180**	65	**65**	58	**58**	65	**65**
Total			140	120	120	**380**	149	**149**	140	**140**	140	**140**
Grand Total			809									

At the slaughterhouses, rectal swabs were collected from pigs after stunning and bleeding but before the scalding. Carcass swabs were also collected from pig carcasses by swabbing an area of about 50 cm2 after the slaughtering process before delivered to the provincial retail markets. Sterile cotton swab was used for each sample and all collected samples were put into transport media and sent to the Veterinary Public Health Department's laboratory, Faculty of Veterinary Science, Chulalongkorn University, Thailand for microbiological analysis. The samples arrived at the laboratory within 36 hours after the sample collection.

A. **B.**

Figure 3: Sample collection from slaughterhouses and retail markets in Thai border provinces **(A)** Sample collection from a municipal slaughterhouse (Nong Khai, Thailand) and **(B)** Sample collection from a municipal slaughterhouse (Chiang Rai, Thailand).

A. B.

Figure 4: Sample collection from slaughterhouses and retail markets **(A)** Sample collection from a municipal slaughterhouse (Sa Kaeo,Thailand) and **(B)** Sample collection from a retail market (Banteay Meanchey, Cambodia).

A. B.

Figure 5: Sample collection from slaughterhouses and retail markets **(A)** Sample collection from a retail market (Vietiane,Lao PDR) and **(B)** Sample collection from a retail market (Tachileik, Myanmar).

2. Isolation and identification of *Salmonella* and *E. coli*

2.1 Isolation and identification of *Salmonella*

The *Salmonella* strains were isolated according to International Organization for Standardization ISO 6579:2002(E) and biochemically confirmed as previously described (Barrow, 1993). Briefly, each cotton swab was put into 5 ml of buffer peptone water (BPW) and inoculated at 37°C for over-night to enrich *Salmonella* species. After incubation, the suspension was taken with a sterile loop and cultured onto the Modified Semi-solid Rappaport-Vassiliadis agar (MSRV) (Difco, MD, USA) and incubated at 42°C for 24 hours. Then, it was transferred and streaked on xylose lysine deoxycholate (XLD) agar (Difco) to get single colonies. Three colonies were picked and streaked on triple sugar iron (TSI) slant (Difco) for biological test and followed by nutrient agar (NA) or Luria-Bertani (LB) agar (Difco) at 37°C for overnight respectively. After which a typical single colony on each plate from tested bacteria was picked and overnight inoculated into 2 ml of LB broth (Difco) at 37°C. Then, 1 ml from inoculated broth was put into 20% sterile glycerol stock. All *Salmonella* isolates were kept in 20% sterile glycerol stocks at -80°C. Steps for *Salmonella* isolation is shown in appendix A.

2.1.1 *Salmonella* serotyping

Three typical colonies were picked up from each *Salmonella* positive sample. Each *Salmonella* isolate was serotyped by using slide agglutination method (Figure 6) according to the Kauffman-White schemes (Tindall et al., 2005). Then, one isolate of each serovar was collected from each positive sample. The specific antiserum was generated by S & A REAGENTS LAB LTD, PART, Lat Phrao, Bangkok, Thailand corresponding to manufacturer's indications.

Firstly, *Salmonella* isolate was cultured onto the NA agar (Difco) and incubated at 37°C for 18 hours. After incubation, one free falling drop of polyvalent O-antisera (OMA, OMB, OMC, etc) was placed and added a typical single colony of *Salmonella* onto a glass slide for agglutination. Then, it was mixed thoroughly, and the agglutination result was examined within 10 seconds. If agglutination occur with one of these three groups, the isolate is positive for that group. Then, it is repeated for further agglutination steps by testing the isolate in each monovalent O-antiserum in this group. e.g.: If the polyvalent antiserum OMA shows agglutination, the mentioned monovalent O-antisera must be tested: O: 1,2; O: 4,5; O: 9; O:3,10,15; O: 46; O: 1,3,19 respectively.

For H-antigen phase 1, a loopful colony of *Salmonella* from NA agar plates was stabbed onto the swarm agar in small petri dish and then incubated at 37°C for 18 hours. After incubation, one free falling drop of polyvalent H-antisera (HMA, HMB, HMC, etc;) was placed onto a glass slide for agglutination and added a typical single colony of *Salmonella* from invasion zone of swarm agar on it. After mixed thoroughly, the agglutination result was examined. If agglutination occur with one of these three groups, the isolate is positive for that group. Then, it is repeated for further agglutination steps by testing the isolate in each monovalent H-antiserum in this group. For example: If the polyvalent antiserum HMA shows agglutination, the described monovalent H-antisera must be tested: a; c; d; 1; z10, respectively.

For H-antigen phase 2, a drop of monovalent H antiserum from phase inversion box was placed into the new small petri dish. After that, the swarm agar was poured into the petri dish and the plate was shaken thoroughly to mix with antisera and agar. The plate was kept at room temperature for 25-30 minutes to dry. A loopful colony of *Salmonella* from H 1 phase was picked and stabbed at the center of the plate and then incubated at 37°C for 18 hours. After incubation, a

culture at the periphery of the invasion zone of the swarm agar was taken and mix with polyvalent H-antisera (HMA, HMB, HMC, etc:) and the result was examined. If agglutination occur with one of these three groups, the isolate is positive for that group. Then, it is repeated for final agglutination step by testing the isolate in each monovalent H-antiserum in this group. Steps for *Salmonella* serotyping is shown in Appendix B.

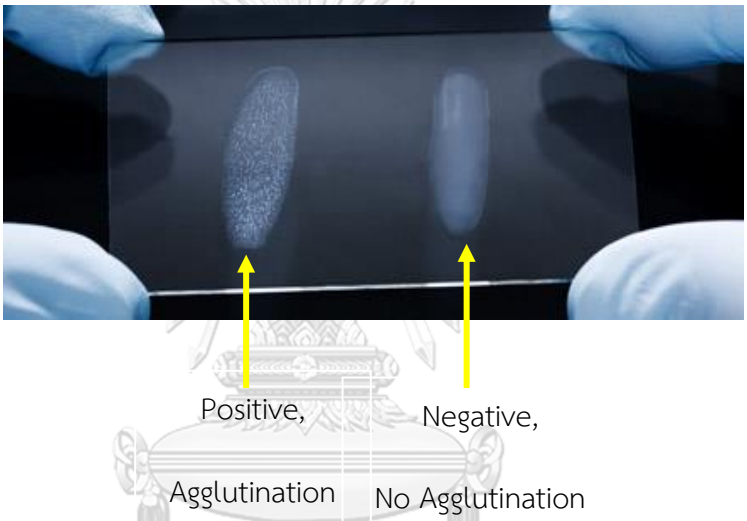

Figure 6: Appearance of agglutination in *Salmonella* serotyping assay

2.2 Isolation and identification of *E. coli*

The *E. coli* strains were grown and biochemically confirmed using the International Organization for Standardization ISO 7251:2005. Briefly, each swab was put into 5 ml of buffer peptone water (BPW) and inoculated at 37°C for over-night to enrich *E. coli* species. After incubation, the suspension was taken with a sterile loop and cultured onto the EC medium (Difco) with Durham tube at 45°C for 24 hours in water-bath. Then, it was streaked on Eosin Methylene Blue (EMB) agar (Difco) at 37°C for over-night. It was transferred and streaked on Mac Conkey agar plate (Difco) at 37°C for 24 hours. Moreover, it was streaked on NA or LB Agar (Difco) at 37°C for 24 hours, respectively. Each isolate was purified to get single colony on each plate and it was transferred and incubated into 2 ml of LB broth (Difco) was put into 20% sterile glycerol stock and mixed well thoroughly. One *E. coli* isolate was collected from each of the positive sample and isolate was stored in 20% glycerol stocks at -80°C. Steps for *E. coli* isolation and confirmation used in this study is shown in Appendix B.

Phase II: Determination of ESBL-production and colistin-resistance of *Salmonella* and *E. coli*

1. Determination of ESBL-production by disk diffusion method

There are two steps of determination of ESBL-production, screening and confirmatory methods as described below.

1.1 ESBL screening method

All the *Salmonella* isolates (n=463) and *E. coli* (n=767) were initially screened for ESBL production by using disk diffusion method according to the Clinical and

Laboratory Standards Institute (CLSI, 2013). *Salmonella* and *E. coli* were grown on Muller Hinton (MHA) agar (Difco) at 37°C for 24 hours. A typical single colony from MHA was picked and suspended in 0.85% NaCl solution (NSS). The turbidity of inoculum was adjusted to the equivalent of a 0.5 McFarland (~108 CFU/ml) by using McFarland Densitometer. The bacterial suspension was streaked thoroughly on to the MHA agar (Difco) using with sterile cotton swab.

Three cephalosporin indicators included ceftazidime (30 µg), cefotaxime (30 µg) and cefpodoxime (10 µg) (Oxoid, Hampshire, England). Then, three cephalosporin disks were placed onto MHA agar (Difco) by using with sterile pointed forceps and incubated at 37°C for 16-18 hours. After incubation, the diameter of inhibition zone was measured by using millimeter scaled ruler and the zone diameter breakpoints for three cephalosporin indicators used to determine the isolates as susceptible or resistant are stated in Table 2. *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) were used as quality control strains during initial screening of ESBL-production.

Table 2: Zone diameter interpretative criteria of cephalosporin indicators used in this study for initial screening of ESBL-production

No.	Antimicrobials	Amount (µg)	Inhibition zone diameter (mm)	
			Susceptible	Resistant
1.	Ceftazidime	30	≥23	≤22
2.	Cefotaxime	30	≥28	≤27
3.	Cefpodoxime	10	≥18	≤17

Source: CLSI (2013)

1.2 ESBL confirmatory method

The isolates that were resistant to at least one of the cephalosporins tested were phenotypically confirmed by a combination disk diffusion method. Ceftazidime (30 µg) (Oxoid) disk and cefotaxime (30 µg) (Oxoid) disk alone and Ceftazidime (30 µg)/clavulanic acid (10 µg) disk (Oxoid) and cefotaxime (30 µg)/clavulanic acid (10 µg) disk (Oxoid) were used and incubated at 37°C for 16-18 hours. Then, the diameter of inhibition zone was measured by using millimeter scaled ruler. The difference of ≥5 mm among the inhibition zones diameters of cephalosporin disks and those of cephalosporin with clavulanate disks were defined as phenotypically positive for the confirmation of ESBL production (Wayne, 2007).

2. Determination of colistin susceptibility

Colistin susceptibility was examined by using two-fold agar dilution method for determining Minimum Inhibitory Concentration (MIC), of which the clinical breakpoint is 4 µg/ml. The results were interpreted based on European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013). Colistin was dissolved in sterile distilled water and its concentration ranges were 0.0625µg/ml, 0.125µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml, respectively. Colistin was purchased from Sigma-Aldrich® (Steinheim, Germany).

All the *Salmonella* (n=463) and *E. coli* (n=767) isolates were grown on MHA agar (Difco) at 37°C for 24 hours. After, a typical single colony from MHA was picked and suspended in 0.85% NSS and the turbidity of each inoculum was adjusted to the equivalent of a 0.5 McFarland (~108 CFU/ml) by using McFarland Densitometer. Then, the bacterial suspension was ten-fold diluted to get ~107 CFU/ml in NSS and it

was transferred into the microtiter plates. Moreover, it was inoculated onto the MHA antibiotic plates with appropriate concentrations of colistin by using multipoint-inoculator and incubated at 37°C for 18-24 hours. After incubation, the MIC value for colistin susceptibility indicated that the lowest concentration of colistin with the inhibition of the visible bacterial growth was detected. *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) were used as quality control strains.

Phase III: Detection of resistance determinants underlying ESBL-production and colistin-resistance of *Salmonella* and *E. coli*

1. Detection of ESBL genes

The detection of ESBL genes were performed by conventional Polymerase Chain Reaction (PCR) assay using specific primers in all ESBL-producing isolates. Template DNA was prepared from all ESBL-producing isolates of *Salmonella* (n=9) and *E. coli* (n=48) by using whole cell boiled lysate method according to the laboratory protocol preciously described (Levesque et al., 1995). *Salmonella* and *E. coli* were grown on LB agar (Difco) at 37°C for over-night. Then, a loopful fresh bacterial colony was picked and suspended in 100 µl of sterilized distilled water. The bacterial suspension was heated in a boiling water bath for 10 minutes and immediately put on ice. Bacterial suspension was then centrifuged at 12,000 rpm for 5 minutes. The DNA supernatant was transferred into a sterile new Eppendorf tube and kept at 20°C until use.

Each PCR reaction included 2 µl of DNA template, 0.75 µl of each primer at 10 µM, 7.5µl of Ge Nei^{TM} MasterMix (Merck, Munich, Germany) and 3.5 µl of RNase-free water to get 24 µl of final volume according to the instructions described by

manufacturer. All specific primers used for each PCR were shown in Table 3. The thermal cycling conditions for each PCR reaction used as the same basic set-up: an initial denaturation at 94°C for 3 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing temperature for $bla_{CTX\text{-}M}$ at 60°C for 1 minute; annealing temperature for bla_{TEM} and bla_{SHV} at 50°C for 1 minute; annealing temperature for $bla_{CMY\text{-}1}$ and $bla_{CMY\text{-}2}$ at 58°C for 1 minute and annealing temperature for bla_{PSE} at 55°C for 1 minute, elongation time at 72°C for 60 seconds and final extension at 72 °C for 10 minutes, respectively.

The gels were then stained with Redsafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology®, Seongnam, South Korea). A 5 µl of PCR product was electrophoresed by using 1.5% agarose gel electrophoresis (Vivantis®, Subang Jaya Malaysia) in 1×Tris-acetate/ EDTA (ethylene diamine teteraacetic acid) (1×TAE) buffer. The PCR product was then visualized under the UV light by Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, USA).

2. Detection of colistin-resistance encoding genes

The detection of plasmid-encoded colistin resistant determinants, *mcr-1*, *mcr-2* and *mcr-3* genes were examined by Multiplex PCR (mPCR) in all *Salmonella* (n=463) and *E. coli* (n=767) isolates. Template DNA was prepared by using the whole cell boiled lysate method according to the laboratory protocol described by (Levesque et al., 1995). The isolates of Salmonella and *E. coli* were grown on LB agar (Difco) plate at 37°C for over-night. Then, a loopful fresh bacterial colony was picked and suspended in 100 µl of sterilized distilled water. The bacterial suspension was heated in a boiling water bath for 10 minutes and immediately put on ice. The bacterial suspension was then centrifuged at 12,000 rpm for 5 minutes. The DNA

supernatant was transferred into a sterile new Eppendorf tube and kept at 20°C until use.

Each PCR reaction included 2 µl of DNA template, 0.37 µl of each primer, 6.3 µl of Ge Nei™ MasterMix (Merck) and 0.25 µl of RNase-free water to get 12 µl of final volume according to the instructions described by manufacturer. The primers used for each multiplex PCR are also shown in Table 3. The thermal cycling conditions for each PCR reaction used as the same basic step: an initial denaturation at 94°C for 15 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing temperature at 58°C for 90 seconds and elongation time at 72°C for 60 seconds, and final extension at 72°C for 10 minutes.

