STUDY OF GENETIC DETERMINANTS TO EXPLAIN ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA* ISOLATED FROM FOOD ANIMALS, MEAT AND HUMANS IN THAILAND

Miss Jiratchaya Puangseree

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Public Health
Department of Veterinary Public Health
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2023

การศึกษาตัวระบุทางพันธุกรรมเพื่ออธิบายการดื้อยาในเอสเชอริเชีย โคไลและซัลโมเนลลาที่แยกได้
จากสัตว์ ที่เลี้ยงเพื่อการบริโภค เนื้อสัตว์และคนในประเทศไทย

น.ส.จิรัชญา พวงเสรี

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาสัตวแพทยสาธารณสุข ภาควิชาสัตวแพทยสาธารณสุข
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2566

Thesis Title	STUDY OF GENETIC DETERMINANTS TO EXPLAIN ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA* ISOLATED FROM FOOD ANIMALS, MEAT AND HUMANS IN THAILAND
By	Miss Jiratchaya Puangseree
Field of Study	Veterinary Public Health
Thesis Advisor	Professor Dr. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

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

.... Dean of the FACULTY OF VETERINARY SCIENCE
(Professor Dr. SANIPA SURADHAT, D.V.M., M.Sc., Ph.D.)

DISSERTATION COMMITTEE

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

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

.... Examiner
(Assistant Professor Dr. SAHARUETAI JEAMSRIPONG, D.V.M., M.P.V.M., Ph.D)

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

.... External Examiner
(Associate Professor Dr. Sunpetch Angkititrakul, D.V.M., Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

จิรัชญา พวงเสรี : การศึกษาตัวระบุทางพันธุกรรมเพื่ออธิบายการดื้อยาในเอสเชอริเชีย โคไลและซัลโมเนลลาที่แยกได้จากสัตว์ ที่เลี้ยงเพื่อการบริโภค เนื้อสัตว์และคนในประเทศไทย. (STUDY OF GENETIC DETERMINANTS TO EXPLAIN ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA* ISOLATED FROM FOOD ANIMALS, MEAT AND HUMANS IN THAILAND) อ.ที่ปรึกษาหลัก : ศ. สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น

การศึกษานี้ประกอบด้วย 4 โครงการวิจัย ซึ่งทำการศึกษาในแบคทีเรียต่างชนิดที่มีจีโนไทป์ต่างกัน ขึ้นกับการออกแบบการทดสองและวัตถุประสงค์ โครงการวิจัยที่ 1 การศึกษาพลาสมิดที่พบในอีโคไลและซัลโมเนลลา เอนเทอริกา ที่แยกได้จากสุกร เนื้อสุกร และคน การศึกษานี้มีวัตถุประสงค์เพื่อจำแนกชนิดของอาร์พลาสมิดในอีโคไล จำนวน 1047 ตัวอย่าง และซัลโมเนลลา จำนวน 816 ตัวอย่าง พบพลาสมิดจำนวน16 ชนิดในอีโคไล (85.3%) โดยพลาสมิดที่พบมากที่สุดคือ พลาสมิดในกลุ่ม IncK-F (23.7%) และพบพลาสมิดจำนวน 11 ชนิดในซัลโมเนลลา (25.7%) โดยพลาสมิดที่พบมากที่สุดคือพลาสมิดในกลุ่ม IncF (46.2%) ชนิดของพลาสมิดและลักษณะปรากฏของการดื้อยามีความสัมพันธ์กันอย่างมีนัยสำคัญ (p<0.05) แต่มีความแตกต่างกันอย่างมีนัยสำคัญ (p<0.05) กับแหล่งที่มาของเชื้อและช่วงปีในการเก็บตัวอย่าง โดยสรุปพบพลาสมิดหลายชนิดในอีโคไลและซัลโมเนลลา และผลการศึกษาของโครงการนี้สามารถใช้เป็นพื้นฐานให้กับการศึกษาในอนาคตเกี่ยวกับวิธีการลดการถ่ายทอดพลาสมิดในแนวขวาง โครงการวิจัยที่ 2 การวิเคราะห์จีโนมของซัลโมเนลลาที่มี class 1 integrons ที่มี *dfrA12-aadA2* ที่แยกได้จากสัตว์ที่เลี้ยงเพื่อการบริโภค เนื้อสัตว์ และคนในประเทศไทย การศึกษานี้มีวัตถุประสงค์เพื่อจำแนกลักษณะทางพันธุกรรมของพลาสมิดที่ถ่ายทอดได้ที่มี class1 integrons ที่มี *dfrA12-aadA2* ในซัลโมเนลลา โดยใช้เทคโนโลยีการถอดรหัสพันธุกรรม Oxford Nanopore Sequencing และ Illumina โดยพบรูปแบบ MLST จำนวน 7 รูปแบบและความสัมพันธ์ทางพันธุกรรมจำนวน 6 clades พบพลาสมิดในกลุ่ม IncFIB(K) ColpVC IncFIB IncHI2 IncHI2A IncX1 IncY และ IncR พบยีนดื้อยาจำนวน 28 ยีนพร้อมกับการคาดคะเนว่าเชื้อจะดื้อยาปฏิชีวนะในหลากหลายกลุ่ม พบพลาสมิดที่มี class1 integrons ที่มี *dfrA12-DUF1010-aadA2* และมียีน *qacE-sul1* ใน conserved region โดยสรุปพบว่า พลาสมิดดื้อยาที่ถ่ายทอดได้เป็นตัวการสำคัญในการแพร่กระจาย class1 integrons ที่มี *dfrA12-aadA2* โครงการวิจัยที่ 3 การศึกษาพื้นฐานทางโมเลกุลที่เกี่ยวข้องกับการคงอยู่ของการดื้อต่อยา chloramphenicol (CHP) ในอีโคไลและซัลโมเนลลาที่แยกได้จากสัตว์เลี้ยงเพื่อการบริโภค เนื้อสัตว์ และคนในประเทศไทย การศึกษานี้มีวัตถุประสงค์เพื่อหากลไกที่อาจจะมีส่วนร่วมในการทำให้มีการคงอยู่ของการดื้อยา CHP ในอีโคไลจำนวน 106 ตัวอย่าง และซัลโมเนลลาจำนวน 57 ตัวอย่าง พบเชื้ออีโคไลจำนวน 67.9% และซัลโมเนลลาจำนวน 64.9% มีการลดลงของค่า MIC ต่อ CHP มากกว่าหรือเท่ากับ 4 เท่าใน PAβN โดย ampicillin tetracycline และ streptomycin คัดเลือก ซัลโมเนลลาและอีโคไลที่ได้รับการถ่ายทอดยีน *cmlA* และมี MIC ต่อยา CHP อยู่ระหว่าง 32-512 ไมโครกรัมต่อมิลลิลิตร พบพลาสมิดในกลุ่ม IncF ทั้งในซัลโมเนลลาและอีโคไลที่ได้รับการถ่ายทอดยีน *cmlA* การวิเคราะห์ผล WGS พบว่า class1 integrons ที่มียีน *cmlA* ในพลาสมิด IncX1 พลาสมิด IncFIA(HI1)/HI1B และพลาสมิด IncFII/FIB ถูกขนาบข้างด้วย IS*26* และ Tn*As1* ส่วนยีน *catA* บนพลาสมิด IncFIA(HI1)/HI1B/Q1 ถูกขนาบข้างด้วย IS*1B* และ Tn*As3* โดยสรุปการคงอยู่ของการดื้อยา CHP อาจมีสาเหตุมาจากการดื้อข้าม (cross resistance) ด้วยกลไก multidrug efflux system และการคัดเลือกร่วม (co-selection) ของยีนดื้อยา CHP จากการใช้ยาต้านจุลชีพอื่นๆ โครงการวิจัยที่ 4 การศึกษาการดื้อยาฆ่าเชื้อและโลหะหนัก และการดื้อข้ามไปยังยาปฏิชีวนะในอีโคไลที่แยกได้จากสุกร เนื้อสุกรและซากสุกร การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการดื้อข้ามระหว่างยาฆ่าเชื้อ โลหะหนัก และยาปฏิชีวนะในอีโคไลที่แยกได้จากสุกร จำนวน 643 ตัวอย่าง เนื้อสุกร จำนวน 111 ตัวอย่าง และซากสุกร จำนวน 110 ตัวอย่าง ในประเทศไทย พบว่าการสัมผัส triclosan benzalkonium chloride และ chlorhexidine ในระดับที่ไม่สามารถฆ่าเชื้อได้จะคัดเลือก spontaneous resistant mutants ที่ดื้อข้ามไปยังยาปฏิชีวนะอย่างน้อยหนึ่งชนิด ค่า MIC ของ chloramphenicol และ trimethoprim ลดลงใน PAβN แต่การเติม PAβN CCCP และ reserpine ไม่ทำให้ค่า MIC ของ ciprofloxacin ใน ciprofloxacin-resistant mutant ที่ไม่มีการกลายพันธ์ของยีน *gyrA* และ *parC* ลดลง โดยสรุปยาฆ่าเชื้อและโลหะหนักเป็น selective pressure ที่ไม่ใช่ยาปฏิชีวนะที่ทำให้มีการเกิดและแพร่กระจายการดื้อยา ควรมีการตรวจติดตามความไวรับต่อยาฆ่าเชื้อและโลหะหนัก สรุปโดยรวมพบว่าการวิจัยนี้ได้เป็นไปตามวัตถุประสงค์ และควรมีการศึกษาวิจัยเพิ่มมากขึ้นเพื่อทำให้เกิดความเข้าใจและการควบคุมเชื้อดื้อยาที่ดียิ่งขึ้น

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต ....
ปีการศึกษา	2566	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5875304331 : MAJOR VETERINARY PUBLIC HEALTH

KEYWORD: antimicrobial resistance Escherichia coli genetic determinants Salmonella Thailand

Jiratchaya Puangseree : STUDY OF GENETIC DETERMINANTS TO EXPLAIN ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA* ISOLATED FROM FOOD ANIMALS, MEAT AND HUMANS IN THAILAND. Advisor: Prof. Dr. RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D.

This study comprised 4 parts. Different bacterial strains with different genotypes were used, depending on the experimental design and objectives. Part 1 - Plasmid profile analysis of *E. coli* and *Salmonella enterica* isolated from pigs, pork, and humans. This part aimed to characterize the profile of R plasmids in *E. coli* (n=1,047) and *Salmonella* (n=816). Sixteen and 11 Inc groups were identified in *E. coli* (85.3%) and *Salmonella* (25.7%), of which IncK-F (23.7%) and IncF (46.2%) were predominant. Plasmid replicon was significantly different among sources of isolates and sampling periods but significantly correlated with resistance phenotype (p<0.05). In conclusion, various plasmids are present in *E. coli* and *Salmonella*. The findings in this part form a basis for future studies to explore the possible methodology to counteract horizontal transfer of plasmids. Part 2 - Genomic analysis of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand. This part aimed to investigate the genetic characteristics of transferable plasmid carrying class 1 integrons with *dfrA12-aadA2* cassette array in 15 *Salmonella* isolates using Oxford Nanopore Sequencing technologies and Illumina platform. Seven MLST types and 6 clades of phylogenetic trees were identified. IncFIB(K), ColpVC, IncFIB, IncHI2, IncHI2A, IncX1, IncY and IncR plasmids were found. All had 28 AMR genes with the prediction to be resistant to various antibiotic groups. All isolates except B82 which was isolated from human, carried class 1 integrons with *dfrA12-DUF1010-aadA2* and *qacE-sul1* in conserved region. In conclusion, transferable R plasmids play an important role in the wide distribution of class 1 integrons with *dfrA12-aadA2* gene array. Part 3 - Molecular basis of the persistence of chloramphenicol (CHP) resistance among *E. coli* and *Salmonella* spp. from food animals, meat and human in Thailand. This study aimed to explore the potential mechanisms associated with the persistence of CHP resistance in *E. coli* (n=106) and *Salmonella* (n=57). Most *E. coli* (67.9%) and *Salmonella* (64.9%) had ≥4-fold CHP MIC decrease in the presence of PAβN. Ampicillin, tetracycline, and streptomycin selective pressure yielded *cmlA*-carrying *Salmonella* and *E. coli*-transconjugants resistant to CHP (MIC 32-512 µg/mL). IncF plasmids were common in *cmlA*-carrying *Salmonella* and *E. coli* transconjugants. The WGS analysis revealed *cmlA1*-class1 integrons flanked by IS*26* and Tn*As1* in IncX1 plasmid, IncFIA(HI1)/HI1B plasmids and IncFII/FIB plasmids, and *catA* flanked by IS*1B* and Tn*As3* in IncFIA(HI1)/HI1B/Q1. In conclusion, the persistence of CHP-resistance was potentially mediated by cross resistance via multidrug efflux systems using proton motif force (pmf) and co-selection of CHP-resistance genes by other antimicrobials. Part 4 - Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in *E. coli* isolated from pigs, pork and pig carcass. This study aimed to determine the possible cross resistance between disinfectants/heavy metals and antibiotics in *E. coli* from pigs (n=643), pork (n=111) and pig carcasses (n=110) in Thailand. Exposure to triclosan (TCS), benzalkonium chloride (BKC) and chlorhexidine (CHX) selected for spontaneous-resistant mutants exhibited cross resistance to at least one antibiotic. The presence of PAβN restored MICs of CHP and trimethoprim in BKC- and TCS-spontaneous resistant mutants but PAβN, CCCP and reserpine could not restore ciprofloxacin MIC in ciprofloxacin-resistant mutants with no *gyrA* and *parC* mutation. In conclusion, the widely used disinfectants and heavy metals serve as non-antibiotic selective pressure for emergence and spread of AMR via cross-resistance involved in pmf-dependent and/or independent mechanisms. Susceptibilities to disinfectants/heavy metals should be routinely monitored. For the overall conclusion, the objectives of this project were successfully achieved. Further studies are suggested for better understanding of AMR and implementing the strategic actions to contain AMR.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Field of Study:	Veterinary Public Health	Student's Signature ....
Academic Year:	2023	Advisor's Signature ....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Professor Dr. Rungtip chuanchuen, for her invaluable guidance and support throughout my PhD's Program. Her expertise and encouragement helped me to complete this research and write this thesis.

I would also like to thank Professor Dr. Alongkorn Amonsin, Associate Professor Dr. Sunpetch Angkittitrakul, Assistant Professor Dr. Taradon Luangtongkum and Assistant Professor Dr. Saharuetai Jeamsripong for serving on my thesis examination committee and provide helpful feedback and suggestions.

I am grateful to Professor Dr. Darren Trott for providing me the opportunity to be visiting PhD student at the Australian Centre for Antimicrobial Resistance Ecology School of Animal and Veterinary Sciences at the University of Adelaide. Along the 6 months period, I have learned a lot from Dr. Anna Sheppard to conduct my research using Bioinformatic analysis and I have been trained by Dr. Abiodun David Ogunniyi and Dr. Hang Thi Nguyen for various laboratory techniques in the project "Testing of New Antibiotics to prevent Infection in Bioluminescent Mouse Infection Models" that related with my AMR research area.

I would like to thanks my friends, colleagues and all staffs at Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, staff of Chulalongkorn University Veterinary Antimicrobial Resistance Cluster (CUVETAMR) for their support and help during my research.

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

Last, I am extremely grateful to my parents and family for their continuous support with unconditional love and encouragement throughout my study. I would like to say thanks to Dr. Vu Thanh My Anh, who support me as the host when I was in Australia.

Jiratchaya Puangseree

Chulalongkorn University

TABLE OF CONTENTS

Page

ABSTRACT (THAI) iii
ABSTRACT (ENGLISH) iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES ix
LIST OF FIGURES xii
LIST OF PUBLICATIONS 1
CHAPTER I 2
1.1 IMPORTANCE AND RATIONALE 2
1.2 QUESTIONS OF STUDY 4
1.3 OBJECTIVES OF STUDY 5
1.4 KEYWORDS (THAI): 5
1.5 KEYWORDS (ENGLISH): 5
1.6 LITERATURE REVIEW 6
1.7 ADVANTAGES OF STUDY 10
CHAPTER II 12
2.1 ABSTRACT 13
2.2 INTRODUCTION 14
2.3 MATERIALS AND METHODS 15
2.4 RESULTS 23
2.5 DISCUSSION 35

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER III 41

3.1 ABSTRACT 42

3.2 INTRODUCTION 43

3.3 MATERIALS AND METHODS 45

3.4 RESULTS 49

3.5 DISCUSSION 62

CHAPTER IV 65

4.1 ABSTRACT 66

4.2 INTRODUCTION 67

4.3 MATERIAL AND METHODS 69

4.4 RESULTS 74

4.5 DISCUSSION 91

CHAPTER V 96

5.1 ABSTRACT 97

5.2 INTRODUCTION 98

5.3 MATERIALS AND METHOD 100

5.4 RESULTS 103

5.5 DISCUSSIONS 120

CHAPTER VI 126

6.1 GENERAL DISCUSSIONS 127

Part 1. Plasmid profile analysis of *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork and humans 128

Part 2. Genomic analysis of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand. 129

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Part 3. Molecular basis of the persistence of chloramphenicol resistance among *Escherichia coli* and *Salmonella* spp. from food animals, meat, and human in Thailand 131

Part 4. Resistance to widely used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass. 132

6.2 CONCLUSIONS 133

6.3 SUGGESTION AND FURTHER STUDIES 135

6.3.1 Applications of the research findings to address AMR: 135

6.3.2 Specific recommendations to address AMR associated with livestock in Thailand. 135

6.3.3 Recommendations for future studies 136

REFERENCES 138

VITA 159

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

LIST OF TABLES

Page

Table 1 Sources and number of Escherichia coli (n=1,047) and Salmonella (n=816) used in this study. 17

Table 2 Serovars of the Salmonella from different sources including pigs, pork and humans that included in this study (n=816)........ 17

Table 3 Antimicrobial resistance and ESBL production in Escherichia coli (n=1,047) and Salmonella (n=816) isolates that included in this study 20

Table 4 Primers used in this study. 22

Table 5 Percentage of Inc group of plasmids of Escherichia coli (n=1,047) and Salmonella (n=816) isolated from pig, pork, and human 26

Table 6 Odds ratio between the presence of replicon types and antimicrobial resistance or ESBLs producing Escherichia coli (n=1,047) and Salmonella (n=816)..... 27

Table 7 Odds ratio between each two replicon types presented in Escherichia coli (n=1,047) and Salmonella (n=816)........ 30

Table 8 Replicon sequence types of Inc F of Escherichia coli (n=26) and Salmonella (n=3) 32

Table 9 Replicon patterns among E. coli (n=1,047) and Salmonella (n=816). 33

Table 10 Characteristics of Salmonella carrying class 1 integrons with dfrA12-aadA2 cassette........ 45

Table 11 Primers used in this study. 47

Table 12 Detection of MLST and plasmids among Salmonella by WGS analysis (n=15) 53

Table 13 Antimicrobial resistance genes identified by ResFinder (n=15) 54

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 14 Antimicrobial resistance phenotypes predicted by ResFinder and defined by clinical breakpoints (n=15) 55

Table 15 Amino acid substitutions of chromosomally encoded genes identified by ResFinder (n=15). 56

Table 16 Salmonella Pathogenicity Islands identified by SPIFinder (n=15) 57

Table 17 Primer used in this study. 70

Table 18 Effect of phenylalanine arginine β-naphthylamide (PAβN) on MIC values of chloramphenicol in Escherichia coli (n=106) and Salmonella (n=57). 74

Table 19 Conjugation rates and chloramphenicol resistance phenotype of transconjugants. 77

Table 20 Plasmid of E. coli (n=11) and Salmonella (n=9) donors and corresponded chloramphenicol resistant transconjugants. 78

Table 21 Quality of whole genome assembly of Escherichia coli (n=3) and Salmonella enterica (n=3) 80

Table 22 Prediction of antimicrobial resistance genes, virulence genes and plasmids from whole genome assembly data in selected Escherichia coli (n=3) and Salmonella enterica (n=3) 85

Table 23 Antimicrobial resistance pattern of Escherichia coli isolated from pigs, pork and pig carcass (n=864). 105

Table 24 Distribution of MIC values for disinfectants and heavy metals in Escherichia coli isolated from pigs, pork and pig carcass (n=864) 106

Table 25 Statistical association between MICs of disinfectants/heavy metals and antibiotic resistance (n=864) 112

Table 26 Statistic correlation between MICs of disinfectants/heavy metals and antibiotic (n=864) 113

Table 27 MICs of disinfectants and heavy metals of Escherichia coli in in vitro exposure experiment (n=24) 114

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 28 MICs of antibiotics and disinfectants of spontaneous resistance mutants and their isogenic parents in in vitro exposure experiment (n=16) 116

Table 29 MICs of ciprofloxacin of spontaneous-resistance mutants and their isogenic parents in in vitro exposure experiment (n=6) 119

LIST OF FIGURES

Page

Figure 1 Phylogenetic tree of Salmonella enterica carrying class 1 integrons with dfrA12- aadA2 cassette array (n=15). 58

Figure 2 Alignment of plasmids carrying class 1 integrons with dfrA12-aadA2 cassette array (n=15). 59

Figure 3 Structural comparison of the IncR plasmids from Salmonella D7 and D15 carrying class 1 integrons with dfrA12-aadA2 cassette array and closely related plasmid. 60

Figure 4 Structural comparison of the IncX1 plasmids from Salmonella B82 carrying class 1 integrons with dfrA12-aadA2 cassette array and closely relative plasmids. 61

Figure 5 Phylogenetic tree by WGS analysis of Escherichia coli (n=3) and Salmonella enterica (n=3). 84

Figure 6 Alignment of plasmids carrying cmlA1 of E. coli (n=3) and Salmonella enterica (n=2). 88

Figure 7 Circular comparison of catA carrying IncFIA(HI1)/HI1B/Q1 plasmid of SA448. 89

Figure 8 Circular comparison of floR-carrying IncA/C plasmid of SA515. 90

Figure 9 Distribution of antimicrobial resistance in Escherichia coli (n=864). 107

Figure 10 MIC distribution of disinfectants in Escherichia coli sorted by years (n=864). 108

Figure 11 MIC distribution of $ZnCl_2$ and $CuSO_4$ in Escherichia coli sorted by years (n=864). 109

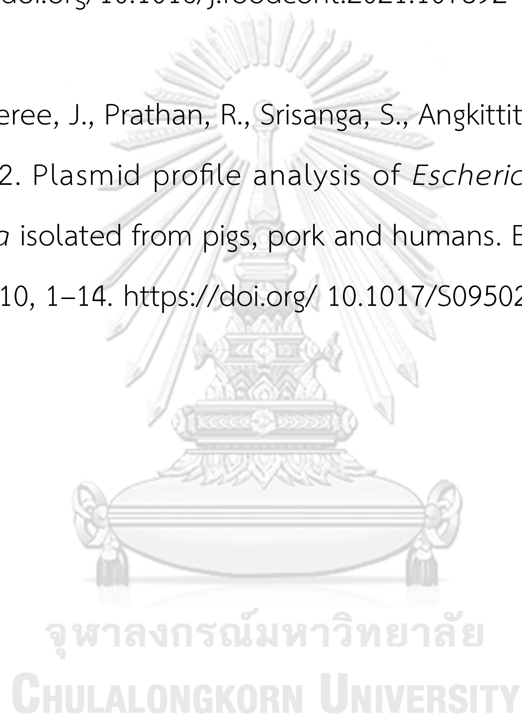
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

LIST OF PUBLICATIONS

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

1. Puangseree, J., Jeamsripong, S., Prathan, R., Pungpian, C. and Chuanchuen, R., 2021. Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass. Food control, 124 (107892), doi: https://doi.org/10.1016/j.foodcont.2021.107892

2. Puangseree, J., Prathan, R., Srisanga, S., Angkittitrakul, S. and Chuanchuen, R., 2022. Plasmid profile analysis of *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork and humans. Epidemiology and Infection 150, e110, 1–14. https://doi.org/ 10.1017/S0950268822000814



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER I

1.1 IMPORTANCE AND RATIONALE

Antimicrobial resistance (AMR) has drastically increased and its prevalence varies across world regions, of which that in Asia is highest (WHO, 2014). Evidently, food animals and their products serve as major reservoirs of resistant bacteria and resistance determinants that could be transferred to food chain, humans, animals and environment (FAO, 2016). It has been pointed out that horizontal transfer of resistance determinants via conjugative plasmids is an important route that promotes the widespread of AMR bacteria (Mathers et al., 2015). Therefore, analysis of plasmid could serve as a tool to describe the genetic linkage and wide distribution of AMR.

In Thailand, *Salmonella* is one of the most common foodborne pathogens and used as safety indicator in meat and meat products. Concurrently, *Escherichia coli* is a commensal bacterium in both human and animal guts and serves as reservoir for resistance determinants. Based on our previous studies, at least 85% of *Salmonella* and *E. coli* from pigs, poultry, pork, chicken and patients were resistant to at least one antibiotic and more than 50% of the isolates were resistant to multiple drugs (; Sinwat et al., 2015; Sinwat et al., 2016). In our laboratory, the research has focused on genetic characterization of AMR and created certain amount of AMR data in food animals and their products in Thailand (Chuanchuan et al., 2009; Trongjit et al., 2017). We have used mobile genetic elements, particularly integrons, as a tool to study genetic characteristics of AMR. The results from our previous studies demonstrated the complexity and dynamics of AMR in animal sector in the country (Sinwat et al., 2015). The widespread distribution of multidrug resistance among foodborne and commensal bacteria originated from animals and meat was predominant (Trongjit et al., 2016; Padungtod et al., 2011). When the results are taken together, the outstanding observations and some comments/questions are raised as follows:

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1. A variety of resistance genes were identified on conjugative plasmids in the *Salmonella* and *E. coli* isolates from different sources and locations in Thailand, indicating that horizontal transfer is a major route of spread of resistance determinants. This highlights the need of identification and classification of R plasmids among the isolates to amplify the understanding of the possible sources and link of AMR in the country.

2. Class 1 integrons was predominant integrons type in the majority of our studies and several resistance gene cassettes have been identified in their variable regions (Chuanchuen et al., 2009; Wannaprasat et al., 2011). The same resistance gene cassettes, particularly *dfrA12-aadA2*, were found in different bacterial species from different sources in Thailand e.g. *Salmonella* from pigs in Khon Kaen and Roi Et, from pork in Chiang Mai, *E. coli* from pigs in Nakhon Ratchasima (Wannaprasat et al., 2011; Lay et al., 2012; Sinwat et al., 2015), *Salmonella enterica* from pet dogs and cats (Srisanga et al., 2017), *Aeromonas hydrophila* from nile tilapia (Lukkana et al., 2012), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from patients (Poonsuk et al., 2012). The findings highlight the importance of horizontal transfer in AMR spreading and the role of the *dfrA12-aadA2* array in spreading of trimethoprim and streptomycin resistance in bacteria of animal origin in the country. These indicate that AMR among food animals, food of animal origin, pets and humans is genetically linked and therefore, the genetic investigation of *dfrA12-aadA2* carrying plasmid may provide the supporting data (Domingues et al., 2012).

3. Chloramphenicol usage has been banned from food animal sector in Thailand since 1999, however, chloramphenicol-resistant bacteria with chloramphenicol-resistance genes are still found in food animals and their meat (Wannaprasat et al., 2011; Lay et al., 2012). This indicate that the persistence of chloramphenicol resistance genes does not always depend on chloramphenicol use. It further suggests that the persistence of AMR genes

may be the results of co-selection with other antibiotics or a consequence of non-antibiotic selection pressure, particularly disinfectants and heavy metals that are commonly used in food animal production (Wales and Davies, 2015). In this case, the use of disinfectants and heavy metals may promote cross resistance to antibiotics and cause persistence of AMR (Chapman et al., 2003; Andersson and Hughes, 2011). In addition, use of other antibiotics can co-select chloramphenicol resistance genes, especially co-locallized of the resistance genes on the same plasmid. Therefore, study of resistance plasmid and cross resistance created by non-antibiotic pressure will help to explain why AMR still persist after the drug ban.

4. Some *Salmonella* isolates carried both resistance genes/class1 integrons with gene cassettes and virulence gene that are probably co-locallized on the same plasmid (Wannaprasat et al., 2011; Sinwat et al., 2015). If so, using of a single antimicrobial drug can simultaneously select both resistance and virulence genes, with the potential to create a serious public health problem in the future. Therefore, understanding of genetics of the cointegrated-resistance and virulence plasmid will provide information that is of public health significance.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Based on the major findings described above, certain questions have been raised and still left unresolved. In this research project, we aim to characterize the resistance determinants, especially plasmid, that were obtained in prior studies to identify the potential answers. The bacterial strains with different AMR phenotype and genotype, and resistance determinants in our strain collection will be used in this project.

1.2 QUESTIONS OF STUDY

1. What is the characteristics and profile of R plasmid in *E. coli* and *Salmonella* isolated from food animals, meat and humans in Thailand?
2. Why is the *dfrA12-aadA2* cassette array widespread in *Salmonella* isolated from food animals, meat and humans in Thailand?

3. Why does the chloramphenicol-resistant *E. coli* and *Salmonella* persist after ban of chloramphenicol usage in food animals?
4. Can disinfectants and heavy metals create selection pressure for AMR?
5. Is there cointegration of virulence and resistance genes on the same plasmid? If so, what is the genetic characteristics of virulence-resistance plasmid in *Salmonella*?

1.3 OBJECTIVES OF STUDY

1. To characterize the profile of R plasmid in *E. coli* and *Salmonella* isolated from food animals, meat and humans in Thailand.
2. To investigate the genetic characteristics of plasmid carrying the *dfrA12-aadA2* cassette array in *Salmonella* isolated from food animals, meat and humans in Thailand.
3. To characterize plasmid carrying chloramphenicol-resistance genes in *E. coli* and *Salmonella* from food animals, meat and humans in Thailand.
4. To determine the possible cross resistance between disinfectants/heavy metals and antibiotics in *E. coli* from food animals in Thailand.
5. To characterize genetic characteristics of plasmid with the cointegration of resistance and virulence genes in *Salmonella*.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.4 KEYWORDS (THAI):

การดื้อยาต้านจุลชีพ เอสเชอริเชีย โคไล ตัวระบุทางพันธุกรรม ซัลโมเนลลา ประเทศไทย

1.5 KEYWORDS (ENGLISH):

antimicrobial resistance, *Escherichia coli*, genetic determinants, *Salmonella*, Thailand

1.6 LITERATURE REVIEW

1.6.1 General characteristic of *Escherichia coli* and *Salmonella enterica*

E. coli is a Gram negative, rod shaped and facultative anaerobe bacterium that commonly found in intestine and feces of warm blooded animals (Tenaillon et al., 2010). Most strains of *E. coli* are harmless, but some strains can cause the illness by producing toxin (Rangel et al., 2005). Therefore, *E. coli* can be both commensal and pathogenic bacterial strain (Tenaillon et al., 2010). *E. coli* can grow easily with optimum temperature, 37°C.

Salmonella is a Gram negative, rod shaped, facultative anaerobe and non-spore forming bacterium in *Enterobacteriaceae* family. There are two species of *Salmonella* including *S. enterica* and *S. bongori* (EFSA, 2010). *S. enterica* has been divided into six subspecies which contains more than 2,500 serotypes base on O somatic and H flagella antigens of Kauffmann-White classification (Brenner et al., 2000). *S. enterica* can infect in warm blooded animals, cold blooded animals and found in the environment. *Salmonella* is a pathogen which some strains, non-typhoidal *Salmonella*, can infect to animals, cause food poisoning in humans and transfer from animal or human to human. However, the other strains, typhoidal *Salmonella*, can infect only in humans and higher primates.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.6.2 Antimicrobial resistance in *E. coli* and *S. enterica*

E. coli has been used as a sentinel for AMR monitoring in fecal bacteria (Tadesse et al., 2012). In addition, *Salmonell*a is one of the pathogenic bacteria suggested to included in monitoring program of AMR (Flanklin et al., 2001). There are several global reports about resistant *E. coli* and *Salmonella* to antimicrobial drugs that have been used in human and veterinary medicine. Up to date, the *Salmonella* and *E. coli* which develop resistance to fluoroquinolone, broad spectrum penicillins and third generation cephalosporins are significant public health impact worldwide (WHO, 2014). Genetic mutations and mobile genetic elements are the importance factors that can be emergence of multidrug resistance (MDR) *E. coli* and *Salmonella*. In

Thailand, MDR *E. coli* and *Salmonella* have been isolated from animals and their food products (Lay et al., 2012; Sinwat et al., 2015).

1.6.3 Transfer of AMR determinants

AMR determinants can spread by both vertical and horizontal transfer. Vertical transfer of AMR occurs when there is clonal spread of particular resistance strain. As for the horizontal genes transfer, three main mechanisms including transduction, DNA transfer by bacteriophages; transformation, obtaining of naked DNA from extracellular compartment; and conjugation, the main mode of AMR genes transfer which require cell to cell contact, have been identified (Mathur and Singh, 2005). The horizontal transfer of resistance genes which frequently found and promote the widespread of AMR among intra- and inter-species is associated with mobile genetic elements such as plasmids, transposons and integrons (Boerlin and Reid-Smith, 2008).

1.6.4 Role of plasmid in AMR distribution

Plasmid is an extra-chromosomal DNA that can self-replicate independently from chromosomal control. Plasmid contains necessary genes for initiation and control of replication (Johnson and Nolan, 2009) and carries many accessory genes including the genes for AMR, virulence and specific functions (Smalla et al., 2015). Normally, plasmids provide host the advantage accessory supplement under pressure situation. Plasmid is one of the most important mobile genetic elements which can promote horizontal transfer of resistance determinants among different bacterial species (Carattoli, 2011). Identification and characterization of plasmid which associated with different bacterial host can provide the understanding of the contribution of plasmid in dissemination of AMR determinants (Rozwandowicz et al., 2018).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.6.4.1 PCR-based replicon typing

Nowadays, the classification of plasmid based on incompatibility of plasmids. The incompatibility is the inability of two plasmids to be stabled in the same cell due to the same plasmid replication control and equipartitioning (Couturier et al., 1988). Incompatible plasmids have been assigned to be the same incompatibility group or

Inc group, while two compatible plasmids in the same cell have been assigned to be different Inc groups (Johnson and Nolan, 2009). The former plasmid incompatibility grouping methods by using conjugation and hybridization are laborious, time consuming, non-standardize and unsuitable for a number of strains. Therefore, PCR based replicon typing was developed to identify and classify the Inc groups of plasmid in Enterobacteriaceae family (Carattoli et al., 2005). This scheme is using the set of primer to detect the specific region in each plasmid group. This method have been revised to detect the 28 major Inc group that associated with the importance AMR genes (Carloni et al., 2017). In addition, this typing method can be used together with other characterization such as resistance gene identification for tracing the spread of specific resistance determinant in the bacterial isolates from different sources (Carattoli, 2013).

1.6.4.2 Plasmid multilocus sequence typing

Plasmid multilocus sequence typing (pMLST) is the additional tool that was developed to differentiate the plasmids within the same Inc group (Carattoli, 2011). This scheme analyzes the different sequence types which are categorized based on DNA sequence of specific loci for each Inc group. Currently, there is only the common family of plasmids, including IncN, IncF, IncI1, IncHI1, IncHI2 and IncA/C are subtyping with this scheme (Carattoli et al., 2014; Hancock et al., 2017).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.6.4.3 Plasmid sequencing

In AMR epidemiology, plasmid mapping and whole plasmid sequencing have been used to characterize the genetic feature of plasmids. Novel in silico plasmid analysis tools which are application for plasmid characterization based on whole genome sequencing studies can help to analyze among large datasets of plasmids (Orlek et al., 2017).

1.6.5 Phenotype after the ban of antimicrobials

The European Union banned some antibiotics, belong to antimicrobial class used in human that use as growth promotion in food producing animals, for example,

avoparcin in 1997 and four antibiotics including bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al., 2003). Because, used of these antibiotics such as avopracin in food animals can generate vancomycin resistance Enterococci which can transfer to human. After banning, the prevalence of bacteria which resistance to these drugs in healthy food animals and humans seem to be decrease, but in patients are still increase (Casewell et al., 2003).

In Thailand, chloramphenicol has been banned since 1999 because of its toxicity (Phongpaichit et al., 2007). However, the phenotypic and genotypic resistance on chloramphenicol have been recently found in bacteria isolated from food animals and their products (Chuanchuen et al., 2009; Wannaprasart et al., 2011; Lay et al., 2012). More than 20% and 50% of prevalence of chloramphenicol resistance *Salmonella* and *E. coli*, respectively have been reported in several studies in 2008-2016 (Nhung et al., 2016). These studies in this period showed the increasing of prevalence of chloramphenicol resistance bacteria when compare to the other studies which reported before 2008 (Nhung et al., 2016). Other studies reported that the co-selection with other AMR genes on the same mobile genetic elements such as plasmid, transposon and integrons are the major factors which drive the chloramphenicol resistance even absence the chloramphenicol use (Bischoff et al., 2005).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

There are two main chloramphenicol resistance mechanisms including chloramphenicol acetyl transferase, an inactivated enzyme encoded by cat gene and chloramphenicol specific exporter encoded by cml gene (Schwarz et al., 2004). In addition, a previous study showed that the *cmlA* gene could be found in gene cassette of class1 integrons which can carry other resistance genes (Chuanchuen et al., 2009).

1.6.6 Contribution of AMR by non-antibiotic factors

Other factor that can contribute the persistence of AMR are using of biocides and heavy metals. There were several studies reported that the biocides used in food

producing animals is the one factor for AMR selection (European Commission, 2009). The biocides such as quaternary ammonium compounds (QACs), formaldehyde, chlorhexidine and triclosan are commonly used for disinfection in farm animals. In food animal production, heavy metals i.e. copper sulfate and zinc chloride are frequently used as growth promoting supplement in animal feed. The bacteria can concurrently against all antibiotics, biocides and heavy metals via some intrinsic resistance mechanisms such as reduction of membrane permeability and over expression of the efflux pump, (Singer et al., 2016). Biocides, heavy metals and antimicrobial agents can attack the same target in multidrug efflux pump which can contribute the cross resistance (Chapman, 2003).

Bacterial antibiotic efflux pumps have been categorized into 5 families, including i) the ATP-binding cassettes (ABC) superfamily, ii) The major facilitator superfamily (MFS), iii) The multi-antimicrobial and toxic compound extrusion family (MATE), iv) the small multidrug resistance (SMR) family, and v) the resistance nodulation cell division (RND) superfamily (Li and Nikaido, 2009). In particular, the antibiotic efflux pumps in RND superfamily are extremely found in Gram negative bacteria and can affect the several substance not only antibiotic drugs, but also disinfectant compounds (Mahamoud et al., 2007). The main efflux pump in *E. coli* is the AcrAB-TolC efflux pump that belongs to RND superfamily (Blanco et al., 2016). AcrAB-TolC efflux pump composes of 3 components including AcrB, the transporter that attach to plasma membrane and encoded by *acrB* gene; AcrA, the side proteins of AcrB that help the movement of substrates through the AcrB transporter and encode by *acrA* gene, and TolC protein which is a channel that connected with AcrB (Amaral et al., 2014).

1.7 ADVANTAGES OF STUDY

1.7.1 Novel knowledge

- The genetic profile of R plasmid in *E. coli* and *Salmonella* in Thailand will be obtained. It will generate the better understanding of link of AMR in the country

- Characteristics of plasmid carrying *dfrA12-aadA2* cassette array will be obtained. This may explain about the distribution and link of AMR.
- Characteristics of plasmid carrying chloramphenicol-resistance encoding genes and determination of the possible cross resistance between disinfectants/heavy metals and antibiotics will be obtained. It will reveal the possible mechanisms for AMR persistence.
- Characteristics of virulence-resistance plasmid in *Salmonella* will be obtained. This will provide more understanding of cointegration of resistance and virulence genes on the plasmid.

1.7.2 Application of knowledge

- Data obtained can be used as a guide for development of control and prevention strategy of AMR dissemination in Thailand and the legislation of antimicrobial use in food animal.
- Data obtained from non-antibiotics selection pressure can be used as part of development of regulation of disinfectants and heavy metals use.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER II

Plasmid profile analysis of *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork, and humans

Jiratchaya Puangseree[a], Rangsiya Prathan[a,b], Songsak Srisanga[a,b], Sunpetch Angkittitrakul[c], Rungtip Chuanchuen[a,b,*]

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

[b]Center for Antimicrobial Resistance Monitoring in Food-borne Pathogens, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand

[c]Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002 Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Epidemiology and Infection Journal (2022)

Plasmid profile analysis of *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork, and humans

2.1 ABSTRACT

This study aimed to determine the epidemiology and association of antimicrobial resistance (AMR) among *Escherichia coli* and *Salmonella* in Thailand. The *E. coli* (n=1,047) and *Salmonella* (n=816) isolates from pigs, pork and humans were screened for 18 replicons including HI1, HI2, I1-γ, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, F, K and B/O using PCR-based replicon typing. The *E. coli* (n=26) and *Salmonella* (n=3) isolates carrying IncF family replicons, ESBL and/or *mcr* genes were determined for FAB formula. IncF represented the major type of plasmids. Sixteen and eleven Inc. groups were identified in *E. coli* (85.3%) and *Salmonella* (25.7%), respectively. The predominant replicon patterns between *E. coli* and *Salmonella* were IncK-F (23.7%) and IncF (46.2%). Significant correlations ($p<0.05$) were observed between plasmid-replicon type and resistance phenotype. Plasmid replicon types were significantly different among sources of isolates and sampling periods. The most common FAB types between *E. coli* and *Salmonella* were F2:A-:B- (30.8%) and S1:A-:B- (66.7%), respectively. In conclusion, various plasmids present in *E. coli* and *Salmonella*. Responsible and prudent use of antimicrobials is suggested to reduce the selective pressures that favor the spread of AMR determinants. Further studies to understand the evolution of R plasmids and their contribution to the dissemination of AMR genes are warranted.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2 INTRODUCTION

Antimicrobial resistance (AMR) constitutes a complex and multifaceted public health challenge that requires a board-integrated one health approach to deal with. AMR monitoring and surveillance has been established across human, animal, and environmental sectors to understand the burden and ecology of the problem. As for AMR monitoring and surveillance in food-animal origin, target bacteria included commensal *Escherichia coli* and *Salmonella* (EFSA, 2012). Commensal *E. coli* normally live in the large intestines of humans and animals, serving as reservoirs of AMR determinants that could spread to bacterial pathogens. *Salmonella* is a foodborne zoonotic bacterial pathogen prevalent in food animals and meat; it is also frequently resistant to multiple antibiotics. Both bacteria possess a vast array of R plasmids, conjugative plasmids conferring on bacteria resistance to one or more antibiotics, that are critical positions for the spread of AMR determinants (Madec J-Y, Haenni M, 2018).

