MONITORING AND CHARACTERIZATION OF COLISTIN RESISTANT *ESCHERICHIA COLI* IN PIG FARM AND ENVIRONMENT FOLLOWING THE CESSATION IN USE OF COLISTIN

Miss Nwai Oo Khine

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Veterinary Science and technology
Common Course
FACULTY OF VETERINARY SCIENCE
Chulalongkorn University
Academic Year 2021
Copyright of Chulalongkorn University

การติดตาม และ คุณลักษณะของเชื้อ*Escherichia coli*
ที่ดื้อต่อยาโคลิสตินในฟาร์มสุกรและสิ่งแวดล้อมหลังการหยุดใช้ยาโคลิสติน

น.ส.ไว โอ คืน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี ไม่สังกัดภาควิชา/เทียบเท่า
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

Thesis Title	MONITORING AND CHARACTERIZATION OF COLISTIN RESISTANT *ESCHERICHIA COLI* IN PIG FARM AND ENVIRONMENT FOLLOWING THE CESSATION IN USE OF COLISTIN
By	Miss Nwai Oo Khine
Field of Study	Veterinary Science and technology
Thesis Advisor	Associate Professor Dr. NUVEE PRAPASARAKUL
Thesis Co Advisor	Professor Dr. PADET TUMMARUK Assistant Professor Dr. Prapat Suriyaphol

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.)

DISSERTATION COMMITTEE

--- Chairman

(Associate Professor Dr. Chanwit Tribuddharat)

--- Thesis Advisor

(Associate Professor Dr. NUVEE PRAPASARAKUL)

--- Thesis Co-Advisor

(Professor Dr. PADET TUMMARUK)

--- Thesis Co-Advisor

(Assistant Professor Dr. Prapat Suriyaphol)

--- Examiner

(Associate Professor Dr. CHANNARONG RODKHUM)

--- Examiner

(Assistant Professor Dr. TARADON LUANGTONGKUM)

--- Examiner

(Associate Professor Dr. Gunnaporn Suriyaphol)

--- External Examiner

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ไว โอ คีน : การติดตาม และ คุณลักษณะของเชื้อ*Escherichia coli* ที่ดื้อต่อยาโคลิสตินในฟาร์มสุกรและสิ่งแวดล้อมหลังการหยุดใช้ยาโคลิสติน. (MONITORING AND CHARACTERIZATION OF COLISTIN RESISTANT *ESCHERICHIA COLI* IN PIG FARM AND ENVIRONMENT FOLLOWING THE CESSATION IN USE OF COLISTIN) อ.ที่ปรึกษาหลัก : ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาร่วม : เผด็จ ธรรมรักษ์,ประพัฒน์ สุริยผล

การเพิ่มขึ้นของการดื้อยาโคลิสตินในเชื้อกลุ่ม *Enterobacteriaceae* จากการส่งผ่านพลาสมิดที่มี *mcr* genes ระหว่างกันถือเป็นวิกฤติในวงการสาธารณสุข การศึกษาในครั้งนี้จึงได้ทำการสำรวจความชุกของเชื้อ *mcr*-positive *Escherichia coli* (MCRPE) ในสุกรสุขภาพดีทั่วประเทศไทย ผลการศึกษาจาก 696 ตัวอย่างที่เก็บจาก 49 จังหวัดในประเทศไทย พบว่ามีอัตราการพบเชื้อดื้อยา MCRPE ที่มียีน *mcr*-1 หรือมียีน *mcr*-1คู่กับ *mcr*-3 ในสุกรในระดับต่ำอยู่ที่ 4.45% และ 0.43% ตามลำดับ โดยพบว่าเชื้อส่วนใหญ่มักดื้อต่อยาปฏิชีวนะหลายชนิด(multidrug-resistant (MDR)) ตั้งแต่ 3-14 ชนิดขึ้นไปและมียีน enterotoxin genes นอกจากนี้ผู้วิจัยยังได้ทำการศึกษาระยะยาว(longitudinal study)เกี่ยวกับความชุกและลักษณะทางพันธุกรรมของเชื้อดื้อยา MCRPEภายในฟาร์มที่มีการหยุดใช้ยาโคลิสตินไปแล้วอย่างต่อเนื่อง ซึ่งผลการศึกษาจาก 170 ตัวอย่างที่เก็บจากแม่สุกรในเล้าคลอด,ลูกสุกรดูดนม,น้ำเสียและคนงานในฟาร์มระหว่างปี 2017-2020 พบว่ายังคงมีเชื้อดื้อยา MCRPE ที่มียีน *mcr*-1 อยู่ในสุกรหลังจากมีการหยุดใช้ยาโคลิสตินนาน 3.5 ปีแต่ในความชุกที่ลดลง ผลจากการทำ DNAfingerprintวิเคราะห์ลายนิ้วมือดีเอนเอของเชื้อเพื่อหาความสัมพันธ์ของเชื้อดื้อยาในแต่ละแหล่งตัวอย่างพบว่ามีเชื้ออีโคไลหลากหลายโคลน (clone) แพร่กระจายอยู่ในฟาร์มและยังดื้อต่อยาปฏิชีวนะหลายชนิด ผลจากการหาลักษณะทางพันธุกรรมของเชื้อด้วยวิธี whole genome sequencing ของเชื้อ MCRPE ที่ถูกเลือกมา 6 สเตน พบว่ายีน *mcr*-1.1 มีตำแหน่งอยู่ที่ IncI2 และ IncX4 plasmid ขณะที่ยีน*mcr*-3(*mcr*-3.2 and *mcr*-3.5) มักพบอยู่ที่ IncFII และ IncHI2 plasmids ซึ่งพลาสมิดเหล่านี้ยังบรรจุยีนดื้อยาหลายชนิดและยีนที่เกี่ยวข้องกับ bacteriocin หรือ efflux pump อีกด้วย โดยพลาสมิดเหล่านี้ถูกค้นพบทั้งในตัวอย่างสุกรและสิ่งแวดล้อมที่ถูกเก็บในปีที่แตกต่างกัน นอกจากยังพบว่าเชื้อ MCRPE ดังกล่าวยังมียีนที่ดื้อต่อกลุ่มยาฆ่าเชื้อและ biocides อีกด้วย จากการศึกษาในครั้งนี้จึงสรุปได้ว่าแม้จะมีการหยุดการใช้ยาโคลิสตินแล้วแต่การพบเชื้ออีโคไลที่มียีน *mcr* ก็ยังคงอยู่ซึ่งอาจเกิดขึ้นจากการส่งผ่านพลาสมิดที่มียีนดื้อยาของเชื้อหรือเกิดจากการใช้ปฏิชีวนะอื่นๆภายในฟาร์มแล้วทำให้เกิดการดื้อยาร่วมกัน (co-selection) เช่น ยากลุ่มอะมิโนไกลโคไซต์และยากลุ่มเซฟาโลสปอริน