The gels were then stained with Redsafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology®). A 5 µl of PCR product was electrophoresed by using 1.5% agarose gel electrophoresis (Vivantis®, Subang Jaya Malaysia) in 1×Tris-acetate/ EDTA (1×TAE) buffer. The PCR products was then visualized under the UV light by Bio-Rad Gel-Documentation System (Bio-Rad Laboratories, USA).

Table 3: Primers used in this study

Primer	Sequence (5'-3')	Gene	Tm (°C)	Size (bp)	Reference
ESBL genes					
CTX-M up	ATGTGCAGYACCAGTAARGTKATGGC	*bla*$_{CTX-M}$	60	593	Batchelor et al., 2005
CTX-M down	TGGGTRAARTARGTSACCAGAAYCAGCGG				
TEM up	GCGGAACCCCTATTTG	*bla*$_{TEM}$	50	343	Hasman et al., 2005
TEM down	TCTAAAGTATATATGAGTAAACTTGGTCTGAC				
SHV up	TTCGCCTGTGTATTATCTCCCTG	*bla*$_{SHV}$	50	854	Hasman et al., 2005
SHV down	TTAGCGTTGCCAGTGYTCG				
CMY-1 up	GTGGTGGATGCCAGCATCC	*bla*$_{CMY-1}$	58	915	Hasman et al., 2005
CMY-1 down	GGTCGAGCCGGTCTTGTTGAA				
CMY-2 up	GCACTTAGCCACCTATACGGCAG	*bla*$_{CMY-2}$	58	758	Hasman et al., 2005
CMY-2 down	GCTTTTCAAGAATGCGCCAGG				
PSE up	GCTCGTATAGGTGTTTCCGTTT	*bla*$_{PSE}$	55	575	Batchelor et al., 2005
PSE down	CGATCCGCAATGTTCCATCC				
MCR genes					
CLR-5F	CGGTCAGTCCGTTTGTTC	*mcr-1*	58	320	Liu et al., 2016
CLR-5R	CTTGGTCGGTCTGTA				
MCR2-IF	TGTTGCTTGTGCCGATTGGA	*mcr-2*	58	725	Xavier et al., 2016
MCR2-IR	AGATGGTATTGTTGGTTGCTG				
MCR3-IF	AAATAAAAATTGTTCCGCTTAT	*mcr-3*	58	929	Yin et al., 2017
MCR3-IR	G AATGGAGATCCCCGTTTTT				

3. Test for transferability by conjugation experiments

All ESBL-producing isolates (*Salmonella*, n=9 and *E. coli,* n=48) were tested by for conjugation experiments using biparental mating method (Woodall, 2003). All the ESBL-producing isolates were used as donor strains. The spontaneous rifampicin-resistant derivatives of *E. coli* K12 strain MG 1655 was used as the recipient strains for ESBL-producing *Salmonella* isolates and the spontaneous rifampicin-resistant derivatives of *S. Entriditis* SE 12 was used as recipient for ESBL-producing *E. coli* isolates.

The donors and recipients were grown in 4 ml LB broth at 37°C for over-night in a shaking incubator. Eighty-µl aliquots of each culture of the donors and the recipients were separately added into 4 ml of fresh LB broth at 37°C for 3-4 hours in a shaking incubator to reach log phase of growth. Each pair of 700 µl of donors and recipients was thoroughly mixed in an Eppendorf tube and then centrifuge at 8,000 rpm for 1 minute. The supernatant was removed, and the pellets were resuspended in 30 µl LB broth warmed at 37°C. The suspension was gently spreaded on a sterile membrane filter (0.45 µm pore size, Millipore, Massachusetts, USA) that was placed on LB agar plate without any antibiotics and incubated at 37°C for over-night.

The inoculated filter paper was carefully taken from LB plate and placed into 1 ml NSS in a new Eppendorf tube. Then, the tube was vortexed to separate the cells and the filter paper was discarded. The suspension was centrifuged at 13,000 rpm for 1 minute and the supernatant was discarded. A hundred-µl of LB broth was added into the bacterial pellet. The conjugation mixture was gently dropped on LB agar supplemented with 32 µg/ml of rifampicin and ampicillin (100 µg/ml) and then the mixture was incubated at 37°C for over-night.

For the *E. coli* recipients, 3-5 *Salmonella* colonies were picked and grown on EMB agar (Difco) with ampicillin. Transconjugants were also confirmed as *E. coli* by growing on Mac Conkey agar (Difco).

For the *Salmonella* recipients, 3-5 E. coli colonies were picked and grown on Brilliant Green (BG) agar (Difco) with ampicillin. Then, transconjugants were also confirmed as *Salmonella* by growing on XLD agar (Difco).

DNA was extracted from each transconjugant using whole cell boiled lysate method and detected for the presence of ESBL genes using PCR as mentioned above.

Statistical Analysis

Statistical analyses were performed by using SPSS 22.0 software (IBM Corp, Armonk, NY, USA). The descriptive statistics was conducted in this study. The significance ($P < 0.05$) of differences between prevalence of *Salmonella* species and *E. coli* and between ESBL-production and colistin resistance occurrence in various populations, locations and sample types were analyzed using Pearson's Chi-square test. A p-value of <0.05 was considered statistically significant.

CHAPTER IV

RESULTS

1. Prevalence of *Salmonella* and *E. coli*

1.1. Prevalence of *Salmonella* and serovars

A total of 809 samples were collected from pork slaughterhouses and retail markets and 403 samples were positive to *Salmonella* (49.8%) (Table 4). The prevalence of *Salmonella* varied among sources of samples and locations of the sampling in different countries. The highest prevalence of *Salmonella* was in Cambodia (Banteay Meanchey province) (98/149, 65.8%), followed by Thailand (Nong Khai, Sa Kaeo and Chiang Rai provinces) (214/380, 56.3%), Lao PDR (Vientiane province) (72/140, 51.4%), and Myanmar (Tachileik province) (19/140, 13.6%). The *Salmonella* isolate was more frequently detected in carcass samples obtained from markets (246/403, 61.04%) than those collected in rectal swabs from slaughterhouse (157/403, 38.96 %) in all countries.

A total of 463 *Salmonella* isolates were obtained from 403 positive samples, of which sixty-one *Salmonella* serovars were identified (Table 5). Among these, serovar Rissen was most commonly detected (165, 35.6%) and followed by Anatum (71, 15.3%), Stanley (26, 5.7%) and Sao (20, 4.3%), respectively. The numbers of identified *Salmonella* serovars in each country are shown in Figure 7. Among the Thailand isolates (n=237), twenty-two serovars were identified, of which, serovar Rissen was most common (103, 43.5%). Serovars Rissen was identified in 46.2% of the isolates from slaughterhouses and 39.4% of the isolates from the markets. Thirty-one serovars were identified among the isolates from Cambodia (n=121) and serovar

Rissen (48, 39.7%) was the most common. The most common serovars were Rissen, 40% of the isolates from slaughterhouses and 39.5 %) from the markets.

Table 4: Prevalence of *Salmonella* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=809)

Country	Sample location	Sample type	No. of samples	No. of positive samples (%)
Thailand	Slaughterhouse	Rectal swab	200	89 (44.5)
	Market	Carcass swab	180	125 (69.4)
	Total		**380**	**214 (56.3)**
Cambodia	Slaughterhouse	Rectal swab	84	39 (46.2)
	Market	Carcass swab	65	59 (90.8)
	Total		**149**	**98 (65.8)**
Lao PDR	Slaughterhouse	Rectal swab	82	28 (34.1)
	Market	Carcass swab	58	44 (75.9)
	Total		**140**	**72 (51.4)**
Myanmar	Slaughterhouse	Rectal swab	75	1 (1.3)
	Market	Carcass swab	65	18 (27.7)
	Total		**140**	**19 (13.6)**
	Grand Total		**809**	**403 (49.8)**

Thirty-four serovars were identified among the 86 isolates from Lao PDR, of which, serovars Stanley was most frequently detected (15, 17.4%). Serovars Stanley in pigs from slaughterhouses (8, 26.7%) and Rissen in pork from markets (11, 19.6%) were the most common serotypes found. Three serovars were identified among the 19 isolates from Myanmar, of which, only one *Salmonella* isolate was obtained from the slaughterhouse and its serovar was Rissen. Serovars Anatum were most common serotypes among the pork isolates from market (14, 77.8%).

Table 5: *Salmonella* serovars isolated from pigs and pork in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=463)

Salmonella serovars	No. of isolates (%)							
	Thailand (n=237)		Cambodia (n=121)		Lao PDR (n=86)		Myanmar (n=19)	
	Pig (n=94)	Pork (n=143)	Pig (n=45)	Pork (n=76)	Pig (n=30)	Pork (n=56)	Pig (n=1)	Pork (n=18)
Typhimurium	12(12.8)	-	2(4.4)	-	1(3.3)	-	-	-
Sao	12(12.8)	8(5.6)	-	-	-	-	-	-
Augustenborg	1(1.1)	1(0.7)	-	-	-	2(3.6)	-	-
Schwarzengrund	2(2.1)	-	-	-	-	1(1.8)	-	-
Derby	2(2.1)	-	3(6.7)	1(1.3)	-	-	-	-
Rissen	37(39.4)	66(46.2)	18(40)	30(39.5)	-	11(19.6)	1(100)	2(11.1)
Saintpaul	2(2.1)	8(5.6)	-	-	-	1(1.8)	-	2(11.1)
Eastbourne	1(1.1)	-	-	-	-	-	-	-
Anatum	8(8.5)	30(20.9)	-	9(11.8)	5(16.7)	5(8.9)	-	14(77.8)
Rideau	1(1.1)	4(2.8)	-	-	-	1(1.8)	-	-
Sanktmarx	5(5.3)	10(6.9)	-	-	1(3.3)	2(3.6)	-	-
Weltevreden	4(4.3)	1(0.7)	1(2.2)	1(1.3)	-	-	-	-
Braenderup	1(1.1)	-	-	2(2.6)	-	-	-	-
Fareham	4(4.3)	2(1.4)	-	-	-	-	-	-
Stanley	2(2.1)	1(0.7)	3(6.7)	5(6.6)	8(26.7)	7(12.5)	-	-
Vijle-1	-	1(0.7)	-	-	-	-	-	-
Norwich	-	2(1.4)	-	-	-	1(1.8)	-	-
Yalding	-	4(2.8)	-	-	-	-	-	-
Calabar	-	1(0.7)	-	-	-	-	-	-
Fareham	-	2(1.4)	-	-	-	-	-	-
Hayindogo	-	3(2.1)	-	1(1.3)	-	6(10.7)	-	-
Muenster	-	1(0.7)	1(2.2)	5(6.6)	-	3(5.4)	-	-
Potto	-	-	-	-	3(10)	-	-	-
Tsevie	-	-	2(4.4)	-	1(3.3)	-	-	-
Brunei	-	-	-	-	4(13.3)	-	-	-
Kissi	-	-	-	-	1(3.3)	-	-	-
Eschberg	-	-	-	-	1(3.3)	-	-	-
Ayinde	-	-	-	-	1(3.3)	-	-	-
Kentucky	-	-	-	-	1(3.3)	2(3.6)	-	-
Rottnest	-	-	-	-	1(3.3)	-	-	-

Table 5 continued.

Salmonella serovars	No. of isolates (%)							
	Thailand (n=237)		Cambodia (n=121)		Lao PDR (n=86)		Myanmar (n=19)	
	Pig (n=94)	Pork (n=143)	Pig (n=45)	Pork (n=76)	Pig (n=30)	Pork (n=56)	Pig (n=1)	Pork (n=18)
Vilvoorde	-	-	-	-	1(3.3)	-	-	-
Kouka	-	-	-	-	1(3.3)	-	-	-
Portanigra	-	-	-	-	-	1(1.8)	-	-
Newlands	-	-	-	-	-	2(3.6)	-	-
Bristol	-	-	-	-	-	1(1.8)	-	-
Sandow	-	-	-	10(13.2)	-	1(1.8)	-	-
Haifa	-	-	-	1(1.3)	-	1(1.8)	-	-
Magumeri	-	-	-	-	-	1(1.8)	-	-
Lika	-	-	-	-	-	1(1.8)	-	-
V	-	-	-	-	-	1(1.8)	-	-
Koenigstuhl	-	-	1(2.2)	-	-	1(1.8)	-	-
II	-	-	-	1(1.3)	-	1(1.8)	-	-
Suberu	-	-	-	-	-	1(1.8)	-	-
Ikayi	-	-	-	-	-	2(3.6)	-	-
Dallgow	-	-	1(2.2)	-	-	-	-	-
Paratyphi-B	-	-	1(2.2)	-	-	-	-	-
Lekke	-	-	1(2.2)	-	-	-	-	-
Herston	-	-	1(2.2)	-	-	-	-	-
Hvittingfoss/II	-	-	1(2.2)	-	-	-	-	-
Stanley ville	-	-	1(2.2)	-	-	-	-	-
Bradford	-	-	1(2.2)	-	-	-	-	-
Yoruba	-	-	1(2.2)	-	-	-	-	-
Rechovot	-	-	4(8.9)	-	-	-	-	-
Bracknell	-	-	1(2.2)	-	-	-	-	-
Idikan	-	-	1(2.2)	-	-	-	-	-
Sinstorf	-	-	-	5(6.6)	-	-	-	-
Paris	-	-	-	1(1.3)	-	-	-	-
Newport	-	-	-	1(1.3)	-	-	-	-
Ituri	-	-	-	1(1.3)	-	-	-	-
Kedougou	-	-	-	1(1.3)	-	-	-	-
Havana	-	-	-	1(1.3)	-	-	-	-

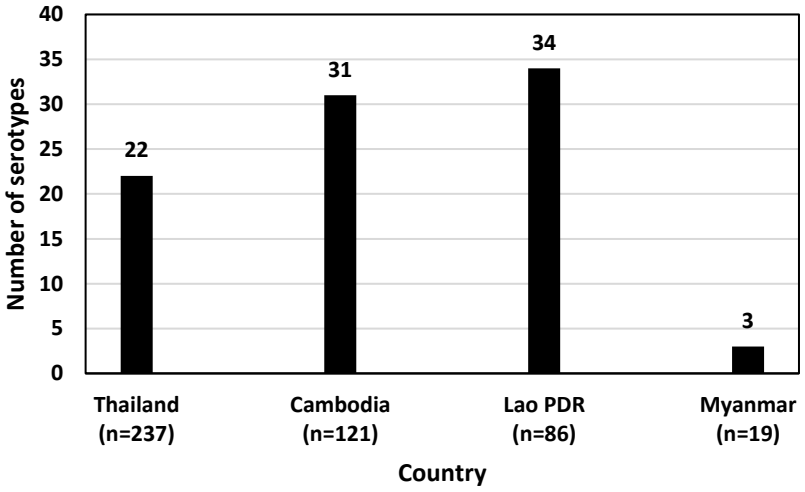

Figure 7: Prevalence of *Salmonella* serovars (n=463)

1.2 Prevalence of *E. coli*

Of the 809 samples, 767 samples (94.8%) were positive to *E. coli* (Table 6). The prevalence of *E. coli* in pork samples from each country was more than 87%. The highest prevalence of positive samples was detected in Thailand (Nong Khai, Sa Kaeo and Chiang Rai provinces) (368/380, 96.8%), followed by Lao PDR (Vientiane province) (133/140, 95.0%), Myanmar (Tachileik province) (130/140, 92.9%) and Cambodia (Banteay Meanchey province) (136/149, 91.3%). The *E. coli* contamination rate in pork (87.7%) was lowest in Cambodia. The percentage of *E. coli* was more frequently detected in pigs from slaughterhouses (424/767, 55.28%) than that in pork from the markets (343/767, 44.72%).