Mobile genetic element (MGE) acquisition, especially plasmid, via horizontal transmission is a major route for the emergence and dissemination of AMR (von Wintersdorff CJ, et al., 2016). Transmissible R plasmids usually carry multiple genes encoding resistance to clinically relevant antibiotics and play an important role in AMR evolution and spread. Certain species-specific association plasmids exist e.g. IncX plasmids in *Salmonella* and *E. coli* (Rozwandowicz M, et al., 2018) and IncF plasmids in *Enterobacteriaceae* (Carattoli A., 2013). Previous studies investigated the dynamics and diversity of AMR among humans, livestock, and food of animal origin (Sinwat N, et al., 2016; Trongjit S, et al., 2017; Trongjit S, et al., 2016). A variety of AMR determinants have been found to be associated with conjugative plasmids. The same genetic elements were detected in different bacterial species from different sources and locations. For example, class1 integrons with *dfrA12-aadA2* cassette were isolated from *Salmonella* in pigs (Sinwat N, et al., 2016; Trongjit S, et al., 2017), poultry (Trongjit S, et al., 2017; Trongjit S, et al., 2016; Sinwat N, et al., 2015) and humans (Sinwat N, et al., 2016; Trongjit S, et al., 2017; Trongjit S, et al., 2016; Sinwat

N, et al., 2015); *E. coli* in pigs (Trongjit S, et al., 2016; Sinwat N, et al., 2015; Lay KK, et al., 2012), poultry (Sinwat N, et al., 2016); *Aeromonas hydrophila* in Nile Tilapia (Lukkana M, et., 2011) and *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in patients (Poonsuk K, et., 2012). These findings underscore the horizontal transfer of plasmids as a major driver for AMR dissemination in Thailand and neighboring countries.

A classical method for plasmid identification and classification is incompatibility (Inc) group testing (Rozwandowicz M, et al., 2018). To date, at least 27 different Inc groups of plasmids have been identified among *Enterobacteriaceae* (Carattoli A, et., 2009). Plasmids in the same Inc group share the same replication control or partitioning mechanisms and can neither coexist in the same bacterial cells nor be co-transferred (Novick RP., 1987). The presence of bacterial strains originated from different sources but carrying plasmids of the same Inc group indicate the horizontal widespread of the plasmids with close-phylogenetic relationship. Accordingly, molecular epidemiological investigation of plasmids has been used to trace the source and potential risk of AMR spread via plasmids.

Data from molecular epidemiological analysis of plasmids will increase knowledge and understanding of plasmid diversity and transmission and benefit the development of strategic action plan to contain AMR. This study aimed to characterize the plasmid profiles in *E. coli* and *Salmonella* from pigs, pork and humans in Thailand.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial isolates and their AMR phenotype and genotype

E. coli (n=1,047) and *Salmonella* (n=816) isolates were included in this study. They originated from our previous epidemiological studies investigating AMR in healthy food animals, meat, and humans during 2005-2019 (Sinwat N, et al., 2016; Sinwat N, et al., 2015; Lay KK, et al., 2012; Pungpian C, et al., 2020; Lay KK, et al., 2021;

Khemtong S, Chuanchuen R., 2008; Wannaprasat W, et al., 2011) (Table 1). The research protocols involving human subjects in these previous studies were approved by Ethics Committee of the Faculty of Medicine of Khon Kaen University (the authorization ID, HE572136). There was no involving of the human sampling in this study, thus the ethical approved was not issued.

All the *E. coli* strains were isolated from rectal swabs of clinically healthy pigs (n=697), pork (n=247) and humans (n=103) from Northern, Northeastern, Central and Western Thailand. A single colony of *E. coli* was collected from each positive sample. The *Salmonella* isolates originated from pigs (n=169), pork (n=510), and humans (n=137) in Northern, Northeastern and Central Thailand (Table 1). *Salmonella* was isolated as described in ISO6579:2017 and serotyped using slide agglutination. A single colony of each serovar was collected from each positive sample. Rissen was the most common serovar among the *Salmonella* isolated from pigs (30.8%, 52/169) and pork (29.2%, 149/510). While *Salmonella* Stanley was the most predominant among the isolates from humans (26%, 19/137) (Table 2).

All *E. coli* and *Salmonella* isolates were previously tested for susceptibilities to 9 antimicrobial agents including ampicillin (AMP), chloramphenicol (CHP), ciprofloxacin (CIP), gentamycin (GEN), streptomycin (STR), sulfamethoxazole (SMZ), tetracycline (TET), Trimethoprim (TMP), colistin (COL) and phenotypically detected for ESBL production (CLSI, 2013) (Table 3). All the isolates were also screened for *mcr-1, mcr-2 and mcr-3*. Ten percent of *E. coli* and 1.5% *Salmonella* carried at least one *mcr*. The ESBL-producing *E. coli* (n= 155) were tested for ESBL genes and found to harbor $bla_{CTX\text{-}M}$ (95.5%), bla_{TEM} (80.6%) and $bla_{CMY\text{-}2}$ (1.3%). The $bla_{CTX\text{-}M}$ group (95.2%) and bla_{TEM} (33.3%) were found in ESBLs-producing *Salmonella* (n=21) (Table 3). The relevant resistance phenotypes are indicated in the text when appropriate.

Table 1 Sources and number of *Escherichia coli* (n=1,047) and *Salmonella* (n=816) used in this study.

Year	No. of *E. coli* isolates			Total	No. of *Salmonella* isolates			Total
	Pig	Pork	Human		Pig	Pork	Human	
2005-2010	309	-	-	309	8	104	52	164
2010-1014	123	223	103	449	67	263	85	415
2015-2019	265	24	-	289	94	143	-	237
Total	697	247	103	1047	169	510	137	816
Grand total	1,047				816			

Table 2 Serovars of the *Salmonella* from different sources including pigs, pork and humans that included in this study (n=816)

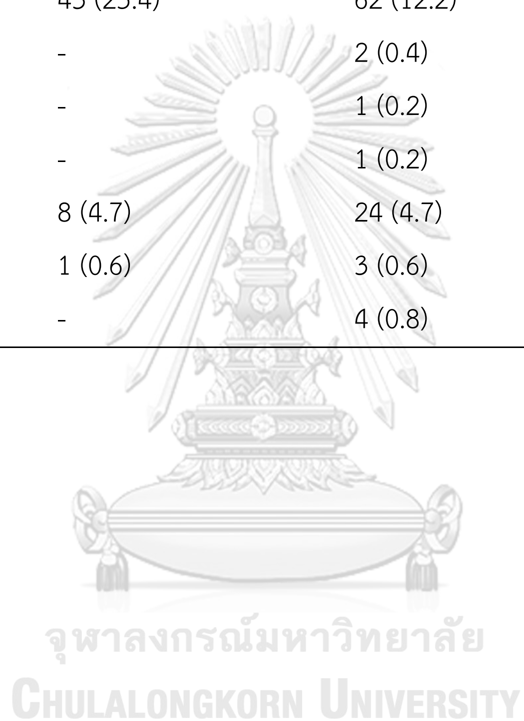
Salmonella serovars	No. (%)		
	Pig (n=169)	Pork (n=510)	Human (n=137)
Agona		1 (0.2)	-
Albany	1 (0.6)	3 (0.6)	-
Anatum	11 (6.5)	110 (21.6)	8 (5.8)
Augustenborg	1 (0.6)	1 (0.2)	-
Baiboukoum	-	1 (0.2)	-
Bardo	-	-	1 (0.7)
Bovismorbificans	-	4 (0.8)	2 (1.5)
Bradford	-	1 (0.2)	-
Braenderup	1 (0.6)	-	-
Brunei	-	-	1 (0.7)
Calabar	-	1 (0.2)	-
Coeln	-	1 (0.2)	-
Corvallis	-	5 (1.0)	9 (6.6)
Cuckmere	-	1 (0.2)	-
Derby	2 (1.2)	4 (0.8)	3 (2.2)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Salmonella serovars	No. (%)		
	Pig (n=169)	Pork (n=510)	Human (n=137)
Eastbourne	1 (0.6)	-	-
Enteritidis	-	-	9 (6.6)
Fareham	4 (2.4)	2 (0.4)	-
Fulda	-	1 (0.2)	-
Give	1 (0.6)	9 (1.8)	2 (1.5)
Hayindogo	-	3 (0.6)	-
Huettwillen	1 (0.6)	-	-
Hvittingfoss	-	2 (0.4)	2 (1.5)
Kedougou	13 (7.7)	21 (4.1)	14 (10.2)
Kingston	1 (0.6)	-	-
Langensalza	-	1 (0.2)	-
Lexington	-	-	4 (2.9)
Lille	-	-	1 (0.7)
Muenster	-	1 (0.2)	-
Newport	-	-	4 (2.9)
Norwich	-	2 (0.4)	-
Orion	1 (0.6)	-	-
Panama	-	5 (1.0)	6 (4.4)
Paratyphi	-	2 (0.4)	-
ParatyphiB	-	1 (0.2)	-
Rideau	1 (0.6)	4 (0.8)	-
Rissen	52 (30.8)	149 (29.2)	4 (2.9)
Saintpaul	2 (1.2)	21 (4.1)	1 (0.7)
Sanktmarx	5 (3.0)	10 (2.0)	-
Sao	12 (7.1)	9 (1.8)	-
Schwarzengrund	2 (1.2)	-	1 (0.7)
Senftenberg	-	1 (0.2)	1 (0.7)
Ser 1,4,5,12	-	-	2 (1.5)
Ser 4,12	-	-	1 (0.7)
Ser 4,12:i	-	-	1 (0.7)
Ser 4,21	-	-	1 (0.7)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Salmonella serovars	No. (%)		
	Pig (n=169)	Pork (n=510)	Human (n=137)
Ser 4,5,12:1	-	-	1 (0.7)
Ser 4,5,12:b:-	-	-	1 (0.7)
Ser 9,12:1,5	-	-	1 (0.7)
Serembah	-	1 (0.2)	-
Singapore	-	2 (0.4)	-
Stanley	5 (3.0)	33 (6.5)	26 (19.0)
Typhimurium	43 (25.4)	62 (12.2)	8 (5.8)
Urbana	-	2 (0.4)	-
Vejle	-	1 (0.2)	-
Virchow	-	1 (0.2)	3 (2.2)
Weltevreden	8 (4.7)	24 (4.7)	19 (13.9)
Worthington	1 (0.6)	3 (0.6)	-
Yalding	-	4 (0.8)	-



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 3 Antimicrobial resistance and ESBL production in *Escherichia coli* (n=1,047) and *Salmonella* (n=816) isolates that included in this study

Antimicrobial drugs/ Enzymes	No. of *E. coli* (%)				No. of *Salmonella* (%)			
	Pig (n=697)	Pork (n=247)	Human (n=103)	Total (n=1047))	Pig (n=169)	Pork (n=510)	Human (n=137)	Total (n=816)
AMP	623 (89.4)	200 (81.0)	62 (60.2)	885 (84.5)	148 (87.6)	395 (77.5)	89 (65.0)	632 (77.5)
CHP	423 (60.7)	83 (33.6)	17 (16.5)	523 (50.0)	25 (14.8)	147 (28.8)	83 (60.6)	255 (31.3)
CIP	220 (31.6)	8 (3.2)	7 (6.8)	235 (22.4)	0 (0)	2 (0.4)	21(15.3)	23 (2.8)
GEN	279 (40.0)	29 (11.7)	14 (13.6)	322 (30.8)	30 (17.8)	69 (13.5)	79 (57.7)	178 (21.8)
STR	453 (65.0	114 (46.2)	11 (10.7)	578 (55.2)	109 (64.5)	323 (63.3)	123 (89.8)	555 (68.0)
SMZ	521 (74.7)	121 (49.0)	38 (36.9)	680 (64.9)	130 (76.9)	408 (80)	103 (75.2)	641 (78.6)
TET	617 (88.5)	169 (68.4)	51 (49.5)	837 (79.9)	140 (82.8)	426 (83.5)	106 (77.4)	672 (82.4)
TMP	475 (68.1)	127 (51.4)	34 (33.0)	636 (60.7)	95 (56.2)	241 (47.3)	64 (46.7)	400 (49.0)
COL	160 (23.0)	15 (6.1)	0 (0)	175 (16.7)	2 (1.2)	7 (1.4)	0 (0)	9 (1.1)
ESBLs	140 (20.1)	7 (2.8)	8 (7.8)	155 (14.8)	2 (1.2)	19 (3.7)	0 (0)	21 (2.6)

AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; STR, streptomycin; SMZ, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; COL, colistin

2.3.2 Plasmid incompatibility grouping by PBRT

Plasmid incompatibility groups were identified by PCR-Based-Replicon-Typing (PBRT) in all *E. coli* and *Salmonella* isolates using 18 targeting replicons using specific primers (Carattoli A, et al., 2005) (Table 4). PCR-DNA templates were prepared by the whole-cell boiling method (Lévesque C, et al., 1995). PCR reactions were prepared using the Toptaq Master Mix kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.3.3 Replicon Sequence Typing (RST)

Since IncF was the most common plasmid, the *E. coli* (n=26) and *Salmonella* (n=3) isolates that carried ESBL and/or *mcr* genes and IncF plasmid were tested using the RST scheme (Villa L, et al., 2010) (Table 4). The RST scheme included the PCR amplification of FIA, using the same primers FIA FW/FIA RV that were used in the PBRT scheme; FII, using FII FW/FII RV for *E. coli* and FIIs FW/FIIs RV for *Salmonella*; and FIB, using FIB FW/FIB RV for E. coli and FIBs FW/FIB RV for *Salmonella*, respectively. PCR products were purified using Nucleospin gel and PCR clean up (McCherey-Nagel, Düren, Germany) and submitted to First Base Laboratories (Selangor Darul Ehsan, Malaysia) for nucleotide sequencing. The obtained sequences were analyzed using the DNA-star program (DNAstar, Madison, WI) and Blast search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and then, compared to alleles available at https://pubmlst.org/plasmid/.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3.4 Statistical analysis

The prevalence of plasmid replicon types was analyzed using Microsoft Excel. Comparisons of the associations between plasmid replicon types and AMR phenotypes were performed separately using odd ratios (OR) by SPSS version 22.0. Comparisons of the replicon type prevalence of *E. coli* and *Salmonella* from different sources and years were conducted using Fisher's exact test. A *p*-value of < 0.05 was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

Table 4 Primers used in this study.

PCR-reaction		Inc group	Name	Sequence of primers	Amplicon size (bp)	Reference
PBRT	Multiplex 1	HI1	HI1 FW	5'-GGAGCGATGGATTACTTCAGTAC-3'	471	[17]
			HI1 RV	5'-TGCCGTTTCACCTCGTGAGTA-3'		
		HI2	HI2 FW	5'-TTTCTCCTGAGTCACCTGTTAACAC-3'	644	
			HI2 RV	5'-GGCTCACTACCGTTGTCATCCT-3'		
		I1-γ	I1-γ FW	5'-CGAAAGCCGGACGGCAGAA-3'	139	
			I1-γ RV	5'-TCGTCGTTCCGCCAAGTTCGT-3'		
	Multiplex 2	X	X FW	5'-AACCTTAGAGGCTATTTAAGTTGCTGAT-3'	376	[17]
			X RV	5'-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3'		
		L/M	L/M FW	5'-GGATGAAAACTATCAGCATCTGAAG-3'	785	
			L/M RV	5'-CTGCAGGGGCGATTCTTTAGG-3'		
		N	N FW	5'-GTCTAACGAGCTTACCGAAG-3'	559	
			N RV	5'-GTTTCAACTCTGCCAAGTTC-3'		
	Multiplex 3	FIA	FIA FW	5'-CCATGCTGGTTCTAGAGAAGGTG-3'	462	[17]
			FIA RV	5'-GTATATCCTTACTGGCTTCCGCAG-3'		
		FIB	FIB FW	5'-GGAGTTCTGACACACGATTTTCTG-3'	702	
			FIB RV	5'-CTCCCGTCGCTTCAGGGCATT-3'		
		W	W FW	5'-CCTAAGAACAACAAAGCCCCCG-3'	242	
			W RV	5'-GGTGCGCGGCATAGAACCGT-3'		
	Multiplex 4	Y	Y FW	5'-AATTCAAACAACACTGTGCAGCCTG-3'	765	[17]
			Y RV	5'-GCGAGAATGGACGATTACAAAACTTT-3'		
		P	P FW	5'-CTATGGCCCTGCAAACGCGCCAGAAA-3'	534	
			P RV	5'-TCACGCGCCAGGGCGCAGCC-3'		
		FIC	FIC FW	5'-GTGAACTGGCAGATGAGGAAGG-3'	262	
			FIC RV	5'-TTCTCCTCGTCGCCAAACTAGAT-3'		
	Multiplex 5	A/C	A/C FW	5'-GAGAACCAAAGACAAAGACCTGGA-3'	465	[17]
			A/C RV	5'-ACGACAAACCTGAATTGCCTCCTT-3'		
		T	T FW	5'-TTGGCCTGTTTGTGCCTAAACCAT-3'	750	
			T RV	5'-CGTTGATTACACTTAGCTTTGGAC-3'		
		FIIAs	FIIAs FW	5'-CTGTCGTAAGCTGATGGC-3'	270	
			FIIAs RV	5'-CTCTGCCACAAACTTCAGC-3'		
	Simplex F	F	F_{repB} FW	5'-TGATCGTTTAAGGAATTTTG-3'	270	[17]

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

PCR-reaction		Inc group	Name	Sequence of primers	Amplicon size (bp)	Reference
			F_{repB} RV	5'-GAAGATCAGTCACACCATCC-3'		
	Simplex K	K	K/B FW	5'-GCGGTCCGGAAAGCCAGAAAAC-3'	160	[17]
			K RV	5'-TCTTTCACGAGCCCGCCAAA-3'		
	Simplex B/O	B	B/O RV	5'-TCTGCGTTCCGCCAAGTTCGA-3'	159	[17]
RST		F	FII FW	5'-CTGATCGTTTAAGGAATTTT-3'	258-262	[19]
			FII RV	5'-CACACCATCCTGCACTTA-3'		
			FIB FW[a]	5'-TCTGTTTATTCTTTTACTGTCCAC-3'	683	[19]
			FIBs FW[b]	5'-TGCTTTTATTCTTAAACTATCCAC-3'	683	[19]
			FIIs FW[c]	5'-CTAAAGAATTTTGATGGCTGGC-3'	259-260	[19]
			FIIs RV[c]	5'-CAGTCACTTCTGCCTGCAC-3'		

[a] Use in a pair with FIB RV for detection of FIB replicon in *E. coli* isolates.

[b] Use in a pair with FIB RV for detection of FIB or FII replicon in *Salmonella* isolates.

[c] Use for detection of FII replicon in *Salmonella* isolates.

2.4 RESULTS

2.4.1. Plasmid replicon types of *Escherichia coli*

Sixteen replicon types (except IncL/M and T) were identified in the *E. coli* isolates (Table 5), of which IncK replicon (60.6%, 634/1047) and IncF (48.9%, 512/1047) were most common. The HI2 (2.7%, 19/697), W (0.1%, 1/697) and X (0.1%, 1/697) replicons were limited to the pig isolates.

The predominant replicon type in the human isolates was IncF (33%, 34/103), while IncK plasmids were predominant in the pigs (73%, 509/697) and pork (42.9%, 106/247) isolates. IncFIIAs (18.2%, 127/697) and K (73%, 509/697) plasmids were significantly higher ($p<0.05$) in the pig isolates than those from other sources. The prevalence of IncHI1, I1-γ, N, FIB, Y, FIIAs, K and F among *E. coli* from pigs (17.2% (120/697), 15.5% (108/697), 13.3% (93/697), 34.4% (240/697), 15.1% (105/697), 18.2%

(127/697), 73% (509/697) and 58.1% (405/697), respectively) were significantly higher ($p < 0.05$) than those from other sources.

When considering years of isolates, IncK and IncF were the most predominant replicons in all periods, 2007-2010 (79.6% (246/309) and 65.4% (202/309)), 2011-2014 (36.6% (163/449) and 31.2% (140/449)) and 2015-2019 (77.9% (225/289) and 58.8% (225/289)), respectively (Figure1). The Inc X (0.3%, (1/289)) and W (0.3% (1/289)) plasmids were identified at a very limited rate and only in the years 2015-2019. The percentage of IncHI1 (20.4% (63/309), 14.2% (41/289)), N (14.9% (46/309), 11.8% (34/289)), FIB (35.9% (111/309), 37.7% (109/289)), FIIAs (10.7% (33/309), 30.8% (89/289)), K (79.6% (246/309), 77.9% (225/289)), and F (65.4% (202/309), 58.8% (170/289)) plasmids among the E. coli isolates during 2007-2010 and 2015-2019, respectively, were significantly higher ($p<0.05$) than those during years 2011-2014. In contrast, the presence of IncP (4.0%, 18/449) and FIC (6.0%, 27/449) plasmids from 2011-2014 were significantly higher than those in other years ($p<0.05$) (Table 5).

Up to 66 replicon patterns were defined (Table 14), of which the K-F replicon pattern was most common (23.7%). Thirty replicon patterns were found in ESBL-producing *E. coli* (n=155), of which I1γ-K-F was the most frequently found (27.3%). The *mcr*-carrying *E. coli* (n=109) had 27 replicon patterns, of which K-F (18.3%) was the most common.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2.4.2 Plasmid replicon types of *Salmonella*

Eleven plasmid replicon types, except IncL/M, X, T, FIA, W, P and K were found among the *Salmonella* isolates (Table 5). Overall, IncFIIAs was the most common replicon type (9.9%, 81/816), followed by IncY (4.9%, 40/816) and IncI1-γ (4.3%, 35/816). The predominant replicon of *Salmonella* isolated from pigs was IncY (20.1%, 34/169), while that among the pork and human isolates were IncFIIAs (7.1% (36/510) and 24.1% (33/137), respectively). The percentage of IncHI1 in the pork isolates (3.5%, (18/510)) and IncI1-γ, FIB, Y and F (10.7% (18/169), 3.6% (6/169), 20.1%

(34/169) and 4.7% (8/169), respectively) among the pig isolates were significantly higher than those from humans (p<0.05). In contrast, the prevalence of IncN, A/C and FIIAs (9.5% (13/137), 4.4% (6/137) and 24.1% (33/137), respectively) among human isolates were significantly higher than those among the pig and pork isolates (p<0.05).

The predominant replicon types in each period varied. IncN (9.1%, 15/164) were the most common plasmids in 2005-2010, while that in 2011-2014 and 2015-2019 were IncFIIAs (12.0%, 50/415) and IncY (13.9%, 33/237), respectively. IncY plasmids in 2015-2019 (13.9%, 33/237) were significantly higher than that in the other periods (p<0.05). The prevalence of IncN and FIC plasmids was the highest during 2005-2010 (9.1% (15/164) and 3.0% (5/164), respectively) (p<0.05).

Fifteen-replicon patterns were found in *Salmonella* (Table 9). The most common replicon pattern was F (46.2%). The ESBLs-producing *Salmonella* (n=21) had five replicon patterns, of which HI1 (42.1%) was the most common.

2.4.3 Association between replicon type and AMR phenotype in *E. coli* and *Salmonella*

Overall, the significant positive associations were more frequently observed than the negative association in both *E. coli* and *Salmonella* (Table 6). In *E. coli*, IncHI1 exhibited the strongest positive associations (OR>1) to AMP, CIP, GEN, STR and TET resistance. For other types of resistance phenotype/replicon associations, the strongest positive associations were between CHP/IncN (OR=2.78), SMZ/FIA (OR=5.22), TMP/B/O (OR=9.47) and COL/HI2 (OR=20.34). IncI1-γ plasmid showed the strongest positive association (OR=6.33) to ESBL production.

As for *Salmonella*, IncHI1 displayed the strongest positive association (OR>1) to CHP resistance (OR=46.8) and ESBL production (OR=159.9) (Table 6). Resistance to CIP, GEN and COL exhibited the highest positive association to IncN, A/C, and FIC, respectively (OR>1).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 5 Percentage of Inc group of plasmids of *Escherichia coli* (n=1,047) and *Salmonella* (n=816) isolated from pig, pork, and human

Target bacteria	Category	Sub category	No. of isolate for each replicon (%)															
			HI1	HI2	I1-γ	X	N	FIA	FIB	W	Y	P	FIC	A/C	FIIAs	K	B/O	F
E. coli	Overall (n=1,047)		137(13.1)	19(1.8)	129(12.3)	1(0.1)	112(10.7)	59(5.6)	290(27.7)	1(0.1)	135(12.9)	23(2.2)	39(3.7)	12(1.1)	146(13.9)	634(60.6)	16(1.5)	512(48.9)
(n=1,047)	By source (n= 1,047)	Pig (n=697)	120(17.2)^{a}	19(2.7)^{a}	108(15.5)^{a}	1(0.1)^{a}	93(13.3)^{a}	49(7)^{a}	240(34.4)^{a}	1(0.1)^{a}	105(15.1)^{a}	9(1.3)^{a}	19(2.7)^{a}	5(0.7)^{a}	127(18.2)^{a}	509(73)^{a}	15(2.2)^{a}	405(58.1)^{a}
		Pork (n=247)	11(4.5)^{b}	0^{b}	14(5.7)^{b}	0^{a}	16(6.5)^{b}	5(2)^{b}	32(13)^{b}	0^{a}	24(9.7)^{b}	7(2.8)^{a,b}	18(7.3)^{b}	6(2.4)^{b}	18(7.3)^{b}	106(42.9)^{b}	0^{b}	73(29.6)^{b}
		Human (n=103)	6(5.8)^{b}	0^{a,b}	7(6.8)^{b}	0^{a}	3(2.9)^{b}	5(4.9)^{a,b}	18(17.5)^{b}	0^{a}	6(5.8)^{b}	7(6.8)^{b}	2(1.9)^{a,b}	1(1)^{a,b}	1(1)^{c}	19(18.4)^{c}	1(1)^{a,b}	34(33)^{b}
	By year	2007-2010 (n=309)	63(20.4)^{a}	19(6.1)^{a}	86(27.8)^{a}	0^{a}	46(14.9)^{a}	28(9.1)^{a}	111(35.9)^{a}	0^{a}	31(10.0)^{a}	0^{a}	5(1.6)^{a}	0^{a}	33(10.7)^{a}	246(79.6)^{a}	11(3.6)^{a}	202(65.4)^{a}
		2011-2014 (n=449)	33(7.3)^{b}	0^{b}	29(6.5)^{b}	0^{a}	32(7.1)^{b}	16(3.6)^{b}	70(15.6)^{b}	0^{a}	46(10.2)^{a}	18(4.0)^{b}	27(6.0)^{b}	10(2.2)^{b}	24(5.3)^{b}	163(36.3)^{b}	1(0.2)^{b}	140(31.2)^{b}
		2015-2019 (n=289)	41(14.2)^{a}	0^{b}	14(4.8)^{b}	1(0.3)^{a}	34(11.8)^{a}	15(5.2)^{ab}	109(37.7)^{a}	1(0.3)^{a}	58(20.1)^{b}	5(1.7)^{b}	7(2.4)^{a}	2(0.7)^{ab}	89(30.8)^{c}	225(77.9)^{a}	4(1.4)^{ab}	170(58.8)^{a}
Salmonella	Overall (n=816)		21(2.6)	2(0.2)	35(4.3)	na	20(2.5)	na	19(2.3)	na	40(4.9)	na	6(0.7)	15(1.8)	81(9.9)	na	1(0.1)	22(2.7)
(n=816)	By source (n= 816)	Pig (n=169)	3(1.8)^{a,b}	0^{a}	18(10.7)^{a}	na	1(0.6)^{a}	na	6(3.6)^{a}	na	34(20.1)^{a}	na	0^{a}	0^{a}	12(7.1)^{a}	na	0^{a}	8(4.7)^{a}
		Pork (n=510)	18(3.5)^{a}	2(0.4)^{a}	14(2.7)^{b}	na	6(1.2)^{a}	na	13(2.5)^{a,b}	na	4(0.8)^{b}	na	6(1.2)^{a}	9(1.8)^{a,b}	36(7.1)^{a}	na	0^{a}	14(2.7)^{a}
		Human (n=137)	0^{b}	0^{a}	3(2.2)^{b}	na	13(9.5)^{b}	na	0^{b}	na	2(1.5)^{b}	na	0^{a}	6(4.4)^{b}	33(24.1)^{b}	na	1(0.7)^{a}	0^{b}
	By year	2005-2010 (n=164)	0^{a}	2(1.2)^{a}	7(4.3)^{ab}	na	15(9.1)^{a}	na	8(4.9)^{a}	na	1(0.6)^{a}	na	5(3.0)^{a}	3(1.8)^{a}	10(6.1)^{a}	na	0^{a}	9(5.5)^{a}
		2011-2014 (n=415)	15(3.6)^{b}	0^{a}	11(2.7)^{a}	na	5(1.2)^{b}	na	10(2.4)^{ab}	na	6(1.4)^{a}	na	1(0.2)^{b}	7(1.7)^{a}	50(12.0)^{b}	na	1(0.2)^{a}	11(2.7)^{ab}
		2015-2019 (n=237)	6(2.5)^{ab}	0^{a}	17(7.2)^{b}	na	0^{b}	na	1(0.4)^{b}	na	33(13.9)^{b}	na	0^{b}	5(2.1)^{a}	21(8.9)^{ab}	na	0^{a}	2(0.8)^{b}

^{a,b,c} Values with different superscripts in the same column and category indicated statistical difference (p <0.05) among *E. coli* or *Salmonella* from different sources or years.

na, No associations due to the lack of the corresponding replicon types.

Table 6 Odds ratio between the presence of replicon types and antimicrobial resistance or ESBLs producing *Escherichia coli* (n=1,047) and *Salmonella* (n=816)

Bacterial strain	Type of replicons	Odd ratio of antimicrobial resistance phenotype (95%CI)									
		AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP	COL	ESBLs producer
E. coli	HI1	4.96(2.0-12.4)[a]	2.62(1.8-3.9)[a]	5.46(3.7-8.0)[a]	5.0(3.4-7.3)[a]	3.7(2.4-5.7)[a]	4.2(2.4-7.4)[a]	12.1(3.8-38.4)[a]	2.56(1.7-3.9)[a]	6.83(4.6-10.1)[a]	2.5(1.6-3.8)[a]
	HI2	na	na	na	-	na	na	na	-	20.34(6.7-62.1)[a]	3.36(1.3-8.7)[a]
	I1-γ	-	-	0.52(0.3-0.9)[b]	4.77(3.2-7.0)[a]	2.5(1.7-3.9)[a]	-	-	0.55(0.4-0.8)[b]	-	6.33(4.2-9.5)[a]
	N	4.95(1.8-13.7)[a]	2.78(1.8-4.3)[a]	3.41(2.3-5.1)[a]	2.34(1.6-3.5)[a]	1.83(1.2-2.8)[a]	2.8(1.6-4.7)[a]	3.3(1.6-6.9)[a]	2.95(1.8-4.8)[a]	1.79(1.1-2.8)[a]	1.82(1.1-2.9)[a]
	FIA	-	-	3.67(2.2-6.3)[a]	-	2.0(1.1-3.6)[a]	5.22(2.1-13.2)[a]	2.61(1.02-6.6)[a]	2.53(1.3-4.8)[a]	-	0.19(0.04-0.8)[b]
	FIB	3.16(1.9-5.3)[a]	1.62(1.2-2.1)[a]	2.0(1.5-2.7)[a]	-	1.56(1.2-2.1)[a]	2.15(1.6-3.0)[a]	2.82(1.8-4.3)[a]	1.58(1.2-2.1)[a]	2.36(1.7-3.3)[a]	-
	Y	3.37(1.5-7.4)[a]	-	1.61(1.1-2.4)[a]	1.92(1.3-2.8)[a]	2.39(1.6-3.6)[a]	-	2.17(1.2-3.9)[a]	1.91(1.3-2.9)[a]	1.93(1.3-3.0)[a]	2.57(1.7-4.0)[a]
	P	-	-	na	0.1(0.01-0.7)[b]	-	-	-	-	na	na
	FIC	-	0.28(0.1-0.6)[b]	-	-	-	-	-	-	-	-
	A/C	na	-	-	-	-	-	-	-	-	-
	FIIAs	-	-	-	0.65(0.4-0.9)[b]	-	-	-	-	1.56(1.02-2.3)[a]	0.51(0.2-0.9)[b]
	K	2.38(1.7-3.4)[a]	2.39(1.9-3.1)[a]	1.77(1.3-2.4)[a]	1.99(1.5-2.6)[a]	2.2(1.7-2.8)[a]	2.38(1.8-3.1)[a]	3.3(2.4-4.6)[a]	2.07(1.6-2.7)[a]	1.47(1.04-2.1)[a]	2.0(1.4-2.9)[a]
	B/O	-	-	-	-	-	-	na	9.47(1.3-72.0)[a]	3.06(1.1-8.5)[a]	na
	F	2.17(1.5-3.1)[a]	1.87(1.5-2.4)[a]	1.4(1.1-1.9)[a]	2.55(1.9-3.3)[a]	1.57(1.2-2.0)[a]	1.59(1.2-2.1)[a]	3.0(2.1-4.2)[a]	-	2.76(1.9-3.9)[a]	3.23(2.2-4.7)[a]
Salmonella	HI1	na	46.8(6.2-350.8)[a]	na	4.04(1.7-9.7)[a]	0.22(0.1-0.6)[b]	na	-	-	11.9(2.3-60.8)[a]	159.9(52.0-491.3)[a]
	HI2	-	-	na	-	-	na	-	-	na	na
	I1-γ	-	-	na	4.5(2.3-9.0)[a]	-	-	-	-	na	5.8(1.8-18.3)[a]
	N	-	44.3(5.9-332.7)[a]	14.4(4.7-43.9)[a]	8.8(3.3-23.2)[a]	-	-	-	-	na	na

Bacterial strain	Type of replicons	Odd ratio of antimicrobial resistance phenotype (95%CI)									
		AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP	COL	ESBLs producer
	FIB	-	4.8(1.8-13.0)[a]	na	-	na	-	na	-	-	na
	Y	0.17(0.1-0.5)[b]	na	-	-	na	na	na	-	na	na
	FIC	0.1(0.02-0.7)[b]	-	na	na	-	-	0.2(0.04-0.98)[b]	-	20.1(2.1-191.6)[a]	na
	A/C	na	14.8(3.3-65.9)[a]	na	14.96(4.2-53.7)[a]	na	na	na	-	na	24.5(7.5-80.0)[a]
	FIIAs	0.15(0.1-0.2)[b]	-	-	-	0.36(0.2-0.6)[b]	-	0.2(0.1-0.3)[b]	-	-	-
	F	-	4.85(2.0-12.0)[a]	na	-	na	-	na	-	-	-

OR>1, The resistance to the drug increased with the presence of corresponding replicon types.

OR<1, The resistance to the drug decreased with the presence of corresponding replicon types.

[a,b] Statistically-significant association (95% CI did not cross 1) between the presence of plasmids in particular Inc groups and resistant or ESBLs producing strains. - No statistically significant association (95% CI cross 1) between the presence of plasmids in particular Inc groups and resistant or ESBLs producing strains.

na, no OR due to the lack of the corresponding replicon types.

AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; STR, streptomycin; SMZ, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; COL, colistin.

2.4.4 Associations between replicon types in *E. coli* and *Salmonella*

Associations between each replicon type were diverse (Table 7). The significant positive association between IncFIB and B/O in *E. coli* was the strongest (OR=41.24). The presence of IncFIB exhibited the strongest positive association with IncF (OR=24.26), FIA (OR=8.85), FIC (OR=2.23) replicons in *E. coli* only. The replicons with the strongest positive associations to IncHI1 (OR=5.58), Y(OR=3.77) and FIIAs (OR=3.86) were IncN, P and K, respectively. The negative association between IncY and F replicons (OR=0.66) was the strongest in *E. coli*. In *Salmonella*, the strongest positive association was observed between IncHI2 and IncN (OR=41.84). IncHI1 was positively associated with IncI1-γ (OR=5.80) and FIIAs (OR=4.87). The positive associations were additionally detected for IncI1-γ /IncY (OR=14.03) and IncA/C/IncN (OR=17.84).

2.4.5 Replicon sequence types of *E. coli* and *Salmonella* carrying *bla* and/or *mcr*

Twenty-six ESBL-producing *E. coli* from pigs (n=11), pork (n=8), and humans (n=7) and three *Salmonella* from a pig (n=1) and pork (n=2) were further subtyped using RST. Seven allele numbers of FII replicon including F-, F46, F18, F2, F29, F100 and S1 were identified. Three alleles including A-, A1,6 and A5,6 were detected in the FIA allele, while seven alleles (i.e. B-, B1, B20, B10, B40, B24 and B13) were observed in the FIB allele. The S1 allele was identified in two *Salmonella* carrying FIIs replicon. Thirteen FAB formulas were assigned (Table 8), of which the most common FAB formula between *E. coli* and *Salmonella* were F2:A-:B- (26.9%, 7/26) and S1:A-:B- (66.7%, 2/3), respectively.

F46:A-:B20 was the FAB formula shared in four *E. coli* isolates (15.4%, 4/26) from pigs (n=3) and one human. F18:A-:B1 was in the *E.coli* isolates (11.5%, 3/26) from pig (n=1) and pork (n=2). While F-:A-:B24 was found in the *E. coli* strains (11.5%, 3/26) isolated from pork (n=3). Two different FAB formulas, S1:A-:B- and F2:A-:B-, were assigned for plasmid in the *Salmonella* isolates.

Table 7 Odds ratio between each two replicon types presented in *Escherichia coli* (n=1,047) and *Salmonella* (n=816)

Bacterial	Replicon	Odd ratio of replicon types (95%CI)													
strain	type	HI1	HI2	I1-γ	N	FIA	FIB	Y	P	FIC	A/C	FIIAs	K	B/O	F
E. coli	HI1	nd	na	-	5.58 (3.60-8.3)[a]	0.11 (0.02-0.79)[b]	1.55 (1.06-2.26)[a]	-	na	-	-	-	1.62 (1.10-2.39)[a]	3.10 (1.06-9.05)[a]	-
	HI2	na	nd	na	na	na	na	na	na	na	na	na	-	na	na
	I1-γ	-	na	nd	0.06 (0.01-0.41)[b]	-	-	-	-	-	-	0.22 (0.09-0.55)[b]	1.65 (1.11-2.47)[a]	3.31 (1.14-9.73)[a]	3.08 (2.05-4.62)[a]
	N	5.58 (3.60-8.3)[a]	na	0.06 (0.01-0.41)[b]	nd	-	0.54 (0.32-0.89)[b]	-	na	-	-	-	2.00 (1.28-3.11)[a]	na	-
	FIA	0.11(0.02-0.79)[b]	na	-	-	nd	8.85 (4.84-16.18)[a]	-	-	-	-	-	-	-	3.96 (2.12-7.43)[a]
	FIB	1.55 (1.06-2.26)[a]	na	-	0.54 (0.32-0.89)[b]	8.85 (4.84-16.18)[a]	nd	-	-	2.32 (1.22-4.42)[a]	-	-	1.34 (1.01-1.78)[a]	41.24 (5.42-313.65)[a]	24.26 (15.43-38.14)[a]
	Y	-	na	-	-	-	-	nd	3.77 (1.57-9.06)[a]	-	-	1.67 (1.05-2.65)[a]	1.64 (1.11-2.43)[a]	na	0.66 (0.46-0.96)[b]
	P	na	na	-	na	-	-	3.77 (1.57-9.06)[a]	nd	-	na	-	-	na	-
	FIC	-	na	-	-	-	2.32 (1.22-4.42)[a]	-	-	nd	na	-	-	na	2.15 (1.09-4.23)[a]
	A/C	-	na	-	-	-	-	-	na	na	nd	na	-	na	-
	FIIAs	-	na	0.22 (0.09-0.55)[b]	-	-	-	1.67 (1.05-2.65)[a]	-	-	na	nd	3.86 (2.45-6.10)[a]	na	-
	K	1.62 (1.10-2.39)[a]	-	1.65 (1.11-2.47)[a]	2.00 (1.28-3.11)[a]	1.19 (0.69-2.06)[a]	1.34 (1.01-1.78)[a]	1.64 (1.11-2.43)[a]	-	-	-	3.86 (2.45-6.10)[a]	nd	9.98 (1.31-75.87)[a]	1.59 (1.24-2.05)[a]
	B/O	3.10 (1.06-9.05)[a]	na	3.33 (1.14-9.73)[a]	na	-	41.24 (5.42-313.65)[a]	na	na	na	na	na	9.98 (1.31-75.87)[a]	nd	16.12 (2.12-122.46)[a]
	F	-	na	3.08 (2.05-4.62)[a]	-	3.96 (2.12-7.43)[a]	24.26 (15.43-38.14)[a]	0.66 (0.46-0.96)[b]	-	2.15 (1.09-4.23)[a]	-	-	1.59 (1.24-2.05)[a]	16.12 (2.12-122.46)[a]	nd
Salmonella	HI1	nd	na	5.80(1.84-18.26)[a]	na	na	na	na	na	na	na	4.87 (1.91-12.45)[a]	na	na	na
	HI2	na	nd	na	41.84 (2.52-694.23)[a]	na	na	na	na	na	na	na	na	na	na
	I1-γ	5.80 (1.84-18.26)[a]	na	nd	na	na	-	14.03 (6.35-31.02)[a]	na	na	na	-	na	na	-
	N	na	41.84 (2.52-694.23)[a]	na	nd	na	na	na	na	na	17.84 (5.13-62.07)[a]	na	na	na	na
	FIB	na	na	-	na	na	nd	na	na	na	-	na	na	na	na
	Y	na	na	14.03	na	na	na	nd	na	na	na	na	na	na	na

Bacterial strain	Replicon type	Odd ratio of replicon types (95%CI)													
		HI1	HI2	I1-γ	N	FIA	FIB	Y	P	FIC	A/C	FIIAs	K	B/O	F
				(6.35-31.02)[a]											
	FIC	na	na	na	na	na	na	na	na	nd	na	-	na	na	na
	A/C	na	na	na	17.84(5.13-62.07)[a]	na	-	na	na	na	nd	na	na	na	-
	FIIAs	4.87(1.91-12.45)[a]	na	-	na	na	na	na	na	-	na	nd	na	na	na
	F	na	na	-	na	na	na	na	na	na	-	na	na	na	nd

OR>1, The presence of the replicon type increased with the presence of corresponding replicon types.

OR<1, The presence of the replicon type decreased with the presence of corresponding replicon types.

[a,b] Statistically-significant association (95% CI did not cross 1) between the presence of plasmids in particular Inc groups and resistant or ESBLs producing strains.

- No statistically significant association (95% CI cross 1) between the presence of plasmids in particular Inc groups and resistant or ESBLs producing strains.

na, no OR due to the lack of the corresponding replicon types.

nd, no OR because the statistics could not be determined.