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต ....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

6175603631 : MAJOR VETERINARY SCIENCE AND TECHNOLOGY
KEYWORD: Colistin, Antimicrobial resistance, Pigs, Plasmid

Nwai Oo Khine : MONITORING AND CHARACTERIZATION OF COLISTIN RESISTANT *ESCHERICHIA COLI* IN PIG FARM AND ENVIRONMENT FOLLOWING THE CESSATION IN USE OF COLISTIN. Advisor: Assoc. Prof. Dr. NUVEE PRAPASARAKUL Co-advisor: Prof. Dr. PADET TUMMARUK,Asst. Prof. Dr. Prapat Suriyaphol

The growing cases of colistin resistance due to plasmid mediated *mcr* genes family in *Enterobacteriaceae* is catastrophic to public health. In this study, our team performed the nation-wide surveillance of *mcr*-positive *Escherichia coli* (MCRPE) in healthy pigs across Thailand. Then we monitored longitudinally over the representative pig farm after colistin was withdrawn and genomic characterization was carried out. Among the 696 samples collected from 49 provinces of Thailand, the low carriage rate of *mcr*-1 or combination of *mcr*-1 and *mcr*-3 (4.45% and 0.43%) were detected. MCRPE isolates were multidrug-resistant (MDR) against 3–14 types of antimicrobial and enterotoxin genes were largely found. For the longitudinal monitoring, 170 samples were collected from farrowing sows and suckling piglets, wastewater, and farm workers from 2017-2020. The results showed that *mcr*-1 were recovered from pig carriages for 3.5 years after withdrawal, but in a declining trend. From DNA fingerprinting methods, diverse *E. coli* clones were distributed on the farm and showed MDR traits. From whole genome sequencing data of 6 selected MCRPE, *mcr*-1.1 was located on the high stability IncI2 and IncX4 plasmid. Whereas, *mcr*-3 variants (*mcr*-3.2 and *mcr*-3.5) were found on IncFII and IncHI2 plasmids which either contained MDR region, bacteriocin or efflux pump. Identical plasmids were discovered between pigs and environment from different investigation years. MCRPE isolates showed both phenotypic and genotypic MDR characteristics as well as antiseptic and biocides resistant genes. Our study concluded that in the absence of colistin selective pressure, the persistence or elimination of the *mcr*-bearing *E. coli* varies depending on the plasmid background and co-selection by other antibiotics usage such as aminoglycosides and cephalosporins as well as farm management.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Field of Study:	Veterinary Science and technology	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....
		Co-advisor's Signature ....
		Co-advisor's Signature ....

ACKNOWLEDGEMENTS

This research was carried out at the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, and was financially supported by the Royal Golden Jubilee Ph.D. (RGJPHD) program, Agricultural Research Development Agency (ARDA) [CRP6205031110], the CHE-TRF Senior Research Fund (RTA6280013), Thailand Science Research and Innovation, and Pathogen Bank, Faculty of Veterinary Science, Chulalongkorn University.