Table 6: Prevalence of *E. coli* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=809)

Country	Sample location	Sample type	No. of samples	No. of positive samples (%)
Thailand	Slaughterhouse	Rectal swab	200	195 (97.5)
	Market	Carcass swab	180	173 (96.1)
	Total		**380**	**368(96.8)**
Cambodia	Slaughterhouse	Rectal swab	84	79 (94.1)
	Market	Carcass swab	65	57 (87.7)
	Total		**149**	**136 (91.3)**
Lao PDR	Slaughterhouse	Rectal swab	82	79 (96.3)
	Market	Carcass swab	58	54 (93.1)
	Total		**140**	**133 (95.0)**
Myanmar	Slaughterhouse	Rectal swab	75	71 (94.7)
	Market	Carcass swab	65	59 (90.8)
	Total		**140**	**130 (92.9)**
Grand Total			**809**	**767(94.8)**

2. Antimicrobial resistance of *Salmonella* and *E. coli*

2.1 Antimicrobial resistance of the *Salmonella* isolates

All *Salmonella* isolates (n=463) obtained from Thailand (n=237), Cambodia (n=121), Lao PDR (n=86) and Myanmar (n=19) were tested for antimicrobial susceptibility of ceftazidime, cefotaxime and cefpodoxime by using disk diffusion method. Among the three cephalosporin antibiotics, the *Salmonella* isolates were resistant to ceftazidime 2.59% (12/463), cefotaxime 2.38% (11/463) and cefpodoxime 2.59% (12/463), respectively (Table 7). Pork isolates from Thailand exhibited the highest prevalence to all third-generation cephalosporins tested (> 3% for all). None

of the pork isolates from Cambodia and pig isolates from Myanmar were resistant to ceftazidime, cefotaxime and cefpodoxime. Concurrently, none of the pork isolates from Myanmar were resistant to cefotaxime and cefpodoxime, with the exception of ceftazidime.

Colistin susceptibility test was assessed in all the *Salmonella* isolates (n=463) for determining MICs. The overall resistance rate was low (12/463, 2.59%) (Table 7). High percentage of colistin-resistant rate was found in isolates from Myanmar (1/19, 5.26%), followed by Cambodia (4/121, 3.31%), Thailand (6/237, 2.53%) and Loa PDR (1/86, 1.16%). Notably, none of the pig isolates from Thailand, Cambodia, Lao PDR and Myanmar were resistant to colistin. In contrast, the colistin-resistant *Salmonella* strains were found in pork samples, of which the resistance rates in different countries were as follows, Myanmar, 5.56%; Cambodia, 5.26%; Thailand, 4.20% and Lao PDR, 1.79%.

Table 7: Antimicrobial resistance rates of *Salmonella* from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar (n=463)

Antimicrobial Agents	No. of isolates (%)												
	Thailand			Cambodia			Lao PDR			Myanmar			Overall
	Pig (n=94)	Pork (n=143)	Total (n=237)	Pig (n=45)	Pork (n=76)	Total (n=121)	Pig (n=30)	Pork (n=56)	Total (n=86)	Pig (n=1)	Pork (n=18)	Total (n=19)	(n=463)
colistin	0(0)	6(4.2)	6(2.53)	0(0)	4(5.26)	4(3.31)	0(0)	1(1.79)	1(1.16)	0(0)	1(5.56)	1(5.26)	12(2.59)
ceftazidime	3(3.19)	5(3.5)	8(3.38)	1(2.22)	0(0)	1(0.83)	1(3.33)	1(1.79)	2(2.33)	0(0)	1(5.56)	1(5.26)	12(2.59)
cefotaxime	2(2.13)	6(4.2)	8(3.38)	1(2.22)	0(0)	1(0.83)	1(3.33)	1(1.79)	2(2.33)	0(0)	0(0)	0(0)	11(2.38)
cefpodoxime	3(3.19)	6(4.2)	9(3.80)	1(2.22)	0(0)	1(0.83)	1(3.33)	1(1.79)	2(2.33)	0(0)	0(0)	0(0)	12(2.59)

2.2 Antimicrobial resistance of *E. coli* isolates

All the *E. coli* isolates (n=767) obtained from Thailand (n=368), Cambodia (n=136), Lao PDR (n=133) and Myanmar (n=130) were carried out for antimicrobial susceptibility of ceftazidime, cefotaxime and cefpodoxime. Among the three cephalosporin antibiotics, 6.39% (49/767), 8.99% (69/767) and 8.74% (67/767) of the *E. coli* isolates, showed positive to the ceftazidime, cefotaxime and cefpodoxime, respectively (Table 8). Resistance rate of these cephalosporin indicators in *E. coli* were higher than those in *Salmonella*. The highest-ceftazidime resistance rate was found among the isolates from Myanmar (12/130, 9.23%), followed Thailand (26/368, 7.07%), Cambodia (7/136, 5.15%) and Lao PDR (4/133, 3.01%), respectively. Resistance rates to cefotaxime and cefpodoxime were not different between different sources and countries.

According to the colistin susceptibility in *E. coli*, the overall resistant rate of colistin was 10.43% (80/767) (Table 8). The percentages of colistin resistance rates for each country were as follows; Lao PDR isolates (38/133, 28.57%), Cambodia (18/136, 13.24%), Thailand (22/368, 5.98%) and Myanmar (2/130, 1.54%), respectively. Among the pig isolates, the isolates from Lao PDR exhibited the highest colistin resistance rate (22.78%), followed by those from Cambodia (13.92%) and Thailand (4.10%), respectively. None of the pig isolates from Myanmar were resistant to colistin. Of the pork isolates, the isolates from Lao PDR showed the highest colistin rate (20/54, 37.04%), followed by those from Cambodia (7/57, 12.28%), Thailand (14/173, 8.09%), and Myanmar (2/59, 3.39%).

Table 8: Antimicrobial resistance rates of *E. coli* from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar (n=767)

Antimicrobial Agents	No. of isolates (%)												
	Thailand			Cambodia			Lao PDR			Myanmar			Overall
	Pig (n=195)	Pork (n=173)	Total (n=368)	Pig (n=79)	Pork (n=57)	Total (n=136)	Pig (n=79)	Pork (n=54)	Total (n=133)	Pig (n=71)	Pork (n=59)	Total (n=130)	(n=767)
colistin	8 (4.10)	14 (8.09)	22 (5.98)	11 (13.92)	7 (12.28)	18 (13.24)	18 (22.78)	20 (37.04)	38 (28.57)	0(0)	2 (3.39)	2 (1.54)	80 (10.43)
ceftazidime	12 (6.15)	14 (8.09)	26 (7.07)	3 (3.80)	4 (7.02)	7 (5.15)	3 (3.80)	1 (1.85)	4 (3.01)	7 (9.86)	5 (8.47)	12 (9.23)	49 (6.39)
cefotaxime	19 (9.74)	15 (8.67)	34 (9.24)	4 (5.06)	1 (1.75)	5 (3.68)	8 (10.13)	1 (1.85)	9 (6.77)	13 (18.31)	8 (13.56)	21 (16.15)	69 (8.99)
cefpodoxime	19 (9.74)	16 (9.25)	35 (9.51)	2 (2.53)	1 (1.75)	3 (2.21)	7 (8.86)	1 (1.85)	8 (6.02)	13 (18.31)	8 (13.56)	21 (16.15)	67 (8.74)

3. Prevalence of ESBL-producing *Salmonella* and *E. coli*

The *Salmonella* (n=15) and *E. coli* (n=78) isolates that were resistant to at least one of the cephalosporin indicators (i.e. ceftazidime, cefotaxime and cefpodoxime) were phenotypically confirmed for ESBL-production by a combination disk diffusion method. The ESBL screening and confirmation results for *Salmonella* and *E. coli* are provided in Table 9 and 10, respectively. Overall, the percentage of ESBL-producing *E. coli* was 6.3% (48/767) that was significantly different from that of *Salmonella* (9/463, 1.9%).

Table 9: Prevalence of ESBL-producing *Salmonella* isolates in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=463)

Country	Source	No. of isolates	No. of ESBL screening isolates (%)	No. of ESBL confirmatory isolates (%)
Thailand	Pig	94	4(4.3)	2(2.1)
	Pork	143	6(4.2)	6(4.2)
	Total	**237**	**10(4.2)**	**8(3.4)**
Cambodia	Pig	45	2(4.4)	0(0)
	Pork	76	0(0)	0(0)
	Total	**121**	**2(1.7)**	**0(0)**
Lao PDR	Pig	30	1(3.3)	0(0)
	Pork	56	1(1.8)	1(1.8)
	Total	**86**	**2(2.3)**	**1(1.2)**
Myanmar	Pig	1	0(0)	0(0)
	Pork	18	1(5.6)	0(0)
	Total	**19**	**1(5.3)**	**0(0)**
Grand Total		**463**	**15(3.2)**	**9(1.9)**

Table 10: Prevalence of ESBL-producing *E. coli* isolates in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=767)

Country	Source	No. of isolates	No. of ESBL screening isolates (%)	No. of ESBL confirmatory isolates (%)
Thailand	Pig	195	20(10.3)	12(6.2)
	Pork	173	18(10.4)	9(5.2)
	Total	**368**	**38(10.3)**	**21(5.7)**
Cambodia	Pig	79	4(5.1)	2(2.5)
	Pork	57	4(7.0)	1(1.8)
	Total	**136**	**8(5.9)**	**3(2.2)**
Lao PDR	Pig	79	8(10.1)	6(7.6)
	Pork	54	1(1.9)	0(0)
	Total	**133**	**9(6.8)**	**6(4.5)**
Myanmar	Pig	71	14(19.7)	13(18.3)
	Pork	59	9(15.3)	5(8.5)
	Total	**130**	**23(17.7)**	**18(13.9)**
Grand Total		**767**	**78(10.2)**	**48(6.3)**

3.1 ESBL producing *Salmonella*

The overall prevalence of ESBL-producing *Salmonella* was 1.9% (9/463), of which the highest prevalence was detected in the isolates from Thailand (8/237, 3.40%) followed, by Lao PDR (1/86, 1.2%). However, none of the isolates from Cambodia and Myanmar were found to be ESBL-producing *Salmonella.* ESBL resistance phenotype of *Salmonella* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar are demonstrated in Figure 8.

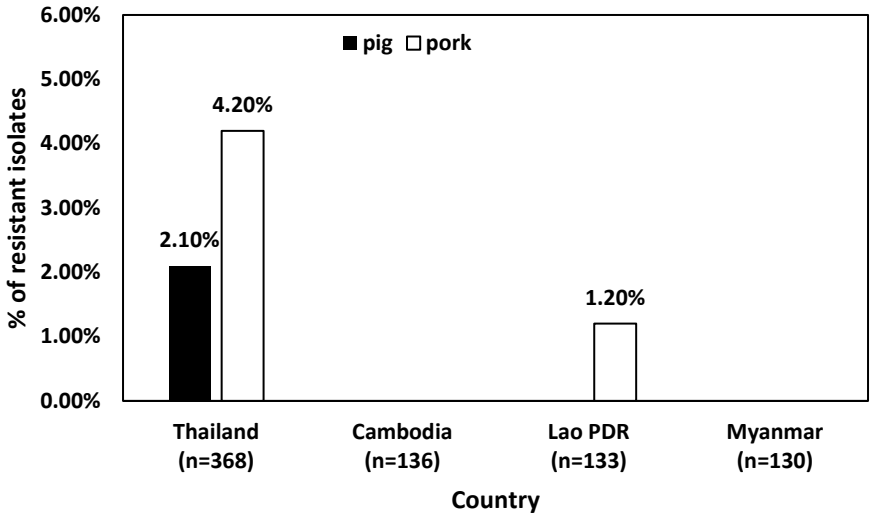

Figure 8: ESBL producing *Salmonella* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=463)

3.2 ESBL producing of *E. coli*

The overall prevalence of ESBL-producing *E. coli* was 6.3% (48/767), of which the highest prevalence *E. coli* was among the isolates from Myanmar (18/130, 13.9%), followed by Thailand (21/368, 5.7%), Lao PDR (6/133, 4.5%) and Cambodia (3/136, 2.2%), respectively (Figure 9). The prevalence of ESBL-*E. coli* varied among sources of sample and locations of sampling. The percentage of ESBL- *E. coli* was higher in pigs from slaughterhouses (33/767, 4.3%) than that in pork from the markets (15/767, 2%). However, none of the isolates from Lao PDR was found to be ESBL-producing *E. coli.* The ESBL production phenotype of *E. coli* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar are demonstrated in Figure 9.

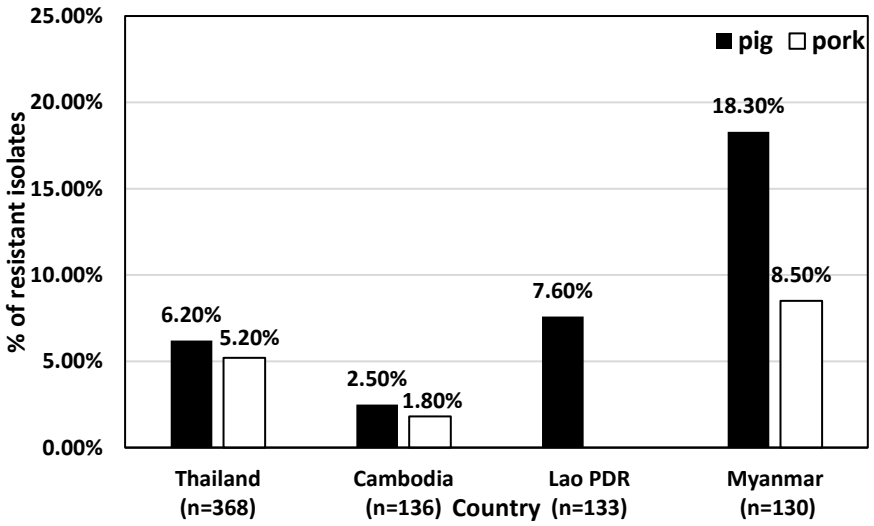

Figure 9: ESBL producing *E. coli* in the border provinces among Thailand, Cambodia Lao PDR and Myanmar (n=767)

3.3 Co-resistance to ESBL-production and colistin resistance in *Salmonella* and *E. coli*

Five isolates (one *Salmonella* isolate and four *E. coli* isolates) were resistant to colistin and able to produce ESBL enzymes simultaneously (Table 11).

Table 11: The isolates resistant to colistin and able to produce ESBL enzymes (*Salmonella*, n=12 and *E. coli*, n=80)

Country	Source	No. of ESBL-producing isolates		*Salmonella*	*E. coli*
		Salmonella	*E. coli*		
Thailand	Pig	2	12	0	1
	Pork	6	9	0	1
Cambodia	Pig	0	2	0	1
	Pork	0	1	0	1
Lao PDR	Pig	0	6	0	0
	Pork	1	0	1	0
Myanmar	Pig	0	13	0	0
	Pork	0	5	0	0
Total		**9**	**48**	**1**	**4**

4. Genotypic detection of ESBL-producing and colistin-resistant *Salmonella* and *E. coli*

4.1 Genotypic detection of ESBL-producing *Salmonella* and *E. coli* isolates

All the ESBL-producing *Salmonella* isolates (n=9) and *E. coli* (n=48) were examined by PCR assay for the presence of ESBL-production encoding genes. Of all

ESBL genes tested, only bla_{CTX-M} and bla_{TEM} genes were observed (Figure 10 and 11). However, the subtype of these genes was not tested.