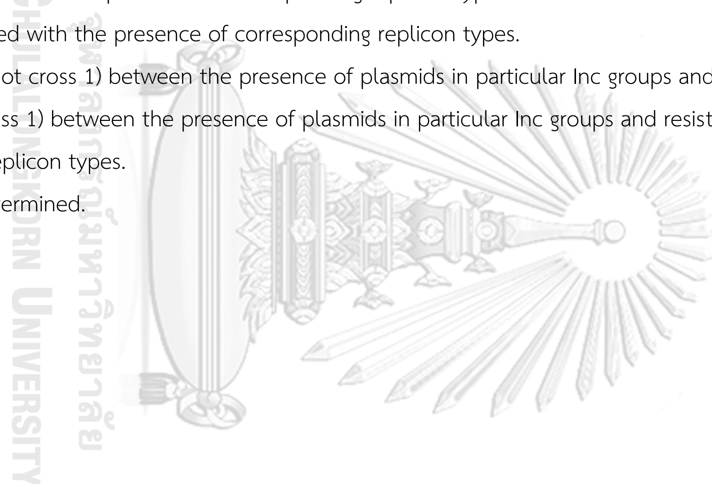


Table 8 Replicon sequence types of Inc F of *Escherichia coli* (n=26) and *Salmonella* (n=3)

Species	Strain name	Regions[a]	Provinces	Sources	Year	Resistance genes	Allele number for replicon			FAB Formula[d]
							FII,FIIs[c]	FIA	FIB	
E. coli	CREM 10	N	CRI	Pork	2016-2017	bla_{CTX-M}, bla_{TEM}	F46	-	-	F46:A-:B-
	CRES 14	N	CRI	Pig	2016-2017	bla_{CTX-M}, bla_{TEM}	F46	-	-	F46:A-:B-
	CRES 7	N	CRI	Pig	2016-2017	*mcr1*	F46	-	B20	F46:A-:B20
	FpCa1	W	RBR	Pig	2015	bla_{CTX-M}, bla_{TEM}, *mcr1*	F46	-	B20	F46:A-:B20
	FpEa24	W	RBR	Pig	2016-2017	bla_{CTX-M}, bla_{TEM}, *mcr1*	F46	-	B20	F46:A-:B20
	NK 253	NE	NKI	Human	2013-2014	bla_{CTX-M}	F46	-	B20	F46:A-:B20
	CRES 20	N	CRI	Pig	2016-2017	*mcr3*	F18	-	B1	F18:A-:B1
	MH 95	NE	MDH	Pork	2013-2014	bla_{CTX-M}, bla_{TEM}	F18	-	B1	F18:A-:B1
	SaEM 37	E	SKW	Pork	2016-2017	*mcr1*	F18	-	B1	F18:A-:B1
	E405	NE	NMA	Pig	2007-2008	bla_{CTX-M}, bla_{TEM}, *mcr3*	F2	-	-	F2:A-:B-
	MH 70	NE	MDH	Human	2013-2014	bla_{CTX-M}	F2	-	-	F2:A-:B-
	SaEM 19	E	SKW	Pork	2016-2017	bla_{CTX-M}, bla_{TEM}	F2	-	-	F2:A-:B-
	SaEM 29	E	SKW	Pork	2016-2017	bla_{CTX-M}	F2	-	-	F2:A-:B-
	SaES 22	E	SKW	Pig	2016-2017	bla_{CTX-M}, bla_{TEM}, *mcr3*	F2	-	-	F2:A-:B-
	NK 261	NE	NKI	Human	2013-2014	bla_{CTX-M}, bla_{TEM}	F2	-	-	F2:A-:B-
	NK 262	NE	NKI	Human	2013-2014	bla_{CTX-M}, bla_{TEM}	F2	-	-	F2:A-:B-
	E431	W	RBR	Pig	2007-2008	*mcr2, mcr3*	F2	-	B40	F2:A-:B40
	MH 227	NE	MDH	Human	2013-2014	bla_{CTX-M}	F29	-	B10	F29:A-:B10
	NK 276	NE	NKI	Human	2013-2014	bla_{CTX-M}	F46	-	B24	F46:A-:B24
	PLCa 7	NE	NMA	Pig	2015	bla_{CTX-M}, bla_{TEM}, *mcr1*	F2	-	B20	F2:A-:B20
	PLEa 14	NE	NMA	Pig	2015	bla_{CTX-M}, bla_{TEM}, *mcr1*	F100	-	B13	F100:A-:B13
	SaES 46	E	SKW	Pig	2016-2017	*mcr1*	F18	A5, A6[e]	B1	F18:A5,6:B1
	NK 267	NE	NKI	Human	2013-2014	bla_{TEM}	-	A1, A6[e]	B1	F-:A1,6:B1
	CREM 48	N	CRI	Pork	2016-2017	bla_{CTX-M}, bla_{TEM}	-	-	B24	F-:A-:B24
	SaEM 15	E	SKW	Pork	2016-2017	bla_{CTX-M}, bla_{TEM}	-	-	B24	F-:A-:B24
	SaEM 57	E	SKW	Pork	2016-2017	bla_{CTX-M}, bla_{TEM}	-	-	B24	F-:A-:B24
S. Weltervreden	MH 178.1	NE	MDH	Pork	2013-2014	$bla_{CTX-M14}$	S1	-	-	S1:A-:B-
S. Yalding	NSM 11.3	NE	NKI	Pork	2016-2017	*mcr1*	S1	-	-	S1:A-:B-
S. Anatum	CRSS 28.1	N	CRI	Pig	2016-2017	bla_{CTX-M}, bla_{TEM} ,*mcr3*	F2	-	-	F2:A-:B-

[a] N, Northern; NE, North-eastern; W, West; E, East.

[b] CRI, Chiangrai; RBR, Ratchaburi; NKI, Nongkhai; MDH, Mukdaharn; SKW, Sakaew; NMA, Nakornratchsrima

[c] Both sequences of FII and FIIs were identified to be allele F.

[d] FAB formula was the combination of the sequence type of FII or FIIs: FIA: FIB.

[e] Exactly matched to more than one references.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 9 Replicon patterns among *E. coli* (n=1,047) and *Salmonella* (n=816).

Replicon pattern[a]	*Escherichia coli*			*Salmonella spp.*		
	No. of isolate (%)	No. of ESBL producing isolate (%)	No. of *mcr* carrying isolate (%)	No. of isolate (%)	No. of ESBL producing isolate (%)	No. of *mcr* carrying isolate (%)
A/C	1 (0.1)	-	-	10 (4.8)	5 (23.8)	-
A/C-F	3 (0.3)	1 (0.6)	-	1 (0.5)	-	-
A/C-K	1 (0.1)	-	-	-	-	-
A/C-K-F	2 (0.2)	-	-	-	-	-
B/O	1 (0.1)	-	-	1 (0.5)	-	-
B/O-K-F	10 (1.1)	-	1 (0.9)	-	-	-
F	129 (14.4)	13 (8.4)	8 (7.3)	97 (46.2)	1 (5.3)	3(60.0)
F-Y-K	36 (4.0)	11 (7.1)	4 (3.7)	-	-	-
HI1	8 (0.9)	2 (1.3)	2 (1.8)	10 (4.8)	8 (42.1)	1 (20.0)
HI1-F	10 (1.1)	1 (0.6)	5 (4.6)	7 (3.0)	1 (5.3)	1 (20.0)
HI1-HI2-F	4 (0.4)	-	4 (3.7)	-	-	-
HI1-HI2-K-F	15 (1.7)	7 (4.5)	11 (10.1)	-	-	-
HI1-I1-γ	-	-	-	4 (1.9)	4 (21.1)	-
HI1-I1-γ-F	2 (0.2)	-	-	-	-	-
HI1-I1-γ-K-B/O-F	5 (0.6)	-	5 (4.6)	-	-	-
HI1-I1-γ-K-F	3 (0.3)	1 (0.6)	1 (0.9)	-	-	-
HI1-K	11 (1.2)	1 (0.6)	7 (6.4)	-	-	-
HI1-K-F	24 (2.7)	2 (1.3)	6 (5.5)	-	-	-
HI1-N	5 (0.6)	-	-	-	-	-
HI1-N-A/C-K	1 (0.1)	-	-	-	-	-
HI1-N-F	8 (0.9)	3 (1.9)	3 (2.8)	-	-	-
HI1-N-K	13 (1.5)	8 (5.2)	-	-	-	-
HI1-N-K-F	11 (1.2)	2 (1.3)	-	-	-	-
HI1-N-Y	1 (0.1)	-	-	-	-	-
HI1-N-Y-F	1 (0.1)	-	-	-	-	-
HI1-N-Y-K	1 (0.1)	-	-	-	-	-
HI1-N-Y-K-F	2 (0.2)	-	-	-	-	-
HI1-Y	1 (0.1)	1 (0.6)	-	-	-	-
HI1-Y-A/C-K	1 (0.1)	1 (0.6)	-	-	-	-
HI1-Y-F	1 (0.1)	1 (0.6)	-	-	-	-
HI1-Y-K	8 (0.9)	8 (5.2)	1 (0.9)	-	-	-
HI1-Y-K-F	1 (0.1)	-	-	-	-	-
HI2	-	-	-	1 (0.5)	-	-
HI2-N	-	-	-	1 (0.5)	-	-
I1-γ	8 (0.9)	1 (0.6)	-	16 (7.6)	-	-
I1-A/C-F	1 (0.1)	-	1 (0.9)	-	-	-
I1-F	16 (1.8)	4 (2.6)	1 (0.9)	3 (1.4)	-	-
I1-γ-F-Y-P	1 (0.1)	-	-	-	-	-
I1-γ-K	9 (1.0)	-	-	-	-	-
I1-γ-K-F	61 (6.8)	42 (27.3)	4 (3.7)	-	-	-

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Replicon pattern[a]	*Escherichia coli*			*Salmonella spp.*		
	No. of isolate (%)	No. of ESBL producing isolate (%)	No. of *mcr* carrying isolate (%)	No. of isolate (%)	No. of ESBL producing isolate (%)	No. of *mcr* carrying isolate (%)
I1-γ-N-K-F	1 (0.1)	-	-	-	-	-
I1-γ-P-F	1 (0.1)	-	-	-	-	-
I1-γ-Y	6 (0.7)	3 (1.9)	-	12 (5.7)	-	-
I1-γ-Y-A/C-K-F	1 (0.1)	-	-	-	-	-
I1-γ-Y-F	3 (0.3)	-	-	-	-	-
I1-γ-Y-K-F	3 (0.3)	2 (1.3)	1 (0.9)	-	-	-
I1-γ-Y-K	8 (0.9)	4 (2.6)	2 (1.8)	-	-	-
K	106 (11.9)	1 (0.6)	3 (2.8)	-	-	-
K-F	212 (23.7)	17 (11.0)	20 (18.3)	-	-	-
N	4 (0.4)	-	1 (0.9)	15 (7.1)	-	-
N-A/C	-	-	-	4 (1.9)	-	-
N-F	9 (1.0)	5 (3.2)	2 (1.8)	-	-	-
N-K	25 (2.8)	1 (0.6)	3 (2.8)	-	-	-
N-K-F	22 (2.5)	4 (2.6)	5 (4.6)	-	-	-
N-Y	1 (0.1)	-	-	-	-	-
N-Y-A/C-K	1 (0.1)	-	-	-	-	-
N-Y-K	1 (0.1)	-	-	-	-	-
N-Y-K-F	5 (0.6)	3 (1.9)	-	-	-	-
P	5 (0.6)	-	-	-	-	-
P-F	3 (0.3)	-	-	-	-	-
P-K	3 (0.3)	-	-	-	-	-
P-K-F	3 (0.3)	-	-	-	-	-
W-K-F	1 (0.1)	-	-	-	-	-
X-F	1 (0.1)	-	-	-	-	-
Y	12 (1.3)	-	3 (2.8)	28 (13.3)	-	-
Y-F	12 (1.3)	2 (1.3)	5 (4.6)	-	-	-
Y-K	21 (2.4)	2 (1.3)	-	-	-	-
Y-P-F	1 (0.1)	-	-	-	-	-
Y-P-K	2 (0.2)	-	-	-	-	-
Y-P-K-F	4 (0.4)	-	-	-	-	-
Positive at least one replicon type	893 (85.3)	154 (99.4)	109 (100)	210 (25.7)	19 (90.5)	5 (41.7)
No replicon pattern	154 (14.7)	1 (0.6)	-	606 (74.3)	2 (9.5)	7 (58.3)
Total	1047	155	109	816	21	12

[a] F, at least one replicon type of IncF family replicon (i.e., FIA, FIB, FIC, FIIAs and F) was found.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.5 DISCUSSION

The *E. coli* and *Salmonella* isolates in this study originated from clinically healthy pigs, pork, and humans previously collected across geographical regions over a long sampling period. It is expected that only healthy animals are slaughtered for human consumption, but their healthy appearance does not guarantee the absence of resistant bacteria. Antimicrobials may be administered to the animals prior to slaughtering for infection treatment, disease prevention or growth promotion and such antimicrobial use could result in AMR acquisition in commensal bacteria and pathogens. Antimicrobial susceptibilities and determinants were investigated among the isolates in this collection. However, they have not been thoroughly investigated for resistance plasmids, despite their important role in resistance traits and resistance gene dissemination.

Up to now, most studies of plasmid Inc groups have been based on the resistance genes identified. Due to the lack of wide screening reports on Inc groups, a direct comparison is rather difficult. In this study, IncK was the most frequently plasmid replicon type present in *E. coli* (60.6%) from pigs, pork and humans. Currently, there are two IncK plasmid subtypes identified, including IncK1, that are commonly found in a variety of mammals, and IncK2 that were predominantly found in poultry (Rozwandowicz M, et al., 2019). While studies of the Inc group are widely available for the *E. coli* isolates from pigs and pork, there is still very limited research covering IncK plasmids. Most IncK studies were conducted in the isolates of humans and poultry originally from European countries (Rozwandowicz M, et al., 2017; Randall LP, et al., 2011). In addition, the absence of IncK in the *Salmonella* isolates in this study supported a previous study demonstrating that some replicon types are specific to certain bacterial hosts (Redondo-Salvo S, et al., 2020).

When considering the sampling period of *E. coli*, IncK plasmid was continuously predominant from 2007 to 2019. In contrast, the prevalence of most of the others fluctuated. For example, HI1, N, FIA, FIB, FIIAs, K, B/O and F decreased from 2011-

2014 and increased between 2015 and 2019. The opposite trend was observed for P, FIC, A/C. Factors that affect the maintenance of some plasmids in each period remain unclear. These changes may be involved in different sampling locations and antimicrobial use. However, the phenomenon was not obvious in *Salmonella*, and this could be due to the limited replicon type observed. In addition, many plasmids of the same Inc group were found in the *E. coli* isolates from pigs, pork, and humans, indicating the circulation of the plasmids in different sectors.

The PBRT primers used for the detection of IncI1 in this study cannot differentiate IncI1 and IncI-γ (Smith H, et al., 2015). Therefore, the IncI1-γ type was used to describe the results obtained. In this study, the coexistence of IncI1-γ type and IncHI1 was observed in *Salmonella* (OR>1), in agreement with a previous study conducted on MDR *Salmonella* Typhi (Mutai WC, et al., 2019). Most *Salmonella* from pigs carried IncY replicon, in line with a previous report (Zhang C, et al., 2017). In addition, IncT and IncW plasmids were unidentified among the isolates in this study. This agrees with the notion that IncT and IncW are rarely detected among bacteria in the *Enterobacteriaceae* family in recent decades (Harada S, et al., 2012; Fernandez-Lopez R, et al., 2006)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

IncL/M, a broad host-range plasmid, was not detected in this study. The L and M plasmids were mistakenly classified together into an incompatibility group due to their high DNA homology and later, they were genetically differentiated to two different groups (Carattoli A, et al., 2005). Therefore, the absence of IncL/M plasmid in this study may be a false-negative result due to PCR primers used (Carattoli A, et al., 2005). Simultaneously, IncX was absent in *Salmonella*. The limited detection of IncX plasmids may be attributable to the uncovered typing scheme. The PCR primers of the PBRT scheme used in this study were specific to IncX2. However, IncX plasmids are diverse and at least nine types of IncX (i.e. X1 to X9) have been identified worldwide (Dobiasova H, Dolejska M., 2016). Therefore, the detection

capacity of the IncX plasmid family should be expanded to enhance the identification and typing of novel AMR-related plasmids in *Enterobacteriaceae*.

It is important to observe that the same Inc plasmids are shared among the *E. coli* and *Salmonella* isolates that originated from different sources (e.g., pigs, pork and humans). Even though the direction of gene flow between different hosts was not investigated, such observations indicate the circulation of plasmids between different hosts.

Multiple plasmids of different Inc groups were found in the same bacterial host strain in this study (Table 9). Since several AMR genes are plasmid mediated and a plasmid could carry several AMR genes, the presence of multiple plasmids agreed with the MDR phenotypes observed. The association between resistance phenotypes and replicon types varied. The significant-positive associations between resistance phenotype and replicon types were commonly observed, highlighting the important role of plasmids in the dissemination of AMR genes in *E. coli* and *Salmonella* in this study. IncHI1 plasmids in *E. coli* exhibited the strongest association with increased resistance rates to AMP, GEN, STR and TET resistance (OR>1), suggesting the existence of corresponding resistance genes on the plasmid of this replicon type. In Salmonella, IncHI1 plasmid was strongly associated with CHP resistance (OR=46.8), inconsistent with a previous study where the strong positive correlation of IncHI1 plasmids to AMP, TMP, SMZ, STR and TET resistance was demonstrated in the pathogen (Mutai WC, et al., 2019). This discrepancy may be from the effects of different antimicrobial-selective pressure in the environment of the bacterial isolates.

Persistent resistance to chloramphenicol after the ban on its use in food-producing animals has been observed in several countries (Frye JG, Jackson CR., 2013; Ibrahim S, et al., 2021; Bischoff KM, et al., 2005). It was linked to co-selection caused by using other antibiotics, of which their resistance genes co-localized on the same plasmid with chloramphenicol-resistance genes. In this study, the chloramphenicol resistance rate in *E. coli* was significantly correlated to IncN (OR=2.78). This plasmid replicon

type was positively associated with resistance to the commonly used antimicrobials including AMP, GEN, STR, SMZ, TET, TMP and COL. In *Salmonella*, in addition to CHP resistance, IncHI1 plasmid was strongly associated with GEN and COL resistance and ESBL production. Such positive associations indicate the possible co-localization on the same plasmids of the resistance genes and serve as evidence that the selective pressure imposed by the use of other antimicrobials commonly used in food animals could promote the co-selection of chloramphenicol resistant bacteria after the ban. However, further studies to analyze the plasmid context are suggested to confirm the co-localization of AMR genes on the same plasmid.

Conversely, negative correlations were observed between some resistance genes and replicon types. For example, IncY in *Salmonella* was significantly associated with reduced frequencies of AMP resistance (OR=0.17). Similarly, IncFIC (OR=0.2) and FIIAs (OR=0.2) plasmids were significantly associated with a reduced prevalence of tetracycline resistance. This indicates that these plasmids do not frequently carry resistance genes for these tested antibiotics. Besides, non-plasmid borne mechanisms (e.g. chromosomally encoded genes, chromosomal mutations) may present and contribute to antibiotic resistance in these bacteria (Jahantigh M, et al., 2020).

Strong positive associations were observed between CIP and IncHI1 plasmids in *E. coli* (OR=5.46) and IncN plasmid in *Salmonella* (OR=14.4). The high quinolone resistance level in bacteria is mediated by chromosomal mutations that alter drug targets and reduce the intracellular concentration of quinolones. The presence of plasmid-mediated quinolone resistance (PMQR) genes provides low-level resistance, not exceeding the clinical breakpoint for susceptibility. However, PMQR genes facilitate higher levels of quinolone resistance if a plasmid carries two or more PMQR genes (Jacoby GA, et al., 2014).

In this study, colistin resistance exhibited a strong positive association with IncHI2 (OR=20.34) and IncHI1 (OR=6.83) in *E. coli* and IncFIC (OR = 20.1) and IncHI1 (OR=11.9) in *Salmonella*, in agreement with a previous study (Zakaria AS, rt al., 2021). Colistin-

resistance encoding genes were previously found on plasmids of several replicon types including IncI2, HI1, HI2, X4, P, F and Y (Sadek M, et al., 2021). A previous study revealed that the IncI2 replicon was the most common plasmid carrying colistin resistance gene in *E. coli* isolated from poultry, food and humans. However, this was not the case for this study (Elbediwi M, et al., 2019).

ESBL genes are usually plasmid-borne. In this study, ESBL production showed the strongest positive association with IncI1-**γ** plasmid (OR=6.33) in *E. coli* and IncHI1 plasmid (OR=159.9) in *Salmonella* (Table 11). This indicates the possible localization of ESBL genes on these plasmid replicon types, in agreement with a previous study in *E. coli* (Dierikx C, et al., 2010) and *Salmonella* (Mutai WC, et al., 2019), respectively. This was supported by the observation that the $bla_{CTX\text{-}M14}$ carrying *Salmonella* from pork (n=4) in this study was postive for IncI1-**γ** and HI1 plasmids (Table 14). Almost all $bla_{CTX\text{-}M\text{-}}$carrying IncI1-**γ** -positive isolates also contained both IncF and IncK plasmids (43/57, 75.4%). When considering ESBL genes, most $bla_{CTX\text{-}M}$ carrying *E. coli* (106/155, 68.3%) were positive for IncK plasmid, in agreement with a previous study in Europe (Cottell JL, et al., 2011). Since these isolates harbored multiple plasmids, the location of $bla_{CTX\text{-}M}$ was uncertain and could be further investigated by plasmid characterization.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The presence of genes encoding ESBLs and colistin resistance were presented in previous study that associated with IncF family plasmids in *Enterobacteriaceae* (Li R, et al., 2018). In this study, the IncF family replicon, including FIA, FIB, FIC, FIIAs and F was the most common in both *E. coli* and *Salmonella* strains. Of all the 13 FAB formulas obtained, the most common FAB formula of *E. coli* was F2:A-:B- as previously observed in many studies (Yang QE, et al., 2015; Chen X, et al., 2014). F plasmid belonging to F46:A-:B20 was identified in the *E. coli* isolates from pigs and humans. This plasmid was previously reported in *Salmonella* Typhimurium from a patient in Taiwan (Chen CY, et al., 2019). The F18:A-:B1 plasmid was also found in *E. coli* from pigs and pork. This plasmid was previously found in *E.coli* from poultry

(Yang QE, et al., 2015). The same FAB formula of IncF plasmid was found among the strains from different pigs, pork and humans from various locations, indicating that the particular plasmids circulate in the food chain. Further studies are suggested to investigate if the circulation was due to horizontal transfer of the plasmid or the bacterial strain dissemination.

In summary, the results revealed a variety of plasmids distributed in pigs, pork, and humans in Thailand. Plasmids were strongly associated with various resistance phenotypes. Multiple plasmids were found in the same host strain, and their major role in the spread of AMR was emphasized. Plasmid analysis serves as an epidemiological marker for AMR surveillance. To the best of our knowledge, this is the first report of plasmid replicon types among *E. coli* and *Salmonella* from pigs, pork and human in Thailand. The findings of the replicon type in this study forms a basis for future studies to explore the possible methodology to counteract horizontal transfer of plasmids.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER III

Genomic analysis of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand

Jiratchaya Puangseree[a], Rungtip Chuanchuen[a,*]

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

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Manuscript is in preparation.

Genomic analysis of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand

3.1 ABSTRACT

Fifteen Salmonella isolates with class1 integrons carrying *dfrA12-aadA2* cassette array on transferable plasmids were included in this study. All were isolated as part of previous studies from healthy pigs (n=8), poultry (n=4), pork (n=2) and human (n=1) during 2020-2022. The presence and transfer of class 1 integrons with *dfrA12-aadA2* cassette array was determined prior to the use of the bacterial isolates. All were detected for Incompatibility (Inc) group using PCR Base Replicon Typing (PBRT) and subjected to Whole genome Sequencing using Oxford Nanopore technologies and Illumina platform Hiseq sequencers. Only two *Salmonella* isolates were positive to plasmid replicons, including SA016 carrying IncY and SA684 carrying IncFIB and IncF plasmids. The WGS analysis showed that the *Salmonella* genome length ranged between 4,692,127 to 5,110,480 bp, of which 7 MLST types including ST29, ST64, ST48, ST96, ST469, ST696 and ST1499 were identified. For the genetic relationship between *Salmonella* isolates, 6 distinct clades were present using phylogenetic trees. Based on WGS analysis, IncFIB(K), ColpVC, IncFIB, IncHI2, IncHI2A, IncX1, IncY and IncR plasmids were identified. Twenty-eight AMR genes were found among all *Salmonella* isolates, of which *aac(6')-Iaa, dfrA12, blaTEM-1B, sul1, qacE* and *aadA2* or *aadA2b* genes were found in all *Salmonella* isolates. According to ResFinder, all were predicted to be resistant to aminoglycosides, β-lactams, folate pathway antagonists, quaternary ammonium compounds, aminocyclitols and quinolones. Chromosomal point mutations that generated amino acid substitutions were observed in *parC, parE, gyrA, pmrA, pmrB* and *acrB*. All *Salmonella*, except B82, carried class 1 integrons with *dfrA12-DUF1010-aadA2* and *qacE-sul1* in conserved region. In conclusion, the results indicate the important role of transferable plasmids as the underline cause for the wide distribution of class 1 integrons with *dfrA12-aadA2* gene array. Decreasing the selective pressure could limit the wide distribution of AMR associated with transferable plasmids.

3.2 INTRODUCTION

Antimicrobial resistance (AMR) in bacteria pathogens is one of the greatest public health challenges globally. Concurrently, *Salmonella* is one of the most common foodborne pathogens and used as safety indicator for meat and meat products for domestic consumption and export. Infection of multidrug-resistant (MDR) *Salmonella* has rapidly increased and had a significant impact on economy, public health, and international food trade in most parts of the world. Over and imprudent use of antibiotics has been blamed as a major cause of emergence and spread of AMR in either commensals or pathogenic bacteria.

There are several methods in which bacteria might develop resistance to antibiotics, but there are three basic ways in which AMR determinants can spread i.e., conjugation, transformation, and transduction. The most frequent type of horizontal transfer is conjugation, which also happens in the natural world. The horizontal transfer of AMR genes through mobile genetic elements is one of the most frequent conjugative processes that might hasten the formation and spread of AMR (MGEs) (e.g., resistance plasmids, integrons and transposons) (von Wintersdorff et al., 2016).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Class 1 integrons is the predominant integrons type and numerous resistance gene cassettes have been found in their variable regions (Chuanchuen et al., 2009; Wannaprasat et al., 2011). Integrons are frequently found on conjugative plasmids, which aid in the spread of AMR genes (Trongjit et al., 2017). In Thailand, there has been a great deal of research done on class 1 integrons of food animal and product origin. It has been observed that the same resistance gene cassettes were identified in class 1 integrons variable region from various bacterial species from different sources in the country.

A particular example is class 1 integrons with *dfrA12-aadA2* gene cassette array that has been previously reported in many bacterial species from different sources e.g.,

Salmonella from pigs in Khon Kaen and Roi Et, from pork in Chiang Mai, *E. coli* from pigs in Nakhon Ratchasima (Wannaprasat et al., 2011; Lay et al., 2012; Sinwat et al., 2015), *Salmonella* from pet dogs and cats (Srisanga et al., 2017), *Aeromonas hydrophila* from nile tilapia (Lukkana et al., 2012), *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from patients (Poonsuk et al., 2012).

Trimethroprim, an analog of dihydrofolate, competitively inhibits dihydrofolate reductase (DHFR). which catalyses the conversion of dihydrofolate to the active coenzyme tetrahydrofolate, the precursor of the DNA synthesis pathway (Huovinen et al, 1995). Trimethoprim resistance is most commonly due to overproduction of trimethroprim-resistant DHFR encoded by *dfr*. The *dfr* gene encodes dihydrofolate reductases (DHFR) enzyme that confers resistance to trimethoprim. There are two main families of *dfr* genes including *dfrA* and *dfrB* genes (van Hoek et al., 2011). To date, over 30 genes in *dfrA* family have been regularly reported, of which the *dfrA1*-group and *dfrA12*-group are most commonly found in Gram-negative bacteria. The *aadA2* gene encodes aminoglycoside adenylyltransferases and confers resistance to antibiotics in aminoglycoside group, including streptomycin and spectinomycin (Michael et al., 2005).

Conjugative plasmid-associated class 1 integrons with *dfrA12-aadA2* cassette array have frequently been found in *Salmonella* isolated from food animals, animal products and human. The *dfra12-aadA2* cassette array was reported approximately 20-60% among class1 integrons- positive *Salmonella* isolated from different sources in many country worldwide such as China (Wang et al., 2010; Li et al., 2013; Zhang et al., 2018; Zhou et al., 2019), Korea (Yu et al., 2003; Noh et al., 2019), Thailand (Padungtod et al., 2011; Wannaprasat et al., 2011; Sinwat et al., 2016; Srisanga et al., 2017), Portugal (Antunes et al., 2005; Antunes et al., 2007) and Egypt (El-Sharkawy et al., 2017). These findings suggest the importance of horizontal transfer in AMR spreading and the role of the *dfrA12-aadA2* array in spreading of trimethoprim and streptomycin resistance in bacteria of animal origin. These also highlight the genetic connection between AMR in people, pets, food animals, and food of animal origin.

Comprehensive genetic data of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* cassette array will help to explain the genetic link and the widespread of class 1 integrons with *dfrA12-aadA2.* Therefore, this study aims to genetically characterize *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand using Whole Genome Sequencing analysis.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial isolates and antimicrobial susceptibility

Fifteen *Salmonella* isolates with class1 integrons carrying *dfrA12-aadA2* cassette array on transferable plasmids were obtained from healthy pigs (n=8), poultry (n=4), pork (n=2) and human (n=1) during 2020-2022 (Table 10) (Khemtong and Chuanchuen, 2008; Wannaprasat et al., 2011; Sinwat et al., 2015; Sinwat et al., 2016). Isolation and identification of *Salmonella* were performed according to ISO6579:2002 (ISO, 2002). Plasmid transfer was tested by Biparental mating method. A single positive serotype of each sample was collected and stored in 20% glycerol at -80°C freezer. All *Salmonella* isolates were multidrug resistant bacteria.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 10 Characteristics of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* cassette.

Salmonella isolate	Serotype	Sample source	Location	AMR phenotype
A2	Anatum	Pork	North-Eastern region	AMP-STR-SUL-TET-TMP
A4	Anatum	Pig - internal organ	North-Eastern region	AMP-STR-SUL-TET-TMP
C76	Anatum	Pig - internal organ	North-Eastern region	AMP-STR-SUL-TET-TMP
D8	Anatum	Pig - internal organ	North-Eastern region	AMP-STR-SUL-TET-TMP
D57	Hindmarsh	Pig - internal organ	North-Eastern region	AMP-CHL-STR-SUL-TET-TMP
NK137.1	Rissen	Pig - rectal swab	North-Eastern region	AMP-CHL-STR-SULTET-TMP
SA012	Schwarzengrund	Broiler - Caecum	Central region	AMP-STR-SUL-TET-TMP
SA016	Stanley	Pig - rectal swab	Central region	AMP-STR-SUL-TET-TMP
SA040	Schwarzengrund	Broiler - Caecum	Central region	AMP-STR-SUL-TET-TMP

Salmonella isolate	Serotype	Sample source	Location	AMR phenotype
SA046	Kentucky	Broiler – Caecum	Central region	AMP-STR-SUL-TET-TMP
SA048	Schwazengrund	Broiler - Caecum	Central region	AMP-STR-SUL-TET-TMP
SA684	Rissen	Pork	Northern region	AMP-CHL-STR-SUL-TET-TMP
B82	Panama	Human	North-Eastern region	AMP-CHL-STR-SUL-TET-TMP
D7	Rissen	Pig - internal organ	North-Eastern region	AMP-STR-SUL-TET-TMP
D15	Rissen	Pig - internal organ	North-Eastern region	AMP-STR-SUL-TET-TMP

AMP, ampicillin; CHl, chloramphenicol; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

3.3.2 Detection of *dfrA12* and *aadA2* genes and PCR Base Replicon Typing (PBRT)

DNA templates of all *Salmonella* isolates (n=15) were prepared using whole cell boiling method (Lévesque et al., 1995). The presence of *dfrA12* and *aadA2* in all *Salmonella* was confirmed using specific primers (Table 11). The PCR cycles comprised one cycle of pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min (Chuanchuen and Padungtod, 2009).

Eighteen Incompatibility (Inc) replicon types, including HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FII_s, F_{repB}, K and B/O were identified using the PCR base replicon typing (PBRT) scheme, of which the specific primers are shown in Table 11. Multiplex PCR amplification conditions included pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing step at 60°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 5 min. For simplex PCR, annealing temperature of 52°C was used. Toptaq Master Mix kit (QIAGEN, Hilden, Germany) was used according to the manufacturer's direction. Size of PCR amplicon was determined using gel electrophoresis. Representative of PCR amplicons was submitted for nucleotide sequencing.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 11 Primers used in this study.

PCR-reaction	Name	Primer sequences	Amplicon size (bp)	Reference
PBRT				
Multiplex 1	HI1 FW	5'-GGAGCGATGGATTACTTCAGTAC-3'	471	(Carattoli et al., 2005)
	HI1 RV	5'-TGCCGTTTCACCTCGTGAGTA-3'		
	HI2 FW	5'-TTTCTCCTGAGTCACCTGTTAACAC-3'	644	
	HI2 RV	5'-GGCTCACTACCGTTGTCATCCT-3'		
	I1 FW	5'-CGAAAGCCGGACGGCAGAA-3'	139	
	I1 RV	5'-TCGTCGTTCCGCCAAGTTCGT-3'		
Multiplex 2	X FW	5'-AACCTTAGAGGCTATTTAAGTTGCTGAT-3'	376	(Carattoli et al., 2005)
	X RV	5'-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3'		
	L/M FW	5'-GGATGAAAACTATCAGCATCTGAAG-3'	785	
	L/M RV	5'-CTGCAGGGGCGATTCTTTAGG-3'		
	N FW	5'-GTCTAACGAGCTTACCGAAG-3'	559	
	N RV	5'-GTTTCAACTCTGCCAAGTTC-3'		
Multiplex 3	FIA FW	5'-CCATGCTGGTTCTAGAGAAGGTG-3'	462	(Carattoli et al., 2005)
	FIA RV	5'-GTATATCCTTACTGGCTTCCGCAG-3'		
	FIB FW	5'-GGAGTTCTGACACACGATTTTCTG-3'	702	
	FIB RV	5'-CTCCCGTCGCTTCAGGGCATT-3'		
	W FW	5'-CCTAAGAACAACAAAGCCCCCG-3'	242	
	W RV	5'-GGTGCGCGGCATAGAACCGT-3'		
Multiplex 4	Y FW	5'-AATTCAAACAACACTGTGCAGCCTG-3'	765	(Carattoli et al., 2005)
	Y RV	5'-GCGAGAATGGACGATTACAAAACTTT-3'		
	P FW	5'-CTATGGCCCTGCAAACGCGCCAGAAA-3'	534	
	P RV	5'-TCACGCGCCAGGGCGCAGCC-3'		
	FIC FW	5'-GTGAACTGGCAGATGAGGAAGG-3'	262	
	FIC RV	5'-TTCTCCTCGTCGCCAAACTAGAT-3'		
Multiplex 5	A/C FW	5'-GAGAACCAAAGACAAAGACCTGGA-3'	465	(Carattoli et al., 2005)
	A/C RV	5'-ACGACAAACCTGAATTGCCTCCTT-3'		
	T FW	5'-TTGGCCTGTTTGTGCCTAAACCAT-3'	750	
	T RV	5'-CGTTGATTACACTTAGCTTTGGAC-3'		
	FII_s FW	5'-CTGTCGTAAGCTGATGGC-3'	270	
	FII_s RV	5'-CTCTGCCACAAACTTCAGC-3'		

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

PCR-reaction	Name	Primer sequences	Amplicon size (bp)	Reference
Simplex F	F_{repB} FW	5'-TGATCGTTTAAGGAATTTTG-3'	270	(Carattoli et al., 2005)
	F_{repB} RV	5'-GAAGATCAGTCACACCATCC-3'		
Simplex K	K/B FW	5'-GCGGTCCGGAAAGCCAGAAAAC-3'	160	(Carattoli et al., 2005)
	K RV	5'-TCTTTCACGAGCCCGCCAAA-3'		
Simplex B/O	K/B FW	5'-GCGGTCCGGAAAGCCAGAAAAC-3'	159	(Carattoli et al., 2005)
	B/O RV	5'-TCTGCGTTCCGCCAAGTTCGA-3'		
Resistance genes				
dfrA12	dfrA12 FW	5'-CTGATCGTTTAAGGAATTTT-3'	330	(Chuanchuen and Padungtod, 2009)
	dfrA12 RV	5'-CACACCATCCTGCACTTA-3'		
aadA2	aadA2 FW[1]	5'-TCTGTTTATTCTTTTACTGTCCAC-3'	500	(Chuanchuen and Padungtod, 2009)
	aadA2 FW[1]	5'-TGCTTTTATTCTTAAACTATCCAC-3'		

3.3.3 Whole genome sequencing (WGS) and bioinformatic analysis

All of *Salmonella* isolates (n=15) were confirmed to carry *dfrA12* and *aadA2* genes prior to WGS analysis. Genomic DNA of all *Salmonella* isolates were extracted by using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's instruction.

All genomic DNA were evaluated for concentration and purity using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scienctific, Delaware, USA) and checked for DNA degradation by running 5 µL of the DNA on 0.8% agarose gel electrophoresis stained with Redsafe nucleic acid staining solution (Thermo Scientific, Deleware, USA) before submitted for sequencing. Then, all were submitted for long read sequencing using Oxford Nanopore technologies (ONT) at Siriraj Long-read Lab, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and for short read sequencing using Illumina platform Hiseq sequencers (Illumina, San Diego, CA, USA) at GENEWIZ China and Suzhou Lab, (GENEWIZ, Suzhou, China).

The raw read quality of long and short read sequence were determined using and Nanoplot (De Coster et al., 2018) and FastQC (Andrews, 2010), respectively. Adapters

were trimmed using Porechop v0.2.4 (https://github.com/rrwick/Porechop). High quality ONT and Illumina reads (n=15) were de novo assembled to create hybrid genome using Unicycler (Wick et al., 2017). The de novo assembly graph of all isolates were visualized by Bandage (Wick et al., 2015) The genomic characteristics including genome size, number of contigs and GC content (%) were identified using QUAST (Gurevich et al., 2013). Taxonomic identification was performed using Kraken2 (Wood et al., 2019) and then, Genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The phenotyping and genotyping using assembled genome/contigs were performed on Center for Genomic Epidemiology services via http://www.genomicepidemiology.org/services/. AMR genes were identified by ResFinder4.1 (Florensa et al., 2022). The multilocus sequence typing (MLST) was performed using MLST 2.0 with the obtained data from http://pubmlst.org. The *Salmonella* serotypes were confirmed by SeqSero 1.2 (Zhang et al., 2015). *Salmonella* pathogenicity islands (SPIs) were identified by SPIFinder 2.0 (Roer et al., 2016). Mobile genetic elements (MGE) and plasmids were identified by MobileElementFinder v1.0.3 (Johansson et al., 2021) and PlasmidFinder2.1 (Camacho et al., 2009; Carattoli et al., 2014), respectively. Plasmid double-locus sequence typing (pDLST) and replicon sequence typing (RST) were analyzed using PubMLST (Jolley et al., 2018). Variant calling and core genome alignment was performed by Snippy (Seemann, 2015) and the phylogenetic trees were generated by IQ-TREE (Nguyen et al., 2015) and visualized by iTOL v6 (Letunic and Bork, 2021). The comparison of genetic environment of *dfrA12-aadA2* and the location of insertion sequence (IS) and mobile genetic elements were generated using Clinker (Gilchrist and Chooi, 2021)and Proksee (Grant et al., 2023).

3.4 RESULTS

3.4.1 Plasmid replicon types and Identification of *dfrA12-aadA2* gene.

Two *Salmonella* isolates were positive to plasmid replicons identified by PBRT. Only one *Salmonella* strain, SA016, carried IncY plasmid. SA684 was found to carry IncFIB

and IncF plasmids. There were no replicon types found among the other *Salmonella* isolates.

3.4.2 Whole genome assembly and genetic relatedness between all *Salmonella* isolates (n=15)

The *Salmonella* genome length ranged between 4,692,127 to 5,110,480 bp. GC contents of all *Salmonella* were between 51.8% to 52.1%. Seven MLST types including ST29, ST64, ST48, ST96, ST469, ST696 and ST1499 were identified (Table 17). Four *Salmonella* isolates (A2, A4, C76 and D8) were in the same MLST, ST64. SA012, SA016 and SA048 were identified as ST96. While NK137.1, SA684, D7 and D15 were in the same MLST ST469, B82 was ST48.

Variation of chromosomal DNA of *Salmonella* was called when compared to reference, *Salmonella* LT2 (accession no. NC003197.2). The genetic relationship between *Salmonella* isolates was shown in Figure 1. Six distinct clades were present using phylogenetic trees. The first clades consisted of *Salmonella* strain SA684, NK137.1, D7 and D15, which were closely related to *Salmonella* Rissen (accession no. SRR26095823). SA046 shared common ancestor with *Salmonella* isolates in the first clade that was closely related to *Salmonella* Kentucky (accession no. SRR26095822). Another clade containing *Salmonella* strain SA012, SA048, and SA040 was genetically related to *Salmonella* Schawarzengrund (accession no. SRR6053709). *Salmonella* B82 was genetically related to *Salmonella* Panama (accession no. SRR25872734). *Salmonella* strain A2, A4, C76 and D8 were in the clade, which were closely related to *Salmonella* Anatum (accession no. SRR26089087). D57, was genetically related to *Salmonella* Hindmarsh (accession no. SRR3665082). The last clade contained *Salmonella* strain SA016, which shared the common ancestor with *Salmonella* LT2 (accession no. NC003197.2)

3.4.3 Plasmid replicons, AMR genes and mutations

Plasmid replicons detected by WGS are shown in Table 12. IncFIB(K) and ColpVC were found to co-exist in four *Salmonella* isolates including A2, A4, C76 and D8.

IncFIB(K) plasmid was found as a single plasmid in SA012, SA040 and SA048. IncHI2 and/or IncHI2A plasmids, which were classified as ST3, were found in D57, SA016, and SA046. B82 also carried IncHI2/HI2A and IncX1 plasmids. IncR plasmids were found in three *Salmonella* isolates (i.e., B82, D7 and D15). IncFIB plasmid, the RST of F46:A-:B20, was found in SA684. In addition, IncY plasmid was found in SA016. There was the absence of plasmid replicon tested in NK137.1.

Twenty-eight AMR genes were found among all *Salmonella* isolates (Table 13). The *aac(6')-Iaa*, *dfrA12*, *bla*$_{\text{TEM-1B}}$, *sul1*, *qacE* and *aadA2* or *aadA2b* genes were found in all *Salmonella* isolates. The *aadA1* was found in all *Salmonella*, except SA016, SA040, and SA048. All *Salmonella* isolates carried *tetA*, except *Salmonella* strain B82, of which *tetB* was found instead. Almost all *Salmonella* isolates carried *cml* or *cmlA1* genes except SA016, SA040, SA046, SA048, D7 and D15. The *sul3* gene was additionally found in almost all isolates, except SA040, SA048, B82, D7 and D15. Similarly, the *qacL* gene was found in all isolates, except SA040, SA048, D7 and D15.

Resistance to 34 antimicrobial drugs were predicted (Table 14). All were predicted to be resistant to aminoglycosides (i.e., amikacin, tobramycin, streptomycin), tetracyclines (i.e. tetracycline and doxycycline), β-lactams (i.e. ampicillin, cephalothin, piperacillin, amoxycillin, and ticarcillin), folate pathway antagonists (i.e., sulfamethoxazole and trimethoprim), quaternary ammonium compounds (i.e. benzylkonium, ethidium bromide, chlorhexidine and cetylpyridinium), aminocyclitols (i.e., spectinomycin) and quinolones (i.e. ciprofloxacin and nalidixic acid). Almost all *Salmonella* isolates were predicted to be chloramphenicol resistant isolates, except SA016, SA040, SA046, SA048 D7 and D15. SA684, D7 and D15 were predicted to be resistant to macrolides (i.e., azithromycin and erythromycin).