I wish to express my sincere gratitude to the people who have supported and helped me in the completion of the dissertation, especially to:

Associate Professor Dr. Nuvee Prapasarakul, my principal advisor for the opportunity and support throughout the course of this dissertation, for believing in me and your involvement, willingness to advise and review the manuscripts.

Associate Professor Dr. Channarong Rodkhum, the Director of The International Graduate Program of Veterinary Science and Technology (VST), for letting me study a doctoral degree in the department of Veterinary Microbiology, Chulalongkorn University.

Professor. Dr. Padet Tummaruk, my co-advisor for guiding me the knowledge upon pig production system.

Assistant Professor Dr. Prapat Suriyaphol, my co-advisor for guiding and giving me suggestions on the bioinformatics work.

Dr. Thidathip Wongsurawat and Dr. Piroon Jenjaroenpun for teaching, guiding, and helping me in whole genome sequencing and bioinformatic analysis.

All my co-authors and collaborators who helped me in throughout the course of this research, especially Mr. Kittitat Lugsomya, Miss Imporn Saenkankam, Mr. Prasert Apiwatsiri, and all my lab members. Without you, life during the period of my study would have been difficult and unpleasant. Thank you very much for creating the good atmosphere and the great friendship. I really appreciate our time spent together.

My parents, my grandma and my brother for their continuous support, love and encouragement throughout this study and survived Covid-19. My dear sister for always being there for me and take care of me all my life.

Nwai Oo Khine

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

TABLE OF CONTENTS

Page

.... iii

ABSTRACT (THAI) iii

.... iv

ABSTRACT (ENGLISH) iv

ACKNOWLEDGEMENTS v

TABLE OF CONTENTS vi

CHAPTER I 14

Importance and rationale 14

Objectives of the study 17

Hypotheses 18

Advantages of study 18

Literature review 19

1. Antimicrobial resistance as a global concern 19

2. *Escherichia coli* 21

3. Colistin resistance 21

4. Maintenance of plasmid encoding resistance genes 24

Conceptual Frame Work 26

CHAPTER II 27

Abstract 28

Introduction 29

Materials and methods 31

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Study area and animal selection 31

Sample collection and bacterial identification....31

Antimicrobial susceptibility determination and *mcr* gene detection....32

Phylogenetic grouping....34

Plasmid replicon typing 34

Detection of virulence genes....35

Data analysis....35

Results....36

Distribution of colistin-resistant *E. coli* containing *mcr* genes 36

Antimicrobial susceptibility testing 38

Phylogenetic grouping....41

Plasmid replicon typing 41

Characterization of the virulent factors....42

Relation analysis between antimicrobials susceptibility and pathogenicity 42

Discussion....45

Conclusion 49

Chapter III....50

Abstract....51

Introduction 52

Materials and methods 53

Study area and farm selection....53

Sample collection and processing 54

Bacterial isolation and identification....55

Antimicrobial susceptibility testing 56

Detection of plasmid-mediated colistin resistance genes 57

Plasmid replicon typing 57

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Detection of virulence genes 58

Conjugation assay 58

Pulsed-field gel electrophoresis (PFGE) 59

Multi-locus sequence typing (MLST) 59

Data analysis....60

Results....60

Detection of colistin resistant *E. coli*....60

Identification of plasmid-mediated colistin resistance genes....62

Antimicrobial susceptibility determination....64

Virulence gene detection....66

Molecular genotypic characterizations 66

Discussion....69

Conclusion 74

Chapter IV....76

Abstract....77

Introduction 79

Materials and methods 81

Sampling and Identification 81

Strain selection and antimicrobial susceptibility testing 81

Whole Genome Sequencing....82

Sequence analysis....82

Nucleotide Sequence Accession Numbers 83

Results....84

Genomic Characterization of *mcr* positive *E. coli* isolates....84

Antibiotic Resistance genes and Virulent genes....86

Genomic insights into the *mcr*-1 *and mcr*-3 positive *E. coli* strains 92

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Discussions 100

Conclusion 107

Chapter V...108

General discussion, conclusion and further recommendations................................108

Appendix 115

Appendix 1 115

Appendix 2 117

Appendix 3 120

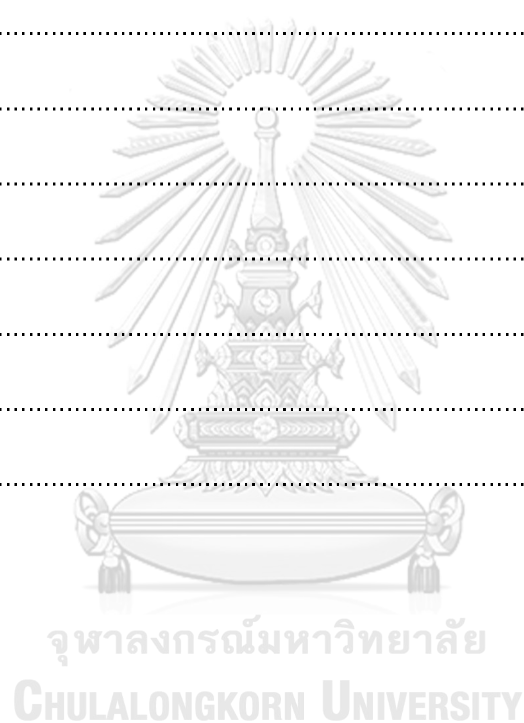
Appendix 4 121

Appendix 5 122

Appendix 6 123

REFERENCES..124

VITA 142



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 1. Geographical distribution of either colistin resistant or susceptible *E. coli* from the surveyed contracted pig farms in Thailand 37