Nine *Salmonella* isolates (1.9%) were resistant to at least one cephalosporin tested and eight isolates (1.7%) were found to be ESBL producers. These included seven isolates from Thailand and one isolate from Lao PDR. Of all *Salmonella* isolates tested, only bla_{CTX-M} (8/463, 1.7%) and bla_{TEM} (7/463, 1.5%) genes were observed (Figure 12). The bla_{CTX-M} was mostly found in the isolates from Thailand (7/237, 3.0%) and only one isolate from Lao PDR (1.2%). None of the bla_{CTX-M} genes were observed in isolates from Cambodia and Myanmar. The bla_{TEM} gene was detected only in the isolates from Thailand (7/237, 3.0%). None of the bla_{TEM} genes were observed in any of the isolates from Cambodia, Lao PDR and Myanmar. Moreover, bla_{SHV}, bla_{CMY-1}, bla_{CMY-2} and bla_{PSE} genes were not found in any ESBL-producing *Salmonella* isolates in all countries.

Forty-Eight *E. coli* isolates (6.3%) were resistant to at least one cephalosporin tested, of which forty-seven isolates (6.1%) were confirmed to be ESBL producers. These included twenty isolates from Thailand, three isolates from Cambodia, six isolates from Lao PDR and eighteen isolates from Myanmar. Of all the isolates tested, the prevalence of bla_{CTX-M} was 6.1% (47/767) and that of the bla_{TEM} was 3.4% (26/767) (Figure 13). When considered the countries, the highest percentage of bla_{CTX-M} gene was in Myanmar (18/130, 13.9%), followed by Thailand (20/368, 5.4%), Lao PDR (6/133, 4.5%) and Cambodia (3/136, 2.2%), respectively. Moreover, the highest percentage of bla_{TEM} was also found in the Myanmar (6/130, 4.6%), followed by Lao PDR (5/133, 3.8%), Thailand (13/368, 3.5%) and Cambodia (2/136, 1.5%), respectively. None of bla_{SHV}, bla_{CMY-1}, bla_{CMY-2} and bla_{PSE} genes were observed in all countries. The percentages of bla_{CTX-M} and bla_{TEM} genes were low in Cambodia isolates (2.2% and 1.5%, respectively) than compared to others.

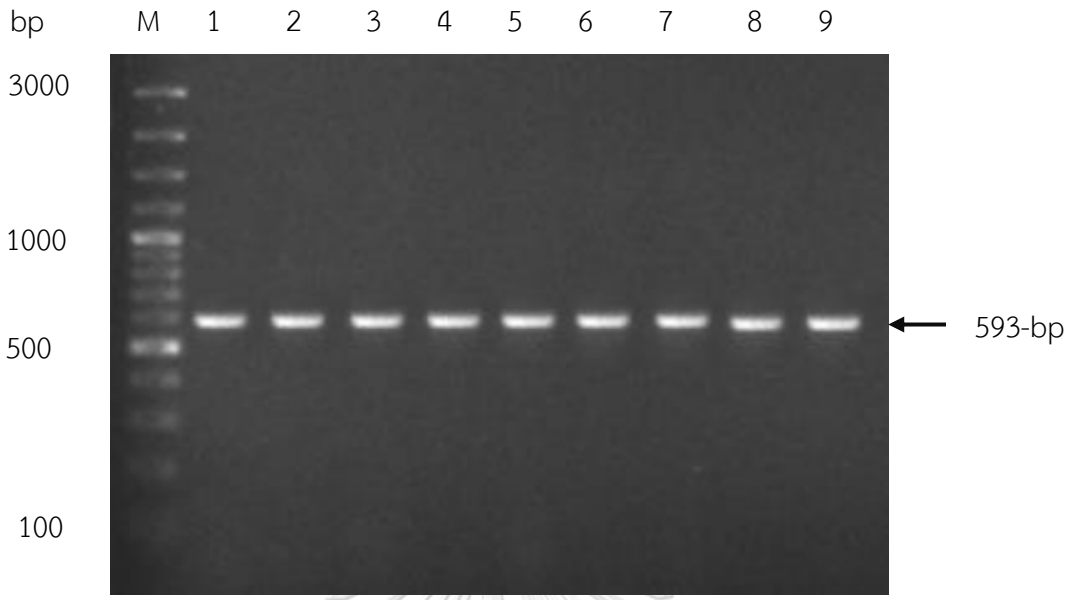

Figure 10: PCR amplicons of *bla*$_{CTX-M}$ gene in *Salmonella* and *E. coli* isolates. Lane M, 100-bp marker; Lane 1-4 *bla*$_{CTX-M}$ positive *Salmonella* isolates, and Lane 4-8 *bla*$_{CTX-M}$ positive *E. coli* isolates and Lane 9 positive control for *bla*$_{CTX-M}$ (593-bp).

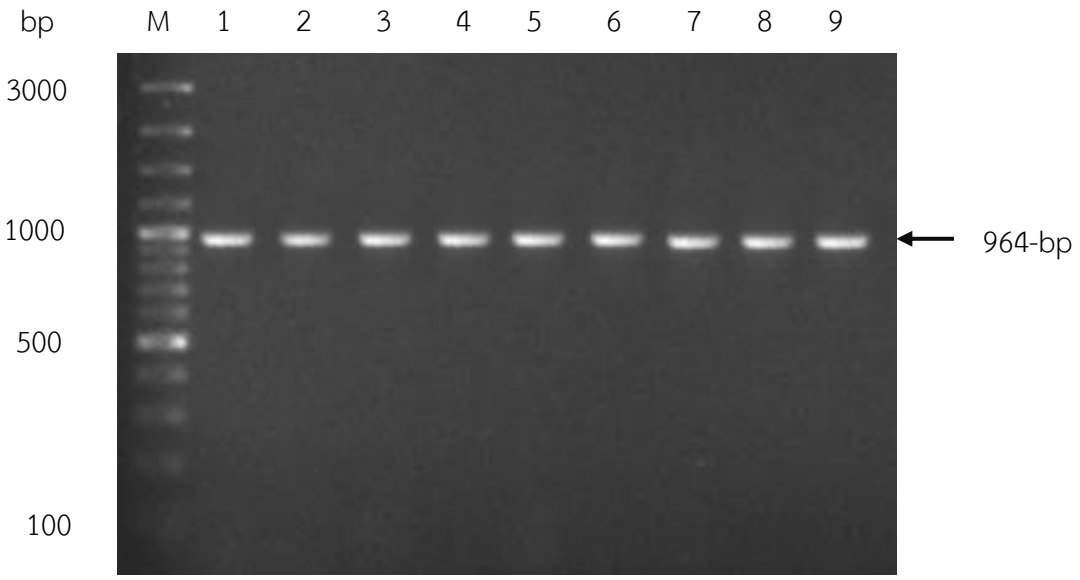

Figure 11: PCR amplicons of *bla*$_{TEM}$ in *Salmonella* and *E. coli* isolates. Lane M, 100-bp marker; Lane 1-4 *bla*$_{TEM}$ positive *Salmonella* strains, and Lane 4-8 *bla*$_{TEM}$ positive *E. coli* strains and Lane 9 positive control for *bla*$_{TEM}$ (964-bp).

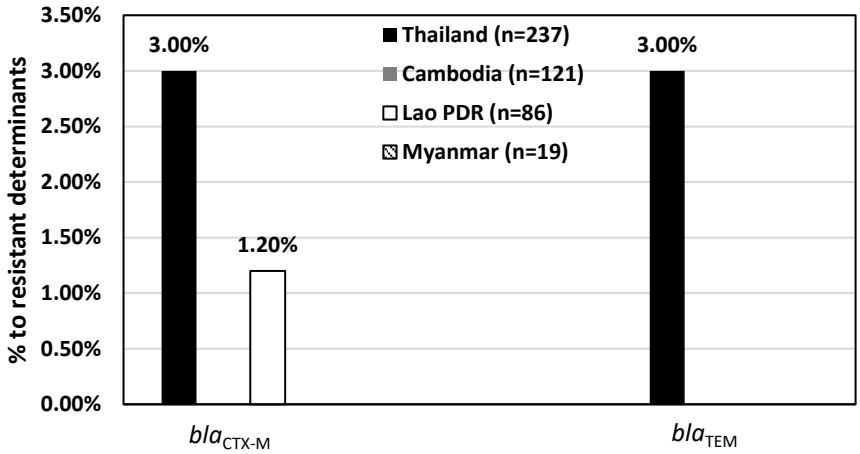

Figure 12: Prevalence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes in *Salmonella* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar

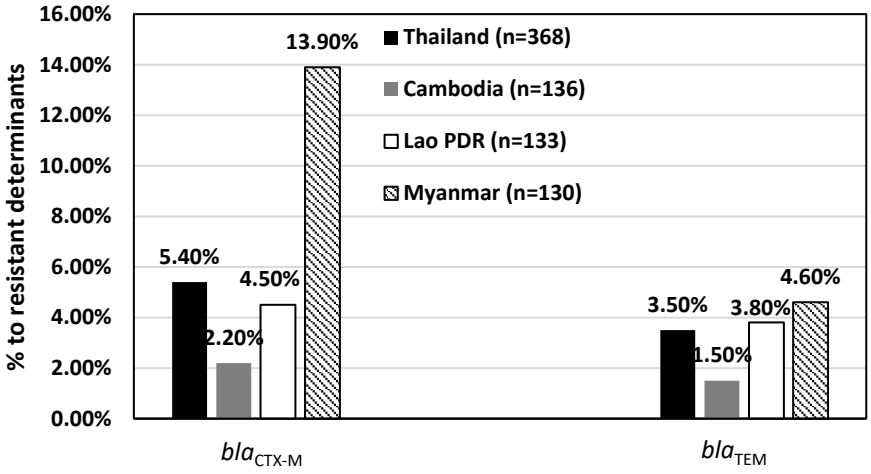

Figure 13: Prevalence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ in *E. coli* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar

4.2 Genotypic detection of colistin-resistant *Salmonella* and *E. coli* isolates

All *Salmonella* isolates (n=463) and *E. coli* (n=767) were tested for the presence of *mcr-1, mcr-2*, and *mcr-3* genes by multiplex PCR. Only *mcr-1* and *mcr-3* genes were observed (Figure 14).

Of the 463 *Salmonella* isolates, twelve isolates (2.6%) were positive to *mcr-1*, including six isolates from Thailand, four isolates from Cambodia, one isolate from Lao PDR and Myanmar (Figure 14). The prevalence of *mcr-1* was commonly found in Myanmar (1/19, 5.3%), followed by Cambodia (4/121, 3.3%), Thailand (6/237, 2.5%) and Lao PDR (1/86, 1.2%), respectively. None of *mcr-2* genes were found in *Salmonella* isolates. The *mcr-3* was detected only the isolate from Thailand (0.4%). None of isolates from Cambodia, Lao PDR and Myanmar were positive to *mcr-3*.

Of the 767 *E. coli* isolates, 68 isolates (8.9%) were positive to *mcr-1*, including seventeen isolates from Thailand, thirteen isolates from Cambodia, thirty-three isolates from Lao PDR and five isolates from Myanmar (Figure 16). The prevalence of *mcr-1* was the most commonly found in the pig and pork isolates in Lao PDR (33/133, 24.8%). The *mcr-1* gene was also found in other countries, including Cambodia (13/136, 9.6%), Thailand (17/368, 4.6%) and Myanmar (5/130, 3.9%), respectively. The *mcr-2* gene was not found in *E. coli* isolates in all countries. The high percentage of *mcr-3* was also detected in Lao PDR (12/133, 9.0%), followed by Cambodia (10/136, 7.3%) and Thailand (9/368, 2.5%) respectively. Notably, none of *mcr-3* was identified from Myanmar.

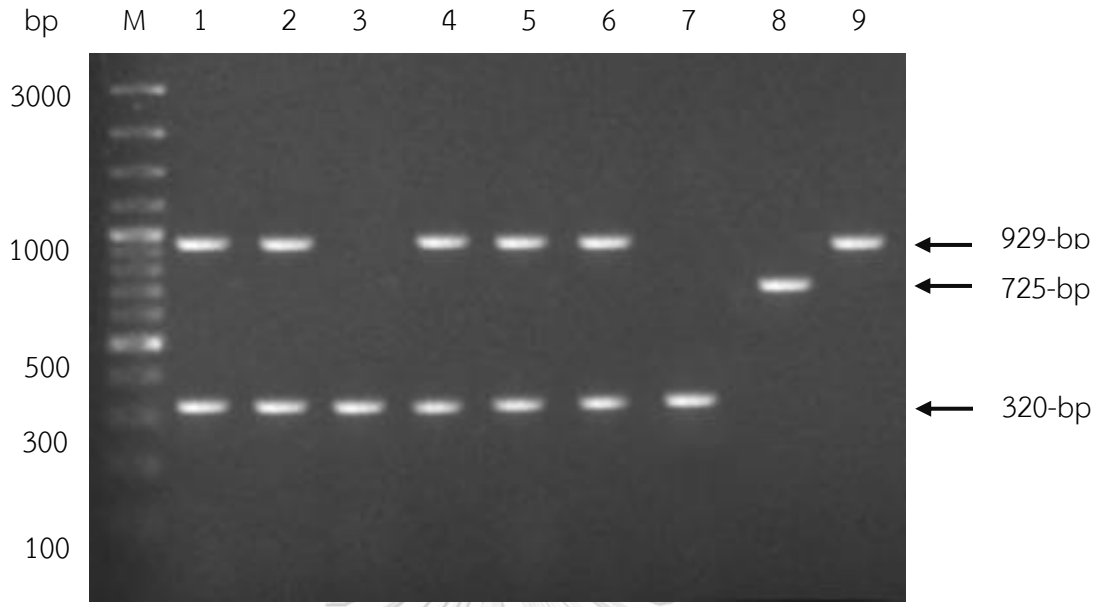

Figure 14. PCR amplicons of *mcr-1, mcr-2,* and *mcr-3* genes in *Salmonella* and *E. coli* isolates. Lane M, 100-bp marker; Lane 1-2, *mcr-1* and *mcr-3* positive *Salmonella* strains, Lane 3, *mcr-1* positive *Salmonella* strains; Lane 4-6, *mcr-1* and *mcr-3* positive *E. coli* strains; Lane 7, positive control for *mcr-1* (320-bp), Lane 8, positive control for *mcr-2* (725-bp) and Lane 8, positive control for *mcr-3* (929-bp).