Chromosomal point mutations that generated amino acid substitutions were observed in *parC*, *parE*, *gyrA*, *pmrA*, *pmrB* and *acrB* genes (Table 15). Amino acid substitution (T57S) in ParC, conferring resistance to nalidixic acid and ciprofloxacin, were found in all *Salmonella* isolates, except D57. In addition, *gyrA* mutations

yielding S83F amino acid substitution conferring resistance to nalidixic acid and ciprofloxacin, were found in SA012, SA040, and SA048. Several point mutations were identified in *parC*, *parE*, *gyrA*, *pmrA*, *pmrB* and *acrB* leading to amino acid substitutions with unknown resistance phenotype. The pathogenicity islands of all *Salmonella* were shown in Table 16.

3.4.4 Plasmids carrying class 1 integrons with *dfrA12- aadA2* cassette array.

Alignment of plasmids/contigs of *Salmonella* carrying class 1 integrons *with dfrA12-aadA2* cassette array was showed in Figure 2. All *Salmonella,* except B82, carried class 1 integrons with *dfrA12*-DUF1010-*aadA2* and *qacE-sul1* in conserved region. *Salmonella* B82 carried class 1 integrons with *dfrA12-DUF101-aadA2-cmlA1-aadA1* gene array and *qacL-IS256* without *sul* gene in conserved region.

Draft of IncR plasmid of *Salmonella* strain D7 and D15 were compared with pS90-2.3 from *Klebsiella pneumoniae* (Figure 3). All carried class 1 integrons with *dfrA12-aadA2-qacE-sul1* cassette array. In *Salmonella* strain D7, the *intI1* and gene cassette array were flanked by *IS6*-like element *IS26* family transposase downstream, and upstream as observed in *Klebsiella pneumoniae* (accession no. CP063884.1) (Figure 3B). The *mphA* and *aadA1* genes were detected among all these IncR plasmids. However, $bla_{TEM\text{-}1B}$ was found in IncR plasmid from D7 and D15, except pS90-2.3 from *K. pneumoniae.*

CHULALONGKORN UNIVERSITY

The structural comparison of IncX1 plasmid carrying *intI1* with *dfrA12-aadA2* cassette array was compared with the closely related IncX1 plasmid, pLAO36 and pRF52-1 from *E. coli* isolated from human stools and swine feces, respectively (Figure 4). The whole plasmid sequence of IncX1 plasmid of *Salmonella* strain B82 was 100% similar to pLAO36 and pRF52-1.

Table 12 Detection of MLST and plasmids among *Salmonella* by WGS analysis (n=15)

Salmonella isolate	Serotype	Sample source	MLST	House-keeping genes							Plasmids	
				aroC	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	Inc group	LST/RST[b]
A2	Anatum	Pork	ST64	10	14	15	31	25	20	33	IncFIB(K), ColpVC	-
A4	Anatum	Intestine	ST64	10	14	15	31	25	20	33	IncFIB(K), ColpVC	-
C76	Anatum	Intestine	ST64	10	14	15	31	25	20	33	IncFIB(K), ColpVC	-
D8	Anatum	Liver	ST64	10	14	15	31	25	20	33	IncFIB(K), ColpVC	-
D57	Hindmarsh	Liver	ST1499	2	59	23	64	38	19	12	IncHI2, IncHI2A,	IncHI2: ST3
NK137.1	Rissen	Rectal swab	ST469	92	107	79	156	64	151	87	-	-
SA012	Schwarzengrund	Chicken	ST96	43	47	49	49	41	15	3	IncFIB(K)	-
SA016	Stanley	Rectal swab	ST29	16	16	20	18	8	12	18	IncHI2A, IncY (IncY[a])	IncHI2: ST3
SA040	Schwarzengrund	Chicken	ST96	43	47	49	49	41	15	3	IncFIB(K)	-
SA046	Kentucky	Chicken	ST696	5	75	54	4	76	109	75	IncHI2, IncHI2A	IncHI2: ST3
SA048	Schwazengrund	Chicken	ST96	43	47	49	49	41	15	3	IncFIB(K)	-
SA684	Rissen	Pork	ST469	92	107	79	156	64	151	87	(IncFIB[a])	IncF: F46:A-:B20
B82	Panama	Human	ST48	22	11	25	21	10	23	23	IncHI2/HI2A, IncR, IncX1	IncHI2/HI2A: ST1
D7	Rissen	Liver	ST469	92	107	79	156	64	151	87	IncR	-
D15	Rissen	Intestine	ST469	92	107	79	156	64	151	87	IncR	-

Bold plasmid Inc groups were presented the results of Incompatibility group of plasmids identified by PBRT.

[a]IncY and IncFIB in parenthesis were detected by PBRT.

[b]LST/RST, Locus Sequence Type/Replicon Sequence Type

Table 13 Antimicrobial resistance genes identified by ResFinder (n=15)

Salmonella isolates	Antimicrobial resistance gene/Contig No.																										
	aac(6')-Iaa	*aac(3)-IV*	*aph(3')-Ia*	*aph(3')-IIa*	*aph(3'')-Ib*	*aph(6)-Id*	*aph(4)-Ia*	*cml*	*cmlA1*	*oqxA*	*oqxB*	$bla_{TEM\text{-}1B}$	*sul1*	*sul3*	*dfrA12*	*tetA*	*tetB*	*tetM*	*qacE*	*qacL*	*aadA1*	*aadA2*	*aadA2b*	*aadA4*	*aadA24*	*bleO*	*mefB*	*mphA*
A2	+							+				+	+	+	+	+			+	+	+	+						
A4	+							+				+	+	+	+	+			+	+	+	+						
C76	+							+				+	+	+	+	+			+	+	+	+						
D8	+							+				+	+	+	+	+			+	+	+	+						
D57	+			+	+	+			+	+	+	+	+	+	+	+		+	+	+	+	+				+		
NK137.1	+							+				+	+	+	+	+			+	+	+	+						
SA012	+	+	+				+		+			+	+	+	+	+			+	+	+		+					
SA016	+				+	+						+	+	+	+	+		+	+	+		+			+	+		
SA040	+	+					+					+	+		+	+			+			+						
SA046	+	+										+	+	+	+	+			+	+	+	+						
SA048	+	+					+					+	+		+	+			+			+						
SA684	+		+					+				+	+	+	+	+			+	+	+	+					+	
B82	+								+			+	+		+		+		+	+	+	+		+				
D7	+											+	+		+	+			+		+	+						+
D15	+											+	+		+	+			+		+	+						+

+, presence of antimicrobial resistance genes.

Table 14 Antimicrobial resistance phenotypes predicted by ResFinder and defined by clinical breakpoints (n=15)

Salmonella isolates	Antimicrobial group/name												
	Aminoglycoside												Amphenicol
	amikacin	tobramycin	streptomycin*	kanamycin	neomycin	bleomycin	hygromycin	gentamycin*	lividomycin	paromomycin	ribostamycin	hygromycin	chloramphenicol*
A2	+	+	+										+
A4	+	+	+										+
C76	+	+	+										+
D8	+	+	+										+
D57	+	+	+	+	+	+							+
NK137.1	+	+	+				+						+
SA012	+	+	+	+	+		+	+	+	+	+	+	+
SA016	+	+	+			+							
SA040	+	+	+				+	+				+	
SA046	+	+	+					+					
SA048	+	+	+				+	+				+	
SA684	+	+	+	+	+				+	+	+		+
B82	+	+	+										+
D7	+	+	+										+
D15	+	+	+										+

Salmonella isolates	Beta-lactam					Folate pathway antagonist		Tetracycline			Quaternary ammonium compound				Aminocyclitol	Quinolone		Macrolide			
	ampicillin*	cephalothin	piperacillin	amoxicillin	ticarcillin	sulfamethoxazole*	trimethoprim*	tetracycline*	doxycycline	minocycline	ethidium bromide	benzylkonium	chlorhexidine	cetylpyridinium	spectinomycin	ciprofloxacin*	nalidixic acid	azithromycin	erythromycin	spiramycin	telithromycin
A2	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
A4	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
C76	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
D8	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
D57	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
NK137.1	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
SA012	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
SA016	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
SA040	+	+	+	+	+	+	+	+	+		+		+	+	+	+	+				
SA046	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
SA048	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
SA684	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+		
B82	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+				
D7	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+
D15	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+

+ , presence of antimicrobial resistance phenotype when using WGS prediction.

+ (black box), presence of antimicrobial resistance phenotype to particular antimicrobials when using WGS prediction and MICs determination.

+ (grey box), presence of antimicrobial resistance phenotype to particular antimicrobials when using WGS prediction but absence of antimicrobial resistance phenotype when MICs were determined.

* Antimicrobial drugs that were determined the MIC values by using agar dilution method according to CLSI standard.

Table 15 Amino acid substitutions of chromosomally encoded genes identified by ResFinder (n=15).

Salmonella isolate	Amino acid substitutions of chromosomal genes					
	parC	*parE*	*gyrA*	*pmrA*	*pmrB*	*acrB*
A2	T57S, T255S, H747P	-	-	-	-	F28L, L40P, Y597C
A4	T57S, T255S, H747P	-	-	-	-	F28L, L40P, Y597C
C76	T57S, T255S, H747P	-	-	-	-	F28L, L40P, Y597C
D8	T57S, T255S, H747P	-	-	-	-	F28L, L40P, Y597C
D57	T255S, A620T	-	-	-	-	F28L, L40P
NK137.1	T57S	-	-	-	-	F28L, L40P
SA012	T57S, T255S, A352V	-	S83F, D759E	-	D284N,	F28L, L40P
SA016	T57S, T255S, N395S, S469A	-	L396M	-	-	F28L, L40P
SA040	T57S, T255S, A352V	-	S83F, D759E	-	D284N	F28L, L40P
SA046	T57S, T255S, N395S	-	-	-	-	F28L, L40P
SA048	T57S, T255S, A352V	-	S83F, D759E	-	D284N	F28L, L40P
SA684	T57S	-	-	-	-	F28L, L40P
B82	T57S, T255S	P231L	T661N, D759E	T89S	V74I, A111T, G73S, I83V, M15T	F28L, L40P
D7	T57S	-	-	-	-	F28L, L40P
D15	T57S	-	-	-	-	F28L, L40P

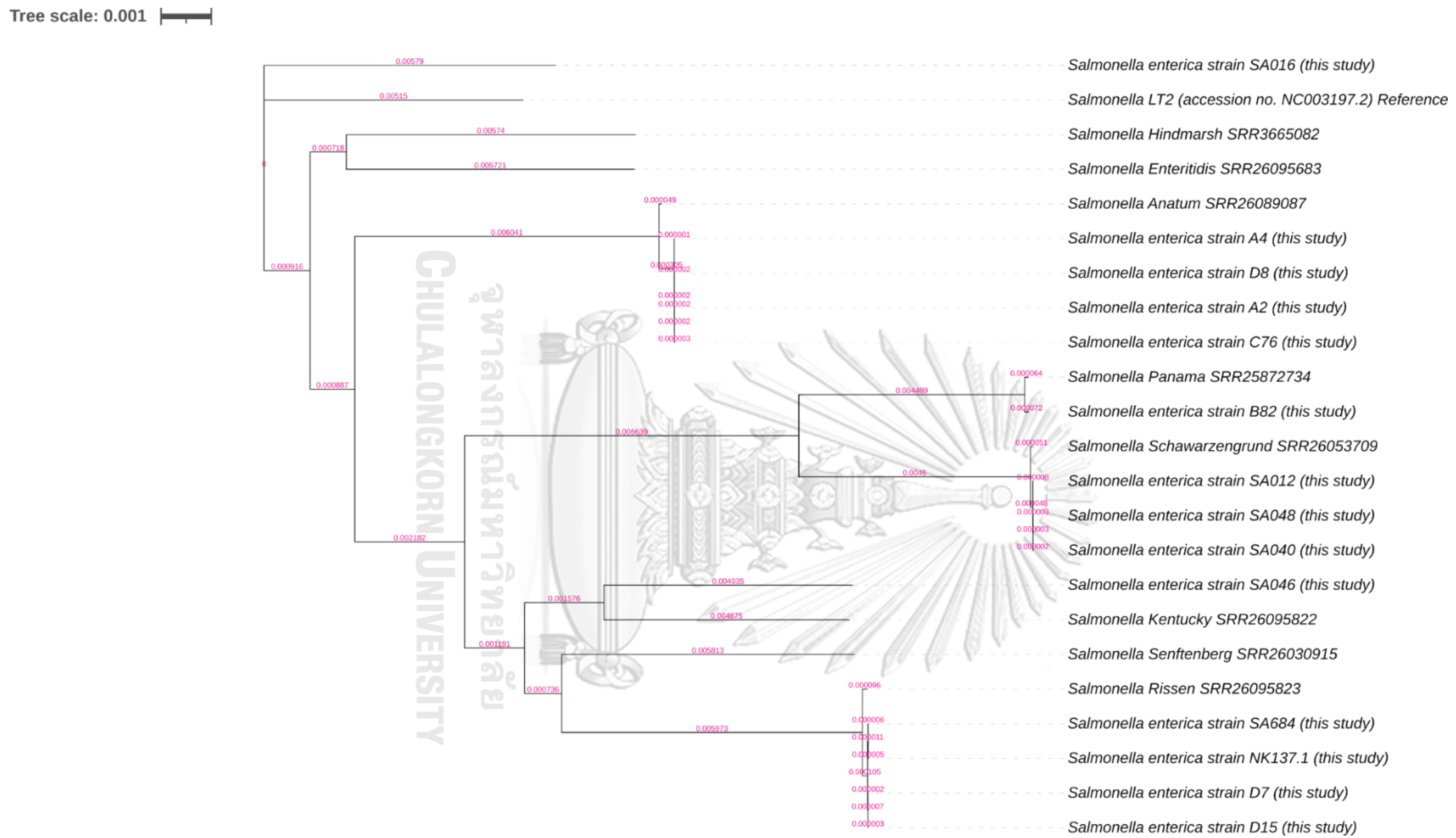
Amino acids substitutions which conferred antimicrobial resistance were presented in bold and underline letters.

A, Alanine; R, Arginine; N, Asparagine; D, Aspartate; C, Cysteine; E, Glutamate; Q, Glutamine; G, Glycine; H, Histidine; I, Isoleucine; L, Leucine; K, Lysine; M, Methionine; F, Phenylalanine; P, Proline; S, Serine; T, Threonine; W, Tryptophan; Y, Tyrosine; V, Valine; and *, Termination.

Table 16 *Salmonella* Pathogenicity Islands identified by SPIFinder (n=15)

Salmonella isolate	*Salmonella* pathogenicity islands										
	SPI-1	*SPI-2*	*SPI-3*	*SPI-4*	*SPI-5*	*SPI-8*	*SPI-9*	*SPI-13*	*SPI-14*	*C63PI*	*CS54_island*
A2	+	+	+	+	+		+	+	+	+	
A4	+	+	+	+	+		+	+	+	+	
C76	+	+	+	+	+		+	+	+	+	
D8	+	+	+	+	+		+	+	+	+	
D57	+	+	+	+	+		+	+	+	+	+
NK137.1	+	+	+	+	+	+	+			+	
SA012	+	+	+	+	+		+	+	+	+	
SA016	+	+	+	+	+		+	+	+	+	+
SA040	+	+	+	+	+		+	+	+	+	
SA046	+	+	+	+	+	+	+			+	
SA048	+	+	+	+	+		+	+	+	+	
SA684	+	+	+	+	+	+	+			+	
B82	+	+	+	+	+		+	+	+	+	
D7	+	+	+	+	+	+	+			+	
D15	+	+	+	+	+	+	+			+	

+, presence of *Salmonella* pathogenicity islands.



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 1 Phylogenetic tree of *Salmonella* enterica carrying class 1 integrons with *dfrA12- aadA2* cassette array (n=15).

Chromosomal DNA sequences of A2, A4, B82, C76, D7, D8, D15, D57, NK137.1, SA012, SA016, SA040, SA046, SA048, SA684 and 8 *Salmonella* reference strains were aligned with *Salmonella* Typhimurium LT2 (accession no. NC003197.2) The single nucleotide polymorphisms (SNPs) were called using Snippy. Phylogenetic trees were generated using Core SNP alignment and visualized by iTOL. The number on the branch indicates genetic changes.

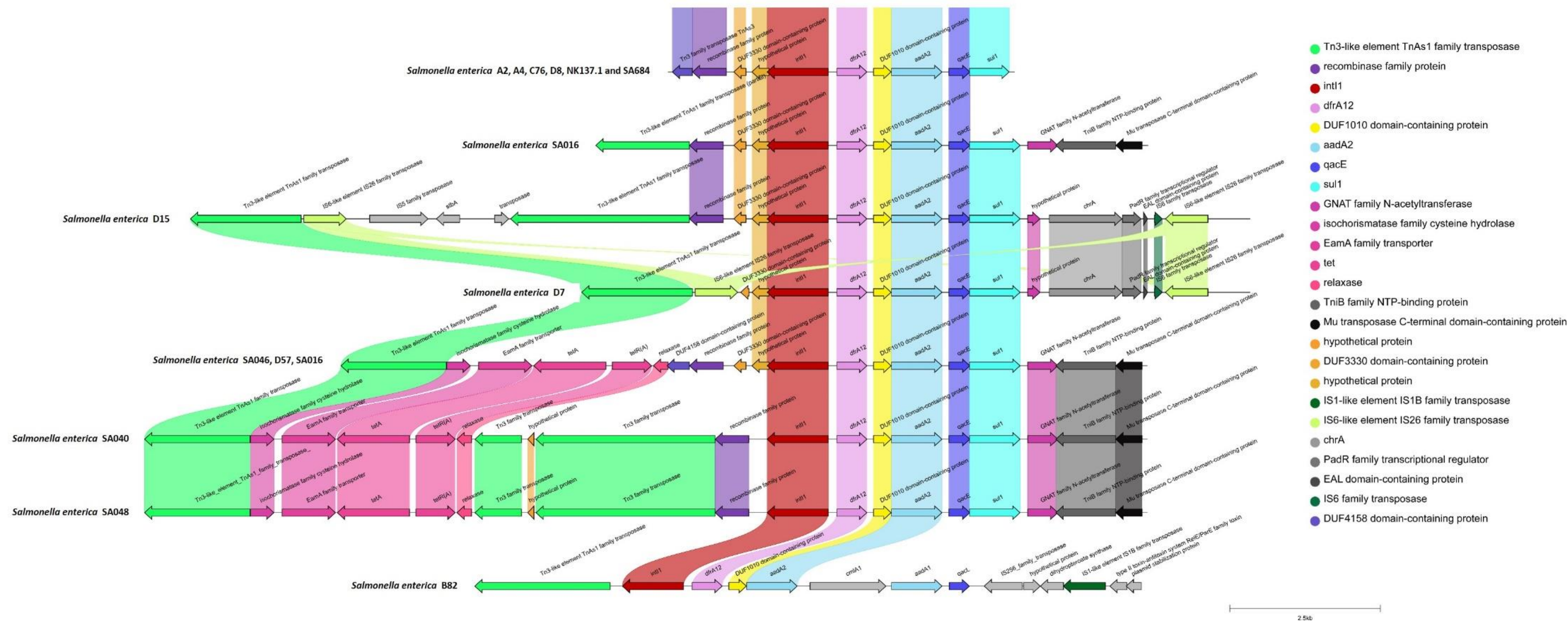


Figure 2 Alignment of plasmids carrying class 1 integrons with *dfrA12-aadA2* cassette array (n=15).

The assembled plasmids from all *Salmonella* carrying class 1 integrons with *dfrA12* and *aadA2* genes were aligned. Arrows indicate the position and direction of genes. Each arrow color represents each gene. Vertical blocks in the same color indicate regions of shared similarity according to BLASTn with at least 90% identity.

Figure 3 Structural comparison of the IncR plasmids from *Salmonella* D7 and D15 carrying class 1 integrons with *dfrA12-aadA2* cassette array and closely related plasmid.

(A) Circular comparison of the IncR plasmids from *Salmonella* D7, and D15 and pS90-2.3 from *Klebsiella pneumoniae* (accession no. CP063884.1). Green, yellow and pink is IncR plasmid of D7, D15 and pS90-2.3, respectively. Dark blue and red denotes insertion sequences/transposase genes and AMR genes from CARD database, respectively. (B) Alignment of a part of IncR plasmids showing the genetic environment of *intI1* and the resistance gene cassette. Arrows indicate the position and direction of genes. The same vertical block color indicates regions of shared similarity according to BLASTn with at least 90% identity.

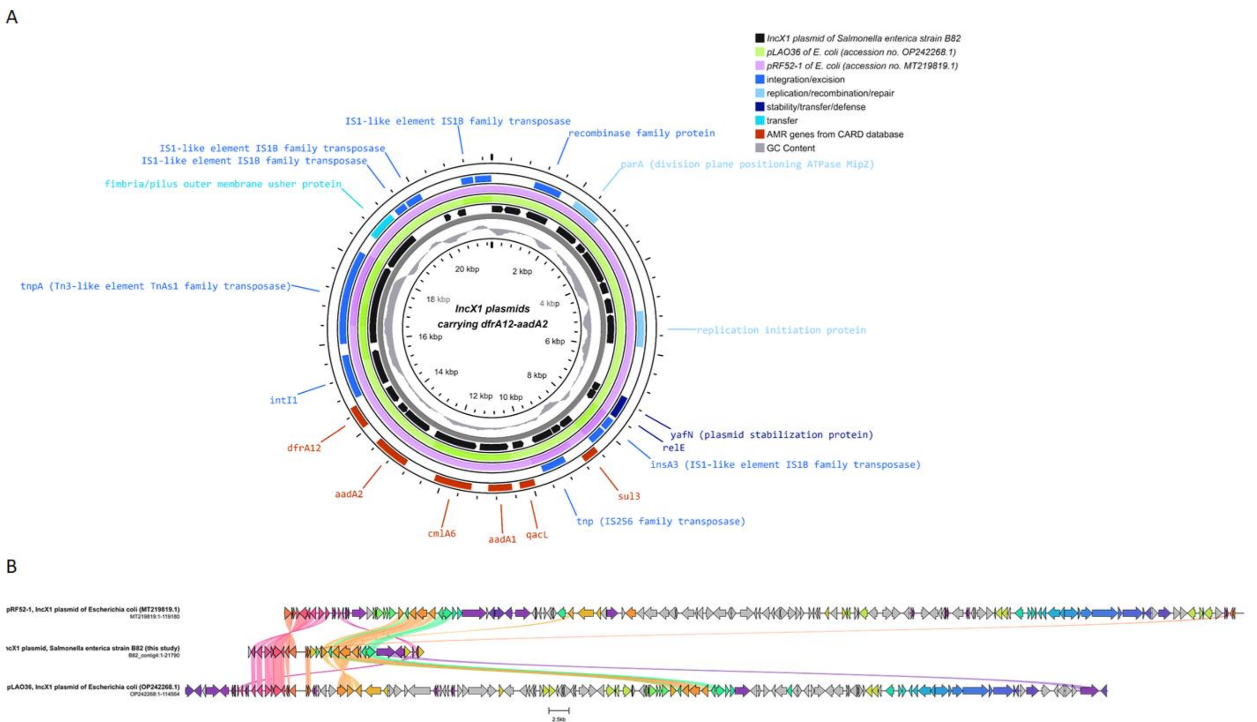


Figure 4 Structural comparison of the IncX1 plasmids from *Salmonella* B82 carrying class 1 integrons with *dfrA12-aadA2* cassette array and closely relative plasmids.

(A) Circular comparison of the IncX1 plasmids of B82, pLAO36 from *E. coli* (accession no. OP242268.1) and pRF52-1 from *E. coli* (accession no.MT219819.1). Black, green and purple are IncX1 plasmid sequences from B82, pLAO36 and pRF52-1, respectively. Blue shade represents integration/excision genes, replication/recombination/repair genes, stability/defense genes and transfer genes. Red indicates AMR genes from CARD database. (B) Alignment of IncX1 plasmids from B82 and closely relative plasmid, pLAO36 and pRF52-1. Each arrow represents each gene. Vertical block in the same color indicates regions of shared similarity according to BLASTn with at least 90% identity.

3.5 DISCUSSION

Salmonella included in this study were derived from previous studies in Thailand. Class1 integrons with*dfrA12-aadA2* cassette array was found among *Salmonella* isolated from different origins including food animal, human and environment. The class1 integrons among *Salmonella* could transfer to *E. coli*. WGS was included in this study to reveal the genetic environment of the *dfrA12-aadA2* cassette array.

Based on the PBRT results, only two *Salmonella* isolates carried plasmid. This may be the result of the PBRT method's limitations, which exclude new plasmids and some plasmid types (such as ColpVC, IncFIB(K), and IncHI2 plasmid). This suggests that the plasmid replicon detection strategy needs to be updated. However, the missing plasmid replicons were additionally discovered by WGS.

According to WGS analysis, IncFIB(K) plasmids commonly found in this study were closely related to pKPN-IT plasmid that was previously reported in *Klebsiella pneumoniae* (Garcia-Fernandez et al., 2012). The pKPN-IT plasmid was found to carry class1 integrons with *dfrA12-orfF-aadA2* encoding resistance to trimethoprim and streptomycin. These may be the explanation for the observation of class1 integrons with *dfrA12-aadA2* cassette array in this study. This is supported by the observation that *Salmonella* A2, A4, C76, D8, SA012, SA040 and SA048 carried class 1 integrons with *dfrA12- aadA2* cassette array that were also located on IncFIB(K).

Beside the IncFIB(K) plasmid, IncHI2 plasmids were previously reported to carry class1 integons containing *dfrA12* and *aadA* genes and could be transferred by conjugation (Shang et al., 2021). This could be another explanation for class1 integrons with *dfrA12* and *aadA* gene array identified in *Salmonella* with IncHI2 plasmid in this study. Regardless, the presence of the same plasmid replicons among *Salmonella* isolated from different sources either poultry or swine in different years indicate the circulation of the plasmids among the *Salmonella* isolates of food producing animal origin.

In addition, pDLST of IncHI2 group detected in *Salmonella* D57 (pig intestinal organ form North-Eastern region), SA016 (Pig intestinal organ form Central region), and SA046 (Chicken form Central region) was ST3, confirming the circulation of this plasmids in Thailand. ColpVC plasmid was found in some *Salmonella* isolates from pork, pig internal organs, in agreement with a previous report conducted in *Salmonella* isolated from an outbreak in human associated with meat consumption in the US (Etter et al., 2019). This again confirms that AMR is borderless and can spread worldwide.

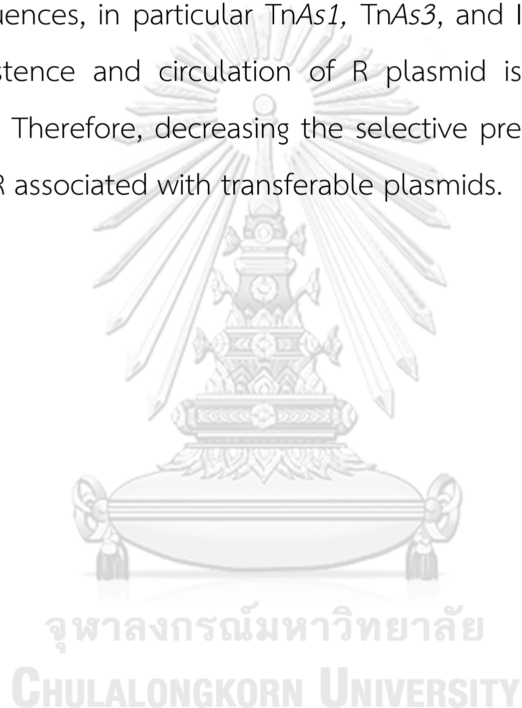
DUF1010 domain-containing protein which encoded by *orfF* and located in between *dfrA12* and *aadA2* in variable region of i*ntI1* were found in previous report (Li et al., 2022). DUF3330 domain-containing protein was reported as a part of conserved region of transposons, which were commonly found on plasmids. This gene involved in modulating the transposition of transposons (Madsen et al., 2018). The Tn*3*/Tn*AS1* and Tn*3*/Tn*As3* which were commonly found at downstream of *intI1* in this study were reported in previous study.

The conserved region of class 1 integrons are usually composed of *qacE1* and *sul1*, in agreement with the observation for the class 1 integrons identified in *Salmonella* isolates from pigs and pork. In contrast, the conserved region of class 1 integrons in the human isolates comprises *qacL* and *sul3*. This may be the result of the different sources and environment of the origin of the isolates. However, the different conserved region may not have an effect on the transferability of the plasmid.

It should be additionally noted that the outputs from the comparison with the ResFinder database indicated that all the isolates contained mutations on *gyrA* and *parC* and predicted that all should be resistant to fluoroquinolones. In contrast, the results from antimicrobial susceptibility testing showed that all were susceptible to ciprofloxacin. This discrepancy is likely due to the difference in interpretive criteria used in these two different methods. The prediction by WGS database is usually based on the presence of mutations and genes. However, the resistance phenotypes

in antimicrobial susceptibility testing are based on clinical breakpoints as interpretive criteria. This indicates the conflict of the phenotypic and genotypic methods. Therefore, the results from genotypic methods, especially genomic technologies, should be carefully applied.

In conclusion, the results indicate the important role of transferable plasmids as the underline cause for the wide distribution of class 1 integrons *dfrA12-aadA2* gene array. The gene structure of the class 1 integrons was closely related to transposons and insertion sequences, in particular Tn*As1,* Tn*As3*, and IS*26* that facilitate efficient mobility. The existence and circulation of R plasmid is maintained by antibiotic selective pressure. Therefore, decreasing the selective pressure could limit the wide distribution of AMR associated with transferable plasmids.


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER IV

Molecular basis of the persistence of chloramphenicol resistance among *Escherichia coli* and *Salmonella* spp. From food animals, meat and human in Thailand

Jiratchaya Puangseree[a], Rangsiya Prathan[a,b], Songsak Srisanga[a,b],
Rungtip Chuanchuen[a,*]

[a]*Research unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary science, Chulalongkorn University, Bangkok, 10330 Thailand*

[b]*Center for Antimicrobial Resistance Monitoring in Food-borne Pathogens, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330*

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Manuscript is in preparation.

Molecular basis of the persistence of chloramphenicol resistance among *Escherichia coli* and *Salmonella* spp. from food animals, meat and human in Thailand

4.1 ABSTRACT

One of the key factors that promote the spread and emergence of antimicrobial resistance (AMR) is antimicrobial usage (AMU). Discontinued antimicrobial use is expected to help decrease the emergence and spread of AMR bacteria. Since 1998, the use of chloramphenicol in food animals has been prohibited in Thailand. However, chloramphenicol-resistant bacteria are consistently isolated from food animals and meat. Therefore, this study aims to investigate the potential mechanisms associated with the persistence of chloramphenicol resistance in *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork, and humans. *E. coli* (n=106) and *Salmonella* (n=57) isolates resistant to chloramphenicol were included and their chloramphenicol susceptibility was determined in the presence and absence of phenylalanine arginine β-naphthylamide (PAβN). Conjugation experiment was conducted to investigate possible co-selection of CHP resistance by using ampicillin, tetracycline, and streptomycin. CHP-resistant transconjugants were screened for chloramphenicol-resistance genes (*catA*, *catB* and *cmlA*) and plasmid replicons. Whole genome sequencing was performed among selected three *E. coli* (E329, E333 and E290) and three *Salmonella* (SA448, SA461 and SA515) isolates with high CHP MIC (32-256 μg/mL) and different plasmid replicon type. Most *E. coli* (67.9%) and *Salmonella* (64.9%) had ≥4-fold CHP MIC decrease in the presence of PAβN. The presence of *cmlA* in *E. coli* was significantly related with <4-fold CHP MIC decrease in the addition of PAβN (25/34, 75%). Nine *E. coli* and 8 *Salmonella* yielded *cmlA*-carrying *Salmonella* and *E. coli*-transconjugants, respectively, with CHP MIC ≥ 32 μg/mL (32-512 μg/mL) in the presence of different selective pressure (i.e., AMP, TET and STR). IncFIIAs plasmids were commonly found in *cmlA*-carrying *Salmonella* transconjugants, while IncFIB and IncF plasmids were mostly found in *cmlA*-carrying *E. coli* transconjugants. The WGS analysis showed that class1 integrons with *cmlA1*

gene cassette flank and flanked by IS*26* and Tn*As1,* were located on IncX1 plasmid of E290, IncFIA(HI1)/HI1B plasmids of E329 and E333 and IncFII/FIB plasmids of SA461 and SA515. While *catA flank*ed by IS*1B* and Tn*As3* was found in IncFIA(HI1)/HI1B/Q1 of SA448. In conclusion, the results demonstrated that the multidrug efflux systems using proton motif force and transferable plasmid play an important role in CHP-resistance. The persistence of CHP-resistance was potentially mediated by cross resistance and co-selection by other antimicrobial drugs.

4.2 INTRODUCTION

Antimicrobial usage (AMU) is one of the key elements contributing to emergence and spread of antimicrobial resistance (AMR). When an antimicrobial agent is used, there is a chance that bacteria adapt and develop resistance, enabling them to survive in it. This phenomenon is exacerbated by inappropriate use of antimicrobials. Reducing or ceasing the use of antimicrobials is expected to lessen the likelihood of AMR bacteria emergence and spread. It is anticipated that if the AMU had been removed, AMR would have disappeared. However, bacteria resistant to restricted antimicrobial drugs have been consistently isolated. For example, the use of chloramphenicol in food animals has been banned in many countries e.g., The US, Canada, Australia, Japan and China since 1994 due to being a cause of aplastic anemia (Hanekamp and Bast, 2015). The antibiotic has been outlawed in Thailand since 1998 (Suwannarak, 2003); nonetheless, chloramphenicol-resistant bacteria have been isolated from food animals and meat e.g., pig (Lay et al., 2012), poultry (Nhung et al., 2016), pork and chicken (Trongjit et al., 2016; Trongjit et al., 2017). It was suggested to be the result of co-selection or cross-resistance brought on by other antimicrobials (Pal et al., 2015; Périchon et al., 2015; Cheng et al., 2019).

Chloramphenicol is a broad-spectrum antibiotic in the amphenicol group, which inhibits bacterial protein synthesis by binding to 50s ribosomal subunit (Cannon et al., 1990; Davis, 2018). One of the most common mechanisms of chloramphenicol resistance is its enzymatic inactivation, particularly by *cat* gene-encoded

acetyltransferases (Sykes and Papich, 2014). Other possible resistance mechanisms include the expression of efflux pumps, which frequently function as multidrug extrusion transporters, reduced outer membrane permeability, and target site mutation or alteration. The *cml* and *floR* genes encode specific exporters of chloramphenicol, while the AcrAB-TolC multidrug efflux system can also export chloramphenicol, but to a lesser degree. (Schwarz et al., 2004). Several chloramphenicol resistance genes (e.g., *catA*, *catB*, *cmlA* and *floR*) were found to be located on either transposon (e.g., Tn*9* and Tn*2424*) and plasmids. In certain plasmids (such as the IncX plasmid), chloramphenicol resistance genes were preserved, rather than in others (Darphorn et al., 2021). These genes were found co-located on the same conjugated plasmid with the other AMR genes such as *tet*, *aadA* and *sul*, conferring multidrug resistance phenotype (Ozawa et al., 2023). Despite being banned in food animals, chloramphenicol is used topically to treat eye infections in humans. Chloramphenicol has antibiofilm activity, hypothetical low impact on ocular microbiota and narrow resistance rate compared to fluoroquinolones (Lorenzo, 2019). There might be the resuscitation of an old antimicrobial medication such as chloramphenicol in the situation that newer medications are not readily available due to the AMR issue. Therefore, investigating the mechanisms behind the persistence of chloramphenicol resistance in the absence of chloramphenicol will be worthwhile.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

In the DNA sequencing era, whole genome sequencing (WGS) has become an indispensable technique for AMR research and control, including the discovery of new antibiotics, identification of AMR in clinical samples, surveillance of AMR, and emergence of AMR (Koser et al., 2014). Information generated by WGS is greatly beneficial for comprehending the origins of AMR and the genetic foundation of AMR mechanisms. Therefore, this study aims to investigate the chloramphenicol resistance and possible mechanisms associated with the persistence of chloramphenicol resistance in *Escherichia. coli* and *Salmonella enterica* isolated from pigs, pork, and humans.

4.3 MATERIAL AND METHODS

4.3.1 Bacterial isolates and antimicrobial susceptibilities

The *E. coli* (n=106) and *Salmonella* (n=57) isolates resistant to chloramphenicol (MIC≥ 32 μg/ml) were obtained from our previous AMR monitoring projects during 2007-2008. The *E. coli* isolates were obtained from cecal content of clinically healthy pigs at slaughterhouses. *E. coli* were isolated and biochemically confirmed (Cole, 1990; Quinn et al., 1994) and a single colony of *E. coli* of each positive sample was collected. The isolates exhibited resistance to ampicillin (AMP, 81.2%), chloramphenicol (CHP,100%), ciprofloxacin (CIP, 43.6%), gentamicin (GEN, 47%), streptomycin (STR, 57.3%), sulfamethoxazole (SMX, 67.5%), tetracycline (TET, 98.3%) and trimethoprim (TMP, 91.5%). The *Salmonella* isolates were isolated from raw pork (n=22) at retail markets, and patient's stools (n=37) at the hospitals using ISO 6579:2002 (ISO, 2002) and serotyped using slide agglutination. One colony of each serovars was collected from each positive sample, The *Salmonella* serovars included Anatum (n=5), Corvallis (n=5), Enteritidis (n=3), Kedougou (n=17), Newport (n=2), Panama (n=4), Rissen (n=7), Stanley (n=7), Typhimurium (n=4) and Weltevreden (n=3). These *Salmonella* were resistant to AMP (89.5%), CHP (100%), CIP (17.5%), GEN (38.6%), STR (89.5%), SMX (63.2%), TET (89.5%) and TMP (91.5%).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Each isolate of *Salmonella* and *E. coli* had varying combinations of at least *catA*, *catB*, and *cmlA*, including *cmlA* only (50.9%, 49%), *catA* only (0, 5.3%), *catB* only (4.7%, 10.5%), *cmlA/catA* (0.9%, 12.3%), *cmlA/catB* (28.3%, 10.5%), *catA/catB* (13.2%, 5.3%) and *cmlA/catA/catB* (1.9%, 7.0%). All bacterial isolates were stored as 20% glycerol at -80°C.

4.3.2 Determination of the effect of efflux system inhibitor on chloramphenicol susceptibility

The MIC value of chloramphenicol (Sigma-Aldrich, Saint Louis, MO) was determined in the presence and absence of 25 μg/mL phenylalanine arginine β-naphthylamide (PAβN, Sigma-Aldrich) using two-fold agar dilution method (CLSI, 2021). The

concentrations of CHP ranged from 1 μg/mL to 1,024 μg/mL. A 4-fold or more change in the chloramphenicol MIC value following the addition of PaβN was defined as significant. Experiments were repeated on two separate occasions.

4.3.3 PCR Based Replicon Typing (PBRT)

DNA templates were prepared using whole cell boiling method (Lévesque et al., 1995). All PCR amplification was performed using Top Taq Master Mix kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. All primers used in this study are listed in Table 17. Eighteen replicons including HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, F_{repB}, K and B/O were PCR amplified using the following thermocycles: one cycle of denaturation at 94°C for 5 min; followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. This was with exception for F simplex PCR using F_{repB} primers where annealing temperature of 52°C was used.

Table 17 Primer used in this study.