Figure 2. Distribution of resistant rates against 18 antimicrobials and presence of extended- spectrum beta-lactamase (ESBL) characteristic among 105 colistin-resistant *E. coli* isolated from contracted pig farms in Thailand 38

Figure 3. The phylogroups detected among 31 MCRPE isolates in contracted pig farms in Thailand. 40

Figure 4. Plasmid replicon types detected among 31 MCRPE isolates in contracted pig farms in Thailand. 41

Figure 5. Comparison of the rate of *mcr* positive *E. coli* isolated from pigs, workers and the environment in four sample collection years (*significant difference; $p \leq 0.05$) 61

Figure 6. Comparison between resistance rates against 18 antimicrobials and extended-spectrum beta-lactamase (ESBL) in MCRPE isolates at four different sample collection times 62

Figure 7. Dendrogram generated from pulsed field gel electrophoresis analysis demonstrating the genetic relatedness among 65 *mcr* positive *Escherichia coli* strains (one to three isolates per positive sample) that were obtained from different sources at each sampling time. 68

Figure 8. The Venn diagram of the various plasmid replicon types detected from 6 MCRPE isolates 85

Figure 9. Heatmap showing the presence or absence of antimicrobial resistance genes of different antibiotic classes in 6 MCRPE from pig, human and environment 91

Figure 10. Sequence alignment of pCP52E-IncX4 *mcr*-1 plasmid with pCSZ4 (GenBank no KX711706) and pHNSHP45 (GenBank no KP347127). The outer circle with red arrows denotes annotation of the plasmid pCP52E-IncX4. 93

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 11. Sequence alignment of pCPA1200-IncI2 *mcr*-1 plasmid with pCPWW7-IncI2 (This study) and pHNSHP45 (GenBank no. KP347127) and p*mcr*1_IncI2 (GenBank no. KU761326). The outer circle with red arrows denotes annotation of the plasmid pCPA1200-IncI2. 94

Figure 12. Comparison of the genetic environment of *mcr*-1.1 gene from MCRPE of this study with references plasmids. The gray area indicates the blast identities, the percentage of identity is indicated in the legend. Open arrows represent coding sequences (green for *mcr*-1.1, blue for PAP2, purple for ISAPl1 and yellow for other genes. The arrow size is proportional to the gene length. The image was generated using EasyFig with default parameters. 95

Figure 13. Sequence alignment of pCPE35-IncFII *mcr*-3.2 plasmid with pCPF6-IncFII *mcr*-3.5 plasmid (This study) and the reference plasmids pECQ4552 (GenBank no. CP077064.1), pBJ114-141 (GenBank no. MF679146). The outer circle with red arrows denotes annotation of the plasmid pCPE35-IncFII. The black bar represents multidrug efflux pump (Tap) from pCPF6. 97

Figure 14. Sequence alignment of pCPF6-IncHI2 mcr-3.2 plasmid with pWJ1 (GenBank no KY924928) . The outer circle with red arrows denotes annotation of the plasmid pCPF6-IncHI2. 98

Figure 15. Comparison of the genetic environment of *mcr*-3 genes from MCRPE isolates of this study with references plasmids. The gray area indicates the blast identities, the percentage of identity is indicated in the legend. Open arrows represent coding sequences (green for antimicrobial resistance genes, blue for dgkA (orf), purple for mobile genetic elements and yellow for other genes. The arrow size is proportional to the gene length. The image was generated using EasyFig with default parameters. 99

Figure 16. Heatmap showing the presence or absence of various virulence factor genes in 6 MCRPE from pig, human and environment 121

Figure 17. Sequence alignment of pCPWWCT-IncX1 plasmid with pCP52E (This study) and the reference plasmid pCSZ4 (GenBank no. KX711706). The outer circle with red arrows denotes annotation of the plasmid pCPWWCT-IncX1 122

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 1. Antibiograms of the 31 MCRPE isolates distributing in 26 pattern types........... 39

Table 2. Presence of virulent profiles including toxin and antigenicity of the 31 MCRPEs 43

Table 3. Relation analysis between MCRPE resistance to the other six antimicrobial groups and their pathogenicity 44

Table 4. Characterization of 33 colistin-resistant *mcr* positive *E. coli* isolates from different years and sources... 63

Table 5. Plasmid replicon types detected in 33 colistin-resistant *E. coli* among the three categories of samples in each sample collection times 65