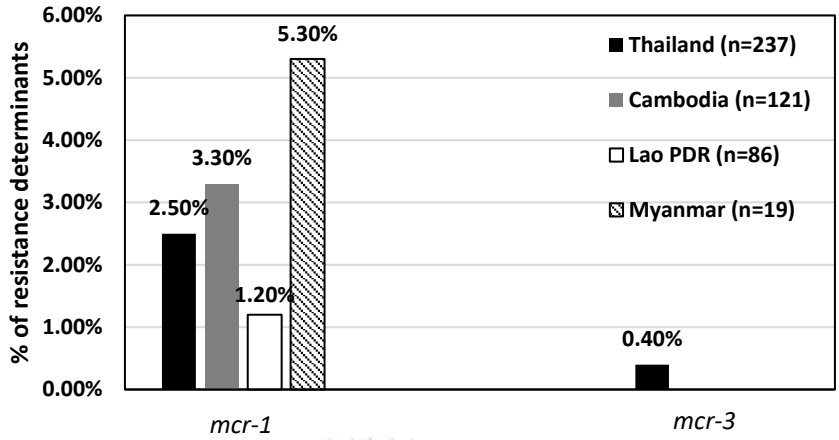

Figure 15. Prevalence of *mcr-1* and *mcr-3* in *Salmonella* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar

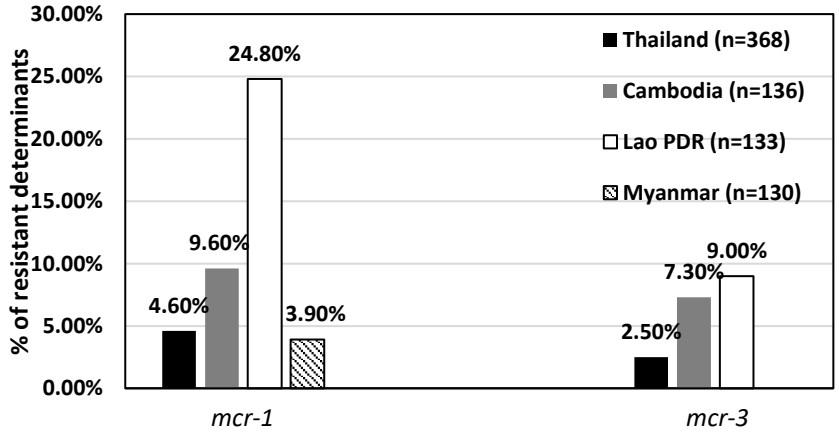

Figure 16. Prevalence of *mcr-1* and *mcr-3* in *E. coli* isolated from pigs and pork in the border provinces among Thailand, Cambodia, Lao PDR and Myanmar

4.3 Co-occurrence of ESBL-producing and colistin-resistant *Salmonella* and *E. coli* isolates

One *Salmonella* isolate from pork in Lao PDR was positive to *mcr-1* and carried bla_{CTX-M}. One *E. coli* isolate from pig in Thailand and two *E. coli* isolates (one from pig and pork) in Cambodia were detected *mcr-3* and bla_{CTX-M}.

4.4 Test for transfer of ESBL genes

The conjugation experiments showed that one *Salmonella* isolate from pig in Thailand was ESBL-producing strain and carried bla_{CTX-M} and also was able to horizontally be transferred *E. coli* recipient (*E. coli* K12 strain MG 1655). The ESBL-producing strains from Cambodia (n=2) (one from pig and one from pork) and Myanmar (n=5) (three from pigs and two from pork) carried bla_{CTX-M} and able to horizontally be transferred *Salmonella* recipients (SE 12). The bla_{TEM} was not transferable in all ESBL-producing *Salmonella* and *E. coli* isolates.

CHAPTER V

DISCUSSION

Antimicrobial agents have been widely used in pig production, mainly for growth promotion, disease prevention and therapy. Of particular concern is that any use of antimicrobials is a key factor in emergence and spread of AMR bacteria and their resistance determinants, including *Salmonella* and *E. coli* (O'Neill, 2015). Recently, the occurrence of cephalosporin and colistin resistance in *Salmonella* and *E. coli* have been increasing found in food-producing animals and humans through food chain worldwide (Falgenhauer et al., 2016; Yao et al., 2016). This raises a particular concern of limitation of antibiotics of choice for bacteria; infection treatment in the future.

One of the main findings of this study was high contaminations of *E. coli* in pigs at slaughterhouses and pork at retail markets, supporting the role of food animal origin as a major reservoir for *E. coli* transmission to humans. In all study areas, the prevalence of *E. coli* in pigs is generally higher than that in pork ($P<0.05$), which was similar to our previous study (Trongjit et al., 2016a). The prevalence of *Salmonella* and *E. coli* varied between sources of samples and locations in this study. Generally, the contamination rate of *Salmonella* is lower than that of *E. coli*, in agreement with a previous study (Silva et al., 2014). All the samples arrived the laboratory in good condition and this could be not interfered the efficacy of the isolation method. *Salmonella* is an important food-borne pathogen and usually the prevalence is less than *E. coli*. The samples can contaminate with commensal *E. coli* from feces and with other *E. coli* from environment and workers in the markets.

In this study, meat products are generally sold in open-air markets and stored at ambient temperatures. In Thailand, retail markets in Bangkok and surrounding areas are under monthly-cleaning routine. The vendors were well-trained and are aware of hygiene and sanitation. Raw meat and carcasses are distributed from slaughterhouses to the shambles by mini-trucks and the unsold carcasses are stored in refrigerator or freezer. In contrast, this procedure may not be strictly applied to retail markets in the border area and the neighboring countries. Hygienic and sanitation practices may not be regularly performed in fresh markets in Banteay Meanchey, Vientiane and Tachileik due to ineffective enforcement. In the markets in the latter, pig carcasses appeared to be distributed in open buckets on tricycle and sold in open air without cooling systems. The unsold meat products are kept in ice-box for re-selling next days. These unhygienic practices are likely a major contributor to the high prevalence of *E. coli* in pork in the provincial retail markets. The results obtained in this study highlighted the importance of microbial contamination of retail meat in provincial markets that may further spread to humans. Therefore, for the effective control of *E. coli* contamination and transmission in the area, the routine-hygienic practices are essential and needs to be enforced. The authority ministries should raise awareness to vendors and consumers for food safety and should support and adopt standards from WHO or from other generally accepted international standards. To minimize the growth and cross-contamination of *E. coli* in pigs and pork, the authorized agencies need to have promotion of safe handling of food management practices and principles of hygiene for food handlers. The border crossings are a major trade route in this region. Traders from Cambodia, Lao PDR and Myanmar routinely cross the border to buy live animals, fresh meat and meat products from Thailand. Therefore, some samples in these three countries may originally come from Thailand. However, the actual geographical sources of samples could not be tracked during the sample collection.

ESBL-production is a major contributor to resistance to new generation cephalosporins that are last choice antibiotics for bacterial infection treatment. In this study, ceftazidime, cefotaxime and cefpodoxime were chosen to be ESBL-production indicators. As three ESBL-production indicators were suggested to be included by CLSI, cefotaxime is a good indicator that can detect most ESBL genes. Ceftazidime is usually included, but its ability to detect ESBL genes is limited. Cefpodoxime is additionally included, however, its specificity to ESBLs is limited.

ESBL-producing *Salmonella* and *E. coli* have also been reported in food products worldwide (Chiaretto et al., 2008), France (Girlich et al., 2007), Denmark (Aarestrup et al., 2006), Norway (Sunde et al., 2008), Spain (Riaño et al., 2006), Japan (Suzuki et al., 2008), China (Zheng et al., 2012), Vietnam (Baker et al., 2009), Cambodia (Trongjit et al., 2016a) and Thailand (Boonyasiri et al., 2014). The present results indicated that foods from animal origin could be potential reservoirs for the emergence of ESBL-producing bacteria and their resistance determinants that may be transmitted to humans and the environment. Therefore, ESBL-producing *Salmonella* and commensal *E. coli* were focused in this study. The prevalence of ESBL-producing *Salmonella* and commensal *E. coli* was lower than previously reported in Thailand (Boonyasiri et al., 2014) and other studies (Horton et al., 2011; Egervärn et al., 2014; Nguyen et al., 2016). Cephalosporins are commercially available in the markets and have been used in animal production for bacterial treatments. The use of these antimicrobials in food-animals may not be common in the areas of this study. The cost of cephalosporins is rather high and use of these antimicrobial drugs issues will increase the investment cost, resulting decreased profit. This could explain the low prevalence of *Salmonella* and *E. coli* observed.

It should be noted that existing of ESBL-producing bacterial isolates may not always be a direct effect of cephalosporins usage and the ESBLs encoding genes

could be possibly co-selected by resistance determinants of other antibiotics. Co-selection of ESBL genes has been previously reported (Guiral et al., 2018). In this study, bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{CMY-1}, bla_{CMY-2}, and bla_{PSE} were focused for the detection of ESBL-related *bla* genes due to their common presence in the previous studies (Hiroi et al., 2012; Trongjit et al., 2016a) . However, only bla_{CTX-M} and bla_{TEM} were detected among the isolates in this collection. The bla_{TEM} gene is generally the most common ESBL produced by *Enterobacteriaceae* family, especially Gram-negative bacteria, of which the bla_{TEM-1} is most common and up to 90% ampicillin resistance in *E. coli* is due to the production of bla_{TEM-1}. The bla_{TEM-1} gene does not encode ESBLs and promotes resistance to narrow and broad-spectrum ß-lactams, such as penicillin and amoxicillin that are commonly used in pig production. However, the subtype of the *bla* genes was not examined in this study. This will be a topic of future study.

The bla_{CTX-M} genes have been recognized as an important cause of new generation cephalosporin (Sasaki et al., 2010; Chen et al., 2014; Nakayama et al., 2015). However, the limited prevalence of these ESBL genes was found in this study. It was previously highlighted that bla_{TEM} positive *E. coli* isolates usually carried *CTX-M* type of ESBL, in agreement with present study. This phenomenon has also been mentioned recently as the common combination found in ESBL-producing isolates (Ojer-Usoz et al., 2013; Tamang et al., 2013). It was additionally reported that people who have closely contact with pigs usually carry ESBL-producing *E. coli* in their feces. This is an evidence of potential role of animals in transmission of ESBL-producing *E. coli* to humans. It also indicated that ESBL genes in bacteria from food animals and their products could be transferred to humans.

Test for resistance gene transfer by biparental mating experiments showed that one *Salmonella* isolate from Thailand carried bla_{CTX-M} on conjugative plasmid

that was able to be transferred to *E. coli* recipient, in agreement with our previous study (Trongjit et al., 2017). In addition, two E. coli isolates from Cambodia and four *E. coli* isolates from Myanmar carried transferable bla_{CTX-M}, in consistent to our previous study (Trongjit et al., 2016a). These results indicated that bla_{CTX-M} are commonly located on conjugative plasmid and horizontal transfer plays a role in spread of the gene intra and inter species.

One of the important findings was the observation that colistin resistance rate in *E. coli* was significantly higher than that in *Salmonella*. The presence of colistin-resistant *E. coli* in pigs could reflect the extensive use of colistin in pig production. The similar phenomenon was observed when compared at country level. The colistin resistance phenotype was common in Salmonella from pork. The reason of such observation is still unclear but may be attributed to cross-contamination that may occur during slaughtering, transportation and in retail markets.

Up to now, the presence of *mcr-1* have also been reported worldwide after first discovery (Arcilla et al., 2016; Du et al., 2016; Zhi et al., 2016). Even though the impact of *mcr-1* on colistin-resistance level is unclear, its significance on wide distribution of colistin-resistant *E. coli* is a particular concern. In the current study, all colistin-resistant *Salmonella* and *E. coli* did not carry *mcr-1*, and the overall prevalence of *mcr-1* was still low. This supports the existence of other colistin-resistance mediated mechanisms.

Interestingly, one *Salmonella* isolate and thirty one *E. coli* isolates were positive to *mcr-3*, a novel-colistin resistance gene, in agreement with previous study (Zhang et al., 2018). The transferable *mcr-3* that was first identified from porcine *E. coli* in Shandong China has already identified worldwide, including Asia (China, Thailand, Malaysia (Bi et al., 2017) and Singapore (Teo et al., 2018), Europe (Denmark

(Litrup et al., 2017), North America (Bi et al., 2017). In Europe, colistin is mostly used to treat bacterial infection in pigs and used in Asian countries as growth promoter. Therefore, the current study suggested that indiscriminate use of antibiotic selected for the emergence of new colistin resistance determinants, like *mcr-3*. Among the colistin-resistant isolates found, one isolate from Thailand, two isolates from Cambodia and three isolates from Lao PDR harbored both *mcr-1* and *mcr-3* genes, in agreement with a previous study (Zhang et al., 2018). Therefore, the current finding highlighted that the global spread of *mcr-1* gene might be a similar chance for *mcr-3* to spread into different geographical regions.

Importantly, one *Salmonella* isolate from pork in Lao PDR carried both *mcr-1* and $bla_{\text{CTX-M}}$ genes, in agreement with previous studies (Falgenhauer et al., 2016; Yao et al., 2016). In addition, one *E. coli* isolate from pigs in Thailand and one *E. coli* isolate from pig and pork from Cambodia carried both *mcr-3* and $bla_{\text{CTX-M}}$ genes. The co-occurrence of *mcr* with ESBLs encoded genes on the same isolate increases the possibility of bacterial resistance to colistin and cephalosporin but reduces the antibiotic choice for treatment of infection with MDR bacteria. The findings underscored the challenges for successful clinical treatment of Gram-negative bacteria and for resistance control strategies in both human and veterinary medicine in this region. Taken together, the results obtained from the present study have alarmed the public about the widespread of ESBL-producing and colistin-resistant *Salmonella* and *E. coli* that might adversely affect both human and veterinary medicine. Therefore, further detection of ESBL-production and colistin resistance in Gram-negative bacteria family is suggested to proof the dynamics of AMR for developing countries.

Conclusions and Suggestions

From the findings of this study, the prevalence of *Salmonella* and *E. coli* varied among sources of samples and locations of sample collection. There was a high prevalence of *Salmonella* in pigs and pork and *E. coli* in pork in all countries. In both *Salmonella* and *E. coli*, the limited prevalence of ESBL-producing and colistin resistance was observed in this study. However, the *Salmonella* and *E. coli* isolates that were resistant to colistin and produced ESBL enzymes, indicating that it could have an impact on human and veterinary medicine. This might possibly create as a superbug, that resistant to most clinically important antibiotics available. Few *Salmonella* and *E. coli* isolates from pigs and pork simultaneously carried *mcr* and ESBL genes, indicating it might potentially distribute to other animals and the environment. Therefore, healthy pigs could serve as reservoirs for ESBL-producing and colistin-resistant *Salmonella* and *E. coli* that might pose a threat to public health.

At a suggestion, the last-resort antimicrobials must be used carefully in food-animal origin and the restrictive policies on the use of these antimicrobials in food-producing animals are mandatory. Role of veterinarian in the control of AMU in food animals, needs to be strengthened and educating the public about the issue of AMR needs to be provoked.

Further studies are suggested and could be as follows:

1. The information from this study confirmed the need for AMR monitoring, surveillance program and preventive strategic plan for AMR in bacteria of food-producing animals at national and regional level.

2. The epidemiological data on *Salmonella* and *E. coli* and their association

obtained from this study could be supported to understand the root cause of AMR, provided information to guide the interventions that are necessary for the region and specify the success of interventions.

3. Regular AMR surveillance and monitoring program in *Salmonella* and *E. coli* in pigs and pork added to other bacteria and other food animals should be conducted in according to fight emergence of AMR determinants between food-producing animals and humans through the food chain.