PCR-reaction	Name	Primer sequences	Amplicon size (bp)	Reference
PBRT				
Multiplex 1	HI1 FW	5'-GGAGCGATGGATTACTTCAGTAC-3'	471	(Carattoli et al., 2005)
	HI1 RV	5'-TGCCGTTTCACCTCGTGAGTA-3'		
	HI2 FW	5'-TTTCTCCTGAGTCACCTGTTAACAC-3'	644	
	HI2 RV	5'-GGCTCACTACCGTTGTCATCCT-3'		
	I1 FW	5'-CGAAAGCCGGACGGCAGAA-3'	139	
	I1 RV	5'-TCGTCGTTCCGCCAAGTTCGT-3'		
Multiplex 2	X FW	5'-AACCTTAGAGGCTATTTAAGTTGCTGAT-3'	376	(Carattoli et al., 2005)
	X RV	5'-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3'		
	L/M FW	5'-GGATGAAAACTATCAGCATCTGAAG-3'	785	
	L/M RV	5'-CTGCAGGGGCGATTCTTTAGG-3'		
	N FW	5'-GTCTAACGAGCTTACCGAAG-3'	559	
	N RV	5'-GTTTCAACTCTGCCAAGTTC-3'		
Multiplex 3	FIA FW	5'-CCATGCTGGTTCTAGAGAAGGTG-3'	462	(Carattoli et al., 2005)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

PCR-reaction	Name	Primer sequences	Amplicon size (bp)	Reference
	FIA RV	5’-GTATATCCTTACTGGCTTCCGCAG-3’		
	FIB FW	5’-GGAGTTCTGACACACGATTTTCTG-3’	702	
	FIB RV	5’-CTCCCGTCGCTTCAGGGCATT-3’		
	W FW	5’-CCTAAGAACAACAAAGCCCCCG-3’	242	
	W RV	5’-GGTGCGCGGCATAGAACCGT-3’		
Multiplex 4	Y FW	5’-AATTCAAACAACACTGTGCAGCCTG-3’	765	(Carattoli et al., 2005)
	Y RV	5’-GCGAGAATGGACGATTACAAAACTTT-3’		
	P FW	5’-CTATGGCCCTGCAAACGCGCCAGAAA-3’	534	
	P RV	5’-TCACGCGCCAGGGCGCAGCC-3’		
	FIC FW	5’-GTGAACTGGCAGATGAGGAAGG-3’	262	
	FIC RV	5’-TTCTCCTCGTCGCCAAACTAGAT-3’		
Multiplex 5	A/C FW	5’-GAGAACCAAAGACAAAGACCTGGA-3’	465	(Carattoli et al., 2005)
	A/C RV	5’-ACGACAAACCTGAATTGCCTCCTT-3’		
	T FW	5’-TTGGCCTGTTTGTGCCTAAACCAT-3’	750	
	T RV	5’-CGTTGATTACACTTAGCTTTGGAC-3’		
	FII_s FW	5’-CTGTCGTAAGCTGATGGC-3’	270	
	FII_s RV	5’-CTCTGCCACAAACTTCAGC-3’		
Simplex F	F_{repB} FW	5’-TGATCGTTTAAGGAATTTTG-3’	270	(Carattoli et al., 2005)
	F_{repB} RV	5’-GAAGATCAGTCACACCATCC-3’		
Simplex K	K/B FW	5’-GCGGTCCGGAAAGCCAGAAAAC-3’	160	(Carattoli et al., 2005)
	K RV	5’-TCTTTCACGAGCCCGCCAAA-3’		
Simplex B/O	K/B FW	5’-GCGGTCCGGAAAGCCAGAAAAC-3’	159	(Carattoli et al., 2005)
	B/O RV	5’-TCTGCGTTCCGCCAAGTTCGA-3’		
Chloramphenicol resistance genes				
catA	catA FW	5’-CCAGACCGTTCAGCTGGATA-3’	454	(Chuanchuen and Padungtod, 2009)
	catA RV	5’-CATCAGCACCTTGTCGCCT-3’		
catB	catB FW	5’-CGGATTCAGCCTGACCACC-3’	461	(Chuanchuen and Padungtod, 2009)
	catB RV	5’-ATACGCGGTCACCTTCCTG-3’		
cmlA	cmlA FW	5’-TGGACCGCTATCGGACCG-3’	641	(Chuanchuen and Padungtod, 2009)
	cmlA RV	5’-CGCAAGACACTTGGGCTGC-3’		

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.3.4 Conjugation experiment

Biparental mating was performed to investigate co-selection of CHP resistance by other antibiotics. All the *E. coli* (n=106) and *Salmonella* (n=57) isolates served as donors. *Salmonella* Enteritidis SE12rif^R (CHP MIC = 4 µg/mL) (Pungpian et al., 2020) and *E. coli* MG1655rif^R (CHP MIC = 4 µg/mL) (Khemtong and Chuanchuen, 2008) were used as recipients for *E. coli* and *Salmonella* donors, respectively. Transconjugants were selected on Luria Bertani agar containing rifampicin (32 µg/mL) and one of the following antibiotics, AMP (150 µg/mL), TET (10 µg/mL) and STR (50 µg/mL). Transconjugants were confirmed to be *E. coli* or *Salmonella* by growing on Eosin Methylene Blue agar (EMB; Difco^{TM}, MI, USA) or Xylose Lysine Deoxycholate agar (XLD; Difco^{TM}, MI, USA), respectively. The transconjugants were determined for their susceptibilities to CHP and corresponding antibiotics (i.e., AMP, TET and STR) and screened for *catA*, *catB* and *cmlA* (Chuanchuen and Padungtod, 2009). CHP MIC changed by at least four times from the recipients was considered significant. One of transconjugant from each selective pressure plate was selected for further plasmid studying. The *E. coli* (n=11) and *Salmonella* (n=9) donors and their corresponded transconjugants with CHP MIC ≥4-fold increase (17 *Salmonella* transconjugants and 18 *E. coli* transconjugants), were subjected to PBRT. *E. coli* MG1655rif^R and *Salmonella* SE12rif^R did not carry any of the 18 replicons tested.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.3.5 Whole Genome Sequencing (WGS) and Bioinformatics Analysis

Genomic DNA was extracted from the *E. coli* (n=3) and *Salmonella* (n=3) isolates that could transfer CHP resistance genes using ZymoBIOMICS^{TM} DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA). The degradation of the isolated genomic DNA was assessed by running 5 µL of the DNA on 0.8% agarose gel. The quality and quantity of the genomic DNA were then assessed using NanoDrop^{TM} 1000 spectrophotometer (Thermo Scientific, Deleware, USA) and submitted for WGS using Oxford Nanopore technologies (ONT) for long read sequencing at Siriraj Long-read Lab, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and using Illumina platform Hiseq sequencers (Illumina, San Diego, CA, USA) for short read sequencing at GENEWIZ China and Suzhou Lab, (GENEWIZ, Suzhou, China). Sequencing analysis was

done as previously described (Arigul et al., 2023). Adapters were trimmed using Porechop v0.2.4 (https://github.com/rrwick/Porechop). ONT and Illumina reads were quality checked using NanoPlot (De Coster et al., 2018) and FastQC (Andrews, 2010), respectively. High quality ONT and Illumina reads were assembled to create hybrid genome using Unicycler (Wick et al., 2017). The genomic characteristics including genome size, number of contigs and % GC content were identified using QUAST (Gurevich et al., 2013). Taxonomic identification was performed using Kraken2 (Wood et al., 2019) and Genome annotation was conducted using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The assembled genome/contigs were then analyzed at Center for Genomic Epidemiology website (http://www.genomicepidemiology.org/services/). Multilocus sequence typing (MLST) was performed using MLST 2.0 with the obtained data from http://pubmlst.org. The serotypes of *E. coli* were classified by SeroTypeFinder (Joensen et al., 2015). The *Salmonella* serotypes were confirmed by SeqSero 1.2 (Zhang et al., 2015). Virulence genes were identified by VirulenceFinder 2.0 (Joensen et al., 2014). AMR genes were identified by ResFinder4.1 (Florensa et al., 2022). Mobile genetic elements (MGE) and plasmids were identified by MobileElementFinder v1.0.3 (Johansson et al., 2021) and PlasmidFinder2.1 (Camacho et al., 2009; Carattoli et al., 2014), respectively. Variant calling and core genome alignment was performed by Snippy (Seemann, 2015). Phylogenetic trees were generated by IQ-TREE (Nguyen et al., 2015) and visualized by iTOL v6 (Letunic and Bork, 2021). The comparison of genetic environment of CHP resistance genes and the location of insertion sequences (ISs) and mobile genetic elements were achieved using EasyFig (Sullivan et al., 2011) and Proksee (Grant et al., 2023).

4.3.6 Statistical analysis

The descriptive statistic including percentage was analyzed by excel program. The chi-squared test and z-test using Bonferroni method with SPSS version 22.0 program was used to compare the effect of PAβN on MIC values of chloramphenicol. A *p*-value of <0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 Effect of efflux pump inhibitor to chloramphenicol MIC

Effects of PAβN on CHP MICs were determined in all *E. coli* (n=106) and *Salmonella* (n=57) (Table 18). Most *E. coli* (67.9%) and *Salmonella* (64.9%) had ≥4-fold CHP MIC decrease in the presence of PAβN. The significant association between the presence of CHP genes and the reduction of CHP MICs in the presence of PAβN was observed only in *E. coli* ($p<0.05$).

In comparison to *E. coli* with ≥4-fold CHP MIC decrease (29/72, 40.3%), the presence of *cmlA* was significantly greater in those with <4-fold CHP MIC decrease (25/34, 75%).

All *E. coli* carrying both *catA and catB* (14/72, 19.4%) exhibited ≥4-fold CHP MIC decrease in the presence of PAβN ($p<0.05$). Neither of *catA* and *catB* were detected among *E. coli* with <4-fold CHP MIC decrease. This was not the case for the *Salmonella* isolates. All the *Salmonella* isolates with <4-fold reduction of CHP MIC in the presence of PAβN (3/20, 15%) carried both *catA* and *catB* genes None of the *Salmonella* isolates with ≥4-fold reduction of CHP MIC contained *catA* and *catB*.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 18 Effect of phenylalanine arginine β-naphthylamide (PAβN) on MIC values of chloramphenicol in *Escherichia coli* (n=106) and *Salmonella* (n=57).

Bacterial species	CHP resistance genes pattern	No. of isolates (% within fold) with indicated fold reduction of CHP MIC when presence of PAβN	
		<4 fold	≥4 fold
Escherichia coli (n=106)	*cmlA* (n=54)	25(73.5)^{a}	29(40.3)^{b}
	catB (n=5)	2(5.9)^{a}	3(4.2)^{a}
	cmlA+catA (n=1)	1(2.9)^{a}	0^{a}
	cmlA+catB (n=30)	6(17.6)^{a}	24(33.3)^{a}
	catA+catB (n=14)	0^{a}	14(19.4)^{b}
	cmlA+catA+catB (n=2)	0^{a}	2(2.8)^{a}
	Total	34(32.1)	72(67.9)

Bacterial species	CHP resistance genes pattern	No. of isolates (% within fold) with indicated fold reduction of CHP MIC when presence of PAβN	
		<4 fold	≥4 fold
Salmonella (n=57)	*cmlA* (n=28)	10(50) [a]	18(48.7) [a]
	catA (n=4)	1(5) [a]	3(8.1) [a]
	catB (n=6)	2(10) [a]	4(10.8) [a]
	cmlA+catA (n=6)	1(5) [a]	5(13.5) [a]
	cmlA+catB (n=6)	2(10) [a]	4(10.8) [a]
	catA+catB (n=3)	3(15) [a]	0 [b]
	cmlA+catA+catB (n=4)	1(5) [a]	3(8.1) [a]
	Total	20(35.1)	37 (64.9)

[a,b] Values with different superscripts within each row, indicated statistical significantly difference (p <0.05) between <4 fold reduction and ≥4 fold reduction of CHP MIC when presence of PAβN.

4.4.2 Co-selection of chloramphenicol resistance by other antibiotics

The *E. coli* isolates could generate CHP resistant *Salmonella*-transconjugants that exhibited ≥ 4-fold CHP MIC increase were obtained when AMP (i.e., E289, E290, E291, E292, E293, E294, E297, E331 and E333), TET (i.e., E290, E291, E292, E293, E294 and E295) and STR (i.e., E329 and E392) were used as selective pressure in conjugation experiment. When considered clinical breakpoint (≥ 32 µg/mL), 9 *E. coli* yielded *Salmonella*-transconjugants with CHP MIC ≥ 32 µg/mL (32-256 µg/mL) in the presence of different selective pressure (AMP, n=6; TET, n=6 and STR, n=1) (Table 19). All the above *E. coli* donors could transfer *cmlA. Salmonella* transconjugants carrying *cmlA* with ≥ 4-fold CHP MIC increase (32-128 µg/mL) were obtained when AMP, TET and STR were used as selective pressure.

Some *Salmonella* produced CHP resistant *E. coli*-transconjugants with ≥ 4-fold CHP MIC increase when AMP (i.e., SA449 and SA606), TET (i.e., SA448, SA449, SA461, SA515, SA633, SA639, SA666 and SA759) and STR (i.e., SA448, SA461, SA515, SA633, SA639, SA666, SA741 and SA759) were used as selective pressure. Among these, 10 *Salmonella* generated CHP resistant *E. coli*-transconjugants that had CHP MIC ≥ 32

μg/mL (32-512 μg/mL) under the selective pressure of AMP (n=1), TET (n=8) and STR (n=7) (Table 24). SA448 and SA741 could transfer *cmlA. E. coli* transconjugants carrying *cmlA* were obtained in the presence of AMP, TET and STR selective pressure and exhibited ≥ 4-fold CHP MIC increase (CHP MIC = 32-256 μg/mL). SA448 additionally produced *catA-E. coli*-transconjugants exhibiting ≥ 4-fold CHP MIC increase (CHP MIC = 256-512 μg/mL) in TET and STR.

4.4.3 Incompatibility groups of transferable plasmids

All *E. coli* donors (n=11) carried at least two plasmids (i.e., IncI1/K/F (n=7), IncI1/F (n=1), IncHI1/ FIIAs/K (n=2) and IncHI1/K/FIB / (n=1)) (Table 20). IncFIIAs plasmids were commonly found in *Salmonella* transconjugants selected by AMP (i.e., AMPE289, AMPE290, AMPE291, AMPE293, AMPE294, AMPE297 and AMPE333) and TET (i.e., TETE290, TETE291, TETE292, TETE293 and TETE295). All *E. coli* donors of these transconjugants carried IncF plasmid, except for E333 containing IncFIIAs plasmid. Two *Salmonella* transconjugants selected by AMP (AMPE331 and AMPE333) and their donors were found to carry IncHI plasmid. Three *Salmonella* transconjugants (i.e., AMPE292, TETE294 and STRE329) acquired *cmlA* from their donors but were not positive to any replicons detected.

The *Salmonella* donors (n=9) carried at least one plasmid including IncHI1/FIIAs (n=2), IncI1/FIB/F (n=1), IncFIB/A/C/F (n=1), IncFIIAs (n=1) IncFIB and F (n=4). Most *E. coli* transconjugants (i.e., SA449T_TET, SA449T_AMP, SA515T_TET, SA515T_STR, SA633T_STR, SA639T_TET, SA639T_STR, SA666T_TET, SA666T_STR, SA759T_TET and SA759T_STR) carried both IncFIB and IncF replicons that were present in their donors. While *E. coli* transconjugants of SA488 (i.e., SA448T_TET and SA448T_STR), SA461 (i.e., SA461T_TET and SA461T_STR), SA606 (i.e., SA606T_AMP) and SA633 (i.e., SA633T_TET) acquired only IncHI1, F, FIIAs and FIB plasmid, from their respective donors.

Table 19 Conjugation rates and chloramphenicol resistance phenotype of transconjugants.

Donor	Antibiotic selective pressure	No (%) isolates with CHP resistance transferability	No (%) of transconjugant with ≥4-fold* increase of CHP MIC			
			MIC of CHP of transconjugants		CHP resistance gene	
			<32 µg/mL	≥32 µg/mL	*cmlA*	*catA*
Escherichia coli (n=106)	AMP	9(8.5)	3/9(27.3)	6/9(54.5)	9/9(100)	-
	TET	6(5.7)	-	6/6(100)	6/6(100)	-
	STR	2(1.9)	1/2(50)	1/2(50)	1/2(50)	-
Salmonella (n=57)	AMP	2(3.5)	1/2(50)	1/2(50)	2/2(100)	-
	TET	8(14.0)	-	8/8(100)	7/8(87.5)	1/8(12.5)
	STR	8(14.0)	1/8(5.9)	7/8(87.5)	6/8(75)	1/8(12.5)

AMP, ampicillin; CHP, chloramphenicol; STR, streptomycin; TET, tetracycline.

*When compared to the original CHP MIC of recipients.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 20 Plasmid of *E. coli* (n=11) and *Salmonella* (n=9) donors and corresponded chloramphenicol resistant transconjugants.

Donor				Selective pressure	Transconjugant			
ID	Inc	CHP resistance gene	CHP MIC (µg/mL)		ID	Inc	CHP resistance gene	CHP MIC (µg/mL)
E289	I1, K, F	*cmlA*	32	AMP	E289T_AMP	FIIAs	*cmlA*	64
E290	I1, K, F	*cmlA*	64	TET	E290T_TET	FIIAs	*cmlA*	32
				AMP	E290T_AMP	FIIAs	*cmlA*	128
E291	I1, K, F	*cmlA*	32	TET	E291T_TET	FIIAs	*cmlA*	64
				AMP	E291T_AMP	FIIAs	*cmlA*	64
E292	I1, K, F	*cmlA*	32	TET	E292T_TET	FIIAs	*cmlA*	64
				AMP	E292T_AMP	-	*cmlA*	16
E293	I1, K, F	*cmlA*	32	TET	E293T_TET	FIIAs	*cmlA*	64
				AMP	E293T_AMP	FIIAs	*cmlA*	32
E294	I1, K, F	*cmlA*	32	TET	E294T_TET	-	*cmlA*	64
				AMP	E294T_AMP	FIIAs	*cmlA*	64
E295	I1, K, F	*cmlA*	64	TET	E295T_TET	FIIAs	*cmlA*	64
E297	I1, F	*cmlA*	32	AMP	E297T_AMP	FIIAs	*cmlA*	64
E329	HI1, K, FIB, F	*cmlA*	64	STR	E329T_STR	-	*cmlA*	32
E331	HI1, FIIAs, K	*cmlA*	32	AMP	E331T_AMP	HI1	*cmlA*	16
E333	HI1, FIIAs, K	*cmlA*	32	AMP	E333T_AMP	HI1, FIIAs	*cmlA*	16
SA448	HI1, FIIAs	*catA*	256	TET	SA448T_TET	HI1	*catA*	256
				STR	SA448T_STR	HI1, K	*catA*	512
SA449	HI1, FIIAs	*catA, cmlA*	128	TET	SA449T_TET	FIB, F	*cmlA*	32
				AMP	SA449T_AMP	FIB, F	*cmlA*	16
SA461	I1, FIB, F	*cmlA*	256	TET	SA461T_TET	F	*cmlA*	32
				STR	SA461T_STR	F	*cmlA*	32
SA515	FIB, A/C, F	*cmlA*	256	TET	SA515T_TET	FIB, A/C, F	*cmlA*	256
				STR	SA515T_STR	FIB, A/C, F	*cmlA*	128
SA606	FIIAs	*catA, cmlA*	64	AMP	SA606T_AMP	FIB, FIIAs, F	*cmlA*	32
SA633	FIB, F	*catA, catB, cmlA*	128	TET	SA633T_TET	FIB	*cmlA*	32
				STR	SA633T_STR	FIB, FIIAs, F	*cmlA*	64
SA639	FIB, F	*catA, catB, cmlA*	128	TET	SA639T_TET	FIB, F	*cmlA*	32
				STR	SA639T_STR	FIB, FIIAs, F	*cmlA*	32

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Donor				Selective pressure	Transconjugant			
ID	Inc	CHP resistance gene	CHP MIC (μg/mL)		ID	Inc	CHP resistance gene	CHP MIC (μg/mL)
SA666	FIB, F	*cmlA*	128	TET	SA666T_TET	FIB, F	*cmlA*	32
				STR	SA666T_STR	FIB, F	*cmlA*	16
SA759	FIB, F	*cmlA*	128	TET	SA759T_TET	FIB, F	*cmlA*	32
				STR	SA759T_STR	FIB, F	*cmlA*	32

AMP, ampicillin; CHP, chloramphenicol; STR, streptomycin; TET, tetracycline

4.4.4 Genomic characteristics of CHP-resistant *E. coli* and *Salmonella*

The quality of genome assembly of selected *E. coli* and *Salmonella* is shown in Table 26. The genome size and GC content of E290 (serotype O8:H16), E329 (serotype O37:H34), and E333 (serotype O37:H34) was 5,203,479 bp; 50.66%; 5,193,590 bp; 50.46% and 5,193,591 bp; 50.46%, respectively (Table 26). E290 was made up of 9 contigs including a chromosome and 8 plasmids. On the other hand, E329's whole genome contained one chromosome and 6 plasmids. Eight contigs, comprising 2 chromosomes and 6 plasmids, were present in E333.

For *Salmonella*, genome size and GC content in *Salmonella* Weltevreden SA448, *Salmonella* Rissen SA461 and *Salmonella* Rissen SA515 were 5,419,175 bp; 51.86%; 5,127,858 bp; 52.05%, and 5,214,816 bp, 52.06%, respectively (Table 21). SA448 and SA461 comprise 4 contigs, including one chromosome and 3 plasmids. There were 11 contigs in SA515, of which 3 were plasmids and 8 were made up of chromosomes.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 21 Quality of whole genome assembly of *Escherichia coli* (n=3) and *Salmonella enterica* (n=3)

Bacterial isolate	GC content (%)	Accession No.	Whole Genome length (bp)	Contigs No.	Chromosomal/plasmid contigs size (bp)
E290	50.66	SAMN35027954	5,203,479	1	4,861,935 (Chromosome)
				2-9	100,162 (pO111 plasmid); 89,517 (IncI1 plasmid); 70,588 (IncX1 plasmid); 68,572 (IncFII plasmid); 4,320; 3,830 (Col440I); 3,003 (Col440I) and 1,552 (Col(MG828))
E329	50.46	SAMN35027955	5,193,590	1	4,849,047 (Chromosome)
				2 -7	275,323 (IncFIA(HI1)/HI1B plasmid); 49,549 (IncX1 plasmid); 9,196 (Col440I); 4,724; 3,295 and 2,459
E333	50.46	SAMN35027956	5,193,591	1	4,849,048 (Chromosome)
				2 and 4	275,323 (IncFIA(HI1)/HI1B plasmid)
				3, 5-8	49,546 (IncX1 plasmid); 9,196 (Col440I); 4,724; 3,295 and 2,459
SA448	51.86	SAMN35027957	5,419,175	1	5,101,868 (Chromosome)
				2	232,899 (IncFIA(HI1)/HI1B/Q1 plasmid); 81,966 (IncFII(s) plasmid) and 2,442
SA461	52.05	SAMN35027958	5,127,858	1	4,936,602 (Chromosome)
				2-4	102,883 (IncFII/FIB plasmid); 83,716 (IncI1 plasmid) and 4,657
SA515	52.06	SAMN35027959	5,214,816	1-8	4,991,361 (Chromosome)
				9-11	114,245 (IncA/C plasmid); 105,743 (IncFII/FIB plasmid) and 4,657

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

4.4.4.1 Genetic relatedness of CHP-resistant *E. coli* and *Salmonella*

Genome was compared by MLST analysis using Whole Genome Sequence data. Using *E. coli* scheme#1, E290, E329, and E333 were identified as ST10, ST156, and ST156, respectively. With E. *coli* scheme#2, they were identified as ST2, ST19, and ST19, respectively. After comparison to *E. coli* LF82 reference strain (accession no. CP082771), variations of chromosomal DNA sequence of selected *E. coli* were called, and genetic trees were generated (Figure 5A). Additional sequences of *E. coli* with similar serotypes (ST10/2, accession no. SRR12903891 and SRR24437713; ST19/156, accession no. SRR3745274 and SRR25176867) were included for the comparison to ensure homogeneity. Two distinct clades of *E. coli* were identified in phylogenetic trees. E290 was closely related to *E. coli* ST10/2 reference (accession no. SRR24437713 and accession no. SRR12903891). E329 and E333 were in the same clade with close relationship to *E. coli* ST156 (accession no. SRR25176867), *E. coli* O8:H16 (accession no. SRR5040873).

SA448, SA461 and SA515 were classified as ST365, ST469 and ST469, respectively. Chromosome DNA variations were identified by comparison with *Salmonella* Typhimurium LT2 (accession no. NC003197.2), Additional sequences of *Salmonella* with similar serotypes and sequence type (*Salmonella* Weltevreden or ST365, accession no. SRR13853517, SRR24258077 and SRR21734369; *Salmonella* Rissen or ST469, accession no. SRR13853514 and SRR24401736) were included for the comparison to ensure homogeneity. Genetic relatedness was demonstrated using phylogenetic trees (Figure 5B), of which 2 distinct clades were identified. SA448 was closely related to *Salmonella* Weltevreden (accession no. SRR24258077) and *Salmonella* ST365 (accession no. SRR21734369). SA461 and SA515 shared a close lineage with *Salmonella* Rissen (accession no. SRR13853514) and *Salmonella* Rissen ST469 (accession no. SRR13853514).

4.4.4.2 Antimicrobial resistance genes and plasmid characteristics

Resistance genes, virulence genes and chromosomal point mutations are presented in Table 22. All three *E. coli* carried class1 integrons with *cmlA.* The *dfrA12*, *aadA2*,

aadA1 genes were found in the same variable region, while *qacL* and *sul3* were present in 3'conserved region. In E290, class1 integrons with *cmlA1* gene cassette were located on IncX1 plasmid that also included $bla_{TEM\text{-}1B}$ and *tetA*. The isolates additionally contained pO111 plasmid carrying $bla_{TEM\text{-}1B}$. The *cmlA1*-carrying class 1 integrons in E329 and E333 were located on IncFIA(HI1)/HI1B plasmids with *aph(3'')-Ib*, *aac(3)-IId*, *mcr3.1*, *mefB*, and $bla_{TEM\text{-}1B}$. These two isolates possessed the chromosomally encoded *tetB* and additionally had IncX1 plasmid without AMR gene but virulence gene, *mrkA*.

Plasmid Multilocus Sequence Typing (pMLST) and replicon sequence type (RST) of IncI1, IncF and IncHI plasmids were identified. IncFII plasmid of the FAB formula F10:A-:B-, and IncI1 plasmid of ST7, were identified in E290 but without resistance genes. The IncFIA(HI1)/HI1B plasmids of E329 and E333 were identified as sequence type (ST) 1 for IncHI1 and the FAB formular F-:A8:B-.

All *Salmonella* isolates carried CHP resistance encoding genes including *catA* in SA448 and *cmlA1* in SA461 and SA515 (Table 22). Class1 integrons with *cmlA1* on IncFII/FIB plasmids was found in both SA461 and SA515 but not SA448. The *aadA2*, *aph(3')-Ia*, *aadA1*, *sul3*, *dfrA12*, $bla_{TEM\text{-}1B}$, *mefB* and *qacL* genes co-localized on the same plasmid. SA515 also contained IncA/C plasmid with *floR,* which deliberates resistance to chloramphenicol, as well as *aph(6)-Id*, *aph(3'')-Ib*, *sul2* and $bla_{CMY\text{-}2}$. Plasmids belonging to IncFIA(HI1)/HI1B/Q1 of SA448 carried *catA* as well as *aph(6)-Id*, *aph(3'')-Ib*, *sul2*, and *tetB*. In addition, *acc(6')-Iaa* was found on chromosomal of all three *Salmonella* isolates. The chromosomally encoded *tetA* gene was exclusively in SA461 and SA515.

Based on pMLST and RST results, IncFIA(HI1)/HI1B/Q1 plasmids in SA448 were identified as ST2 and F-:A8:B-. In the same isolate, IncFII(s) plasmid belonged to S1:A-:B- FAB formula was found with the absences of resistance and virulence genes. IncFII/FIB plasmids carrying *cmlA1* in in SA461 and SA515 was in F46:A-:B- FAB

formula. SA461 additionally carried IncI1plasmid without AMR and virulence genes. IncA/C plasmid of ST3 lacking *floR* was found in SA515.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

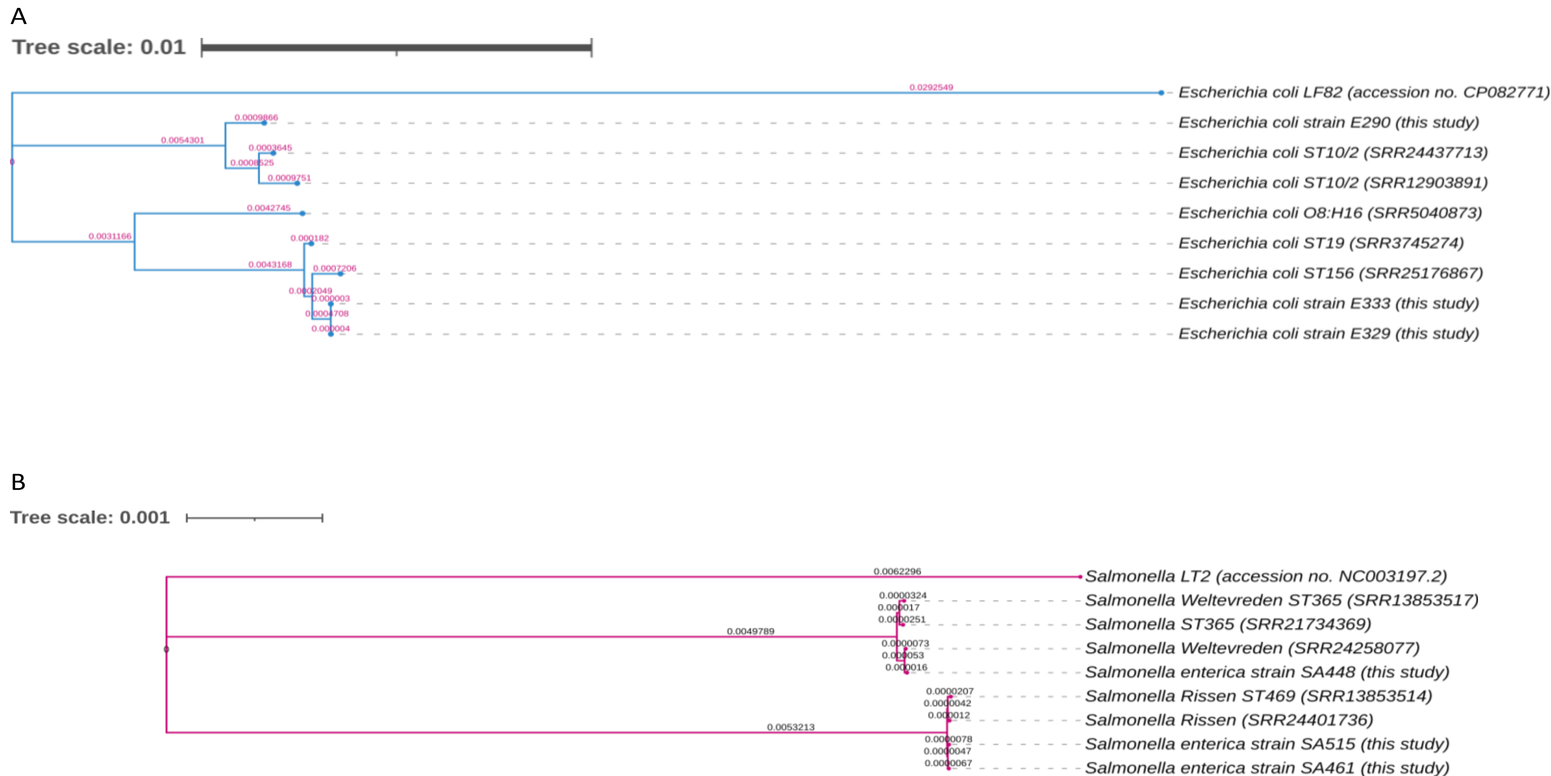


Figure 5 Phylogenetic tree by WGS analysis of *Escherichia coli* (n=3) and *Salmonella enterica* (n=3).

Chromosomal sequences of (A) E290, E329, E333, reference strains (*E. coli* ST10/2; *E. coli* O8:H16; *E. coli* ST19 and *E. coli* ST156) were aligned with *E. coli* LF82 and (B) SA448, SA461, SA515, *Salmonella* Weltevreden, *Salmonella* ST365, *Salmonella* Rissen and *Salmonella* ST469 were aligned with *Salmonella* Typhimurium LT2. The single nucleotide polymorphisms (SNPs) were called using Snippy. Phylogenetic trees were generated using Core SNP alignment and visualized by iTOL. The number on the branch indicates genetic changes.

Table 22 Prediction of antimicrobial resistance genes, virulence genes and plasmids from whole genome assembly data in selected *Escherichia coli* (n=3) and *Salmonella enterica* (n=3)

Bacterial species	Strain	Serotype	Sample source	CHP MIC (μg/mL)	MLST	Gene mutation	Contig No.	Plasmid		Acquire antimicrobial resistance genes	Virulence genes
								Inc group	pMLST / FAB formula		
Escherichia coli	E290	O8:H16	Pig	64	10[a]/2[b]	- *parC*:p.E62K, - *gyrB*:p.A306S	1[c]	-	-	-	*yehD, terC, fimH, hlyE, iss, AslA, csgA, fdeC, fyuA, hha, fimH, yehB, gad, yehC, nlpl, astA, irp2, yehA*
							2	pO111	-	bla_{TEM-1B}	-
							3	I1	7	-	-
							4	X1	-	*aadA1, aadA2, dfrA12, sul3, tetA, tetM,* bla_{TEM-1B}*, qacL, cmlA*	-
							5	FII	F10:A-:B-	-	*traT, sepA*
	E329	O37:H34	Pig	64	156[a]/19[b]	- *gyrA*:p.S83L*, *gyrA*:p.D87H*, - *parC*:p.S80I*, *parC*:p.E62K, - *ampC*-promoter:g.-18G>A, *ampC*-promoter:g.-1C>T, - *pmrB*:p.Y358N, *pmrB*:p.D283G, - *pmrA*:p.G144S,	1[c]	-	-	*tetB*	*hra, gad, hha, fimH, terC, hlyE, yehC, csgA, yehB, nlpl, fdeC, lpfA, yehA, yehD, iss,*
							2	HI1A, HI1B(R27) and FIA(HI1)	1(HI1)/ F-:A8:B-	*aadA1, aadA2, aph(3'')-Ib, aac(3)-IId, mcr3.1, sul3, dfrA12, tetM, blaTEM-1B, mefB, qacL, cmlA1*	-
							3	X1	-	-	*mrkA*
	E333	O37:H34	Pig	512	156[a]/19[b]	- *gyrA*:p.S83L*, *gyrA*:p.D87H*, - *parC*:p.S80I*, *parC*:p.E62K, - *ampC*-promoter:g.-18G>A, *ampC*-promoter:g.-1C>T, - *pmrB*:p.D283G, *pmrB*:p.Y358N,	1[c]	-	-	*tetB*	*nlpl, fdeC, iss, yehB, lpfA, yehC, gad, terC, hha, hra, yehA, hlyE, csgA, fimH, yehD*
							2&4	HI1A, HI1B (R27),	1 (HI1)/ F-:A8:B-	*aac(3)-IId, aadA1, aph(3")-Ib, aadA2, mcr3.1, sul3, dfrA12, tetM,* bla_{TEM-1B}*, mefB,*	

Bacterial species	Strain	Serotype	Sample source	CHP MIC (μg/mL)	MLST	Gene mutation	Contig No.	Plasmid		Acquire antimicrobial resistance genes	Virulence genes
								Inc group	pMLST / FAB formula		
						- *pmrA*:p.G144S		FIA(HI1)		*qacL, cmlA1*	
							3	X1	-	-	*mrkA*
Salmonella enterica	SA448	Weltevreden	Pork	256	365	- *acrB*:p.L40P, *acrB*:p.F28L, - *parC*:p.T57S*	1[c]	-	-	*aac(6')-Iaa*	*nlpl*
							2	HI1A, HI1B (R27), FIA(HI1), Q1	2 (HI1)/ F-:A8:B-	*aph(6)-Id, aph(3")-Ib, sul2, tetB, catA1*	-
							3	FII(s)	S1:A-:B-	-	-
	SA461	Rissen	Pork	256	469	- *parC*:p.T57S*, - *acrB*:p.L40P, *acrB*:p.F28L	1[c]	-	-	*aac(6')-Iaa, tetA*	*nlpl*
							2	FII/FIB	F46:A-:B-	*aadA2, aph(3')-Ia, aadA1, dfrA12, sul3,* bla_{TEM-1B}, *mefB, qacL, cmlA1*	*traT, traJ, anr*
							3	I1	113[d] and 115[d]	-	-
	SA515	Rissen	Pork	256	469	- *parC*:p.T57S*, - *acrB*:p.L40P, *acrB*:p.F28L	1, 3, 5, 7-14[c]	-	-	*aac(6')-Iaa, tetA*	*nlpl*
							2	A/C	3	*aph(6)-Id, aph(3")-Ib, sul2, blaCMY-2, floR*	-
							4	FII/FIB	F46:A-:B-	*aadA2, aph(3')-Ia, aadA1, dfrA12, sul3, tetA, blaTEM-1B, mefB, qacL, cmlA1*	*traT, traJ, anr*

[a] MLST results which were typed using MLST *Escherichia coli* scheme #1

[b] MLST results which were typed using MLST *Escherichia coli* scheme #2

[c] chromosomal DNA contig

[d] Nearest results that obtained from pMLST 2.0

CHP, chloramphenicol; MIC, minimum inhibitory concentration; MLST, muti locus sequence typing.

4.4.4.3 Structural comparison of plasmid carrying *cmlA1, catA* and *floR*

IncFIA(HI1)/HI1B plasmids from E329 and E333 and IncFII/FIB plasmid from SA461 and SA515 shared some similar structure and sequences (Figure 6). AMR genes and ISs in two regions located upstream and downstream of IncFIA(HI1)/HI1B plasmids are distributed in IncFII/FIB and IncX1 plasmid. In the upstream region, class1 integrons with *dfrA12-aadA2-cmlA1-aadA1* gene cassette array and *qacL*-IS*256-sul3* conserved region were identified in all plasmids. Class 1 integrons were flanked by Tn*3*-like element *Tn3* or Tn*As1* family transposase at upstream and IS*6*-like element of IS*26* family transposase at downstream. The downstream region with $bla_{TEM\text{-}1B}$ and ISs/transposons (i.e., IS*6*-like element of IS*26* family transposase, IS*256* and *Tn3*) were identified in all plasmids.

IncFIA(HI1)/HI1B/Q1 plasmid from SA448 had the highest sequence similarity with p30155-1 plasmid from *Salmonella* Derby originated from swine (accession no., CP053049.1). The *tetB*, *aph(6)-Id, aph(3'')-Ib, sul2* and *catA1* genes were found in all plasmids, except CP022495.1. Horizontal gene transfer (HGT) regions were present only in IncFIA(HI1)/HI1B/Q1 plasmid of SA448, CP053049.1 and CP022495.1 (Figure 7A and 7B). The *catA1* gene was located in the HGT region and flanked by IS*1*-like element IS*1B* family transposase and Tn*3*-like element Tn*As3* family transposase.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

IncA/C plasmid carrying *floR* identified in *Salmonella* strain SA515 was closely related to pSANI-1736 from *Salmonella* Anatum isolated from bovine (accession no., CP014658.1) and pF18S036-1 from *Salmonella* Ohio isolated from swine (accession no., CP082407.1) (Figure 8). The common resistance genes found in all plasmids included *tetA*, *aph(6)-Id*, *aph(3'')-Ib*, *sul2* and $bla_{CMY\text{-}2}$. The *floR* gene was flanked by IS*91*-like element IS*Vsa3* family transposase and IS*91* family transposase. The *int2* gene of class 2 integrons were additionally identified.

Figure 6 Alignment of plasmids carrying cmlA1 of *E. coli* (n=3) and *Salmonella enterica* (n=2).

(A) Whole plasmid sequences of IncFIA(HI1)/HI1B plasmid from E329 and E333, IncFII/FIB plasmid from SA461, SA515, and IncX1 plasmid from E290 were compared. Green arrows indicate the position and direction of genes. Blue vertical blocks indicate regions of shared similarity shaded according to BLASTn (dark blue for matches in the same direction and red for inverted matches). (B) A zoomed-in view (yellow dashed line-box) shows the area containing class1 integrons with *cmlA1* gene cassette, which is flanked by Tn*3*-like element *Tn3* or Tn*As1* family transposase at upstream and IS*6*-like element of IS*26* family transposase at downstream. (C) A zoomed-in view (gray dashed line-box) shows the position of *bla*$_{TEM-1B}$ and IS*6*-like element of IS*26* family transposase that are located downstream of class1 integrons in IncFIA(HI1)/HI1B plasmid of E329 and E333 and IncX1 plasmid of E290 and upstream in IncFII/FIB plasmid from SA461 and SA515.

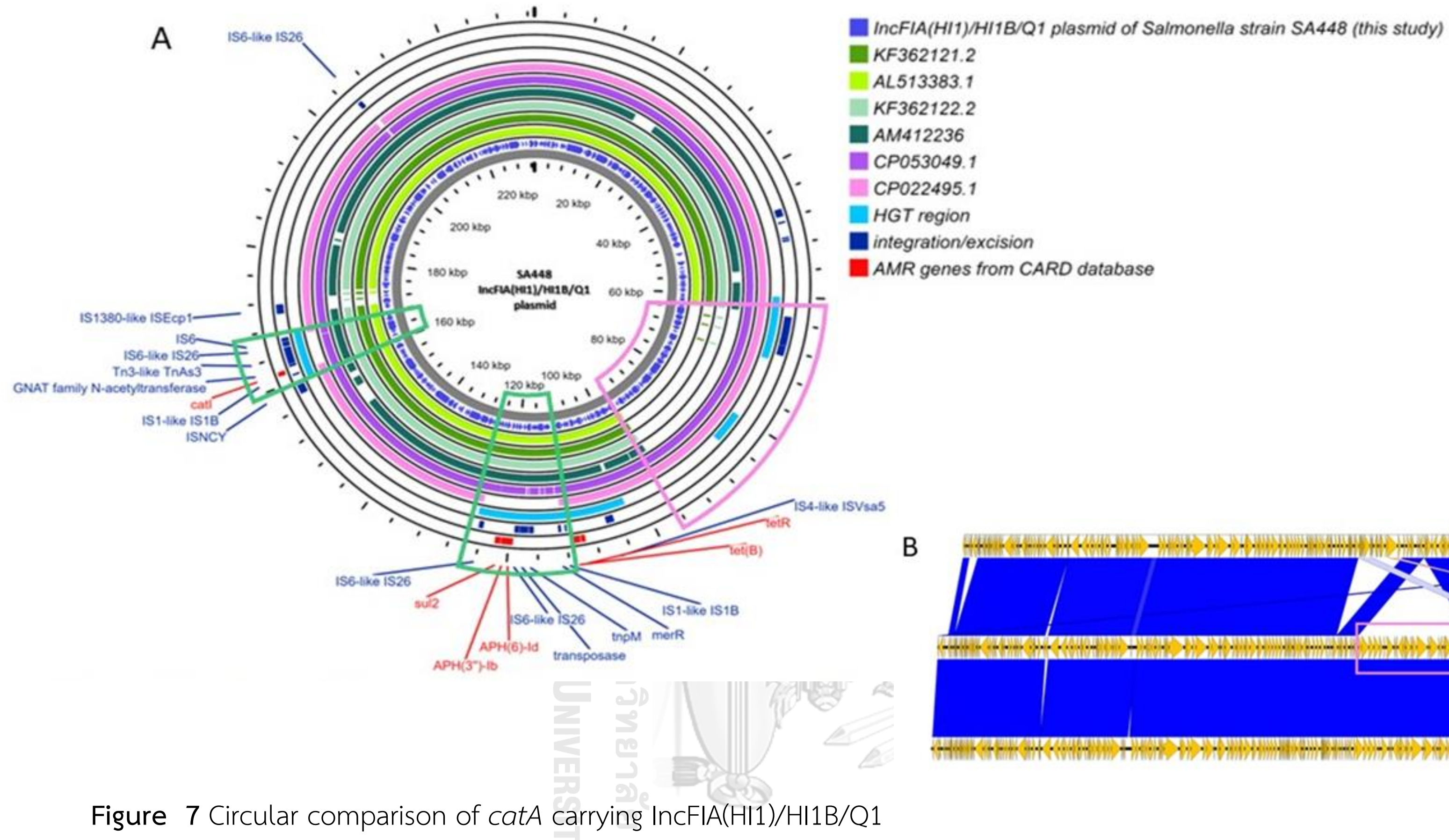


Figure 7 Circular comparison of *catA* carrying IncFIA(HI1)/HI1B/Q1

(A) The comparison was against the highest similarity sequence obtained from NCBI database (accession no., AL513383.1, KF362121.2, KF362122.2, AM412236, CP053049.1 and CP022495.1). The outer red circle indicates AMR genes from CARD database. Green, pink and purple color are sequences of the high degree similarity of reference plasmids. The light blue and navy blue denotes the location of horizontal gene transfer (HGT) region and integration/excision genes, respectively. Green block arcs show the area containing AMR genes found in among all plasmids, except for CP022495.1. Pink block arc indicates the area containing HGT region and integration/excision region that are found only in IncFIA(HI1)/HI1B/Q1 plasmid of SA448, CP053049.1 and CP022495.1. (B) The whole plasmid sequence comparison of IncFIA(HI1)/HI1B/Q1, AL513383.1 and CP022495.1. Yellow arrows indicate the position and direction of the genes. Vertical blocks between sequences indicate regions of shared similarity shaded according to BLASTn (dark blue for matches in the same direction or red for inverted matches). Green and pink rectangles correspond to green and pink block mentioned above.

Figure 8 Circular comparison of *floR*-carrying IncA/C plasmid of SA515.

The comparison was made to the sequence with the highest similarity obtained from NCBI database (pSANI-1736, accession no., CP014658.1 and pF18S036-1, CP082407.1). Blue and red circles indicate the integration/excision genes and AMR genes from CARD database, respectively. The aligned sequences in yellow and green circles show a significant degree of similarity of IncFIA(HI1)/HI1B/Q1 plasmid to pSANI-1736 from bovine-*Salmonella* Anatum and pF18S036-1 from swine-*Salmonella* Ohio.

4.5 DISCUSSION

The findings in this study demonstrated the potential mechanisms involved in the persistence of CHP resistance in *E. coli* and *Salmonella*, including cross-resistance mediated by the expression of multidrug efflux systems using proton motif force and co-selection of R plasmids by other antimicrobial agents.