Table 6. Genomic features including MLST, serotypes, resistance, virulence profiles of the six *E. coli* strains tested by whole genome sequencing 87

Table 7. Details of the sample types and numbers collected at five different sampling times between 2017 and 2020, and numbers of samples found positive for MCRPE ... 115

Table 8. Antibiogram patterns detected from the 33 MCRPE isolates from samples collected in four different years... 117

Table 9. MLST sequence types and source and number of the MCRPE isolates that were representatives of the 34 PFGE patterns 120

Table 10. Heavy metal resistance genes detected among 6 MCRPE isolates 123

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

CHAPTER I

Importance and rationale

Antimicrobial resistance (AMR) is one of the most global public health problems since there have been reports of new antibiotic resistance mechanisms or resistance genes against the last resort drugs. Certain antimicrobial classes for human medicine are commonly used in livestock productions as growth promoter for prophylaxis indication, which can accelerate the development of resistant bacteria (Nhung T Nguyen et al., 2016). The decreasing efficacy of those antibiotics can affect not only to clinical aspects but also impact to raise of budget in food production. Moreover, the resistant bacteria in farms can potentially spread to farmers or environment resulting the existence of antibiotic resistance genes (ARG) (Xia et al., 2019). *Escherichia coli* are one of sentinel bacteria act as pathogen and commensal in animal hosts and widely contaminate into environment especially sewage in livestock farm. Additionally, these bacteria have the high capability to accumulate resistant genes which are used as the representative for monitoring of AMR and ARG (Kaspar, 2006).

Colistin (polymyxin E antibiotic) has been regarded as the critically important antibiotic (WHO 2019) for treating multidrug-resistant bacterial infections (Biswas, Brunel, Dubus, Reynaud-Gaubert, & Rolain, 2012). However, the first report of plasmid mediated colistin resistance gene (*mcr*-1) *E. coli* (MCRPE) from China raised a massive awareness with colistin usage on pig farms. Moreover, there was the subsequent

discovery of other *mcr* variants including *mcr*-2 to *mcr*-9 mainly from *Enterobacteriaceae* family (AbuOun et al., 2018; L. M. Carroll et al., 2019; X. Wang et al., 2018; Y. Q. Yang, Li, Lei, Zhang, & Wang, 2018) from different geographical areas (Duggett et al., 2018). More recently, the novel *mcr*-10 had also been identified in colistin susceptible *Enterobacter* clinical strain (C. Wang et al., 2020). Among them, the *mcr*-1, being the most prevalent, have been reported worldwide not only from livestock animals but also in multiple cases of bacterial infections in human (Skov & Monnet, 2016).

In Thailand, majority of pig farming systems are contract farming between the primary producers and the agribusiness companies. The routine use of antibiotics in commercial pig farms could increase the resistance of critically important antibiotics higher than therapeutic usage (K. Lugsomya, T. Chatsuwan, et al., 2018). Although there were reports regarding a high prevalence of multidrug resistant (MDR) *E. coli* in pigs in Thailand, the *mcr* genes situation in large scale study for pigs is still limited. However, *mcr*-1 was found in several cases of MDR human patients and more commonly found in *E. coli* than in *K. pneumoniae* isolates from human cases of Thailand (Eiamphungporn et al., 2018). Besides, co-selection of multiple antimicrobial resistance genes and enterotoxin production had been found among several clinical isolates (Eiamphungporn et al., 2018; Garcia-Menino et al., 2018). Plasmids carrying resistance genes which also encode virulence genes is worrisome problem.

CHULALONGKORN UNIVERSITY

Because of the importance of colistin usage in clinical infections, many countries had begun to ban colistin as a prophylaxis usage (Walsh & Wu, 2016). Since 2017, colistin has been prohibited for using as prophylaxis in farms by the Department of Livestock Development, Ministry of Agriculture and Cooperatives of Thailand. However, the consequence after withdrawal on the control of the emergence and spread of *mcr*-1 remain unclear (Xia et al., 2019). Moreover, it is practically not feasible for plasmids be eliminated completely from bacterial populations (de Toro, Garcillaon-Barcia, & De La Cruz, 2014). Many studies also stated that even drastic reduction of antibiotic use could be an ineffective strategy for removing antibiotic resistance plasmids (Brolund, Sundqvist, Kahlmeter, & Grape, 2010; Sundqvist et al., 2010; Yates, Shaw, Roe, Woolhouse, & Amyes, 2006). Therefore, the understanding of plasmid adaptation and co-evolution between plasmid and host could be a new insight in tackling antibiotic resistance and for strategic control implement.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

This study will be investigated the plasmid mediated colistin resistance genes and their virulent potential in large scale pig farms across Thailand. Then, it will be followed by the longitudinal monitoring of the representative pig farm where was reported as *mcr*-1 gene positive in workers, pig carriages and environment. According to the farm history, the farm had voluntarily banned colistin sulfate as a feed additive since the beginning of 2017. However, the cessation in use of colistin in the farm could not assure that the pig carriages would lack the resistant genes. Thus, to follow up *mcr*

genes detection in pigs, workers and environment in that farm could be helpful in term of policy and management beneath one health concept. The current study is to survey the incidence of *mcr* positive *E. coli* in contract farms and follow up the occurrence of mcr genes in pigs, workers and environment in a high prevalent farm after colistin withdrawal policy. The representative *E. coli* from different hosts and years of isolation will be genetically characterized and compared for their possible adaptation analysis.