4. Similar research work and surveillance program should be continuously extended in every country in the region for deep understanding of their spread and impact of AMR.

5. Use of standardized and harmonized methods of sampling and antimicrobial susceptibility testing should be motivated in all regions to provide critical-comparable information to the global surveillance system.

REFERENCES

Aarestrup FM, Hasman H, Agerso Y, Jensen LB, Harksen S and Svensmark B 2006. First description of *bla*$_{CTX-M-1}$-carrying *Escherichia coli* isolates in Danish primary food production. J Antimicrob Chemother. 57(6): 1258-1259.

Agbaje M, Begum R, Oyekunle M, Ojo O and Adenubi O 2011. Evolution of *Salmonella* nomenclature: a critical note. Folia Microbiol. 56(6): 497-503.

Andreu A and Planells I 2008. Etiology of community-acquired lower urinary infections and antimicrobial resistance of *Escherichia coli*: a national surveillance study. Medicina clinica. 130(13): 481-486.

Angkititrakul S, Chomvarin C, Chaita T, Kanistanon K and Waethewutajarn S 2005. Epidemiology of antimicrobial in *Salmonella* isolated from pork, chicken meat and humans in Thailand. Southeast Asian J Trop Med Public Health. 36(6): 1510.

Angulo FJ, Collignon P, Powers JH, Chiller TM, Aidara-Kane A and Aarestrup FM 2009. World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies for the use of antimicrobials in food production animals. Clin Infect Dis. 49(1): 132-141.

Anjum MF, Duggett NA, AbuOun M, Randall L, Nunez-Garcia J, Ellis RJ, Rogers J, Horton R, Brena C and Williamson S 2016. Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. J Antimicrob Chemother. 71(8): 2306-2313.

Archawakulathep A, Kim T, Ta C, Meunsene D, Hassim H, Rovira HR, Myint KS, Baldrias LR, Sothy M and Aung M 2014. Perspectives on antimicrobial resistance in livestock and livestock products in ASEAN countries.

Arcilla MS, van Hattem JM, Matamoros S, Melles DC, Penders J, de Jong MD and

Schultsz C 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis. 16(2): 147-149.

Baker S, Thuy CT, Nga TTT, Hoang NVM, Campbell JI, Yen LM, Hieu NT, Chau NVV, Farrar J and Schultsz C 2009. High prevalence of plasmid-mediated quinolone resistance determinants in commensal members of the *Enterobacteriaceae* in Ho Chi Minh City, Vietnam. J Med Microbiol. 58(12): 1585-1592.

Barber AE, Norton JP, Spivak AM and Mulvey MA 2013. Urinary tract infections: current and emerging management strategies. Clin Infect Dis. 57(5): 719-724.

Barrow G 1993. Feltham, 1~ KA: Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, London.

Barton MD 2014. Impact of antibiotic use in the swine industry. Curr Opin Microbiol. 19: 9-15.

Beringer P 2001. The clinical use of colistin in patients with cystic fibrosis. Curr Opin Pul Med. 7(6): 434-440.

Bi Z, Berglund B, Sun Q, Nilsson M, Chen B, Tärnberg M, Ding L, Lundborg CS, Bi Z and Tomson G 2017. Prevalence of the *mcr-1* colistin resistance gene in extended-spectrum ß-lactamase-producing *Escherichia coli* from human faecal samples collected in 2012 in rural villages in Shandong Province, China. Int J Antimicrob Agents. 49(4): 493-497.

Blanc V, Mesa R, Saco M, Lavilla S, Prats G, Miró E, Navarro F, Cortés P and Llagostera M 2006. ESBL-and plasmidic class C ß-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. Vet Microbiol. 118(3-4): 299-304.

Bonnet R 2004. Growing group of extended-spectrum ß-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother. 48(1): 1-14.

Boonyasiri A, Tangkoskul T, Seenama C, Saiyarin J, Tiengrim S and Thamlikitkul V 2014. Prevalence of antibiotic resistant bacteria in healthy adults, foods, food animals,

and the environment in selected areas in Thailand. Pathog Glob Health. 108(5): 235-245.

Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I and Malorny B 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. enterica serovar Paratyphi B. J Antimicrob Chemother. 72(12): 3317-3324.

Brinas L, Moreno MA, Teshager T, Sáenz Y, Porrero MC, Domínguez L and Torres C 2005. Monitoring and characterization of extended-spectrum ß-lactamases in *Escherichia coli* strains from healthy and sick animals in Spain in 2003. Antimicrob Agents Chemother. 49(3): 1262-1264.

Butaye P and Wang C 2018a. Colistin resistance, beyond the current knowledge. EBio Med.

Butaye P and Wang C 2018b. Colistin resistance, beyond the current knowledge. EBio Med. 34: 16-17.

Callaway T, Edrington T, Anderson R, Byrd J and Nisbet D 2008. Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella* 1 2. J Ani Sci. 86(14_suppl): E163-E172.

Canton R, Novais A, Valverde A, Machado E, Peixe L, Baquero F and Coque T 2008. Prevalence and spread of extended-spectrum ß-lactamase-producing *Enterobacteriaceae* in Europe. Clin Microbiol Infect. 14(s1): 144-153.

Carattoli A 2008. Animal reservoirs for extended spectrum ß-lactamase producers. Clin Microbiol Infect. 14: 117-123.

Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G and Magistrali CF 2017. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. Euro Surveill.

22(31).

Caspar Y, Maillet M, Pavese P, Francony G, Brion J-P, Mallaret M-R, Bonnet R, Robin F, Beyrouthy R and Maurin M 2017. *mcr-1* colistin resistance in ESBL-producing *Klebsiella pneumoniae*, France. Emerg Infect Dis. 23(5): 874.

Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A, Jukes H, Liebana E, Navas AL and Mackay D 2015. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. Int J Antimicrob Agents. 46(3): 297-306.

Chaudhary AS 2016. A review of global initiatives to fight antibiotic resistance and recent antibiotics' discovery. Acta Pharmaceutica Sinica B. 6(6): 552-556.

Chen LF, Freeman JT, Nicholson B, Keiger A, Lancaster S, Joyce M, Woods CW, Cook E, Adcock L and Louis S 2014. Widespread dissemination of CTX-M-15 genotype extended-spectrum-ß-lactamase-producing *Enterobacteriaceae* among patients presenting to community hospitals in the southeastern United States. Antimicrob Agents Chemother. 58(2): 1200-1202.

Chiaretto G, Zavagnin P, Bettini F, Mancin M, Minorello C, Saccardin C and Ricci A 2008. Extended spectrum ß-lactamase SHV-12-producing *Salmonella* from poultry. Vet Microbiol. 128(3-4): 406-413.

Chong Y, Shimoda S, Yakushiji H, Ito Y, Miyamoto T, Kamimura T, Shimono N and Akashi K 2013. Community spread of extended-spectrum ß-lactamase-producing *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*: a long-term study in Japan. J Med Microbiol. 62(7): 1038-1043.

Cloeckaert A, Praud K, Doublet B, Bertini A, Carattoli A, Butaye P, Imberechts H, Bertrand S, Collard J-M and Arlet G 2007. Dissemination of an extended-spectrum-ß-lactamase bla_{TEM-52} gene-carrying IncI1 plasmid in various

Salmonella enterica serovars isolated from poultry and humans in Belgium and France between 2001 and 2005. Antimicrob Agents Chemother. 51(5): 1872-1875.

Collignon PC, Conly JM, Andremont A, McEwen SA, Aidara-Kane A, World Health Organization Advisory Group BMoISoAR, Agerso Y, Andremont A, Collignon P and Conly J 2016. World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies to control antimicrobial resistance from food animal production. Pages 1087-1093 *in* Clin Infect Dis. Vol. 63.

Coque T, Baquero F and Canton R 2008. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. Euro Surveill. 13(47): 19044.

Damjanova I, Tóth Á, Pászti J, Hajbel-Vékony G, Jakab M, Berta J, Milch H and Füzi M 2008. Expansion and countrywide dissemination of ST11, ST15 and ST147 ciprofloxacin-resistant CTX-M-15-type ß-lactamase-producing *Klebsiella pneumoniae* epidemic clones in Hungary in 2005—the new 'MRSAs'? J Antimicrob Chemother. 62(5): 978-985.

Deris ZZ, Akter J, Sivanesan S, Roberts KD, Thompson PE, Nation RL, Li J and Velkov T 2014. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. J Antibiot. 67(2): 147.

Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H and Mevius D 2010. Increased detection of extended-spectrum ß-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet Microbiol. 145(3-4): 273-278.

Du H, Chen L, Tang Y-W and Kreiswirth BN 2016. Emergence of the *mcr-1* colistin resistance gene in carbapenem-resistant *Enterobacteriaceae*. Lancet Infect Dis. 16(3): 287-288.

DuPont HL and Steele JH 1987. Use of antimicrobial agents in animal feeds: implications for human health. Rev Infect Dis. 9(3): 447-460.

Dyar OJ, Hoa NQ, Trung NV, Phuc HD, Larsson M, Chuc NT and Lundborg CS 2012. High prevalence of antibiotic resistance in commensal *Escherichia coli* among children in rural Vietnam. BMC Infect Dis. 12(1): 92.

Egervärn M, Börjesson S, Byfors S, Finn M, Kaipe C, Englund S and Lindblad M 2014. *Escherichia coli* with extended-spectrum ß-lactamases or transferable AmpC ß-lactamases and *Salmonella* on meat imported into Sweden. Int J Antimicrob Agents. 171: 8-14.

Empel J, Baraniak A, Literacka E, Mrówka A, Fiett J, Sadowy E, Hryniewicz W, Gniadkowski M and Group B-PS 2008. Molecular survey of ß-lactamases conferring resistance to newer ß-lactams in *Enterobacteriaceae* isolates from Polish hospitals. Appl Enrviron Microbiol. 52(7): 2449-2454.

Falgenhauer L, Waezsada S-E, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, Michael GB, Schwarz S, Werner G and Kreienbrock L 2016. Colistin resistance gene *mcr-1* in extended-spectrum ß-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis. 16(3): 282-283.

Gal-Mor O, Boyle EC and Grassl GA 2014. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. Front Microbiol. 5: 391.

Geser N, Stephan R and Hächler H 2012. Occurrence and characteristics of extended-spectrum ß-lactamase (ESBL) producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk. BMC Vet Res. 8(1): 21.

Girlich D, Poirel L, Carattoli A, Kempf I, Lartigue M-F, Bertini A and Nordmann P 2007. Extended-spectrum ß-lactamase CTX-M-1 in *Escherichia coli* isolates from healthy poultry in France. Appl Enrviron Microbiol. 73(14): 4681-4685.

Guiral E, Pons MJ, Vubil D, Marí-Almirall M, Sigaúque B, Soto SM, Alonso PL, Ruiz J, Vila J and Mandomando I 2018. Epidemiology and molecular characterization of multidrug-resistant *Escherichia coli* isolates harboring bla_{CTX-M} group 1 extended-spectrum ß-lactamases causing bacteremia and urinary tract infection in Manhiça, Mozambique. Infect Drug Resist. 11: 927.

Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agersø Y, Zankari E, Leekitcharoenphon P, Stegger M and Kaas RS 2015. Detection of *mcr-1* encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. Euro Surveill. 20(49).

Hasman H, Mevius D, Veldman K, Olesen I and Aarestrup FM 2005. ß-Lactamases among extended-spectrum ß-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. J Antimicrob Chemother. 56(1): 115-121.

Hiroi M, Yamazaki F, Harada T, Takahashi N, Iida N, Noda Y, Yagi M, Nishio T, Kanda T and Kawamori F 2012. Prevalence of extended-spectrum ß-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals. J Vet Med Sci. 74(2): 189-195.

Horton R, Randall L, Snary E, Cockrem H, Lotz S, Wearing H, Duncan D, Rabie A, McLaren I and Watson E 2011. Fecal carriage and shedding density of CTX-M extended-spectrum ß-lactamase-producing *Escherichia coli* in cattle, chickens, and pigs: implications for environmental contamination and food production. Appl Enrviron Microbiol. 77(11): 3715-3719.

Humphrey T 2000. Public-health aspects of *Salmonella* infection. *Salmonella* Domestic Anim. 1: 245-263.

Jacoby GA and Munoz-Price LS 2005. The new ß-lactamases. New England J Med.

352(4): 380-391.

Johansen HK, Moskowitz SM, Ciofu O, Pressler T and Høiby N 2008. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. J Cystic Fibrosis. 7(5): 391-397.

Kempf I, Fleury MA, Drider D, Bruneau M, Sanders P, Chauvin C, Madec J-Y and Jouy E 2013. What do we know about resistance to colistin in *Enterobacteriaceae* in avian and pig production in Europe? Int J Antimicrob Agents. 42(5): 379-383.

Korten V, Ulusoy S, Zarakolu P, Mete B and Group TMS 2007. Antibiotic resistance surveillance over a 4-year period (2000–2003) in Turkey: results of the MYSTIC Program. Diagn Microbiol Infect Dis. 59(4): 453-457.

Kubitschek H 1990. Cell volume increase in *Escherichia coli* after shifts to richer media. J Bacteriol. 172(1): 94-101.

Landman D, Georgescu C, Martin DA and Quale J 2008. Polymyxins revisited. Clin Microbiol Rev. 21(3): 449-465.

Lay KS, Vuthy Y, Song P, Phol K and Sarthou JL 2011. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. J Vet Med Science. 73(3): 325-329.

Le HV, Kawahara R, Khong DT, Tran HT, Nguyen TN, Pham KN, Jinnai M, Kumeda Y, Nakayama T and Ueda S 2015. Widespread dissemination of extended-spectrum ß-lactamase-producing, multidrug-resistant *Escherichia coli* in livestock and fishery products in Vietnam. Int J Antimicrob Agents. 2(1): 17.

Li B, Zhao Z-c, Wang M-h, Huang X-h, Pan Y-h and Cao Y-p 2014. Antimicrobial resistance and integrons of commensal *Escherichia coli* strains from healthy humans in China. J Chemother. 26(3): 190-192.

Litrup E, Kiil K, Hammerum AM, Roer L, Nielsen EM and Torpdahl M 2017. Plasmid-borne colistin resistance gene *mcr-3* in *Salmonella* isolates from human

infections, Denmark, 2009–17. Euro Surveill. 22(31).

Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B and Huang X 2016a. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 16(2): 161-168.

Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H and Shen J 2016b. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet Infectious Diseases. 16(2): 161-168.

Livermore DM 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis. 34(5): 634-640.

Manarungsan S 2010. Thailand's Trade Relations with GMS and CLMV. BRC Res Rep. (3): 206-228.

Marshall BM and Levy SB 2011. Food animals and antimicrobials: impacts on human health. Clin Microbiol Rev. 24(4): 718-733.

McKenna M 2013. The last resort. Nature. 499(7459): 394.

Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Grundmann H, Pantosti A and Rossolini G 2014. Colistin-resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill. 19(42): 20939.

Moubareck C, Daoud Z, Hakimé NI, Hamzé M, Mangeney N, Matta H, Mokhbat JE, Rohban R, Sarkis DK and Doucet-Populaire F 2005. Countrywide spread of community-and hospital-acquired extended-spectrum ß-lactamase (CTX-M-15)-producing *Enterobacteriaceae* in Lebanon. J Clin Microbiol. 43(7): 3309-3313.