PAβN is one of the efflux pump inhibitors that is broad spectrum to many kind of efflux pump family that use proton motif force as energy, including resistance nodulation-cell division (RND) family, the major facilitator superfamily (MFS), ATP-binding cassette (ABC) transporters (Davin-Regli et al., 2021). As all *E. coli* and *Salmonella* in this study were resistant to CHP, most *E. coli* (67.9%) and *Salmonella* (56.1%) exhibited ≥4 fold CHP MICs decrease in the presence of PAβN, indicated the involvement of multidrug efflux systems in their CHP resistance phenotype. In this case, any antimicrobial molecules that can turn on the expression of multidrug efflux pump (s) may promote cross resistance to CHP, leading to CHP resistance. At the same time, most of the CHP-resistant *E. coli* and *Salmonella* isolates with <4 fold reduction of CHP MICs (0 to ≤ 2 folds) in the presence of PAβN, carried *cmlA* gene (*E. coli,* 25/54 isolates and *Salmonella*, 10/28 isolates) ($p<0.05$). The *cmlA* gene encodes the proton motif force multidrug efflux pump that belongs to major facilitator superfamily (Amoah Barnie, 2014). This indicates the limited contribution of CmlA to CHP extrusion.

A plasmid- or chromosome-linked *cat* gene encodes chloramphenicol acetyltransferase (CAT) enzyme that prevents the antibiotic molecules unable to interfere with translation. The significant finding of *E. coli* carrying both *catA* and *catB* genes with ≥4 fold CHP MIC decrease was observed, indicating the accumulative effects of enzymatic and non-enzymatic mechanisms on CHP resistance.

In conjugation experiment, AMP, TET and STR selective pressure could result in CHP-resistant transconjugants either *E. coli* or *Salmonella* transconjugants, indicating the

contribution of co-selection in the persistence of CHP resistant bacteria, at least in *in vitro* condition. It was observed that almost all CHP-resistant *E. coli* or *Salmonella* transconjugants carried *cmlA* while only one CHP-resistant *Salmonella* transconjugants contained *cat,* indicating the horizontal transfer of *cmlA* and *cat.* The *cmlA* and *catA* genes are usually located on multi resistant integrons or associated with transposons on transferable and non-transferable plasmids (Roberts and Schwarz, 2009; McMillan et al., 2020). The CHP MICs of CHP-resistant *Salmonella* transconjugants were 16-128 µg/ml and 16-512 µg/ml, respectively. The MIC increased from 4 to 128 folds (mostly 8 and 16 folds). This seems contradicted to the observations in the PAβN experiment that the limited contribution of *cmlA* was observed. The possible explanation could be the contribution of *cmlA* to CHP resistance level that could be more clearly observed in the *in vitro* setting where the recipients with low CHP MIC were used (CHP MIC of 4 µg/ml for both *E. coli* MG1655rif^r and *Salmonella* Enteritidis SE12 Rif^r) in the absence of accumulative effects. Taken together, the findings suggest the involvement of cross resistance and co-selection as the mechanisms responsible for CHP resistance in *E. coli* and *Salmonella*.

In the PBRT experiment, no plasmids were detected in some *cmlA* carrying *E. coli* (E292T_AMP, E294T_TET and E329T_STR), in agreement with their corresponded donors. The possible explanations could be the gene were located on other plasmids that were not included in the PBRT scheme used in this study. While IncF plasmids were mostly found in transconjugants with *cmlA*. This may not be surprising because IncF plasmids are most found in *Enterobacterales* including *E. coli* and *Salmonella.* This is in agreement with a previous study where *cmlA* was predominantly located on IncF plasmid (McMillan et al., 2020). Besides the IncF plasmids, IncHI1 and IncA/C plasmids were additionally transferred (E331T_AMP, E333T_AMP, SA448T_TET, SA448T_STR, SA515T_TET, SA515T_STR). A previous study reported that the *cmlA* gene was located on IncA/C plasmids isolated from *Salmonella* clinical isolates (Garcia et al., 2011). However, it was not clear which plasmid exactly contained *cmlA*. This could be a subject for future study.

The WGS analysis was additionally included to further elucidate the genetics underlying chloramphenicol resistance in particular isolates. In *E. coli*, E290 carrying IncFIIAs plasmid and *cmlA1* was selected for further investigation by WGS and the results indicated that IncX1 plasmid carried *cmlA* gene. However, IncX1 plasmid was not detected in transconjugants by PBRT. This confirmed the limitation of IncX1 detection using PBRT as previously described (Carloni et al., 2017). E329 carried no plasmid based on PBRT scheme as the responsible for transfer the *cmlA1*. WGS analysis showed that E329 had IncFIA(HI1)/HI1B plasmid carrying class1 integrons with *cmlA1*. This plasmid contained multiple replicons including IncHI1A, HI1B and FIA(HI1), which might have been undetected by PBRT due to the partial replicon sequences. E333 was further tested by WGS to confirm because it carries *cmlA1* and IncHI1 and IncFIIAs plasmids by PBRT. The results from WGS showed that class1 integrons with *cmlA1* was located on IncFIA(HI1)/HI1B plasmid carried. For *Salmonella*, SA461 was selected as the representative of IncF plasmid carrying *cmlA1*. IncFII/FIB plasmid and IncI1 plasmid were found by using WGS. Class1 integrons with *cmlA* were located on IncFII/FIB plasmid. SA515 was further confirmed due to the possession of *cmlA1* together with IncA/C and IncF plasmid and class1 integrons with *cmlA* were located on IncFII/FIB plasmid.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

To further analysis, structural comparison was performed. All plasmids carrying *cmlA1* (IncFIA(HI1)/HI1B, IncFII/FIB and IncX1 plasmids) carried class 1 integrons with *dfrA12-aadA2-cmlA1-aadA1 dfrA12* cassette array (Figure 6B). The genes at the upstream and downstream regions of IncFIA(HI1)/HI1B of E329 and E333 were also found in IncFII/FIB of SA461 and SA515 with new arrangement (Figure 6C). In addition to the *cmlA1*-class 1 integrons, *aph(3'')-Ib*, *mcr3.1, tetM, aac(3)-IId* and $bla_{\text{TEM-1}}$ were found at the downstream region of IncFIA(HI1)/HI1B of E329 and E333. The $bla_{\text{TEM-1}}$ gene was found in all plasmids including IncFII/FIB plasmid of SA461 and SA515 and IncX1 plasmid of E290. These WGS analysis results reveal the existence of several resistance genes on *cmlA* or *catA*-carrying plasmids in selected *E. coli* and *Salmonella* and confirm the co-selection of CHP-resistance by other antibiotics.

The class 1 integrons with *dfrA12-aadA2-cmlA1-aadA1 dfrA12* cassette array are flanked by Tn*As1*/ Tn*3* at upstream and IS*6*/ IS*26* at downstream as described in previous studies (Pfeiffer et al., 2018; Harmer and Hall, 2019). Tn*3* family is divided into 7 clades, of which the representative Tn from each clade is Tn*3*, Tn*4651*, Tn*4430*, Tn*3000*, Tn*1071*, Tn*21* and Tn*163* (Nicolas et al., 2014). Tn*As1* that was found in this study is a member of Tn*21* as described in a transposon database, TnCentral (https://tncentral. proteininformationresource.org/). Beside Tn*As1*, Tn *As3* is also included as subgroup of the Tn*21* that was commonly found with the presence of *cmlA1* and *intI1*. Flanking by insertion sequences could facilitate the mobilization of the genes, leading to the spread of the AMR bacteria. There are eleven primary clades among the IS*6* family members found in ISfinder (https://www-is.biotoul.fr/scripts/search-db.php), with IS*26* belonging to clade b. According to a prior study, IS*26* and other IS*6* family members improve the capacity for replicon fusions, or cointegration between donor and target replicons. (Varani et al., 2021).

SA448 carried *catA* on transferable plasmid. From the WGS analysis results, *catA* was located between IS*1B* on IncFIA(HI1)/HI1B/Q1 plasmid. However, the genetic environment of *catA* was different from that of *cmlA* that was located in variable region of class1 integrons. IS*1*-like element IS*1B* family transposase was found next to *catA*, similar to a previous study (Partridge and Hall, 2004). However, one side of *catA* on IncFIA(HI1)/HI1B/Q1 plasmid was flanked by gene encoding GCN5-related N-acetyltransferase (GNAT), which is the superfamily of enzymes that use acetyl-CoAs to acylate their substrates. The first members of GNAT were aminoglycoside acetyltransferases conferring resistance to gentamicin and kanamycin (Vetting et al., 2005). Interestingly, resistance mechanism of *catA* encoding chloramphenicol acetyltransferases enzyme is closely related to GNAT family. Chloramphenicol, aminoglycosides and streptogramins are the three main types of antimicrobials that acetyltransferase enzymes can acetylate with, and the bacteria can become resistance to (Alcala et al., 2020). In addition, another side of the GNAT gene in IncFIA(HI1)/HI1B/Q1 plasmid were flanked by Tn*3*/Tn*As3* followed by IS*6*/IS*26*. In a

previous study, Tn*As3* was previously shown to be *cat* genes (Ross et al., 2021). Even though the transposons confer phenicol resistance in common, they could carry different genes such as *cat* in Tn*As3*, *cmlA* in Tn*21* (Ross et al., 2021). From the structural comparison, almost entire sequence of IncFIA(HI1)/HI1B/Q1 plasmid of SA448 and the reference plasmid (accession no. CP022495.1) were similar, except for 2 positions that contained AMR genes (Green block arcs in Figure 2). Both AMR gene-containing area flanked by ISs were horizontal gene transfer regions (HGT region).

SA448, SA461 and SA515 also carried point mutations in chromosomally encoded *acrB,* leading to amino acid substitution of AcrB (L40P and F28L). Due to the lack of information of this mutation in the ResFinder database, it is not able to predict the relation of amino acid substitution to any specific antimicrobial resistance.

In conclusion, cross resistance by multidrug efflux system and co-selection of R plasmids by other antimicrobial drugs mediates plasmids containing CHP resistance genes are the potential mechanisms responsible for the persistence of CHP resistance in *E. coli* and *Salmonella*. It clearly demonstrates that prohibiting a single antimicrobial agent is not enough to address the AMR problem. The fact is that reducing the use of antibiotics will not be accomplished by a simple solution. Instead, an integrated, multidisciplinary effort from several stakeholders is required. In addition to antibiotic ban, several measures and activities that lower the need for antimicrobials and thus slow the spread of AMR are required e.g., farm biosecurity, infection control, vaccination program, prudent antimicrobial use etc.

CHAPTER V

Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass

Jiratchaya Puangseree[a], Saharuetai Jeamsripong[a], Rangsiya Prathan[a], Chanika Pungpian[a], Rungtip Chuanchuen[a,*]

[a]*Research unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary science, Chulalongkorn University, Bangkok, 10330 Thailand*

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Food Control (2021)

Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass

5.1 ABSTRACT

A total of *Escherichia coli* isolated from pigs (n=643), pork (n=111) and pig carcasses (n=110) in Thailand during 2008-2018 were included. Susceptibilities to antibiotics, disinfectants (i.e. triclosan (TCS), chlorhexidine digluconate (CHX), benzalkonium chloride (BKC), glutaraldehyde (GLU), formaldehyde (FOR)) and heavy metals (i.e. zinc chloride and copper sulfate) were examined. The *E. coli* isolates susceptible to all antibiotics (n=24) were included for *in vitro* exposure experiment. The effect of phenylalanine arginine β-naphthylamide (PAβN) on the susceptibilities was determined. The results showed that the majority of *E. coli* (89%) from pigs, pig carcass and pork were multidrug resistant strains. The MICs of all disinfectants and heavy metals, except triclosan were clustered under 1 or 2 concentrations, indicating that there was no development or development at limited degree of resistance to these substances. TCS MICs for *E. coli* isolated in 2011-2014 were significantly higher than that of the strains in 2008-2010. The weak correlation between biocide MICs (BKC, GLU, TCS, CHX and $CuSO_4$) and antibiotic resistance was observed ($p<0.05$). Exposure to triclosan, benzalkonium chloride and chlorhexidine digluconate selected for spontaneous-resistant mutants exhibited cross resistance to at least one antibiotic. The cross resistance was observed between TCS and all eight antimicrobial drugs; BKC and chloramphenicol/ciprofloxacin/sulfamethoxazole/tetracycline; and CHX and ciprofloxacin/gentamicin/streptomycin. The presence of PAβN restored MICs of chloramphenicol and trimethoprim in some BKC- and TCS-spontaneous resistant mutants, highlighting the important role of multidrug efflux system as cross-resistance mediated mechanism. The presence of PAβN, carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine could not restore ciprofloxacin MIC in some ciprofloxacin-resistant mutant derivatives with no mutation in neither *gyrA* nor *parC*, suggesting the existence of proton motive force-independent mechanisms. In conclusions, the widely-used disinfectants and heavy

metals play an important role as non-antibiotic selective pressure for emergence and spread of AMR. Therefore, susceptibilities to disinfectants/heavy metals should be routinely monitored.

5.2 INTRODUCTION

Antimicrobial resistance (AMR) is a serious public health threat with global impact. The unregulated and excessive use of antimicrobials is considered a major contributor to emergence and spread of both AMR bacteria and their determinants. World health organization (WHO) have recommended that decreasing of overall antimicrobial use is the most important action that could do as the control of AMR crisis (Collignon, et al., 2016). Campaigns and implementations to reduce use of antibiotics in human and animal sectors have been launched in many countries worldwide, however, it is still unclear if reduction of antimicrobial use (AMU) could resolve the AMR issue (Wales & Davies, 2015).

In veterinary medicine, antimicrobial drugs, particularly antibiotics, have been widely used for treatment and prevention of bacterial infections in livestock production (Nhung, Cuong, Thwaites, & Carrique-Mas, 2016). In response to the global effort to reduce AMR by minimizing antibiotic use, biocides (e.g. disinfectants, antiseptics) have been increasingly applied as an integral part of infection control, and those were commonly used in farm and slaughterhouses including quaternary ammonium compound, bisphenol and biguanide (Long, et al., 2016). At the same time, triclosan, a bisphenol biocide, has been generally used as a preservative in water-based formulations of many consumer products for personal hygiene. For animal husbandry, triclosan is commonly formulated in veterinary hygiene biocidal products (e.g. hands scrub, teat dip, dry teat sealant) (ECHA, 2017; SCCP, 2010). However, its use in food and feed production is not advised (Rodricks, Swenberg, Borzelleca, Maronpot, & Shipp, 2010). To date, cross resistance between biocides (e.g. benzalkonium chloride, chlorhexidine, triclosan) and antibiotics has been infamously known in bacterial pathogens and become a particular concern (Braoudaki & Hilton,

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2004; Carey & McNamara, 2014). Among these, contribution of triclosan to antibiotic resistance has been extensively studied in bacterial pathogens of human origin. Exposure to triclosan could cause regulatory mutations of multidrug (MDR) efflux pumps, resulting in multidrug resistance phenotype (Carey & McNamara, 2014; Chuanchuen, et al., 2001).

Heavy metals (e.g. Cu^{2+}, Zn^{2+}) are added into animal feed at very low concentration to balance micro-minerals, increase antimicrobial effect and promote healthy growth (Yazdankhah, Rudi, & Bernhoft, 2014). A particular public concern is that accumulation of these trace minerals in animal manure could cause residue pollution that are possibly attributed to emergence and spread of AMR by triggering either co-selection or co-regulation of resistance genes (Singer, Shaw, Rhodes, & Hart, 2016). A well-known example is the expression of AcrAB-TolC multidrug efflux pump that is upregulated by *SoxS*. The *SoxS* gene was upregulated by Cu^{2+} under the oxidative stress, and thus, subsequently increased the expression of AcrAB-TolC (Harrison, 2009). These data point out the contribution of non-antibiotic selective pressures to the persistence of AMR bacteria and their resistance determinants in food animals despite the reduction of antibiotic use.

Routine monitoring of antibiotic resistance is encouraged and initiated throughout the world. This is not the case for insusceptibility to non-antibiotic selective pressure that has gained less attention. Currently, the usage of disinfectants and heavy metals is not strictly regulated and susceptibility to these biocides is not regularly monitored in most world regions. Knowledge of the current situation and distribution of biocide-insusceptible bacteria of food animal origin is still limited. Such data is indispensable for the development of future interventions to control emergence and spread of AMR attributed to non-antibiotic substances. The susceptibility to disinfectants/heavy metals and its correlation to antibiotic resistance was previously demonstrated in *Salmonella* from livestock. In this study, the research was expanded to commensal *E. coli* that is one of the target bacterial species for AMR monitoring (EFSA, 2013). Commensal *E. coli* are indicators of the Gram-negative commensal intestinal flora

that are commonly isolated from animal intestinal content and faeces. Most AMR phenotype present in food animal population appears in commensal intestinal flora (EFSA, 2013). At the same time, *E. coli* are indicators of faecal contamination food of animal origin. The aims of this study were to i) determine the susceptibility to selected disinfectants, heavy metals and antibiotics in *E. coli* isolated from pigs and their products in Thailand ii) investigate the correlation and possible cross resistance between disinfectants/heavy metals and antibiotics in *E. coli* isolates iii) investigate the involvement of multidrug efflux pumps as a possible mechanism for cross resistance between biocides and antibiotics.

5.3 MATERIALS AND METHOD

5.3.1 Bacterial isolates

A total of 864 *E. coli* (n=864) isolates previously collected as part of AMR monitoring in Thailand were included in this study. They were isolated from rectal swab of clinically healthy pigs (n=643), pork (n=111) and pig carcasses (n=110) from 11 provinces in Thailand including Nongkhai, Mukdahan, Udon Thani, Nakhon Ratchasima, Buriram, Suphan Buri, Ang Thong, Kanchana Buri, Ratchaburi, Chachoengsao and Chon Buri during 2008-2018. These provinces are the important pig production sites for either domestic consumption or exports. Disinfectants have been routinely used and pigs are usually fed with feed containing Cu^{++} and Zn^{++} as trace minerals. Isolation of *E. coli* was conducted according to Diagnosis Procedures in Veterinary Bacteriology and Mycology (Cole, 1990). Pre-enrichment in buffered peptone water (Difco^{TM}, MI, USA) was firstly performed. The *E. coli* strains were isolated on Eosin-Methylene Blue agar (Difco^{TM}) and MacConkey agar (Difco^{TM}). The typical *E. coli* colonies were biochemically confirmed by Indole test. A single colony of *E. coli* was collected from each positive sample and stored in 20% glycerol at -80°C freezer.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5.3.2 Determination of antimicrobials susceptibility

All *E. coli* isolates were tested for their susceptibility to 8 antimicrobial drugs by determination of minimum inhibitory concentrations (MICs) using two-fold agar dilution method (CLSI, 2013; Singer, et al., 2016; Yazdankhah, et al., 2014). The antibiotics tested were chosen to be the representatives of different antimicrobial classes and based on their high resistance prevalence previously reported (Lay, Koowattananukul, Chansong, & Chuanchuen, 2012; Pungpian, Sinwat, Angkititrakul, Prathan, & Chuanchuen, 2020). The reference strains were *Escherichia coli* ATCC29522, *Staphylococcus aureus* ATCC25923 and *Pseudomonas aeruginosa* ATCC27853.

5.3.3 Determination of MICs of disinfectants and heavy metals

MICs of disinfectants and heavy metals were examined by using serial two-fold agar dilution method according to CLSI (CLSI, 2013) with some modifications. Five disinfectants tested and their concentration ranges (in parenthesis) included triclosan (TCS, 0.0156-128 µg/mL), benzalkonium chloride (BKC, 0.5-512 µg/mL), chlorhexidine digluconate (CHX, 0.25-2048 µg/mL), glutaraldehyde (GLU, 1-4096 µg/mL), formaldehyde (FOR, 1-2048 µg/mL). Two heavy metals tested were zinc chloride ($ZnCl_2$) and copper sulfate ($CuSO_4$), of which the concentration range was 2-2048 µg/mL. $ZnCl_2$ and $CuSO_4$ powder were dissolved in distilled water. The pH of $ZnCl_2$ solution was adjusted to 5.5. BKC, CHX, GLU and FOR were obtained from Sigma-Aldrich (Saint Louis, MO, USA). $ZnCl_2$ and $CuSO_4$ were purchased from UNILAB (Sydney, NSW, Australia). *E. coli* ATCC29522, *S. aureus* ATCC25923 and *P. aeruginosa* ATCC27853 were used as control strains for disinfectant and heavy metal susceptibility testing (Beier, et al., 2015). The MIC ranges of BKC, CHX, FOR, GLU, TCS, $CuSO_4$ and $ZnCl_2$ for *E. coli* ATCC29522, *S. aureus* ATCC25923 and *P. aeruginosa* ATCC27853 that served as control strains were 4-16, 4-16, 128-256 µg/mL; 2-4, 1-2, 16-32 µg/mL; 64-128, 32-64, 64-128 µg/mL; 1024-2048, 1024-2048, 2048-4096 µg/mL; 0.03125-0.0625, <0.0156, >16 µg/mL; 1024-2048, 128-512, 1024-2048 µg/mL and 512-1024, 128-256, 1024-2048 µg/mL respectively.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5.3.4 In vitro exposure experiment and determination of effects of active efflux inhibitors

The *E. coli* isolates susceptible to all antimicrobial agents tested with low MICs to the disinfectants and heavy metals (n=24) were selected. All isolates were exposed to gradually-increasing concentrations of five disinfectants and two heavy metals individually as previously described (Chuanchuen, Pathanasophon, Khemtong, Wannaprasat, & Padungtod, 2008; Gradel, Randall, Sayers, & Davies, 2005). The *E. coli* strains were grown on Luria-Bertani agar (LBA, Difco^{TM}) overnight at 37°C. A single colony was picked and grown in Luria-Bertani broth (LBB, Difco^{TM}) containing each disinfectant or heavy metal at the concentration of ¼ MIC value overnight at 37°C. The bacterial suspensions with growth (visible turbidity) were transferred to fresh LBB with increasing concentration of disinfectant /or heavy metal by a factor of 1.5. The procedure was repeated daily until no growth was observed. The *E. coli* isolates that grew at the concentration ≥4 fold of their original MICs of disinfectants or heavy metals were considered spontaneous resistant mutants. The spontaneous resistant mutants and their parent strains were subjected to determination of antibiotic MICs. The spontaneous resistant mutants were sub-cultured on fresh LBA for 20 consecutive days and determined for their antibiotic MICs. The similarity of mutant strains to their parents were confirmed by repetitive sequence based polymerase chain reaction (Rep-PCR) (Jonas, Spitzmüller, Weist, Rüden, & Daschner, 2003).

The spontaneous-resistant mutant derivatives that exhibited at least 4 fold increase in antibiotic MICs (n=16), and their isogenic parents (pre-exposure) were examined for susceptibilities to antibiotics and corresponded disinfectants/heavy metals in the presence and absence of 25 µg/mL phenylalanine arginine **β**-naphthylamide (PA**β**N; Sigma-Aldrich, Saint Louis, MO) (Pannek, et al., 2006). At least 4-fold difference in the MIC value of antibiotics was considered significant.

Six spontaneous resistant mutants exhibiting decreased CIP susceptibility (≥ 4 fold MIC increase) with no change in CIP MIC level after the addition of PA**β**N were

selected for further examination. The isolates were obtained from exposure to BKC (n=1), CHX (n=1) and TCS (n=4). All were examined for CIP MICs in the presence and absence of 50 µM of carbonyl cyanide m-chlorophenylhydrazone (CCCP) or 20 µg/mL of reserpine. All were tested for mutations in quinolone resistance-determining region (QRDR) of *gyrA* and *parC* by DNA sequencing analysis (Chuanchuen & Padungtod, 2009). The *gyrA* and *parC* gene were PCR amplified using primers gyrA-F (5'-GCTGAAGAGCTCCTATCTGG-3'), gyrA-R (5'-GGTCGGCATGACGTCCGG-3') parC-F (5'- GTACGTGATCATGGATCGTG-3') and parC-R (5' TTCCTGCATGGTGCCGTCG-3'). The PCR products were submitted to First Base Laboratories (Selangor Darul Ehsan, Malaysia) for nucleotide sequencing and the obtained sequences were analyzed by using Molecular Evolutionary Genetic Analysis version 6.0.

5.3.5 Statistical Analysis

Antimicrobial resistance rates and MIC distribution of biocides were analyzed by using Microsoft Excel. The association between antimicrobial resistance and MIC of disinfectants were determined using Fisher's Exact test. Statistical correlations between MICs of disinfectants/heavy metals and MICs of antibiotics were determined by Spearman's rank order correlation with SPSS version 22.0. A *p-value* of <0.05 was considered statistically significant. In all experiments, a ≥4 fold different in MIC value was considered significant difference.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

5.4 RESULTS

5.4.1 Susceptibilities to antibiotics, disinfectants and heavy metals

Almost all *E. coli* isolates (97.2%) were resistant to at least one antibiotic and 88.8% were multidrug resistant (MDR, being resistant to at least 3 antibiotics in different classes). Most *E. coli* isolates were resistant to ampicillin (88.4%), tetracycline (86.2%) and sulfamethoxazole (73.4%). Resistance rates to chloramphenicol, ciprofloxacin, gentamicin, streptomycin, and trimethoprim were 56%, 23.8%, 35.1%, 63.9% and 68.3%, respectively (Figure 9). The *E. coli* isolates from pigs exhibited highest resistance rates to all antibiotics tested i.e. AMP (90.5%), CHP (63.3%), CIP

(34.1%), GEN (42.9%), STR (69.4%), SMZ (79.8%) TET (91.9%) and TMP (72.9%). AMP-CHP-STR-SMZ-TET-TMP was the most common AMR pattern identified among the isolates from pigs (11.2%) and pig carcasses (11.8%) (Table 23). The most common AMR pattern among the pork isolates was AMP (9%).

Overall, MICs for all biocides tested, except TCS, clustered under one to two consecutive concentrations. For disinfectants, the most predominant MIC concentrations of BKC, CHX, GLU, FOR were 16-64 μg/mL (95.3%), 2-4 μg/mL (84.2%), 2048 μg/mL (79.6%) and 64 μg/mL (94.5%), respectively. The majority of the *E. coli* isolates had $CuSO_4$ and $ZnCl_2$ MICs of 1,024 μg/mL (94.9%) and 512 μg/mL (75.1%), respectively (Table 24).

Most of the isolates exhibited TCS MIC of ≤0.0156 – 0.125 μg/mL (91.8 %) (Table 24). Forty percent of the isolates had TCS MIC of ≤0.0156 μg/mL (47.1%), while almost the same number of the isolates had the MIC between 0.03125-0.125 μg/mL (44.7%).

The isolates were sorted by years of isolation, 2008-2010 (n=309); 2011-2014 (n=343) and 2015-2018 (n=212) (Figure 10 and 11). The isolates from these three periods displayed the similar MICs for CHX (2-4 μg/mL); BKC (32-64 μg/mL); GLU (2048 μg/mL); $CuSO_4$ (1024 μg/mL) and $ZnCl_2$ (512 μg/mL). The isolates from year 2008-2010 had TCS MIC ranging from ≤0.0156 – 0.5 μg/mL, of which most isolates (25.6%) had TCS MIC of ≤0.0156 μg/mL. The number of the isolates with TCS MIC of 0.03125 μg/mL in this period was much less (6%). For the isolates from year 2011-2014, the TCS MIC ranged from ≤0.0156 to 256 μg/mL. The percentages of the isolates exhibiting TCS MIC of ≤0.0156 μg/mL (9.7%), 0.0625 μg/mL (11%) and 0.125 μg/mL (8.9%) were not significantly different. The isolates from year 2011-2014 had TCS MIC significantly higher than those of the isolates from year 2008-2010 (p<0.05). Some isolates from year 2015-2018 exhibited high TCS MICs (32-256 μg/mL) but at low occurrence (0.9%).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 23 Antimicrobial resistance pattern of *Escherichia coli* isolated from pigs, pork and pig carcass (n=864).

AMR pattern	No. (%)			
	Pig (n=643)	Pork (n=111)	Pig carcass (n=110)	Total (n=864)
AMP	5 (0.8)	10 (9)	11 (10)	26 (3.0)
AMP-CHP-CIP-GEN-SMZ-TET-TMP	19 (3.0)	-	-	19 (2.2)
AMP-CHP-CIP-GEN-STR-SMZ-TET	13 (2.0)	-	-	13 (1.5)
AMP-CHP-CIP-GEN-STR-SMZ-TET-TMP	65 (10.1)	-	-	65 (7.5)
AMP-CHP-CIP-GEN-STR-TET-TMP	15 (2.3)	-	-	15 (1.7)
AMP-CHP-CIP-SMZ-TET-TMP	15 (2.3)	-	1 (0.9)	16 (1.9)
AMP-CHP-CIP-STR-SMZ-TET-TMP	31 (4.8)	3 (2.7)	1 (0.9)	35 (4.0)
AMP-CHP-GEN-STR-SMZ-TET-TMP	38 (5.9)	3 (2.7)	1 (0.9)	42 (4.9)
AMP-CHP-GEN-STR-SMZ-TET	11 (1.7)	-	1 (0.9)	12 (1.4)
AMP-CHP-SMZ-TET-TMP	29 (4.5)	9 (8.1)	5 (4.5)	43 (5.0)
AMP-CHP-STR-SMZ-TET-TMP	72 (11.2)	6 (5.4)	13 (11.8)	91 (10.5)
AMP-CHP-TET	9 (1.4)	1 (0.9)	-	10 (1.2)
AMP-CHP-TET-TMP	7 (1.1)	3 (2.7)	1 (0.9)	11 (1.3)
AMP-CIP-GEN-STR-SMZ-TET-TMP	19 (3.0)	-	-	19 (2.2)
AMP-GEN-STR-SMZ-TET	25 (3.9)	4 (3.6)	-	29 (3.4)
AMP-GEN-STR-SMZ-TET-TMP	15 (2.3)	2 (1.8)	2 (1.8)	19 (2.2)
AMP-SMZ-TET	8 (1.2)	3 (2.7)	1 (0.9)	12 (1.4)
AMP-SMZ-TET-TMP	16 (2.5)	3 (2.7)	7 (6.4)	26 (3.0)
AMP-STR-SMZ-TET	24 (3.7)	1 (0.9)	7 (6.4)	32 (3.7)
AMP-STR-SMZ-TET-TMP	34 (5.3)	9 (8.1)	9 (8.2)	52 (6.0)
AMP-STR-TET	7 (1.1)	3 (2.7)	6 (5.5)	16 (1.9)
AMP-TET	4 (0.6)	3 (2.7)	3 (2.7)	10 (1.2)
CHP-TET-TMP	11 (1.7)	4 (3.6)	-	15 (1.7)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 24 Distribution of MIC values for disinfectants and heavy metals in *Escherichia coli* isolated from pigs, pork and pig carcass (n=864)

Substance	No (%) of isolates with MICs (μg/ml)																		
	≤0.0156	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096
TCS	407 (47.1)	134 (15.5)	152 (17.6)	100 (11.6)	38 (4.4)	19 (2.2)	4 (0.5)	-	1 (0.1)	-	1 (0.1)	6 (0.7)	-	-	2 (0.2)	-	-	-	-
CHX	-	-	-	-	-	-	66 (7.6)	469 (54.3)	258 (29.9)	20 (2.3)	39 (4.5)	11 (1.3)	1 (0.1)	-	-	-	-	-	-
BKC	-	-	-	-	-	-	-	13 (1.5)	17 (2.0)	3 (0.3)	108 (12.5)	480 (55.6)	235 (27.2)	7 (0.8)	1 (0.1)	-	-	-	-
GLU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6 (0.7)	688 (79.6)	170 (19.7)
FOR	-	-	-	-	-	-	-	-	-	-	-	30 (3.5)	815 (94.5)	18 (2.1)	1 (0.1)	-	-	-	-
$CuSO_4$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	820 (94.9)	44 (5.1)	-
$ZnCl_2$	-	-	-	-	-	-	-	-	-	-	-	-	1 (0.1)	6 (0.6)	150 (17.4)	649 (75.1)	57 (6.6)	2 (0.2)	-

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

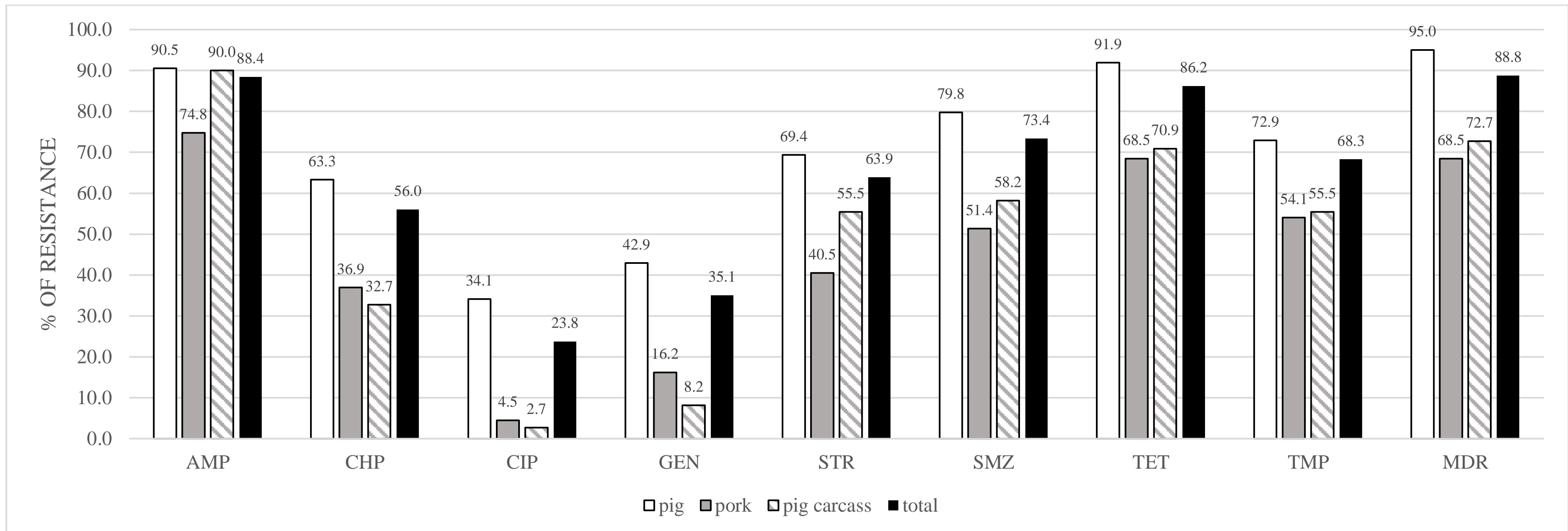


Figure 9 Distribution of antimicrobial resistance in *Escherichia coli* (n=864).

For each antimicrobials, the antimicrobial resistance among Escherichia coli isolated from pig (□), pork(■), pig carcass(▨) and all sources (■).

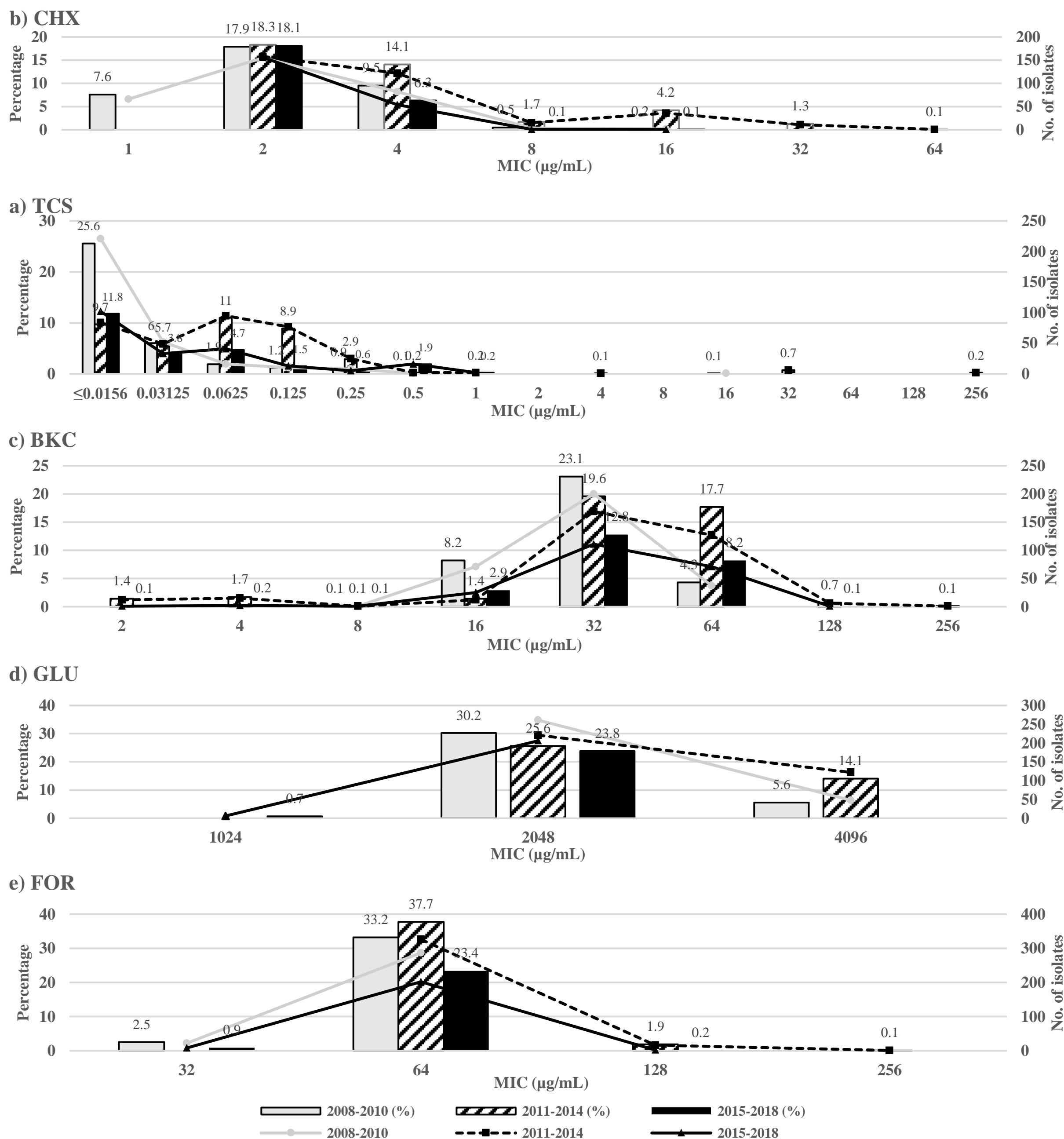
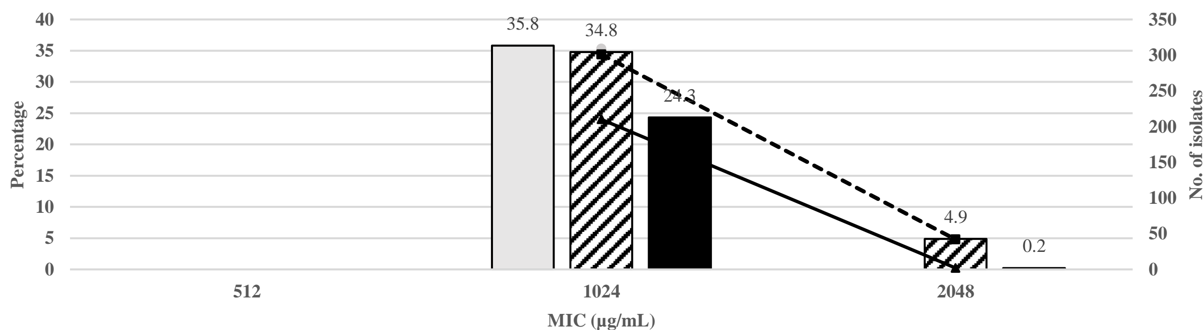
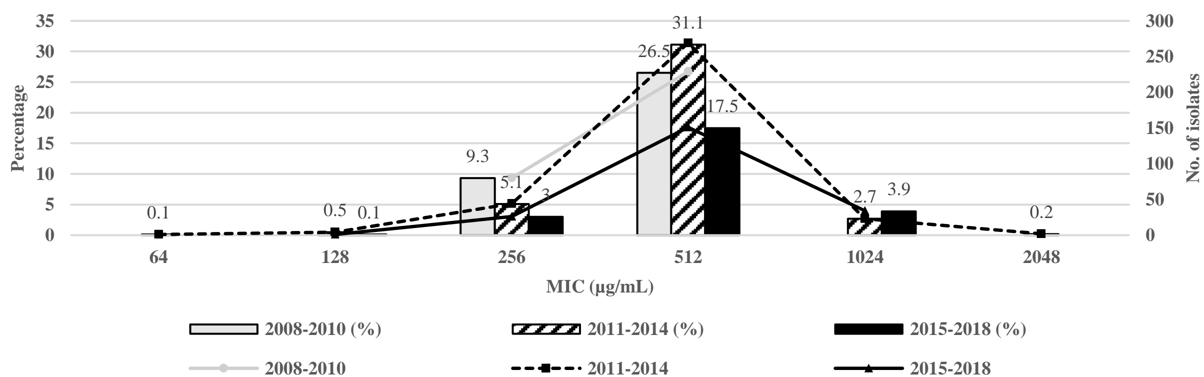


Figure 10 MIC distribution of disinfectants in *Escherichia coli* sorted by years (n=864). Different style of bar charts represents different period of bacterial isolation including 2008-2010( ), 2011-2014 ( ) and 2015-2018 ( ). The lines show trend of the MIC values sorted by year including 2008-2010( ), 2011-2014 ( ) and 2015-2018 ( ).

a) $CuSO_4$



b) $ZnCl_2$



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 11 MIC distribution of $ZnCl_2$ and $CuSO_4$ in *Escherichia coli* sorted by years (n=864).

Different style of bar charts represents different period of bacterial isolation including 2008-2010(▬), 2011-2014 (▨) and 2015-2018 (■). The lines show trend of the MIC values sorted by year including 2008-2010(—), 2011-2014 (⋯) and 2015-2018 (—).

5.4.2 Association among susceptibilities of antibiotics, disinfectants and heavy metals

The association between antibiotic resistance (as defined by clinical breakpoints) and MIC values of disinfectants/heavy metals was examined. The MICs of BKC, GLU, TCS, CHX and $CuSO_4$ were significantly associated with resistance to 7 antimicrobial drugs including CHP, CIP, GEN, STR, SMZ, TET and TMP (p <0.05). Only MIC values of TCS and CHX showed the association with AMP resistance (Table 25). In addition, the MIC values of $ZnCl_2$ were associated with resistance to 3 antimicrobial agents including CIP, GEN and TMP (p <0.01). The MICs of FOR was statistically associated with CIP and TET resistance (p <0.05).

The correlation between MICs of disinfectants/heavy metals and antibiotics was determined (Table 26). The significant-positive (correlation coefficient range of 0.069-0.182) and negative (correlation coefficient range of 0.069-0.256) correlations were observed at weak level. The weak negative correlations were observed between the MICs of three biocides (i.e. $CuSO_4$, CHX and GLU) and that of all antibiotics tested (p <0.05). The MICs of these disinfectants/heavy metals may increase, while that of antibiotics decreased, and vice versa (Table 31). While the MIC of TCS was negatively correlated to that of AMP, STR, and TMP at weak (p <0.05), the positive correlation at weak level was observed between the MICs of $ZnCl_2$ - CHP, CIP and GEN and BKC - CHP, CIP, TET and TMP (p <0.05). The MIC of FOR was in the same direction with STR and TMP (p <0.05) but not with TET.

5.4.3 Cross resistance between disinfectants/heavy metals and antibiotics

Twenty-four *E. coli* isolates susceptible to all antimicrobial drugs tested and with low MICs to disinfectants and heavy metals were chosen for *in vitro* exposure experiment (Table 27). Exposure to gradually-increasing concentrations of TCS, BKC and CHX yielded spontaneous-resistant mutant derivatives exhibiting increase MICs to antibiotics (≥4 fold) (Table 28).