Objectives of the study

1. To surveillance and characterize the antibiogram, virulent traits of *mcr* genes positive *E. coli* (MCRPE) from the fecal samples of healthy pigs derived from the contract farming system across Thailand.
2. To monitor and clonal characterization of MCRPE in pigs, workers and environment in a selected pig farm positive with *mcr* from 2017 to 2020.
3. To analyze in genomic comparison and characterization of mobile genetic elements related with *mcr* genes in different hosts along investigation year.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Hypotheses

1. There will be a specific distribution of MCRPE in particular areas of Thailand related with intensity of the pig production.
2. The reduction of MCRPE in pig carriages can be detected after colistin withdraw and there is diverse distribution of *E. coli* clone types.
3. The persistence of *mcr* genes is favored by co-selection with other antibiotic resistance, heavy metals or biocides genes allocation.

Advantages of study

1. This study can provide the awareness of *mcr* gene mediated colistin resistant *E. coli* distributing in high intensity pig farms in Thailand.
2. The supporting information after colistin withdraw policy use that provide a critical point and risk of *mcr* genes persistence in the pigs and farm environment.
3. The gene analysis of MCRPE isolates can be able to provide window of opportunity to combat antibiotic resistance and being applicable in the field.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Keywords (in Thai): การดื้อยาโคลิสติน *เอสเชอริเชีย โคไล* การปรับตัวของยีน สุกร

Keywords (in English): colistin resistance, *Escherichia coli*, gene adaptation, pigs

Literature review

1. Antimicrobial resistance as a global concern

The problems regarding antimicrobial resistance are global issues since resistance bacteria can arise from any sectors and able to spread between intra-species, inter-species and across borders (Kemp, 2019). Increasing demands of antimicrobial usage in human and animal sectors led to the rapid development of new resistance mechanisms and AMR genes. Spreading of these genes from one place to another through movements of animals and/or people, also farm environment plays an important aspect. Therefore, tackling the AMR problems require a multisectoral or One Health approach.

Generating and transmission of AMR genes can be occurred in different ways, in which horizontal gene transfer being the most critical concern (Jindal, Pandya, & Khan, 2015). In the presence of stress environment like antibiotic selection pressure, bacteria evolve to enhance their fitness by acquiring and expressing resistance genes (Holmes et al., 2016). Moreover, AMR genes could be transferred through conjugation, transformation, or transduction, via mobile genetic elements (MGE) which helps in incorporation into another bacteria genome or plasmids (Tenover, 2006). Moreover, if MGEs contain several antibiotic resistance cassettes which lead to the recipient bacteria to resist numerous other antibiotic genes and the potential for multidrug resistance bacteria (Kemp, 2019).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The certain antimicrobial classes for human medicine are also used in livestock productions. With the increasing demand for intensive animal production, antimicrobials are massively applied in the livestock industry as treatment or prophylaxis for bacterial diseases (Landers, Cohen, Wittum, & Larson, 2012). Despite the use of antibiotics is important to treat animal diseases, inappropriate usages can lead into arising of resistance problems (Tangcharoensathien, Chanvatik, & Sommanustweechai, 2018). The most controversial antibiotic usage is administering antimicrobials as growth promoters in food animals. Since antimicrobials are applied at subtherapeutic doses for prolonged periods, this usage acts as the selective pressure for both commensal and pathogenic bacteria (FAO, 2016). Therefore, livestock farms became the hotspot for resistant bacteria and/or genes to accumulate and spread from farms to the environment and possibly to human (Van den Meersche et al., 2019). AMR happening on a variety of infectious agents represents public health awareness since new resistance genes are emerging and spreading globally. The consequences of AMR comprise lack of successful treatment which in turn lead to increase mortality or prolonged illness in clinical aspects. Moreover, the losses in animal production and if resistant bacteria contaminate in meat or food products that can affect food safety (FAO, 2016).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

2. *Escherichia coli*

E. coli is a member of the *Enterobacteriaceae* family which are commensal organisms of humans and animals. Since they are the integral component of the gastrointestinal (GI) tract, frequent exposure to antibiotics taken by the host might lead a pool of resistant commensal bacteria (Aarestrup, 2015). Moreover, some strains of *E. coli* are pathogenic and associated with intestinal or extraintestinal diseases (Bok et al., 2018). Especially in neonatal and weaned piglets, diarrhea due to *E. coli* is an economically important disease. Several authors have reported that commensal *E. coli* with pathogenic potential carrying virulence genes and able to exchange resistance genes both in human and animal cases (Bok et al., 2018; Madoshi et al., 2016). *E. coli* strains with their relatively easy to isolate, investigate and genome plasticity nature, are being recognized as model organisms for monitoring resistance genes, especially for horizontal gene transfer (Kaspar, 2006).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3. Colistin resistance

Colistin (polymyxin E) is recognized as the last-resort drug to combat multidrug resistant bacteria. In pig productions, colistin was used for different purposes; therapeutically, prophylactically, and even for growth promotion (Katsunuma et al., 2007). Some Gram-negative bacteria such as *Serratia* spp., *Proteus* spp. and *Burkholderia* spp. are naturally resistant to polymyxin. However, the acquired

resistance occurring in *Enterobacteriaceae*, especially *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp. became drastic problems.