Nakayama T, Ueda S, Huong BTM, Tuyen LD, Komalamisra C, Kusolsuk T, Hirai I and

Yamamoto Y 2015. Wide dissemination of extended-spectrum ß-lactamase-producing *Escherichia coli* in community residents in the Indochinese peninsula. Infect Drug Resist. 8: 1.

Newton-Foot M, Snyman Y, Maloba MRB and Whitelaw AC 2017. Plasmid-mediated *mcr-1* colistin resistance in *Escherichia coli* and *Klebsiella* spp. clinical isolates from the Western Cape region of South Africa. Antimicrob Resist Infect 6(1): 78.

Nguyen DP, Nguyen TAD, Le TH, Tran NMD, Ngo TP, Dang VC, Kawai T, Kanki M, Kawahara R and Jinnai M 2016. Dissemination of extended-spectrum ß-lactamase-and AmpC ß-lactamase-producing *Escherichia coli* within the food distribution system of Ho Chi Minh City, Vietnam. BioMed Res Int. 2016.

O'Neill J 2015. Tackling a global health crisis: initial steps. Rev Antimicrob Resist.

Ojer-Usoz E, González D, Vitas AI, Leiva J, García-Jalón I, Febles-Casquero A and de la Soledad Escolano M 2013. Prevalence of extended-spectrum ß-lactamase-producing *Enterobacteriaceae* in meat products sold in Navarra, Spain. Meat Sci. 93(2): 316-321.

Olaitan AO, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Khounsy S, Morand S and Rolain J-M 2014. Clonal transmission of a colistin. Microb Drug Resist. 20: 310-315.

Paterson DL and Harris P 2016. Colistin resistance: a major breach in our last line of defence. Lancet Infect Dis. 16(2): 132.

Poirel L, Jayol A and Nordmann P 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 30(2): 557-596.

Poirel L, Kieffer N, Liassine N, Thanh D and Nordmann P 2016. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. Lancet Infect Dis. 16(3): 281.

Prestinaci F, Pezzotti P and Pantosti A 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health. 109(7): 309-318.

Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM, Guerra B, Malorny B, Borowiak M and Hammerl JA 2018. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. Euro Surveill. 23(6).

Rhouma M, Beaudry F, Theriault W and Letellier A 2016. Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. Front Microbiol. 7: 1789.

Riaño I, Moreno MA, Teshager T, Sáenz Y, Domínguez L and Torres C 2006. Detection and characterization of extended-spectrum ß-lactamases in *Salmonella enterica* strains of healthy food animals in Spain. J Antimicrob Chemother. 58(4): 844-847.

Robins-Browne RM and Hartland EL 2002. *Escherichia coli* as a cause of diarrhea. J Gastroenterol Hepatol 17(4): 467-475.

Robinson T, Bu D, Carrique-Mas J, Fèvre E, Gilbert M, Grace D, Hay S, Jiwakanon J, Kakkar M and Kariuki S 2016. Antibiotic resistance is the quintessential One Health issue. Trans Royal Soc Trop Med Hyg. 110(7): 377-380.

Sasaki T, Hirai I, Niki M, Nakamura T, Komalamisra C, Maipanich W, Kusolsuk T, Sanguankiat S, Pubampen S and Yamamoto Y 2010. High prevalence of CTX-M ß-lactamase-producing *Enterobacteriaceae* in stool specimens obtained from healthy individuals in Thailand. J Antimicrob Chemother. 65(4): 666-668.

Schierack P, Steinrück H, Kleta S and Vahjen W 2006. Virulence factor gene profiles of *Escherichia coli* isolates from clinically healthy pigs. Appl Enrviron Microbiol. 72(10): 6680-6686.

Senok AC, Botta GA and Soge OO 2012. Emergence and spread of antimicrobial-

resistant pathogens in an era of globalization. Int Discipli Perspect Infect Dis. 2012.

Silva FFPd, Horvath MB, Silveira JG, Pieta L and Tondo EC 2014. Occurrence of *Salmonella* spp. and generic *Escherichia coli* on beef carcasses sampled at a brazilian slaughterhouse. Braz J Microbiol. 45(1): 17-24.

Sinwat N, Angkittitrakul S, Coulson KF, Pilapil FMIR, Meunsene D and Chuanchuen R 2016. High prevalence and molecular characteristics of multidrug-resistant *Salmonella* in pigs, pork and humans in Thailand and Laos provinces. J Med Microbiol. 65(10): 1182-1193.

Skov MN, Andersen JS, Aabo S, Ethelberg S, Aarestrup FM, Sørensen AH, Sørensen G, Pedersen K, Nordentoft S and Olsen KE 2007. Antimicrobial drug resistance of *Salmonella* isolates from meat and humans, Denmark. Emerg Infect Dis. 13(4): 638.

Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, Haesebrouck F and Butaye P 2010. Broad-spectrum β-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. FEMS Microbiol Rev. 34(3): 295-316.

Smith J, Drum D, Dai Y, Kim J, Sanchez S, Maurer J, Hofacre C and Lee M 2007. Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichia coli* strains colonizing broiler chickens. Appl Enrviron Microbiol. 73(5): 1404-1414.

Sunde M, Tharaldsen H, Slettemeås JS, Norström M, Carattoli A and Bjorland J 2008. *Escherichia coli* of animal origin in Norway contains a bla_{TEM-20}-carrying plasmid closely related to bla TEM-20 and bla TEM-52 plasmids from other European countries. J Antimicrob Chemother. 63(1): 215-216.

Suzuki S, Shibata N, Yamane K, Wachino J-i, Ito K and Arakawa Y 2008. Change in the prevalence of extended-spectrum-β-lactamase-producing *Escherichia coli* in

Japan by clonal spread. J Antimicrob Chemother. 63(1): 72-79.

Tamang MD, Nam H-M, Kim S-R, Chae MH, Jang G-C, Jung S-C and Lim S-K 2013. Prevalence and molecular characterization of CTX-M β-lactamase–producing *Escherichia coli* isolated from healthy swine and cattle. Foodborne Pathog Dis. 10(1): 13-20.

Teo JW, Kalisvar M, Venkatachalam I, Ng OT, Lin RT and Octavia S 2018. *mcr-3* and *mcr-4* variants in carbapenemase-producing clinical *Enterobacteriaceae* do not confer phenotypic polymyxin resistance. J Clin Microbiol. 56(3): e01562-01517.

Thacker PA 2013. Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Technol. 4(1): 35.

Timofte D, Dan M, Maciuca I, Ciucu L, Dabija E, Guguianu E and Panzaru C 2015. Emergence of concurrent infections with colistin-resistant ESBL-positive *Klebsiella pneumoniae* and OXA-23-producing *Acinetobacter baumannii* sensitive to colistin only in a Romanian cardiac intensive care unit. Euro J Clin Microbiol Infect Dis. 34(10): 2069-2074.

Tindall B, Grimont P, Garrity G and Euzeby J 2005. Nomenclature and taxonomy of the genus *Salmonella*. Int J Syst Evol Microbiol. 55(1): 521-524.

Trongjit S, Angkititrakul S, Tuttle RE, Poungseree J, Padungtod P and Chuanchuen R 2017. Prevalence and antimicrobial resistance in *Salmonella enterica* isolated from broiler chickens, pigs and meat products in Thailand–Cambodia border provinces. Microbiol Immunol. 61(1): 23-33.

Trongjit S, Angkittitrakul S and Chuanchuen R 2016a. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. Microbiol Immunol. 60(9): 575-585.

Trongjit S, Angkittitrakul S and Chuanchuen R 2016b. Occurrence and molecular

characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. Microbiol Immuno. 60(9): 575-585.

Wayne P 2007. Clinical and laboratory standards institute. Perform Std Antimicrob Suscept Testing. 17.

Wieler L, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klös H, Wengert D and Baljer G 2001. Prevalence of enteropathogens in suckling and weaned piglets with diarrhoea in southern Germany. J Vet Med B. 48(2): 151-159.

Woerther P-L, Burdet C, Chachaty E and Andremont A 2013. Trends in human fecal carriage of extended-spectrum ß-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev. 26(4): 744-758.

Woodall CA 2003. DNA transfer by bacterial conjugation. In: *E. coli* Plasmid Vectors. Springer. 61-65.

Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H and Malhotra-Kumar S 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. Euro Surveill. 21(27): 30280.

Yang Y-Q, Zhang A-Y, Ma S-Z, Kong L-H, Li Y-X, Liu J-X, Davis MA, Guo X-Y, Liu B-H, Lei C-W and Wang H-N 2016. Co-occurrence of *mcr-1* and ESBL on a single plasmid in *Salmonella enterica*. J Antimicrob Chemother. 71(8): 2336-2338.

Yao X, Doi Y, Zeng L, Lv L and Liu J-H 2016. Carbapenem-resistant and colistin-resistant *Escherichia coli* co-producing *NDM-9* and *MCR-1*. Lancet Infect Dis. 16(3): 288-289.

Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Shen J and Wang Y 2017. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. MBio. 8(3): e00543-00517.

Yumuk Z, Afacan G, Nicolas-Chanoine M-H, Sotto A and Lavigne J-P 2008. Turkey: a

further country concerned by community-acquired *Escherichia coli* clone O25-ST131 producing CTX-M-15. J Antimicrob Chemother. 62(2): 284-288.

Zhang J, Chen L, Wang J, Yassin AK, Butaye P, Kelly P, Gong J, Guo W, Li J and Li M 2018. Molecular detection of colistin resistance genes (*mcr-1, mcr-2* and *mcr-3*) in nasal/oropharyngeal and anal/cloacal swabs from pigs and poultry. Scientific reports. 8(1): 3705.

Zheng H, Zeng Z, Chen S, Liu Y, Yao Q, Deng Y, Chen X, Lv L, Zhuo C and Chen Z 2012. Prevalence and characterisation of CTX-M β-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. Int J Antimicrob Agents. 39(4): 305-310.

Zhi C, Lv L, Yu L-F, Doi Y and Liu J-H 2016. Dissemination of the *mcr-1* colistin resistance gene. Lancet Infect Dis. 16(3): 292-293.

APPENDICES

Appendix A

Steps for bacterial isolation and identification

A 1. Steps for *Salmonella* isolation and identification

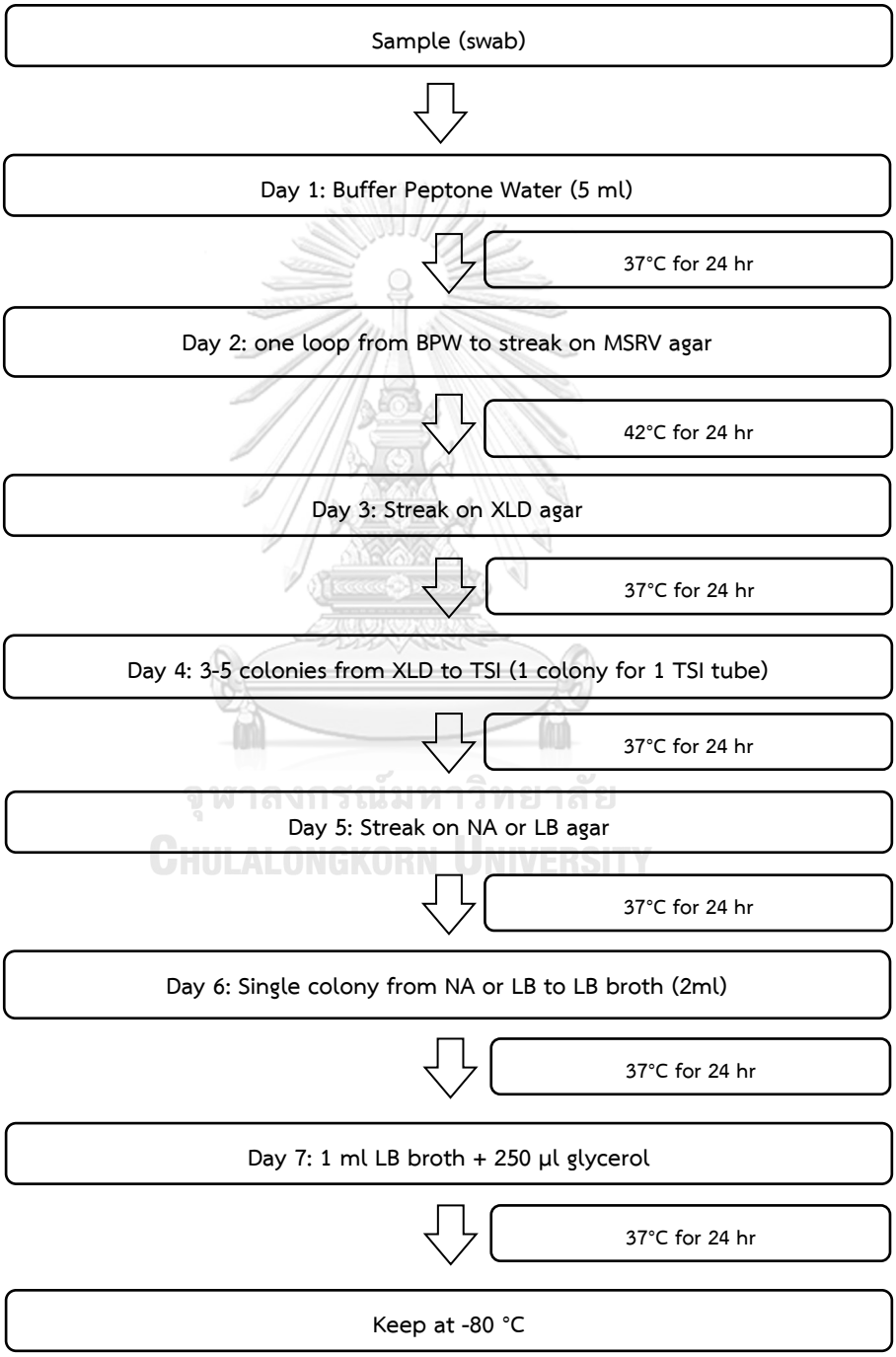

Figure A 1. Diagram for *Salmonella* isolation (ISO 6579:2002(E), Barrow,1993).