Twenty-two *E. coli* isolates yielded TCS-spontaneous resistant mutants that grew in the presence of TCS at the concentrations of 6 to ≥1,000 folds of their original MICs (Table 27). Among these, 13 isolates had increase MIC ≥4 fold to at least one antimicrobial drug i.e. AMP, CHP, CIP, GEN, STR, SMZ, TET and TMP (Table 28). MH21T1, MH33T1 and MH58T1 exhibited 4-fold increase MICs to most antibiotics including CHP, CIP, TET and TMP, while NK32T1 showed 8-fold increase MICs to CHP, CIP, SMZ and TMP.

Five TCS-spontaneous resistant mutants including NK32T1, MH21T1, MH33T1, MH43T1 and MH58T1 developed increase MICs of both CHP (8-16 fold) and CIP (4-8 fold), simultaneously. Exposure to TCS raised the CHP MIC value from 4 to 16 fold in 9 isolates, of which three mutant strains, NK26T1, NK32T1 and MH33T1, had the increase MIC over clinical breakpoint of CHP (≥32 µg/mL). The NK32T1 and NK26T1 additionally had increase MICs ≥4 fold to SMZ and TMP. Most TCS-spontaneous resistant mutants had increase MIC ≥4 fold to TET (4-16 fold) and/or TMP (4-8 fold). The NK69T1, TCS derivative mutant strain of NK69, developed resistance to AMP (64 µg/mL) above clinical breakpoint (32 µg/mL). An increase MIC of GEN and STR ≥4 fold was observed in E250.1 that additionally developed resistance to CHP.

Exposure to BKC resulted in two BKC-spontaneous resistant mutants, NK26B1 (16 fold) and NK32B1 (32 fold). NK26B1 developed resistance to only CHP (8 fold), while NK32B1 developed resistance to CHP, CIP, SMZ and TET (4 - 8 fold) (Table 28).

Only one CHX-spontaneous resistant mutant was obtained. This mutant had high CHX MIC up to 1,557 µg/mL (Table 27) and enhanced MIC (≥4 fold) to CIP, GEN and STR (Table 28).

Table 25 Statistical association between MICs of disinfectants/heavy metals and antibiotic resistance (n=864)

Substance	**MIC (µg/mL) range**	**Antimicrobial resistance**							
		AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP
$ZnCl_2$	64-2048	-	-	+	+	-	-	-	+
$CuSO_4$	1024-2048	-	+	+	+	+	+	+	+
TCS	≤0.0156-256	+	+	+	+	+	+	+	+
CHX	1-64	+	+	+	+	+	+	+	+
BKC	2-256	-	+	+	+	+	+	+	+
GLU	1024-4096	-	+	+	+	+	+	+	+
FOR	32-256	-	-	+	-	-	-	+	-

+ Significant association between MICs of disinfectants/heavy metals and antibiotic resistance ($p<0.05$).

- No significant association between MICs of disinfectants/heavy metals and antibiotic resistance ($p>0.05$).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 26 Statistic correlation between MICs of disinfectants/heavy metals and antibiotic (n=864)

Substance	Correlation coefficients of MICs							
	AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP
$ZnCl_2$	+0.053	+0.156*	+0.167*	+0.174*	+0.029	+0.035	+0.047	+0.006
$CuSO_4$	-0.256*	-0.104*	-0.110*	-0.077*	-0.130*	-0.156*	-0.160*	-0.168*
TCS	-0.089*	-0.006	-0.033	+0.039	-0.074*	-0.046	+0.001	-0.069*
CHX	-0.263*	-0.073*	-0.082*	-0.075*	-0.147*	-0.247*	-0.118*	-0.168*
BKC	+0.027	+0.147*	+0.086*	+0.041	-0.024	+0.037	+0.118*	+0.182*
GLU	-0.219*	-0.118*	-0.137*	-0.113*	-0.198*	-0.167*	-0.216*	-0.095*
FOR	-0.057	-0.043	-0.039	+0.029	+0.073*	+0.059	-0.181*	+0.069*

+ positive correlation (a value greater than 0) between MIC values of disinfectants/heavy metals and antibiotics that is the value of increase or decrease together, strong correlation means correlation coefficient close to 1.

- negative correlation (a value less than 0) between MIC values of disinfectants/heavy metals and antibiotics that is the one variable increase, the value of the other variable decrease, strong correlation means correlation coefficient close to -1.

*Significant correlation between MIC values of disinfectants/heavy metals and antibiotics ($p<0.05$).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 27 MICs of disinfectants and heavy metals of *Escherichia coli* in in vitro exposure experiment (n=24)

Strain	MIC (μg/ml)*													
	$ZnCl_2$		$CuSO_4$		TCS		CHX		BKC		GLU		FOR	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST
E 247	512	288	1024	864	< 0.0156	**>0.34**	1	2.85	32	18	4096	2304	64	81
E 248	512	288	1024	864	< 0.0156	**>1.35**	1	2.85	32	12	4096	2304	64	81
E 250	512	432	1024	864	< 0.0156	**>1.35**	1	2.85	32	8	2048	1152	64	81
E 254	512	432	1024	864	< 0.0156	**>0.34**	1	2.85	32	12	4096	2304	64	121.5
E 255	512	288	1024	864	< 0.0156	**>0.67**	1	2.85	32	12	2048	1152	64	121.5
NK 26	256	486	1024	864	0.0625	**10.1**	4	3.4	4	**25.6**	4096	1536	64	121.5
NK 32	256	486	2048	768	0.25	**18.2**	32	27	4	**38.4**	4096	1536	64	182.3
NK 35	256	216	1024	864	256	486	32	12	64	54	2048	1152	64	36
NK 69	256	486	2048	1152	0.0625	**51.1**	32	**1557**	64	121.5	4096	1536	64	121.5
NK 119	512	288	1024	864	0.0625	**6.7**	2	3.8	32	27	2048	2592	64	121.5
NK 121	512	288	1024	864	0.03125	**0.45**	2	2.5	32	18	2048	2592	64	182.3
NK 221	512	432	1024	864	0.0625	**10.1**	2	3.8	64	24	2048	2592	64	121.5
MH 13	512	192	2048	1152	0.125	**0.8**	16	9	32	91.1	2048	2592	64	81
MH 21	512	192	1024	864	0.125	**6.1**	16	6	32	60.75	2048	1728	64	81
MH 22	512	192	1024	1296	0.125	**0.8**	4	5.7	32	18	2048	2592	64	121.5
MH 33	512	972	2048	1152	0.5	**10.8**	16	30.3	32	40.5	4096	2304	64	81
MH 43	256	216	1024	1296	0.125	**69.2**	8	4.5	32	40.5	2048	1152	64	54

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Strain	MIC (μg/ml)*													
	$ZnCl_2$		$CuSO_4$		TCS		CHX		BKC		GLU		FOR	
	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE	POST
MH 44	1024	384	1024	864	256	486	16	13.5	64	36	2048	2592	64	54
MH 58	512	384	2048	1152	4	**61.6**	8	34.1	32	91.1	4096	1536	64	81
MH 128	512	384	1024	864	0.03125	**5.12**	2	3.8	64	54	2048	2502	64	162
MH 168	512	1458	1024	1296	<0.0078	**55.4**	2	2.5	64	24	4096	1024	64	121.5
MH 174	512	972	1024	864	<0.0078	**4.9**	2	2.5	32	18	4096	1024	64	182.3
MH 189	512	432	1024	864	<0.0078	**4.9**	2	2.5	64	24	4096	1024	64	121.5
MH 193	512	972	1024	864	0.0156	**43.8**	2	2.5	32	27	2048	1152	64	182.3

*Bold letters indicate the post-exposure MICs at least 4-fold higher than the pre-exposure MICs

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 28 MICs of antibiotics and disinfectants of spontaneous resistance mutants and their isogenic parents in in vitro exposure experiment (n=16)

Substance	Strain	Source	Exposure	PAβN	MIC*	MIC (µg/mL)							
						AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP
BKC	NK26	pork	Pre	-	4	8	2	0.03125	1	4	8	1	1
	NK26B1		Post	-	**64**	8	**16**	0.0156	1	4	4	2	1
				+	**1**	16	**2**	0.0156	1	4	4	1	**≤0.25**
	NK32	pig	Pre	-	4	16	4	0.03125	0.25	2	64	2	1
	NK32B1		Post	-	**128**	32	**16**	**0.125**	0.25	2	**512**	**16**	2
				+	128	32	**4**	0.0625	0.25	4	**8**	8	**0.5**
CHX	NK69	pork	Pre	-	32	8	2	0.0156	0.25	2	8	1	1
	NK69C1		Post	-	**256**	16	4	**0.0625**	**1**	**8**	8	2	0.5
				+	256	16	2	0.03125	1	8	8	4	0.5
TCS	E250	pig	Pre	-	0.0039	2	2	0.0078	0.5	2	8	1	2
	E250.1		Post	-	**0.125**	4	**8**	0.0078	**2**	**8**	8	1	4
				+	**≤0.0078**	2	**1**	0.0039	1	4	**2**	0.5	**≤0.25**
	NK26	pork	Pre	-	0.5	4	2	0.03125	1	4	8	1	1
	NK26T1		Post	-	**4**	2	**32**	0.0625	0.5	4	**32**	2	**4**
				+	**0.25**	2	**2**	0.0625	1	4	**≤4**	4	**0.5**
	NK32	pig	Pre	-	0.25	16	4	0.03125	0.25	2	64	2	1
	NK32T1		Post	-	**4**	16	**32**	**0.25**	0.25	2	**512**	4	**8**
				+	**0.5**	32	**2**	0.125	0.25	2	**8**	4	**1**
	NK69	pig	Pre	-	0.0625	8	2	0.0156	0.25	2	8	1	1
	NK69T1		Post	-	**8**	**64**	**16**	0.03125	0.5	2	16	**8**	1
				+	**1**	**8**	**2**	0.03125	0.5	2	**4**	4	**≤0.25**
	NK221	pork	Pre	-	0.0625	4	4	0.0078	1	4	16	2	0.5
	NK221T1		Post	-	**1**	8	4	0.0078	2	8	≤4	1	**2**
				+	**0.0625**	**2**	**≤0.5**	≤0.0039	1	4	≤4	0.5	**≤0.25**
	MH21	Pig carcass	Pre	-	0.125	16	2	0.0078	0.25	2	256	1	0.5
	MH21T1		Post	-	**0.5**	32	**16**	**0.03125**	0.25	2	512	**16**	**2**
				+	**0.03125**	64	**2**	0.0156	≤0.125	2	**4**	**2**	**≤0.25**
	MH33	Pig	Pre	-	0.5	16	4	0.0156	0.25	2	16	2	≤0.25
	MH33T1		Post	-	**2**	32	**64**	**0.125**	0.25	2	32	**8**	**8**
				+	**0.0625**	64	**2**	**0.03125**	0.25	1	**≤4**	8	**0.5**

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Substance	Strain	Source	Exposure	PAβN	MIC[*]	MIC (µg/mL)							
						AMP	CHP	CIP	GEN	STR	SMZ	TET	TMP
	MH43	Pork	Pre	-	0.125	32	1	0.0039	0.25	2	16	0.5	0.5
	MH43T1		Post	-	**128**	32	**16**	**0.03125**	0.5	4	32	**4**	1
				+	**8**	64	**2**	0.0156	0.25	4	**2**	2	**≤0.25**
	MH58	Pork	Pre	-	0.25	32	4	0.0078	0.5	4	32	2	1
	MH58T1		Post	-	**16**	32	**16**	**0.0625**	0.25	2	64	**16**	**4**
				+	**4**	32	**2**	0.03125	0.25	2	**8**	8	**≤0.25**
	MH128	pig	Pre	-	0.0625	4	4	0.0078	1	4	16	1	8
	MH128T1		Post	-	**1**	4	8	0.0078	1	8	16	**4**	16
				+	**0.0078**	4	**2**	0.0078	0.5	4	8	**1**	**≤0.25**
	MH168	Pig carcass	Pre	-	0.0039	4	2	0.0078	0.5	4	8	1	≤0.25
	MH168T1		Post	-	**16**	8	**8**	0.0078	1	4	8	1	≤0.25
				+	**2**	8	**1**	0.0078	0.5	4	≤4	1	≤0.25
	MH174	Pig carcass	Pre	-	0.0039	8	4	0.0078	0.5	4	2	1	2
	MH174T1		Post	-	**0.0156**	16	4	0.0078	0.5	4	**16**	1	4
				+	0.0078	8	**1**	0.0078	0.5	4	**≤4**	1	**≤0.25**
	MH189	pork	Pre	-	0.0039	4	4	≤0.0039	4	4	≤4	1	1
	MH189T1		Post	-	**0.0625**	4	4	0.0078	4	8	≤4	1	**4**
				+	**0.0078**	4	**1**	≤0.0039	**1**	4	≤4	1	**≤0.25**

+, - Presence and absence of PAβN

[*]MICs (µg/mL) of the corresponded disinfectants.

[**]Bold letters indicate at least 4-fold increase MICs of mutant derivatives in comparison to their isogenic parents. Underlined letters indicate at least 4-fold decrease MICs of mutant derivatives in the presence of PAβN in comparison to that in the absence of PAβN

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5.4.4 Inhibitory effect of PAβN

The addition of 25 μg/mL PAβN decreased TCS MIC from 2 to 128 fold in all TCS-spontaneous resistant mutants (n=13) (Table 28). The presence of PAβN restored MICs of CHP and TMP in all TCS-spontaneous resistant isolates but did not affect MICs of GEN and STR in any isolates. The AMP MIC decreased 8 fold in only one isolate, NK69T1, and the SMZ MIC reduced 4-128 fold in 9 isolates (i.e. E250.1, NK26T1, NK32T1, NK69T1, MH21T1, MH33T1, MH43T1, MH58T1 and MH174T1). A decrease of MIC to TET was observed in MH21T1 (4 fold) and MH128T1 (4 fold). The CIP MIC was restored only in one isolate (MH33T1, 4-fold reduction).

Of two BKC-spontaneous resistant mutants, the presence of PAβN could restore BKC sensitivity in NK26B1 (64-fold reduction). The MIC values of CHP and TMP decreased ≥4 fold in both NK26B1 and NK32B1. Only NK32B1 developed resistance to SMZ after BKC exposure and its SMZ MIC was reduced up to 64 fold in the presence of PAβN. No changes were observed in CHX-spontaneous resistant isolates.

The addition of PAβN did not affect CIP MIC in 4 TCS-spontaneous resistant mutants (NK32T1, MH21T1, MH43T1 and MH58T1), a BKC-spontaneous resistant mutant (NK32B1) and a CHX-spontaneous resistant mutant (NK69C1) that had the increase CIP MIC ≥4 fold. The presence of CCCP and reserpine did not alter the CIP MIC in these isolates (Table 29). All these six spontaneous resistant mutants did not carry mutations in *gyrA* and *parC*.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 29 MICs of ciprofloxacin of spontaneous-resistance mutants and their isogenic parents in in vitro exposure experiment (n=6)

Substance	Strain	Exposure	EPI**	MIC of CIP (μg/mL)
BKC	NK32	Pre	-	0.03125
	NK32B1	Post	-	**0.125**
			+CCCP	0.0625
			+reserpine	0.125
CHX	NK69	Pre	-	0.0156
	NK69C1	Post	-	**0.0625**
			+CCCP	0.03125
			+reserpine	0.03125
TCS	NK32	Pre	-	0.03125
	NK32T1	Post	-	**0.25**
			+CCCP	0.125
			+reserpine	0.25
	MH21	Pre	-	0.0078
	MH21T1	Post	-	**0.03125**
			+CCCP	0.03125
			+reserpine	0.03125
	MH43	Pre	-	0.0039
	MH43T1	Post	-	**0.03125**
			+CCCP	0.03125
			+reserpine	0.03125
	MH58	Pre	-	0.0078
	MH58T1	Post	-	**0.0625**
			+CCCP	0.0625
			+reserpine	0.0625

*Bold letters indicate at least 4-fold increase MICs of mutant derivatives in comparison to that of the isogenic parents.

**+,- Presence and absence of CCCP or reserpine (indicated)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5.5 DISCUSSIONS

High resistance rates to antibiotics were observed among the *E. coli* isolates in this study. Resistance to ampicillin (88.4%), tetracycline (86.2%), sulfamethoxazole (73.4%), streptomycin (63.9%), and trimethoprim (68.3%) and chloramphenicol (56%) was common, in agreement with previous studies (Aniroot Nuangmek, 2018; Lugsomya, et al., 2018). This is not surprising because these antibiotics have been widely used in livestock production in Thailand for a long time. The percentage of MDR *E. coli* (88.8%) in this study was higher than the previous studies (Lugsomya, et al., 2018; Trongjit, Angkittitrakul, & Chuanchuen, 2016). It is likely because the isolates in this study were originated from the pig farming area with high pig population density where antibiotics are more commonly used. In contrast, resistance rates to CIP (23.8%) and GEN (35.1%) were lower than previous studies in other countries in Asia (Nguyen, et al., 2016).

In this study, susceptibility to disinfectants and heavy metals was examined by determination of MICs using agar dilution method. In general, the antimicrobial activity of disinfectants is tested by determination of survival curves that is laborious and more time consuming. Even though MIC determination is not the best method for detection of the efficacy of disinfectants and heavy metals, the method was shown to be practical for detecting susceptibility of a large number of bacterial isolates. Detection of MICs of disinfectants and heavy metals using the CLSI agar dilution methods were previously conducted in many studies (Aarestrup & Hasman, 2004; Kawamura-Sato, Wachino, Kondo, Ito, & Arakawa, 2010; Morrissey, et al., 2014). Therefore, MIC distribution was analyzed and used to identify trend of the susceptibility. Currently, there is no standard method available to detect the MIC values of disinfectants/heavy metals and no standard breakpoints available to define whether a bacterial species is susceptible or resistant to the substances. However, *E. coli* ATCC29522, *S. aureus* ATCC25923 and *P. aeruginosa* ATCC27853 were included as control for the susceptibility testing (Beier, et al., 2015).

From MIC distribution, at least two distinct groups of bacterial population at high and low MIC values in each compound is expected if there is the development of resistant population. The congregation of the *E. coli* isolates under a narrow MIC range was observed for CHX, BKC, GLU, FOR, $CuSO_4$ and $ZnCl_2$ (Table 29), indicating that there was no development or development at limited degree of resistance to these substances among the isolates, in agreement with a previous study (Aarestrup & Hasman, 2004). The CHX MIC range in this study (1-64 µg/mL) was in agreement with a previous study in Thailand (2-64 µg/mL) and Spain (0.5-64 µg/mL) but was much broader than a previous study conducted in livestock in Denmark (1-2 µg/mL) (Aarestrup & Hasman, 2004). However, most isolates (54.3%) in this study had MIC CHX value of 2 µg/mL. The BKC MIC ranged between 2-256 µg/mL, of which the most common MICs were 16-64 µg/mL (95.3%), in agreement with a study in livestock in Denmark (Aarestrup & Hasman, 2004) and Spain (Morrissey, et al., 2014). Both CHX and BKC MIC distribution was similar to our previous study in *Salmonella* isolated from poultry and swine (Chuanchuen, et al., 2008). A previous study in China reported a wider BKC MIC range (16-1024 µg/mL) but with the similar-common MIC value (32 µg/mL) (Zhang, et al., 2016). In addition, high MICs of GLU (2,048 – 4,096 µg/mL) and FOR (32 - 128 µg/mL) were obtained, in agreement with previous studies in Brazil (Priscila Gava Mazzola, 2009) and Belgium (Maertens, De Reu, Meyer, Van Coillie, & Dewulf, 2019).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The majority of the *E. coli* isolates were clustered into two groups by their TCS MIC values that were ≤0.0156 µg/mL (47.1%) and 0.03125-0.125 µg/mL (44.7%). This is inconsistent with a previous study demonstrating one large *E. coli* population with TCS MIC range of 0.03-0.5 µg/mL (Morrissey, et al., 2014). When considered the TCS MIC range, the range in this study (≤0.0156-256 µg/mL) was slightly higher than a previous study in Spain (≤0.0156-64 mg/L) (Morrissey, et al., 2014). Interestingly, TCS MIC values increased by years from ≤0.0156- 0.25 µg/mL in 2008-2010 to 32-256 µg/mL in 2015-2018. This may be a warning call for emergence of triclosan-resistant *E. coli* and the potential for effectiveness loss of triclosan in the future.

Epidemiological cut-off values (ECOFFs) of biocides were previously evaluated and applied to define *E. coli* wild-type and mutant strains and the ECOFF values of 2, 64 and 64 mg/L were proposed for TCS, CHX and BKC, respectively (Morrissey, et al., 2014). If the ECOFFs are used as interpretative criteria of the *E. coli* isolates in this study, resistance rates to TCS, CHX and BKC will be 1.1, 0.1 and 30.1%, respectively.

Zinc compounds have been formulated as feed additives to promote gut health and animal immunity especially in piglets. The zinc compounds including zinc oxide, ZnO; zinc chloride, $ZnCl_2$; zinc sulphate, $ZnSO_4$ are recommended as safe sources of Zn^{++} for all animal species (EFSA, 2015). ZnO is more commonly used due to its less toxicity to skin and mucosae. $ZnCl_2$ is a soluble form of zinc that could be absorbed in animal gut (Tacnet, Lauthier, & Ripoche, 1993). Therefore, $ZnCl_2$ is used as a source of Zn^{++} in this study. Almost all *E. coli* isolates had $ZnCl_2$ and $CuSO_4$ MICs of 1,024 and 256 - 512 µg/mL, respectively, similar to a previous study in Denmark (Aarestrup & Hasman, 2004).

Resistance to all antibiotics tested (defined by clinical breakpoints) was significantly associated with TCS and CHX MICs ($p < 0.05$), suggesting sharing resistance mechanisms among the antibiotics and biocides. The similar weak correlation between CHX and BKC MICs and resistance to some antibiotics (i.e. GEN and CIP) was previously reported in *Pseudomonas* spp.(Lavilla Lerma, Benomar, Casado Munoz Mdel, Galvez, & Abriouel, 2015) and *Acinetobacter* spp. (Kawamura-Sato, et al., 2010). This indicates that the isolates with high antibiotic MICs do not always have high disinfectant/ heavy metal MICs and vice versa, suggesting the variety and difference of mechanisms mediated resistance to antibiotics and biocides. Due to a lack of breakpoints for defining resistance to disinfectants and heavy metals, those with high MICs to certain disinfectants or heavy metals may not be defined as being clinically resistant. The associations between antibiotic resistance and disinfectant/heavy metal resistance cannot be analyzed.

Cross resistance between antibiotic and non-antibiotic substances has become a particular concern due to the possible contribution to the persistence of AMR despite antibiotic withdrawn. Expression of multidrug efflux pump has been recognized as a major mechanism mediating such cross resistance (Blanco, et al., 2016). In this study, exposure to CHX, BKC and TCS at sub-lethal concentration level resulted in cross resistance to antibiotics.

Two BKC-spontaneous resistant derivatives i.e. NK26B1 and NK32B1 were obtained. NK26B1 developed resistance to CHP, in agreement with previous studies (Langsrud, Sundheim, & Holck, 2004; Soumet, Fourreau, Legrandois, & Maris, 2012). NK32B1 additionally developed resistance to CIP, SMZ and TET, consistent to a previous study (Braoudaki & Hilton, 2004). Similarly, a CHX-spontaneous resistant mutant NK69C1 developed resistance to CIP, GEN and STR, in agreement with a previous study (Sheridan, Lenahan, Duffy, Fanning, & Burgess, 2012). In contrast, exposure to GLU promoted cross resistance to antibiotics in two previous studies (Karatzas, Webber, et al., 2007; Nhung, et al., 2015) but the phenomenon was not observed in this study. A previous study also demonstrated that exposure to commercial disinfectant products containing quaternary ammonium compound, formaldehyde and glutaraldehyde promoted cross resistance to TET, CHP, CIP and AMP in *Salmonella* (Karatzas, Randall, et al., 2007), supporting the *in vitro* results from this study.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Cross resistance generated by TCS has been extensively studied (Braoudaki and Hilton, 2004). In the present study, exposure to TCS promoted cross resistance to CHP, TET and TMP, in agreement with previous study (Braoudaki & Hilton, 2004). NK69T1 reduced susceptibility to AMP, as similar to a previous study in *Salmonella* Typhimurium (Karatzas, Webber, et al., 2007). On the other hand, previous study showed that no TCS-tolerant E. coli were resistant to antibiotics but significantly more susceptible to drugs in aminoglycoside group, AMP, CIP and TMP (Cottell, Denyer, Hanlon, Ochs, & Maillard, 2009).

Heavy metals e.g. Zn++ and Cu++ could promote cross resistance to antibiotics via multidrug efflux system and reduction in membrane permeability (Baker-Austin, Wright, Stepanauskas, & McArthur, 2006). However, this phenomenon was not observed in this study.

Involvement of multidrug efflux pumps in cross resistance was examined by determination of MICs in the presence and absence of PAβN, a well-studied efflux pump inhibitor (EPI) for multidrug efflux pumps in the resistance-nodulation division (RND) family. PAβN is one of the most common EPIs that have been used for examining the contribution of the RND efflux pumps to MDR phenotype. The presence of PAβN enhanced the potency of multiple antimicrobial drugs (e.g. ciprofloxacin, β-lactam drugs) in E. coli (Karczmarczyk, Martins, Quinn, Leonard, & Fanning, 2011; Lomovskaya, et al., 2001). Its ability to block TolC, an outer membrane protein for the AcrAB system, resulted in increased susceptibility to multiple antibiotics was demonstrated (Pu, et al., 2016). The results in this study showed that PAβN restored the susceptibility of CHP (93.8%), SMZ (62.5%), TMP (87.5%) and TCS (81.3%) in almost all spontaneous resistant mutant derivatives. The effects of the PAβN addition on MICs varied. MICs of CHP among two BKC-spontaneous resistant mutants (NK26B1, NK32B1) and nine TCS-spontaneous resistant mutants (E250.1, NK26T1, NK32T1, NK69 T1, MH21T1, MH33T1, MH43T1, MH58T1 and MH168T1) were restored (4-16 fold) by the presence of PAβN in agreement with a previous study in Vietnam (Nhung, et al., 2015). The presence of PAβN could reduce the MICs of SMZ (4-64 fold) in all three TCS-spontaneous resistant mutants (NK26T1, NK32T1 and MH174T1) and one BKC- spontaneous resistant mutants (NK32B1) and reduced that of TMP (8-16 fold) in seven TCS-spontaneous resistant mutants (NK26T1, NK32T1, NK221T1, MH21T1, MH33T1, MH58T1and MH189T1). These results highlighted important role of multidrug efflux system as cross-resistance mediated mechanism. The reduced MIC values of CHP, TMP, SMZ, TET and quinolones as a result of the presence of PAβN were similar to previous studies (Barrero, et al., 2014; Kvist, Hancock, & Klemm, 2008). Interestingly, the presence of PAβN, CCCP and reserpine did not restore the CIP MIC in some TCS-, BKC- and CHX-spontaneous

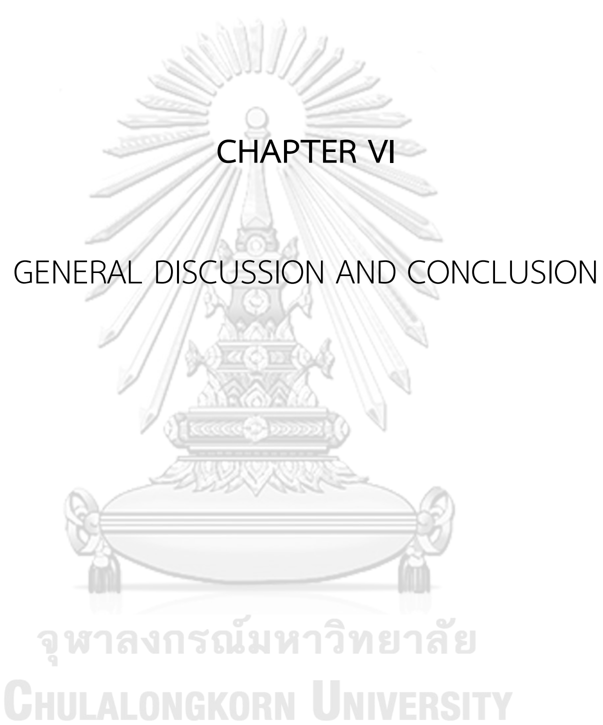
resistant mutants lacking mutation in other *gyrA* or *parC*. Interestingly, the presence of PAβN, CCCP and reserpine did not restore the CIP MIC in some TCS-, BKC- and CHX-spontaneous resistant mutants lacking mutation in either *gyrA* or *parC*. Mutations in the quinolone resistance-determining regions (QRDR) of *gyrA* and *parC* are one the major mechanisms for fluoroquinolone resistance (Karczmarczyk, et al., 2011) and a previous study demonstrated that exposure to commercial biocides selected for ciprofloxacin-resistant mutants (Webber, et al., 2015). However, this was not the case for the present study. These results suggest the existence of proton motive force-independent mechanisms.

In conclusion, the results highlight the important role of widely-used disinfectants and heavy metals as non-antibiotic selective pressure for emergence and spread of AMR. These emphasize that AMR is a complicated issue that requires complex-systematic strategic actions to deal with. Standardized and harmonized methods for detecting of biocide efficacy are needed. Simultaneously, monitoring resistance to biocides should be systematically and routinely conducted. Regulation on use of disinfectants and heavy metals should be enforced and prudent use of the substances need to be encouraged.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION



6.1 GENERAL DISCUSSIONS

Infections with MDR bacteria have rapidly increased and created a significant impact on economy, public health, and international food trade in most parts of the world. Using One Health approach, AMR monitoring and surveillance has been established among human, animal, and environmental sectors to understand the situation and track the progress of the problem. Action plans for the Control and preventative measures of AMR in food animals have been introduced in almost all countries, including Thailand, for example, restriction of antibiotic use, inhibition of antibiotic growth promoter etc.. However, it was evident that the politically driven ban of antimicrobial use in food animals is not the absolute solution for addressing AMR. A well-known example in Thailand is the persistence of chloramphenicol resistance. This antibiotic has been banned from being used in food animal since 1998. Bacteria in Enterobacteriaceae family resistant to chloramphenicol are still frequently isolated.

AMR monitoring data and several restudies in Thailand revealed that *Salmonella* and *E. coli* from pigs, poultry, pork, chicken and patients are usually resistant to multiple drugs. These studies also demonstrated the genetic link of resistance determinants among food animals, food of animal origin, and humans. Bacteria from different sources in Thailand and other Southeast Asian nations, especially *Salmonella* and *E. coli*, are known to have specific resistance determinants (e.g., *dfrA12-aadA2* gene cassette). The findings of the same resistance determinants from different samples, different sources and different locations suggest the involvement of horizontal transfer. It has been pointed out that antibiotics may not be the only selective pressure for emergence and spread of AMR, while the presence of non-antibiotic selective pressure has been suggested. In addition, cross resistance between antibiotics and disinfectants was demonstrated as a mechanism for multidrug resistance phenotype and the persistence of resistance. Contribution of non-antibiotic substances to AMR spread should be considered.

Even with the extensive research on AMR, there are still several unanswered questions. These include the persistence and spread of AMR bacteria and resistance determinants as well as the factors that determine their resistance. Therefore, this research project was designed to provide solutions for the unanswered questions of interest. The results were discussed in each individual chapter. General discussion is made to cover missing or additional messages as follows.

Part 1. Plasmid profile analysis of *Escherichia coli* and *Salmonella enterica* isolated from pigs, pork and humans.

Mobile genetic elements (MGE), especially plasmid, are a major route for the emergence and dissemination of AMR by horizontal transfer. Transmissible R plasmids normally carry multiple genes encoding resistance to antibiotics and play an important role in AMR evolution and spread. A variety of AMR determinants have been found to be associated with conjugative plasmids. The same genetic elements such as class1 integrons were detected in different bacterial species from different sources and locations. These findings highlight the horizontal transfer of plasmids as a major driver for AMR dissemination in Thailand and neighboring countries.

The presence of bacterial strains originated from different sources but carrying plasmids of the same Inc group indicate the horizontal widespread of the plasmids with close-phylogenetic relationship. The knowledge and understanding of plasmids diversity and transmission could be useful to develop the strategic action plan to contain AMR.

The plasmid replicon types were identified using PBRT (Carattoli et al., 2005). The commercial PBRT-KIT is currently available with increased sensitivity and specificity and capable of detecting up to 28 replicons (Carloni et al., 2017). However, the PBRT scheme that was used in this study had limitation in detection of some replicons in IncX plasmid group.

Most *E. coli* replicon types varied when the isolation times were considered. Factors that affect the maintenance of some plasmids in each period remain unclear. Different sampling location and antimicrobial use might be involved. However, the same plasmid replicon among *E. coli* from several origins including pig, pork, and humans, pointed out the circulation of plasmid in different sectors.

It is important to note that the association between resistance phenotype and replicon type varied. The significant-positive associations between resistance phenotype and replicon types were commonly observed, highlighting the important role of plasmids in the dissemination of AMR genes in *E. coli* and *Salmonella* in this study. In addition, the same FAB formula of IncF plasmid was found among the strains from different pigs, pork and humans from various locations, indicating that the particular plasmids circulate in the food chain.

In this part, R-plasmid profile of *E. coli* and *Salmonella* isolated from pigs, pork and humans in Thailand was investigated. The results could describe the answers for the first question about the characteristics and profile of R plasmid in *E. coli* and *Salmonella* isolated from food animals, meat and humans in Thailand. The results revealed a variety of plasmids distributed in pigs, pork, and humans in Thailand. Plasmid analysis serves as an epidemiological marker for AMR surveillance. To the best of our knowledge, this is the first report of plasmid replicon types among *E. coli* and *Salmonella* from pigs, pork and human in Thailand. The findings were extended by the study of the possible mechanisms associated with WGS analysis of *Salmonella* carrying *dfrA12* from food animals, meat and human in Thailand in part 2 and the persistence of chloramphenicol resistance in *Salmonella* and *E. coli* from food animals and humans in Thailand in part 3.

Part 2. Genomic analysis of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand.

The *dfra12-aadA2* cassette array was commonly reported among class1 integrons-positive bacterial species isolated from different sources in many countries worldwide. Class1 integrons with *dfrA12-aadA2* cassette array were commonly found in different bacterial species especially *Salmonella* from different sources in Thailand and the genetic connection between AMR in human, pets, food animals, and food of animal origin was highlighted. Comprehensive genetic data of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* cassette array is expected to explain the genetic link and the widespread of class 1 integrons with *dfrA12-aadA2.* Therefore, genetic characterization of *Salmonella* carrying class 1 integrons with *dfrA12-aadA2* gene cassette array isolated from food animals, meat and human in Thailand were done by using WGS analysis.

According to WGS analysis, IncFIB(K) plasmids commonly found in this study and were closely related to pKPN-IT plasmid. Beside IncFIB(K) plasmid, IncHI2 plasmids were previously reported to carry class1 integons containing *dfrA12* and *aadA* genes and could be transferred by conjugation (Shang et al., 2021). The presence of the same plasmid replicons among *Salmonella* isolated from different sources in different years indicate the circulation of the plasmids among the *Salmonella* isolates of food producing animal origin. The Tn*3*/Tn*AS1* and Tn*3*/Tn*As3* were commonly found at downstream of *intI1* in this study. These transposons might be another evidence to support the spread of mobile genetic element.

The findings were to partially explain the widespread of the *dfrA12-aadA2* cassette array widespread in *Salmonella* isolated from food animals, meat and humans in Thailand. The results indicate the important role of transferable plasmids as the underline cause for the wide distribution of class 1 integrons *dfrA12-aadA2* gene array. The gene structure of the class 1 integrons was closely related to transposons and insertion sequences, in particular Tn*As1,* Tn*As3*, and IS*26* that facilitate efficient mobility. The existence and circulation of R plasmid is maintained by antibiotic selective pressure. Therefore, decreasing the selective pressure could limit the wide distribution of AMR associated with transferable plasmids.

Part 3. Molecular basis of the persistence of chloramphenicol resistance among *Escherichia coli* and *Salmonella* spp. from food animals, meat, and human in Thailand

Use of antimicrobial agents is one of the key factors contributing to the emergence and spread of AMR. Reducing the use of antimicrobials is expected to decrease the likelihood of AMR bacteria emergence and spread. It is continuously observed that bacteria resistant to restricted antimicrobial drugs have been consistently isolated (Hanekamp and Bast, 2015). The persistence of chloramphenicol resistance is an outstanding example. It was proposed to be the result of co-selection or cross-resistance brought on by other antimicrobials (Pal et al., 2015; Périchon et al., 2015; Cheng et al., 2019). Therefore, conjugation experiment and WGS were included in this project to determine the possible mechanism associated with co-selection. In addition, the possible contribution of efflux pumps were investigated by using PA**β**N. efflux pump inhibitor.

The *cmlA* encodes a putative multidrug efflux pump from the MATES and is capable of extrude CHP. The results showed that the contribution of *cmlA* in CHP resistance was rather limited (<4-fold CHP MIC decrease in the presence of Pa**β**N). On the other hand, *E. coli* that had both *catA* and *catB* showed a ≥4-fold drop in CHP MIC when PA**β**N was present. These highlight the accumulative effects of enzymatic and non-enzymatic mechanisms on CHP resistance. The *cmlA* and *catA* genes are usually located on multi resistant integrons or associated with transposons on transferable and non-transferable plasmids (Roberts and Schwarz, 2009; McMillan et al., 2020). Taken together, the findings suggest the involvement of cross resistance and co-selection in CHP resistance in *E. coli* and *Salmonella*.

From the WGS analysis revealed that all plasmids identified, including IncFIA(HI1)/HI1B, IncFII/FIB and IncX1 plasmids, carried class 1 integrons with *dfrA12-aadA2-cmlA1-aadA1 dfrA12* cassette array. Besides class 1 integrons with *dfrA12-*

aadA2-cmlA1-aadA1 dfrA12 cassette array were flanked by Tn*As1*/ Tn*3* at upstream and IS*6*/ IS*26* at downstream, while IncFIA(HI1)/HI1B/Q1 plasmid carrying *catA* showed quite different the genetic environment when compared to *cmlA*. IS*1*-like element IS*1B* family transposase was found next to *catA*. Both *cmlA* and *catA* containing area were flanked by ISs of HGT region. Flanking by insertion sequences could facilitate the mobilization of the genes, leading to the spread of the AMR bacteria. In addition, WGS analysis results revealed the existence of several resistance genes on *cmlA* or *catA*-carrying plasmids and confirmed the co-selection of CHP-resistance by other antibiotics.

The results from this part exhibited that the persistence of CHP-resistant *E. coli* and *Salmonella* after the chloramphenicol ban from use in food animals was due to co-selection by using the other antibiotics and cross resistance vis the expression of multidrug efflux pump. Therefore, prohibiting a single antimicrobial agent is not enough to address the AMR problem. Several measures and activities that lower the need for antimicrobials and thus slow the spread of AMR are required (e.g., farm biosecurity, infection control, vaccination program, prudent antimicrobial use etc.).

Part 4. Resistance to widely used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass.

Chulalongkorn University

The above results have led to the need to explore non-antibiotic selective pressure, in particular disinfectants and heavy metals, contributing to AMR. In food animal production, disinfectants are used extensively for disinfection. Heavy metals i.e. copper sulfates and zinc chlorides are formulated as the growth-promoting feed supplements. Bacteria may develop resistance to disinfectants and heavy metals and promote cross resistance to antibiotics. One of the mechanisms underlying insusceptibility to biocide is the presence of an active efflux pump. In *Salmonella*, the RND family AcrAB-TolC system has been shown to confer resistance to multiple antibiotics and disinfectants, resulting in multiple antibiotic resistance phenotypes (Lacroix et al., 1996; White et al., 1997). In this study, susceptibility to disinfectants

and heavy metals was examined in *E. coli* isolated from clinically healthy pig by determination of MICs using agar dilution method. There is no standard method available to detect the MIC values of disinfectants/heavy metals and no standard breakpoints available to define whether a bacterial species is susceptible or resistant to the substances. It was observed that the isolates with high antibiotic MICs do not always have high disinfectant/ heavy metal MICs and vice versa, suggesting the variety and difference of mechanisms mediated resistance to antibiotics and biocides.

Exposure to CHX, BKC and TCS at sub-lethal concentration level resulted in cross resistance to antibiotics. It was evident by the observation that a CHX-spontaneous resistant mutant developed resistance to CIP, GEN and STR. At the same time, exposure to TCS also promoted cross resistance to CHP, TET and TMP.

The presence of PAβN enhanced the potency of multiple antimicrobial drugs. Its ability to block TolC, an outer membrane protein for the AcrAB system, resulted in increased susceptibility to multiple antibiotics. The results in this study showed that PAβN restored the susceptibility of CHP, SMZ, TMP and TCS in almost all spontaneous resistant mutant derivatives. These results highlighted the important role of multidrug efflux system as cross-resistance mediated mechanism.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

This part was to describe the role of disinfectants and heavy metals as non-antibiotic selective pressure for AMR development and spread. The findings unequivocally show that AMR is a complex problem that calls for sophisticated-systematic strategic approaches to be solved. It emphasizes the necessity of conducting systematic and regular monitoring of resistance to biocides. Enforcing regulations for and encouraging the prudent use of disinfectants and heavy metals is necessary.

6.2 CONCLUSIONS

The research was conducted as planned and the objectives of this study were achieved. The summary could be described for each objective as follows:

Objective 1: To characterize the profile of R plasmid in *E. coli* and *Salmonella* isolated from food animals, meat and humans in Thailand.

Plasmid replicon was significantly correlated with resistance phenotype but significantly different among sources of isolates and sample collecting periods. The same plasmid was found among the strains from pigs, pork and humans from various locations, indicating that the particular plasmids circulate in the food chain and confirming that AMR is a One Health issue.

Objective 2: To investigate the genetic characteristics of plasmid carrying the *dfrA12-aadA2* cassette array in *Salmonella* isolated from food animals, meat and humans in Thailand**.**

Class1 integrons with *dfrA12-aadA2* cassette array are located on various transferable R plasmids. Transposons and insertion sequences exist to facilitate the movement of class 1 integrons. Transferable plasmids play an important role in the wide distribution of class 1 integrons with *dfrA12-aadA2* gene array.

Objective 3: To characterize plasmid carrying chloramphenicol-resistance genes in *E. coli* and *Salmonella* from food animals, meat and humans in Thailand.

Cross-resistance via multidrug efflux pump and co-selection by other antimicrobial agents mainly contribute to the persistence of CHP-resistance.

Objective 4: To determine the possible cross resistance between disinfectants/heavy metals and antibiotics in *E. coli* from food animals in Thailand.

Exposure to disinfectants and heavy metals could promote cross resistance to antimicrobial drugs. Disinfectants and heavy metals play an important role as non-antibiotic selective pressure for emergence and spread of AMR.