Colistin's mechanism of action is mainly from the electrostatic binding to the bacterial outer membrane, the phosphate groups of the lipid A region of lipopolysaccharide (LPS) (Sun, Zhang, Liu, & Feng, 2018). Thereby interrupt the LPS structure and increase the permeability of the bacterial membrane leading cell death (Poirel, Jayol, & Nordmann, 2017). For several years, resistance to colistin occurred due to the mutations in chromosomes; PmrAB and PhoPQ two component systems which result in modifications of the bacterial outer membrane (Olaitan, Morand, & Rolain, 2014). However, the first discovery of plasmid mediated colistin resistant gene called *mcr*-1 in China, (Liu et al., 2016) became a significant concern for public health. The *mcr*-1 protein confers colistin resistance by addition of phosphoethanolamine (PEtN) to lipid A, similarly to the chromosomal mutations. In a short time, *mcr*-1 and several other homologs (*mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5, *mcr*-6, *mcr*-7 and *mcr*-8) were subsequently identified in various *Enterobacteriaceae* of different origins around the world (X. Wang et al., 2018). More recently, the two novel *mcr*-9 and *mcr*-10 genes had been identified and, of note, these genes are phenotypically susceptible to colistin (L. M. Carroll et al., 2019; C. Wang et al., 2020). The discovery of two novel *mcr* genes in colistin-susceptible strains might be due to low-level gene expression and may act as a silent spreading of the genes (Lei, Zhang, Wang, & Wang, 2020). Among 10 *mcr* variants, the

CHULALONGKORN UNIVERSITY

mcr-1 being the most predominantly found from various origins and mainly identified in *Escherichia coli* species. Moreover, many of these isolates were detected from livestock animals, primarily pigs. Similarly, *mcr*-3 has been widely identified in *Enterobacteriaceae* (mainly *E. coli*) and *Aeromonas* spp. from Asia, Europe, and North America (Y. Xu et al., 2018).

Historically, colistin was a legal to use antibiotic for pig production, mainly for the treatment and prevention of post-weaning diarrhea (PWD) in piglets (Kempf et al., 2013). However, usage of colistin in pigs feed for prophylactic purposes serve as the selective pressure for the development of resistant bacteria. This usage did not come under particular concern in the several years ago since resistance to colistin was mainly due to chromosomal mutation and not likely to disseminate rapidly (M. Rhouma et al., 2019). After the first discovery of *mcr*-1 in 2016, and its rapid evolution of variants and worldwide spread, the global actions are needed for colistin usage in animals feed (Y. Wang et al., 2020). Many countries have been practiced the withdrawal plan regarding the colistin in feed additives, including Brazil (2016), Thailand (2017), China (2017), Japan (2018), Malaysia (2019), Argentina (February, 2019), and India (July, 2019) (Y. Wang et al., 2020). In Thailand, limitation of antibiotics as a growth promoter in livestock animals has been started by the Ministry of Agriculture and Cooperatives since 2015 (Poolperm, Tangkoskul, Seenama, Maknakhon, & Thamlikitkul, 2020). There are few studies concerned with the effect of colistin withdraw in farms (Randall et al., 2018; Xia

CHULALONGKORN UNIVERSITY

et al., 2019) and found out the likely beneficial of controlling the emergence of *mcr*-1 on pig farms. However, the variations between time points of *mcr* genes persistence and re-occurrence of these genes in farm even no selective pressure are still concerning. The possible of co-selection between *mcr* genes and other types of antibiotic resistant genes cannot be ignored.

4. Maintenance of plasmid encoding resistance genes

Generally, acquisition of antibiotic resistance related to mobile genetic elements, including plasmids, impair fitness costs on the bacterial host (Andersson, 2006). However, this burden is much dependent upon plasmid backbone and on the host (Humphrey et al., 2012). Several studies had been found that no considerable fitness cost regarding the acquisition of *mcr*-1 plasmids in *E. coli* (Zhang et al., 2017), (Tietgen et al., 2018; R. Wang, Y. Liu, et al., 2018) and (Ma, Feng, & Zong, 2018) but impaired it in *K. pneumoniae*. Certain genes in the *mcr* bearing plasmid may compensate for the fitness cost and this findings could be consistent with rapid dissemination and persistence of *mcr*-positive strains (Choi et al., 2020; Sun et al., 2018). Moreover, several factors could favor the plasmid maintenance, such as mutational or evolution events, by reducing the plasmid fitness cost to the host and improve its stability (Gama, Zilhão, & Dionisio, 2020). The previous study had stated that *E. coli* strains, ST131, with almost identical core genome could contain different plasmids through rapid plasmid adaptation (Lanza et al., 2014). In case of comparison between ESBL positive *E. coli*