A 2. Steps for *E. coli* isolation and identification

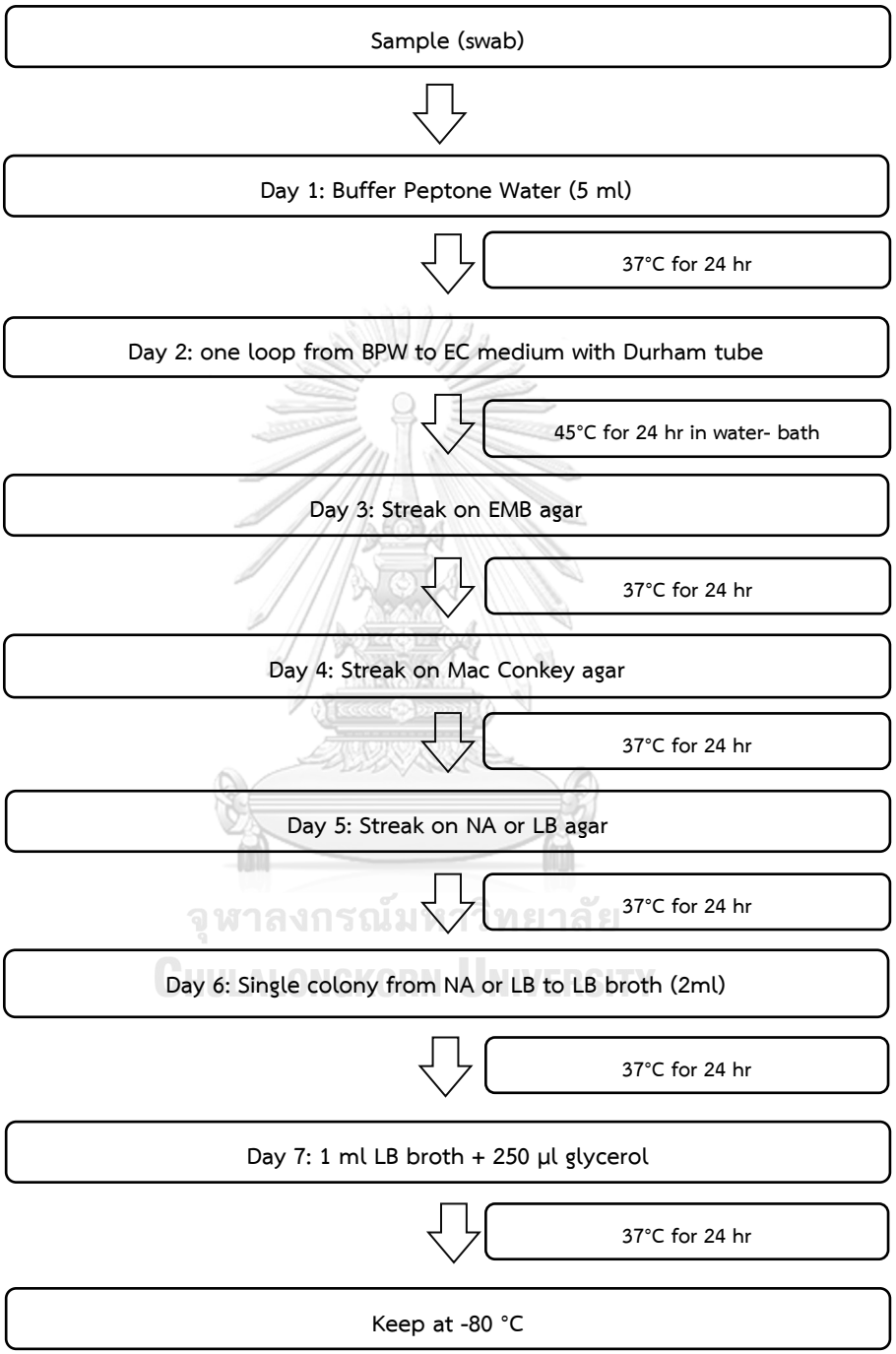

Figure A 2. Diagram for *E. coli* isolation (ISO 7251:2005)

Appendix B

Steps for *Salmonella* serotyping

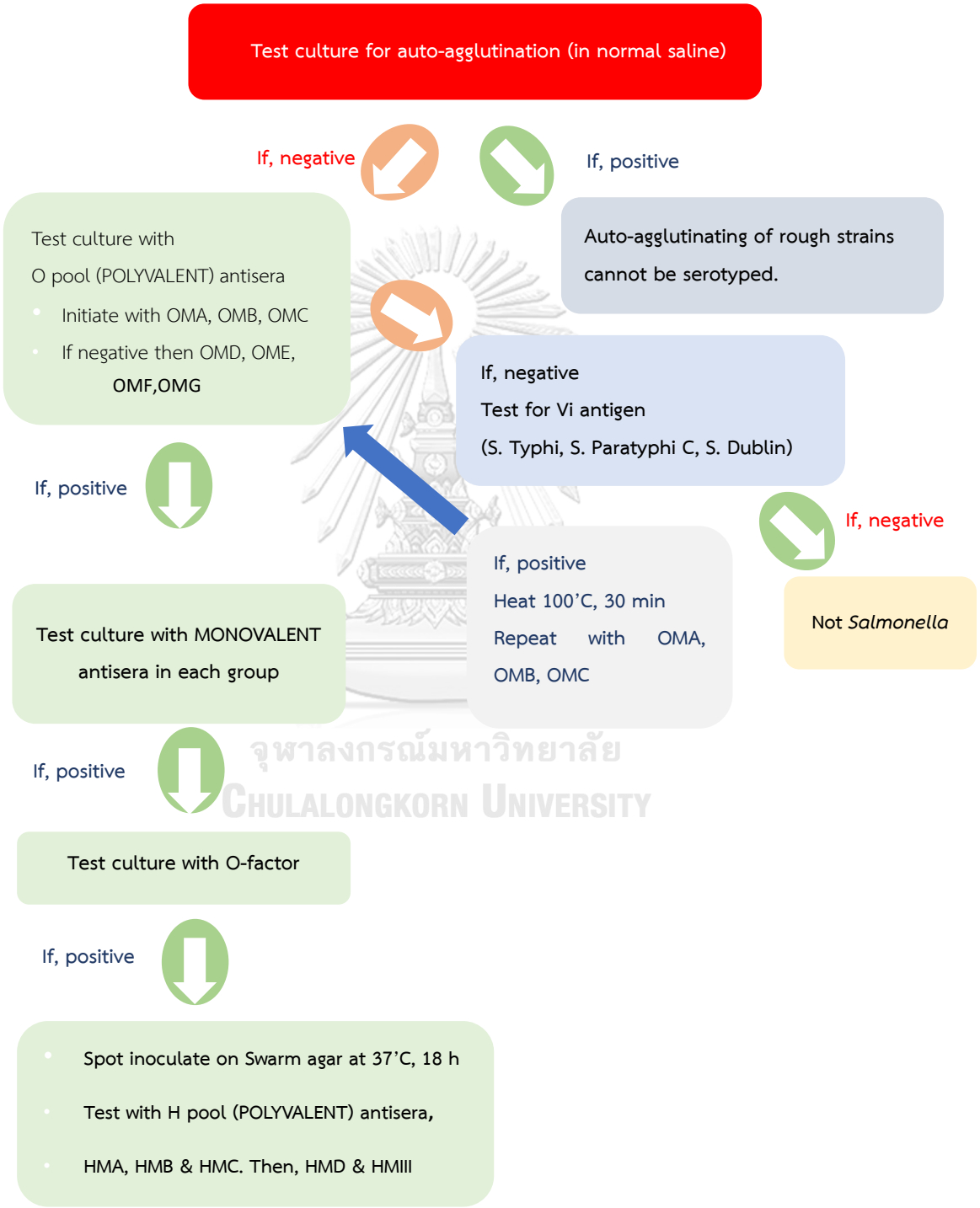

Figure B 1. Flow of the *Salmonella* serotyping

Figure B 1. Flow of *Salmonella* serotyping (continued).

Appendix C

ESBL Screening and confirmation test

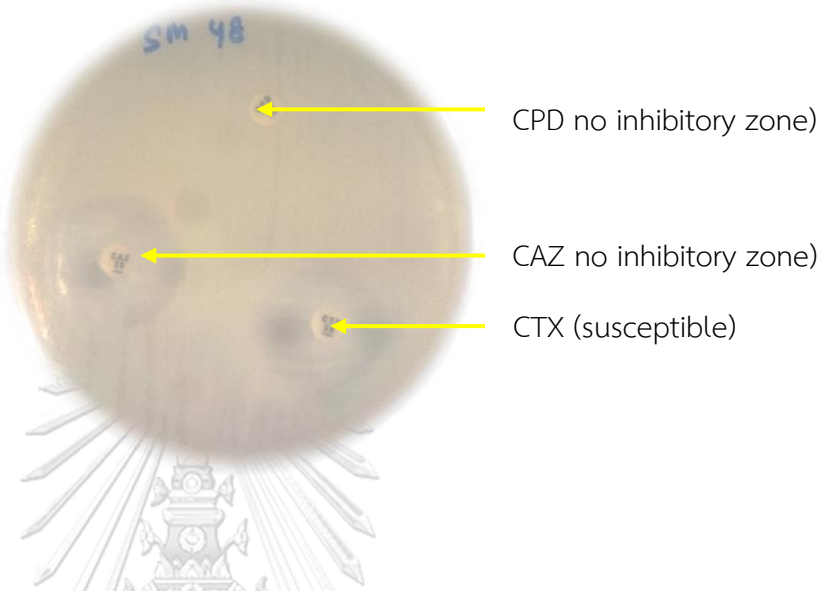

Figure C 1. ESBL screening test result

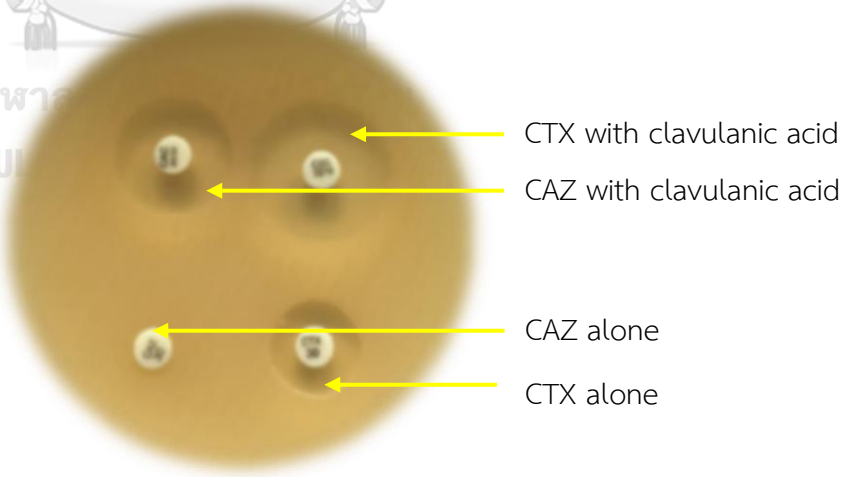

Figure C 2. ESBL confirmation test result

Appendix D

Solvent, concentration and breakpoint of colistin

Antibiotic	Solvent	Concentration range (µg/ml)	Breakpoint(µg/l)
colistin	Sterile distilled water	0.0625,0.125,0.25,0.5,1,2,4,8,16,32,64	4

Source: EUCAST (2013)

Appendix E

Bacterial growth media, PCR assay and chemicals

E 1. Bacterial growth media

- Buffer Peptone Water

Peptone	10.0g
Sodium chloride	5.0g
Disodium phosphate	3.5g
Potassium dihydrogen phosphate	1.5g

- Modified Semi-solid Rappaport-Vassiliadis Agar (Difco)

Tryptose	4.59g
Casein hydrolysate (acid)	4.59g
Sodium chloride	7.34g
Monpotassium phosphate	1.47g
Magnesium chloride (anhydrous)	10.93g
Malachite green oxalate	37.0g
Agar	2.70g

- Xylose Lysine Deoxycholate Agar (Difco)

Xylose	3.5g
L-lysine	5g
Lactose	7.5g
Saccharose	7.5g
Sodium chloride	15.0g
Yeast extract	3.0g
Phenol red	0.08g

Sodium Deoxycholate	2.5g
Ferric Ammonium Citrate	0.8g
Sodium Thiosulfate	6.8g
Agar	13.5g

- Triple Sugar Iron agar (Difco)

Beef extract	3.0g
Yeast extract	3.0g
Pancreatic Digest of casein	15.0g
Proteose Peptone no.3	5.0g
Dextrose	1.0g
Lactose	10.0g
Sucrose	10.0g
Ferrous sulfate	0.2g
Sodium chloride	5.0g
Sodium thiosulfate	0.3g
Agar	12.0g
Phenol red	24.0mg

- Luria Bertani agar (Difco)

Typhone	10.0g
Yeast extract	5.0g
Sodium chloride	10.0g
Agar	15.0g

- Luria Bertani broth (Difco)

Typhone	10.0g
Yeast extract	5.0g
Sodium chloride	10.0g

- EC medium (Difco)

Pancreatic digest of casein	20.0g
Bile salt no.3	1.12g
Lactose	5.0g
Dipotassium phosphate	4.0g
Monopotassium phosphate	1.5g
Sodium chloride	5.0g

- Eosin Methylene Blue agar, Modified (Difco)

Pancreatic digest of gelatin	10.0g
Lactose	5.0g
Sucrose	5.0g
Dipotassium phosphate	2.0g
Eosin Y	0.4g
Methylene blue	65.0g
Agar	13.5g

- MacConkey agar (Difco)

Peptone	20.0g
Lactose	10.0g
Bile salts	5.0g
Agar	12.0g
Neutral red	75.0mg

- Brilliant Green agar (Difco)

Proteose peptone no.3	10.0g
Yeast extract	3.0g
Lactose	10.0g
Saccharose	10.0g
Sodium chloride	5.0g
Agar	20.0g
Brilliant Green	12.5mg
Phenol red	0.08g

- Muller Hninton agar (Difco)

Beef extract powder	2.0g
Acid digest of casein	17.5g
Starch	1.5g
Agar	17.0g

E 2. PCR assay

- Ge Nei ™ MasterMix
  - Taq DNA polymerase in reaction buffer 0.05units/µl
  - dNTPs (dATP, dCTP, dTTP, dGTP) 0.04mM of each
  - $MgCl_2$ 4mM
- DNA marker (Fermentas®)
- Loading Dye (Fermentas®)
- Agarose gel (Vivantis®)
  - Agarose (ultra-pure) 1.2/ 0.8g
  - 1x TAE buffer

- 50x TAE buffer

    - Tris-base 242.0g
    - Glacual acetic acid 57.1g
    - 0.5M EDTA (pH 8.0) 100.0ml
    - Distilled water 1000.0ml

E 3. Other chemicals

- TE buffer

    - Tris (10 mM)
    - EDTA (1 mM)

- Sodium Dodecyl Sulfate (Vivantis®)

OUTPUTS

The results from this study were presented as poster presentation at the 17th Chulalongkorn University Veterinary Conference (CUVC, 2018) from 25 April - 27 April, IMPACT Forum Building, Nonthaburi, Bangkok. The abstract for our research was published in the proceeding of Thai Journal of Veterinary Medicine.

Kyaw Phyoe Sunn, Sunpetch Angkititrakul and Rungtip Chuanchuen 2018. Phenotypic and genotypic characteristics of ESBL-producing and colistin resistance in Salmonella enterica and Escherichia coli isolated from pigs in the border provinces between Thailand and Cambodia, Lao PDR and Myanmar. Thai J Vet Med. Suppl 48: 191-192.

VITA

NAME	Mr. Kyaw Phyoe Sunn
DATE OF BIRTH	3rd September 1987
PLACE OF BIRTH	Minbya Township, Rakhine State, Myanmar
INSTITUTIONS ATTENDED	University of Veterinary Science, Yezin, Myanmar.
HOME ADDRESS	Building (1354), Room (33), Dhana Theidhi Quarter, Zabu Thiri Township, Nay Pyi Taw, Myanmar
PUBLICATION	Kyaw Phyoe Sunn, Sunpetch Angkititrakul and Rungtip Chuanchuen 2018. Phenotypic and genotypic characteristics of ESBL-producing and colistin resistance in Salmonella enterica and Escherichia coli isolated from pigs in the border provinces between Thailand and Cambodia, Lao PDR and Myanmar. Thai J Vet Med. Suppl 48: 191-192.
AWARD RECEIVED	-