6.3 SUGGESTION AND FURTHER STUDIES

6.3.1 Applications of the research findings to address AMR:

A mix of short- and long-term measures must be taken into account in order to successfully reduce AMR derived from livestock and support an ethical and sustainable approach to animal husbandry while safeguarding public health. As several measures have been implemented in Thailand, only additional activities are recommended as follows:

1. The results may serve as evidence in favor of legislation or the development of guidelines pertaining to the usage of antibiotics.
2. The findings of non-antibiotics selective pressure can be used for raising awareness of antibiotic resistance that emerges and spreads due to using disinfectants and heavy metals.
3. The findings of non-antibiotics selective pressure can be utilized to advocate for the legislation limiting the use of heavy metals and disinfectants as well as prudent use specific for the use of the substances.
4. Conduct seminars and training to educate veterinary students and other relevant stakeholders about AMR spread associated with mobile genetic elements and non-antibiotic selective pressure.
5. Increase communication to public, farmers and other stakeholder about AMR risks.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

6.3.2 Specific recommendations to address AMR associated with livestock in Thailand.

Several measures have been implemented in Thailand to contain AMR associated with livestock (e.g., farm biosecurity, good animal husbandry, improved nutrition, vaccination, use of antibiotic alternatives and prudent use of antimicrobials). Therefore, only additional activities, as derived from the findings in this study, are recommended as follows.

1. Encourage strict hygiene, cleaning and appropriate disinfection protocols to minimize the accumulation of AMR bacteria.

2. Enforce the effective use of guidelines to reduce and regulate the unnecessary use of antibiotics in animal husbandry.
3. As Thailand currently lacks specific guidelines for disinfectant use, manual or guideline for effective and appropriate use of disinfectants and antimicrobial drugs should be developed.
4. Initiate monitoring of resistance to disinfectants and heavy metals.
5. Promote the development and use of the harmonized and standardized protocol for determination of disinfect efficacy.
6. Department of Livestock Development should consider include mobile genetic elements to national AMR surveillance as well as Integrated One Health AMR surveillance.

6.3.3 Recommendations for future studies

The fact is that a substantial quantity of AMR remains unclear and should be addressed for a comprehensive understanding. Considering the findings of this study, the following recommendations are given for more research:

1. Expand the study of plasmids to cover other mobile genetic elements and additional sectors e.g., humans, small animals, aquatic animals etc.
2. Expand the scope of non-antibiotic selective pressure research to include other animal and agricultural sectors, such as aquaculture, slaughterhouses, animal hospitals, and poultry.
3. Research compounds that can prevent or inhibit R plasmids from being transferred.
4. Investigate for efflux pump inhibitors to be used in combination of antibiotics that bacteria develop resistance to.
5. Explore the evidence and correlation between AMR/mobile genetic elements and antimicrobial use.
6. Study the mobile genetic elements in the poultry or pig production chain or from farm to fork perspective.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

7. Develop risk assessment models to predict the potential human and animal health risks associated with AMR due to mobile genetic elements and the use of disinfectants and heavy metals.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

REFERENCES

Aarestrup, F. M., & Hasman, H. (2004). Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. Vet Microbiol, 100(1-2), 83-89.

Alcala A, Ramirez G, Solis A, Kim Y, Tan K, Luna O, Nguyen K, Vazquez D, Ward M, Zhou M, Mulligan R, Maltseva N and Kuhn ML 2020. Structural and functional characterization of three Type B and C chloramphenicol acetyltransferases from Vibrio species. Protein Sci. 29(3): 695-710.

Amaral L, Martins A, Spengler G and Molnar J 2014. Efflux pumps of Gram-negative bacteria: what they do, how they do it, with what and how to deal with them. Front Pharmacol. 4: 168.

Amoah Barnie P 2014. Development of Efflux Pumps and Inhibitors (EPIs) in *A. baumanii*. Clinical Microbiology: Open Access. 03(01).

Andersson DI and Hughes D 2011. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev. 35(5): 901-911.

Andrews S 2010. FASTQC. A quality control tool for high throughput sequence data.

Nuangmek A, Rojanasthien S, Chotinun S, Yamsakul P, Tadee P, Thamlikitkul V, Tansakul N and Patchanee P 2018. Antimicrobial Resistance in ESBL-Producing *Escherichia coli* Isolated from Layer and Pig Farms in Thailand. Acta Scientiae Veterinariae, 46, 1538.

Arigul T, Jenjaroenpun P, Wankaew N, Vespiromc J, Thuamkaew P, Wongsurawat T and Ledlod S 2023. Complete Genome Sequences of Three *Salmonella* Strains Obtained from a Poultry Production Farm in Thailand. Journal of Bacteriology. 12(2).

Baker-Austin C, Wright MS, Stepanauskas R and McArthur JV 2006. Co-selection of antibiotic and metal resistance. Trends in Microbiology, 14(4), 176-182.

Barrero MAO, Pietralonga PAG, Schwarz DGG, Silva A, Jr AS, Paula SO and Moreira MAS 2014. Effect of the inhibitors phenylalanine arginyl ss-naphthylamide (PAssN) and 1-(1-naphthylmethyl)-piperazine (NMP) on expression of genes in multidrug efflux systems of *Escherichia coli* isolates from bovine mastitis. Res Vet Sci, 97(2), 176-181.

Beier RC, Foley SL, Davidson MK, White DG, McDermott PF, Bodeis-Jones S, Zhao S, Andrews K, Crippen TL, Sheffield CL, Poole TL, Anderson RC and Nisbet DJ 2015. Characterization of antibiotic and disinfectant susceptibility profiles among *Pseudomonas aeruginosa* veterinary isolates recovered during 1994-2003. J Appl Microbiol, 118(2), 326-342.

Bischoff KM, White DG, Hume ME, Poole TL and Nisbet DJ 2005. The chloramphenicol resistance gene *cmlA* is disseminated on transferable plasmids that confer multiple-drug resistance in swine *Escherichia coli*. FEMS Microbiol Lett. 243(1): 285-291.

Blanco P, Hernando-Amado S, Reales-Calderon JA, Corona F, Lira F, Alcalde-Rico M, Bernardini A, Sanchez MB and Martinez JL 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. Microorganisms. 4(1): 14.

Boerlin P and Reid-Smith RJ 2008. Antimicrobial resistance: its emergence and transmission. Anim Health Res Rev. 9(2): 115-126.

Bonnin RA, Poirel L, Carattoli A and Nordmann P 2012. Characterization of an IncFII plasmid encoding NDM-1 from *Escherichia coli* ST131. PLoS One. 7(4): e34752.

Braoudaki M and Hilton AC 2004. Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-Resistance to Antimicrobial Agents. Journal of Clinical Microbiology, 42(1), 73-78.

Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B 2000. *Salmonella* nomenclature. J Clin Microbiol. 38 (7): 2465-2467.

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K and Madden TL 2009. BLAST+: architecture and applications. BMC Bioinformatics. 10: 421.

Cannon M, Harford S and Davies J 1990. A comparative study on the inhibitory actions of chloramphenicol, thiamphenicol and some fluorinated derivatives. Journal of Antimicrobial Chemotherapy. 26: 307-317.

Carattoli A. 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrobial Agents and Chemotherapy 2009; 53(6): 2227-2238.

Carattoli A 2011. Plasmids in Gram negatives: molecular typing of resistance plasmids. Int J Med Microbiol. 301(8): 654-658.

Carattoli A 2013. Plasmids and the spread of resistance. Int J Med Microbiol. 303(6-7): 298-304.

Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL and Threlfall EJ 2008. Identification of plasmids by PCR-based replicon typing. Journal of Microbiological Methods 2005; 63(3): 219-228.

Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL and Threlfall EJ 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 63(3): 219-228.

Carattoli A, Seiffert SN, Schwendener S, Perreten V and Endimiani A 2015. Differentiation of IncL and IncM Plasmids Associated with the Spread of Clinically Relevant Antimicrobial Resistance. PLoS One 2015; 10(5): e0123063.

Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F and Hasman H 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 58(7): 3895-3903.

Carey DE and McNamara PJ 2014. The impact of triclosan on the spread of antibiotic resistance in the environment. Front Microbiol, 5, 780.

Carloni E, Andreoni F, Omiccioli E, Villa L, Magnani M and Carattoli A 2017. Comparative analysis of the standard PCR-Based Replicon Typing (PBRT) with the commercial PBRT-KIT. Plasmid. 90: 10-14.

Casewell M, Friis C, Marco E, McMullin P and Phillips I 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J Antimicrob Chemother. 52(2): 159-161.

Chapman JS 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. Int Biodeterior Biodegradation. 51(4): 271-276.

Chen CY, Hsieh PH, Chang CY, Yang ST, Chen YH, Chang K and Lu PL 2019. Molecular epidemiology of the emerging ceftriaxone resistant non-typhoidal *Salmonella* in southern Taiwan. Journal of Microbiology, Immunology and Infection 2019; 52(2): 289-296.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Cheng G, Ning J, Ahmed S, Huang J, Ullah R, An B, Hao H, Dai M, Huang L, Wang X and Yuan Z 2019. Selection and dissemination of antimicrobial resistance in Agri-food production. Antimicrob Resist Infect Control. 8: 158.

Chen X, He L, Li Y, Zeng Z, Deng Y, Liu Y and Liu J-H 2014. Complete sequence of a F2:A-:B- plasmid pHN3A11 carrying *rmtB* and *qepA*, and its dissemination in China. Veterinary Microbiology 2014; 174(1-2): 267-271.

Chu C, Chiu CH, Wu WY, Chu CH, Liu TP and Ou JT 2001. Large drug resistance virulence plasmids of clinical isolates of *Salmonella enterica* serovar Choleraesuis. Antimicrob Agents Chemother. 45(8): 2299-2303.

Chuanchuen R and Padungtod P 2009. Antimicrobial resistance genes in *Salmonella enterica* Isolates from poultry and swine in Thailand. J. Vet. Med. Sci. 71(10): 1349–1355.

Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR and Schweizer HP 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother, 45(2), 428-432.

Chuanchuen R, Pathanasophon P, Khemtong S, Wannaprasat W, & Padungtod P 2008. Susceptibilities to Antimicrobials and Disinfectants in *Salmonella* Isolates Obtained from Poultry and Swine in Thailand. J. Vet. Med. Sci., 70(6), 595-601.

CLSI 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI 2021. Performance Standards for Antimicrobial Susceptibility Testing. CLSI supplement M100. 41.

Cole GRCaJR 1990. Diagnostic Procedures in Veterinary Bacteriology and Mycology. Academic press, Inc. fifth edition.

Collignon PJ, Conly JM, Andremont A, McEwen SA, Aidara-Kane A, World Health Organization Advisory Group BMoISoAR, Agerso Y, Andremont A, Collignon P, Conly J, Dang Ninh T, Donado-Godoy P, Fedorka-Cray P, Fernandez H, Galas M, Irwin R, Karp B, Matar G, McDermott P, McEwen S, Mitema E, Reid-Smith R,

Chulalongkorn University

Scott HM, Singh R, DeWaal CS, Stelling J, Toleman M, Watanabe H and Woo GJ 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. Clin Infect Dis, 63(8), 1087-1093.

Cottell A, Denyer SP, Hanlon GW, Ochs D and Maillard JY 2009. Triclosan-tolerant bacteria: changes in susceptibility to antibiotics. J Hosp Infect, 72(1), 71-76.

Cottell JL, Webber MA, Coldham NG, Taylor DL, Cerdeno-Tarraga AM, Hauser H, Thomson NR, Woodward MJ and Piddock LJV 2011. Complete sequence and molecular epidemiology of IncK epidemic plasmid encoding *bla*CTX-M-14. Emerging Infectious Diseases 2011; 17(4): 645-652.

Couturier M, Bex F, Bergquist PL and Maas WK 1988. Identification and classification of bacterial plasmids. Microbiol Rev. 52(3): 375-395.

Darphorn TS, Bel K, Koenders-van Sint Anneland BB, Brul S and Ter Kuile BH 2021. Antibiotic resistance plasmid composition and architecture in *Escherichia coli* isolates from meat. Sci Rep. 11(1): 2136.

Davin-Regli A, Pages JM and Ferrand A 2021. Clinical Status of Efflux Resistance Mechanisms in Gram-Negative Bacteria. Antibiotics (Basel). 10(9).

Davis JL 2018. Pharmacologic Principles. In: Equine Internal Medicine. ed. (ed). 79-137.

De Coster W, D'Hert S, Schultz DT, Cruts M and Van Broeckhoven C 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics. 34(15): 2666-2669.

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

Dobiasova H, Dolejska M. 2016. Prevalence and diversity of IncX plasmids carrying fluoroquinolone and beta-lactam resistance genes in *Escherichia coli* originating from diverse sources and geographical areas. Journal of Antimicrobial Chemotherapy 2016; 71(8): 2118-2124.

จุฬาลงกรณ์มหาวิทยาลัย

Domingues S, da Silva GJ and Nielsen KM 2012. Integrons: vehicles and pathways for horizontal dissemination in bacteria. Mob Genet Elements. 2(5): 211-223.

ECHA. (2017). Guidance on the BPR: Volume II Efficacy - Assessment and Evaluation (Parts B+C). In: European Chemicals Agency.

EFSA 2010. Scientific opinion on monitoring and assessment of the public health risk of *Salmonella* Typhimurium-like strains. EFSA J. 8 (10): 7-8.

EFSA 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. EFSA J. 10: 1–64.

EFSA. (2013). Annual Report of the EFSA Journal 2012. EFSA Supporting Publicaton, EN-418, 1-9.

EFSA. (2015). Scientific Opinion on the safety and efficacy of zinc compounds (E6) as feed additives for all animal species (zinc acetate, dihydrate; zinc chloride, anhydrous; zinc oxide; zinc sulphate, heptahydrate; zinc sulphate, monohydrate; zinc chelate of amino acids, hydrate; zinc chelate of glycine, hydrate), based on a dossier submitted by FEFANA asbl. EFSA Journal, 13(4).

European Commission 2009. “Assessment of the antibiotic resistance effects of biocides” [Online] Available: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/ scenihr_o_021.pdf Accessed April 3, 2018.

EFSA 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. bacteria transmitted through food. European Food Safety Authority 2012; 10(6).

Elbediwi M, Li Y, Paudyal N, Pan H, Li X, Xie S, Rajkovic A, Feng Y, Fang W, Rankin SC and Yue M 2019. Global Burden of Colistin-Resistant Bacteria: Mobilized Colistin Resistance Genes Study (1980-2018). Microorganisms 2019; 7(10).

FAO 2016. “Drivers, dynamics and epidemiology of antimicrobial resistance in animal production” [Online] Available: http://www.fao.org/3/a-i6209e.pdf Accessed March 18, 2018.

CHULALONGKORN UNIVERSITY

Fernandez-Lopez R, Garcillan-Barcia MP, Revilla C, Lazaro M, Vielva L and Cruz Fdl 2006. Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS Microbiology Reviews 2006; 30(6): 942-966.

Flanklin A, Acar J, Anthony F, Gupta R, Nicholls T, Tamaru Y, Thomson S, Threlfall EJ, Vose D, Vuuren MV, White DG, Wegener HC and Costarrica ML 2001. Antimicrobial resistance: harmonisation of national antimicrobial resistance monitoring and surveillance programmes in animals and in animal-derived food. Rev sci tech Off int Epiz. 20(3): 859-870.

Florensa AF, Kaas RS, Clausen P, Aytan-Aktug D and Aarestrup FM 2022. ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. Microb Genom. 8(1).

Fluit AC 2005. Towards more virulent and antibiotic-resistant *Salmonella*? FEMS Immunol Med Microbiol. 43(1): 1-11.

Frye JG, Jackson CR. 2013. Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica*, *Escherichia coli*, and *Enteroccocus* spp. isolated from U.S. food animals. Frontiers in Microbiology 2013; 4: 135.

Garcia-Fernandez A, Chiaretto G, Bertini A, Villa L, Fortini D, Ricci A and Carattoli A 2008. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. J Antimicrob Chemother. 61(6): 1229-1233.

Garcia P, Guerra B, Bances M, Mendoza MC and Rodicio MR 2011. IncA/C plasmids mediate antimicrobial resistance linked to virulence genes in the Spanish clone of the emerging *Salmonella enterica* serotype 4,[5],12:i. J Antimicrob Chemother. 66(3): 543-549.

Garcia P, Hopkins KL, Garcia V, Beutlich J, Mendoza MC, Threlfall J, Mevius D, Helmuth R, Rodicio MR, Guerra B 2014. Diversity of plasmids encoding virulence and resistance functions in *Salmonella enterica* subsp. enterica serovar Typhimurium monophasic variant 4,[5],12:i:- Strains Circulating in Europe. PLoS ONE 9(2): e89635.

Gestal AM, Stokes HW, Partridge SR and Hall RM 2005. Recombination between the *dfrA12-orfF-aadA2* cassette array and an *aadA1* gene cassette creates a hybrid cassette, *aadA8b*. Antimicrob Agents Chemother. 49(11): 4771-4774.

Gradel KO, Randall L, Sayers AR and Davies RH 2005. Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of mar. Vet Microbiol. 107(1-2): 127-138.

Grant JR, Enns E, Marinier E, Mandal A, Herman EK, Chen CY, Graham M, Van Domselaar G and Stothard P 2023. Proksee: in-depth characterization and visualization of bacterial genomes. Nucleic Acids Res. 51(W1): W484-W492.

Guerra B, Soto S, Helmuth R and Mendoza MC 2002. Characterization of a self-transferable plasmid from *Salmonella enterica* Serotype Typhimurium clinical isolates carrying two integron-borne gene cassettes together with virulence and drug resistance genes. Antimicrob Agents and Chemother. 46(9): 2977-2981.

Guiney DG and Fierer J 2011. the role of the *spv* genes in *Salmonella* pathogenesis. Front Microbiol. 2: 129.

Gurevich A, Saveliev V, Vyahhi N and Tesler G 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics. 29(8): 1072-1075.

Hancock SJ, Phan MD, Peters KM, Forde BM, Chong TM, Yin WF, Chan KG, Paterson DL, Walsh TR, Beatson SA and Schembri MA 2017. Identification of IncA/C Plasmid Replication and Maintenance Genes and Development of a Plasmid Multilocus Sequence Typing Scheme. Antimicrob Agents Chemother. 61(2) e01740-16.

Hanekamp JC and Bast A 2015. Antibiotics exposure and health risks: chloramphenicol. Environ Toxicol Pharmacol. 39(1): 213-220.

Harada S, Ishii Y, Saga T, Kouyama Y, Tateda K and Yamaguchi K 2012. Chromosomal integration and location on IncT plasmids of the blaCTX-M-2 gene in *Proteus mirabilis* clinical isolates. Antimicrobial Agents and Chemotherapy 2012; 56(2): 1093-1096.

Harmer CJ and Hall RM 2019. An analysis of the IS*6*/IS*26* family of insertion sequences: is it a single family? Microb Genom. 5(9).

Harrison JT, Valentina T, Stan MA, Chan CS, Vacchi-Suzzi C, Heyne BJ, Parsek MR, Ceri H, and Turner RJ 2009. Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. Environmental microbiology, 11, 2491-2509.

Ibrahim S, Hoong LW, Siong YL, Mustapha Z, Zalati CWSCW, Aklilu E, Mohamad M and Kamaruzzaman NF 2021. Prevalence of Antimicrobial Resistance (AMR) *Salmonella* spp. and *Escherichia coli* Isolated from Broilers in the East Coast of Peninsular Malaysia. Antibiotics (Basel) 2021; 10(5).

ISO 2002. Microbiology of food and animal feeding stuffs— horizontal method for the detection of *Salmonella* spp: ISO6579 Fourth Edition Vol. Reference number ISO 6579:2002(E).

ISO. 2017. Microbiology of the food chain — horizontal method for the detection, enumertion and serotyping of *Salmonella*: ISO6579 First Edition. Reference number ISO 6579-1:2017(E) 2017.

Jacoby GA, Strahilevitz J and Hooper DC. 2014. Plasmid-mediated quinolone resistance. Microbiology Spectrum 2014; 2(5).

Jahantigh M, Samadi K, Dizaji RE and Salari S 2020. Antimicrobial resistance and prevalence of tetracycline resistance genes in *Escherichia coli* isolated from lesions of colibacillosis in broiler chickens in Sistan, Iran. BMC Veterinary Research 2020; 16(1): 267.

Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM and Aarestrup FM 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. J Clin Microbiol. 52(5): 1501-1510.

Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM and Scheutz F 2015. Rapid and Easy In Silico Serotyping of *Escherichia coli* Isolates by Use of Whole-Genome Sequencing Data. J Clin Microbiol. 53(8): 2410-2426.

Johansson MHK, Bortolaia V, Tansirichaiya S, Aarestrup FM, Roberts AP and Petersen TN 2021. Detection of mobile genetic elements associated with antibiotic resistance in *Salmonella enterica* using a newly developed web tool: MobileElementFinder. J Antimicrob Chemother. 76(1): 101-109.

รณ์มหาวิทยาลัย

Johnson TJ and Nolan LK 2009. Plasmid replicon typing. Methods Mol Biol. 551: 27-35.

Jonas D, Spitzmüller B, Weist K, Rüden H and Daschner FD 2003. Comparison of PCR-based methods for typing *Escherichia coli*. Clinical Microbiology and Infection, 9(8), 823-831.

Karatzas KA, Randall LP, Webber M, Piddock LJV, Humphrey TJ, Woodward MJ and Coldham NG 2007. Phenotypic and Proteomic Characterization of Multiply Antibiotic-Resistant Variants of *Salmonella enterica* Serovar Typhimurium Selected Following Exposure to Disinfectants. Applied and Environmental Microbiology, 74(5), 1508-1516.

Karatzas KA, Webber MA, Jorgensen F, Woodward MJ, Piddock LJ and Humphrey TJ 2007. Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J Antimicrob Chemother, 60(5), 947-955.

Karczmarczyk M, Martins M, Quinn T, Leonard N and Fanning S 2011. Mechanisms of fluoroquinolone resistance in *Escherichia coli* isolates from food-producing animals. Appl Environ Microbiol, 77(20), 7113-7120.

Kawamura-Sato K, Wachino J-i, Kondo T, Ito H and Arakawa Y 2010. Correlation between reduced susceptibility to disinfectants and multidrug resistance among clinical isolates of *Acinetobacter* species. Journal of Antimicrobial Chemotherapy, 65(9), 1975-1983.

Khemtong S and Chuanchuen R 2008. Class 1 integrons and *Salmonella* genomic island 1 among *Salmonella enterica* isolated from poultry and swine. Microbe Drug Resist. 14(1): 65–70.

Koser CU, Ellington MJ and Peacock SJ 2014. Whole-genome sequencing to control antimicrobial resistance. Trends Genet. 30(9): 401-407.

Kvist M, Hancock V and Klemm P 2008. Inactivation of efflux pumps abolishes bacterial biofilm formation. Appl Environ Microbiol, 74(23), 7376-7382.

Langsrud S, Sundheim G and Holck AL 2004. Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. Journal of Applied Microbiology, 96(1), 201-208.

Lavilla Lerma L, Benomar N, Casado Munoz Mdel C, Galvez A and Abriouel H 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiol, 51, 33-44.

Lay KK, Koowattananukul C, Chansong N and Chuanchuen R 2012. Antimicrobial resistance, virulence, and phylogenetic characteristics of *Escherichia coli* isolates from clinically healthy swine. Foodborne Pathog Dis. 9(11): 992-1001.

Lay KK, Jeamsripong S, Sunn KP, Angkititrakul S, Prathan R, Srisanga S and Chuanchuen R 2021. Colistin Resistance and ESBL Production in *Salmonella* and *Escherichia coli* from Pigs and Pork in the Thailand, Cambodia, Lao PDR, and Myanmar Border Area. Antibiotics (Basel) 2021; 10(6).

Letunic I and Bork P 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49(W1): W293-W296.

Lévesque C, Piché L, Larose C and Roy PH 1995. PCR Mapping of Integrons Reveals Several Novel Combinations of Resistance Genes. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY. 39(1): 185-191.

Li R, Yu H, Xie M, Chen K, Dong N, Lin D, Chan EWC and Chen S 2018. Genetic basis of chromosomally-encoded *mcr-1* gene. International Journal of Antimicrobial Agents 2018; 51(4): 578-585.

Li XZ and Nikaido H 2009. Efflux-mediated drug resistance in bacteria: an update. Drugs. 69(12): 1555-1623.

Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H and Lee VJ 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob Agents Chemother, 45(1), 105-116.

Long M, Lai H, Deng W, Zhou K, Li B, Liu S, Fan L, Wang H and Zou L 2016. Disinfectant susceptibility of different *Salmonella* serotypes isolated from chicken and egg production chains. J Appl Microbiol, 121(3), 672-681.

Lorenzo D 2019. Chloramphenicol Resurrected: A Journey from Antibiotic Resistance in Eye Infections to Biofilm and Ocular Microbiota. Microorganisms. 7(9).

Lugsomya K, Yindee J, Niyomtham W, Tribuddharat C, Tummaruk P, Hampson DJ and Prapasarakul N 2018. Antimicrobial Resistance in Commensal *Escherichia coli* Isolated from Pigs and Pork Derived from Farms Either Routinely Using or Not Using In-Feed Antimicrobials. Microb Drug Resist, 24(7), 1054-1066.

Lukkana M, Wongtavatchai J and Chuanchuen R 2012. Class 1 integrons in *Aeromonas hydrophila* Isolates from farmed Nile Tilapia (*Oreochromis nilotica*). J Vet Med Sci. 74(4): 435-440.

Lukkana M, Wongtavatchai J and Chuanchuen R 2011. Expression of AheABC Efflux System and Plasmid Profile of *Aeromonas hydrophila* Isolates from Farmed Nile Tilapia (*Oreochromis niloticus*). Thai Journal of Veterinay Medicine 2011; 41(4): 529-533.

Madec J-Y, Haenni M 2018. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid 2018; 99: 72-81.

Maertens H, De Reu K, Meyer E, Van Coillie E and Dewulf J 2019. Limited association between disinfectant use and either antibiotic or disinfectant susceptibility of *Escherichia coli* in both poultry and pig husbandry. BMC Vet Res, 15(1), 310.

Mahamoud A, Chevalier J, Alibert-Franco S, Kern WV and Pages JM 2007. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J Antimicrob Chemother. 59(6): 1223-1229.

Marchant M, Vinue L, Torres C and Moreno MA 2013. Change of integrons over time in *Escherichia coli* isolates recovered from healthy pigs and chickens. Vet Microbiol. 163(1-2): 124-132.

Mathers AJ, Peirano G and Pitout JDD 2015. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. Clin Microbiol Rev. 28(3): 565-591.

Mathur S and Singh R 2005. Antibiotic resistance in food lactic acid bacteria--a review. Int J Food Microbiol. 105(3): 281-295.

McMillan EA, Jackson CR and Frye JG 2020. Transferable Plasmids of *Salmonella enterica* Associated With Antibiotic Resistance Genes. Front Microbiol. 11: 562181.

Morrissey I, Oggioni MR, Knight D, Curiao T, Coque T, Kalkanci A, Martinez JL and Consortium B 2014. Evaluation of epidemiological cut-off values indicates that biocide resistant subpopulations are uncommon in natural isolates of clinically-relevant microorganisms. PLoS One, 9(1), e86669.

Mutai WC, Waiyaki PG, Kariuki S and Muigai AWT 2019. Plasmid profiling and incompatibility grouping of multidrug resistant *Salmonella enterica* serovar Typhi isolates in Nairobi, Kenya. BMC Research Notes 2019; 12(1): 422.

Nguyen L-T, Schmidt HA, von Haeseler A and Minh BQ 2015. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Molecular Biology and Evolution. 32(1): 268-274.

Nguyen NT, Nguyen HM, Nguyen CV, Nguyen TV, Nguyen MT, Thai HQ, Ho MH, Thwaites G, Ngo HT, Baker S and Carrique-Mas J 2016. Use of Colistin and Other Critical Antimicrobials on Pig and Chicken Farms in Southern Vietnam and Its Association with Resistance in Commensal *Escherichia coli* Bacteria. Appl Environ Microbiol, 82(13), 3727-3735.

Nhung NT, Cuong NV, Thwaites G and Carrique-Mas J 2016. Antimicrobial Usage and Antimicrobial Resistance in Animal Production in Southeast Asia: A Review. Antibiotics. 5(4): 37.

Nhung NT, Thuy CT, Trung NV, Campbell J, Baker S, Thwaites G, Hoa NT and Carrique-Mas J 2015. Induction of Antimicrobial Resistance in *Escherichia coli* and Non-Typhoidal *Salmonella* Strains after Adaptation to Disinfectant Commonly Used on Farms in Vietnam. Antibiotics (Basel), 4(4), 480-494.

Nicolas E, Lambin M, Dandoy D, Galloy C, Nguyen N, Oger CA and Hallet B 2014. The Tn*3*-family of Replicative Transposons. Microbiol Spectrum. 3(4): 1-32.

Normark BH and Normark S 2002. Evolution and spread of antibiotic resistance. J Intern Med. 252: 91–106.

Novick RP. 1987. Plasmid Incompatibility. Microbiological Reviews 1987; 51(4): 381-395.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Nuangmek A, Rojanasthien S, Chotinun S, Yamsakul P, Tadee P, Thamlikitkul V, Tansakul N and Patchanee P 2018. Antimicrobial Resistance in ESBL-Producing *Escherichia coli* Isolated from Layer and Pig Farms in Thailand. Acta Scientiae Veterinariae, 46, 1538.

Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, Crook D, Woodford N, Walker AS, Phan H and Sheppard AE 2017. Plasmid classification in an era of whole-genome sequencing: application in studies of antibiotic resistance epidemiology. Front Microbiol. 8: 182.

Ozawa M, Shirakawa T, Moriya K, Furuya Y, Kawanishi M, Makita K and Sekiguchi H 2023. Role of Plasmids in Co-Selection of Antimicrobial Resistances Among *Escherichia coli* Isolated from Pigs. Foodborne Pathog Dis.

Padungtod P, Kadohira M and Hill G 2008. Livestock production and foodborne diseases from food animals in Thailand. J. Vet. Med. Sci. 70(9): 873–879.

Padungtod P, Tribuddharat C and Chuanchuen R 2011. Widespread presence of *dfra12* and its association with *dfra12-aada2* cassette in *Salmonella enterica* isolates from swine. Southeast Asian J Trop Med Public Health. 42(6):1471-1476.

Pal C, Bengtsson-Palme J, Kristiansson E and Larsson DG 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics. 16: 964.

Pannek S, Higgins PG, Steinke P, Jonas D, Akova M, Bohnert JA, Seifert H and Kern WV 2006. Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-beta-naphthylamide. J Antimicrob Chemother. 57(5): 970-974.

Partridge SR and Hall RM 2004. Complex multiple antibiotic and mercury resistance region derived from the r-det of NR1 (R100). Antimicrob Agents Chemother. 48(11): 4250-4255.

Périchon B, Courvalin P and Stratton CW 2015. Antibiotic Resistance. In: Reference Module in Biomedical Sciences. ed. (ed).

Pfeiffer F, Zamora-Lagos MA, Blettinger M, Yeroslaviz A, Dahl A, Gruber S and Habermann BH 2018. The complete and fully assembled genome sequence

of Aeromonas salmonicida subsp. pectinolytica and its comparative analysis with other Aeromonas species: investigation of the mobilome in environmental and pathogenic strains. BMC Genomics. 19(1): 20.

Phongpaichit S, Liamthong S, Mathew AG and Chethanond U 2007. Prevalence of Class 1 integrons in commensal *Escherichia coli* from pigs and pig farmers in Thailand. J Food Prot. 70(2): 292–299.

Poonsuk K, Tribuddharat C and Chuanchuen R 2012. Class 1 integrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical isolates. Southeast Asian J Trop Med Public Health. 43(2):376-84.

Mazzola P, Jozala AF, Novaes LCdL, Moriel P, Christina T and Penna V (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents. Brazilian Journal of Pharmaceutical Sciences, 45(2), 241-248.

Pungpian C, Sinwat N, Angkititrakul S, Prathan R and Chuanchuen R 2020. Presence and Transfer of Antimicrobial Resistance Determinants in *Escherichia coli* in Pigs, Pork, and Humans in Thailand and Lao PDR Border Provinces. Microbial Drug Resistance.

Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB, Ge H, Sun Y, Xie XS and Bai F 2016. Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant Bacterial Cells. Mol Cell, 62(2), 284-294.

Quinn PJ, Carter ME, Markey BK and Carter GB 1994. *Enterobacteriaceae*. In: Bacteriology: Clinical Veterinary Microbiology. ed. SA Grafos (ed). Spain: Mosby-Year Book Europe. 209-236.

Randall LP, Clouting C, Horton RA, Coldham NG, Clifton-Hadley FA, Davies RH and Teale CJ 2011. Prevalence of *Escherichia coli* carrying extended-spectrum beta-lactamases (CTX-M and TEM-52) from broiler chickens and turkeys in Great Britain between 2006 and 2009. Journal of Antimicrobial Chemotherapy 2011; 66(1): 86-95.

Redondo-Salvo S, Fernandez-Lopez R, Ruiz R, Vielva L, Toro Md, Rocha EPC, Garcillan-Barcia MP and Cruz Fdl 2020. Pathways for horizontal gene transfer in bacteria revealed by a global map of their plasmids. Nature Communications 2020; 11(1): 3602.

Rangel JM, Sparling PH, Crowe C, Griffin PM and Swerdlow DL 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. Emerg Infect Dis. 11(4): 603–609.

Roberts MC and Schwarz S 2009. Tetracycline and Chloramphenicol Resistance Mechanisms. In: Antimicrobial Drug Resistance. ed. (ed). 183-193.

Rodricks JV, Swenberg JA, Borzelleca JF, Maronpot RR and Shipp AM 2010. Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. Crit Rev Toxicol, 40(5), 422-484.

Ross K, Varani AM, Snesrud E, Huang H, Alvarenga DO, Zhang J, Wu C, McGann P and Chandler M 2021. TnCentral: a Prokaryotic Transposable Element Database and Web Portal for Transposon Analysis. mBio. 12(5): e0206021.

Rotger R and Casadesús J 1999.The virulence plasmids of *Salmonella.* Internatl Microbiol. 2:177-184.

Rozwandowicz M, Brouwer MSM, Zomer AL, Bossers A, Harders F, Mevius DJ, Wagenaar JA and Hordijk 2017. Plasmids of Distinct IncK Lineages Show Compatible Phenotypes. Antimicrobial Agents and Chemotherapy 2017; 61(3).

Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, Mevius DJ and Hordijk J 2018. Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae.* J Antimicrob Chemother.73(5): 1121-1137.

Rozwandowicz M, Brouwer MSM, Mughini-Gras L, Wagenaar JA, Gonzalez-Zorn B, Mevius DJ and Hordijk J 2019. Successful Host Adaptation of IncK2 Plasmids. Frontiers in Microbiology 2019; 10: 2384.

Sadek M, et al. 2021. Genomic Features of MCR-1 and Extended-Spectrum beta-Lactamase-Producing Enterobacterales from Retail Raw Chicken in Egypt. Microorganisms 2021; 9(1).

SCCP. (2010). Opinion on Triclosan (antimicrobial resistance). In: European Commission.

Schubert S, Darlu P, Clermont O, Wieser A, Magistro G, Hoffmann C, Weinert K, Tenaillon O, Matic I and Denamur E 2009. Role of intraspecies recombination in the spread of pathogenicity islands within the *Escherichia coli* species. PLoS Pathog. 5(1): e1000257.

Schwarz S, Kehrenberg C, Doublet B and Cloeckaert A 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev. 28(5): 519-542.

Seemann T 2015. snippy: fast bacterial variant calling from NGS reads.

Sheridan À, Lenahan M, Duffy G, Fanning S and Burgess C 2012. The potential for biocide tolerance in *Escherichia coli* and its impact on the response to food processing stresses. Food Control, 26(1), 98-106.

Singer AC, Shaw H, Rhodes V and Hart A 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. Front Microbiol. 7: 1728.

Sinwat N, Angkittitrakul S and Chuanchuen R 2015. Characterization of antimicrobial resistance in *Salmonella enterica* isolated from pork, chicken meat, and humans in Northeastern Thailand. Foodborne Pathog Dis. 12(9): 759-765.

Sinwat N, Angkittitrakul S, Coulson KF, Pilapil FM, 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.

Smalla K, Jechalke S and Top EM 2015. Plasmid detection, characterization, and ecology. Microbiol Spectr. 3(1): PLAS-0038-2014.

Smith H, Bossers A, Harders F, Wu G, Woodford N, Schwarz S, Guerra B, Rodriguez I, van Essen-Zandbergen A, Brouwer M and Mevius D 2015. Characterization of epidemic IncI1-Iγ plasmids harboring ambler class A and C genes in *Escherichia coli and Salmonella enterica* from animals and humans. Antimicrobial Agents and Chemotherapy 2015; 59(9): 5357-5365.

Soumet C, Fourreau E, Legrandois P and Maris P 2012. Resistance to phenicol compounds following adaptation to quaternary ammonium compounds in *Escherichia coli*. Vet Microbiol, 158(1-2), 147-152.

Srisanga S, Angkititrakul S, Sringam P, Le Ho PT, AT TV and Chuanchuen R 2017. Phenotypic and genotypic antimicrobial resistance and virulence genes of *Salmonella enterica* isolated from pet dogs and cats. J Vet Sci. 18(3): 273-281.

Sullivan MJ, Petty NK and Beatson SA 2011. Easyfig: a genome comparison visualizer. Bioinformatics. 27(7): 1009-1010.

Suwannarak W 2003. Food safety controls from farm to table with emphasis on aquaculture shrimp in Thailand. Fifth World Fish Inspection and Quality Control Congress, The Hague, Netherlands.

Tacnet F, Lauthier F and Ripoche P 1993. Mechanisms of zinc transport into pig small intestine brush-border membrane vesicles. Journal of physiology, 465, 57-72.

Tadesse DA, Zhao S, Tong E, Ayers S, Singh A, Bartholomew MJ and McDermott PF 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. Emerg Infect Dis. 18(5): 741-749.

Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M and Ostell J 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 44(14): 6614-6624.

Tenaillon O, Skurnik D, Picard B and Denamur E 2010. The population genetics of commensal *Escherichia coli*. Nat Rev Microbiol. 8(3): 207-217.

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

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: 23–33.

Varani A, He S, Siguier P, Ross K and Chandler M 2021. The IS6 family, a clinically important group of insertion sequences including IS26. Mob DNA. 12(1): 11.

Vetting MW, LP SdC, Yu M, Hegde SS, Magnet S, Roderick SL and Blanchard JS 2005. Structure and functions of the GNAT superfamily of acetyltransferases. Arch Biochem Biophys. 433(1): 212-226.

Villa L, Garcia-Fernandez A, Fortini D and Carattoli A 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. Journal of Antimicrobial Chemotherapy 2010; 65(12): 2518-2529.

von Wintersdorff CJ, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM and Wolffs PFG 2016. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Frontiers in Microbiology 2016; 7: 173.

Wales AD and Davies RH 2015. Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens. Antibiotics. 4: 567-604.

Wannaprasat W and Chuanchuen R 2013. Mutations in topoisomerase genes and expression of *acrAB* multidrug efflux system in fluoroquinolone-resistant *Salmonella enterica* from pork and patients. The Thai Journal of Veterinary Medicine. 43(2): 243-249.

Wannaprasat W, Padungtod P and Chuanchuen R 2011. Class 1 integrons and virulence genes in *Salmonella enterica* isolates from pork and humans. Int J Antimicrob Agents. 37(5): 457-461.

Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ and Piddock LJ 2015. Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. J Antimicrob Chemother, 70(8), 2241-2248.

WHO 2014. "Antimicrobial resistance global report on surveillance" [Online] Available: http://apps.who.int/iris/bitstream/handle/10665/112642/978924154748_eng.pdf?sequence=1 Accessed April 2, 2018.

Wick RR, Judd LM, Gorrie CL and Holt KE 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 13(6): e1005595.

Wood DE, Lu J and Langmead B 2019. Improved metagenomic analysis with Kraken 2. Genome Biol. 20(1): 257.

Yang QE, Sun J, Li L, Deng H, Liu BT, Fang LX, Liao XP and Liu YH 2015. IncF plasmid diversity in multi-drug resistant *Escherichia coli* strains from animals in China. Frontiers in Microbiology 2015; 6: 964.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Yazdankhah S, Rudi K, and Bernhoft A 2014. Zinc and copper in animal feed - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. Microb Ecol Health Dis, 25.

Zakaria AS, Edward EA and Mohamed NM 2021. Genomic Insights into a Colistin-Resistant Uropathogenic *Escherichia coli* Strain of O23:H4-ST641 Lineage Harboring mcr-1.1 on a Conjugative IncHI2 Plasmid from Egypt. Microorganisms 2021; 9(4).

Zhang A, He X, Meng Y, Guo L, Long M, Yu H, Li B, Fan L, Liu S, Wang H and Zou L 2016. Antibiotic and Disinfectant Resistance of *Escherichia coli* Isolated from Retail Meats in Sichuan, China. Microb Drug Resist, 22(1), 80-87.

Zhang C, Feng Y, Liu F, Jiang H, Qu Z, Lei M, Wang J, Zhang B, Hu Y, Ding J and Zhu B 2017. A Phage-Like IncY Plasmid Carrying the mcr-1 Gene in *Escherichia coli* from a Pig Farm in China. Antimicrobial Agents and Chemotherapy 2017; 61(3): e02035-02016.

Zhang S, Yin Y, Jones MB, Zhang Z, Deatherage Kaiser BL, Dinsmore BA, Fitzgerald C, Fields PI and Deng X 2015. *Salmonella* serotype determination utilizing high-throughput genome sequencing data. J Clin Microbiol. 53(5): 1685-1692.

Zhang, A., He, X., Meng, Y., Guo, L., Long, M., Yu, H., Li, B., Fan, L., Liu, S., Wang, H., & Zou, L. (2016). Antibiotic and Disinfectant Resistance of *Escherichia coli* Isolated from Retail Meats in Sichuan, China. Microb Drug Resist, 22(1), 80-87.

CHULALONGKORN UNIVERSITY

VITA

NAME	Jiratchaya Puangseree
DATE OF BIRTH	17 December 1990
PLACE OF BIRTH	Nakhon Pathom
INSTITUTIONS ATTENDED	Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2015
HOME ADDRESS	74 Charoennakorn 13, Charoennakorn Road, Klongtonsai, Klongsan, Bangkok, 10600
PUBLICATION	1. Trongjit, S., Angkititrakul, S., Tuttle, R., Puangseree, J., Padungtod, P. and Chuanchuen, R. (2017). Prevalence and antimicrobial resistance in Salmonella enterica isolated from broiler chickens, pigs and meat products in the Thailand-Cambodia border provinces: AMR in Salmonella enterica. Microbiology and Immunology. 61. 10.1111/1348-0421.12462. 2. Sinwat, N., Puangseree, J., Angkittitrakul, S. and Chuanchuen, R. (2018). Mutations in QRDRs of DNA gyrase and topoisomerase IV genes in nalidixic acid and ciprofloxacin-resistant Salmonella enterica isolated from chicken meat, pork and humans. Thai Journal of Veterinary Medicine. 48. 79-84. 3. Puangseree, J., Jeamsripong, S., Prathan, R., Pungpian, C. and Chuanchuen, R. (2021). Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in Escherichia coli isolated from pigs, pork and pig carcass. Food Control. 124. 107892. 4. Puangseree, J., Prathan, R., Srisanga, S., Angkittitrakul, S., and Chuanchuen, R. (2022). Plasmid profile analysis of Escherichia coli and Salmonella enterica isolated from pigs, pork and humans. Epidemiology and Infection. 150. E110. 1-14.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University