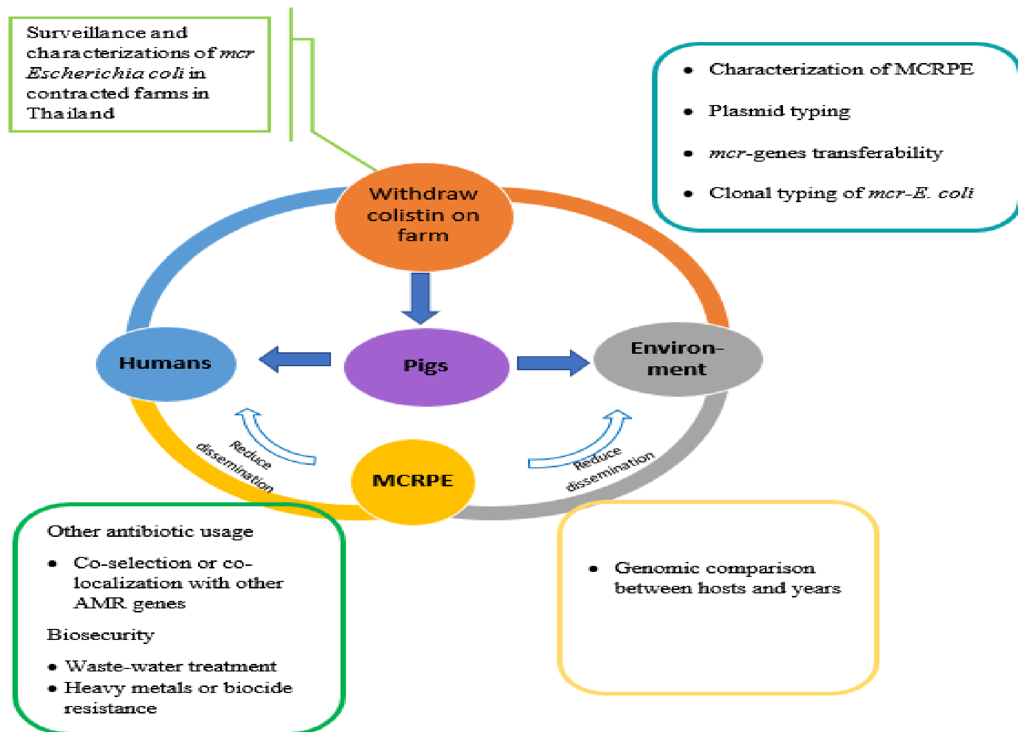
จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

from animals and human in Dutch showed that ESBL transmission was not strain transfer but plasmid transfer by identical plasmids of the IncI and IncK types (de Been et al., 2014b).

Plasmids encoding the *mcr* genes which co-exist with other antimicrobial resistance genes is burdensome for public health. To date, *mcr* genes have been identified in varieties of plasmid and be able to locate and/or transfer with other resistant genes by conjugation (M. Rhouma et al., 2019). Moreover, several reports had been indicated the cross-resistance of colistin by usage of biocides or other antibiotics (Wand, Bock, Bonney, & Sutton, 2017; Xu, Zeng, Hinenoya, & Lin, 2018). This is worrisome because the use of antimicrobials other than polymyxins can participate in the co-selection of isolates carrying *mcr*-1 and favor their spread. Moreover, the plasmid-mediated *mcr*-1 gene has been identified in carbapenemase producing *Enterobacteriaceae* isolates which might lead to no antibiotic option to select for treatment (Mendes et al., 2018). Growing resistant to colistin might not cause health problems immediately, however, infections with no antibiotic options become disperse, which will lead a great threat to clinical practice. Therefore, a One Health approach to monitor and decrease the inappropriate usage of colistin in livestock sector is the goal to reduce these genes spread.

CHULALONGKORN UNIVERSITY

Conceptual Frame Work



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

CHAPTER II

Multidrug-resistance and virulence factors of *Escherichia coli* harboring plasmid-mediated colistin-resistance; *mcr-1* and *mcr-3* genes in contracted pig farms in Thailand

Nwai Oo Khine[1], Kittitat Lugsomya[2], Benjarong Kaewgun[1], Lertrob Honhanrob[1], Panupong Pairojrit[1], Suthipat Jermprasert[1], Nuvee Prapasarakul[1,3,*]

[1]Department of Veterinary Microbiology, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, 10330, Thailand

[2]Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon Tong Hong Kong, Hong Kong SAR

[3]Diagnosis and Monitoring of Animal Pathogen Research Unit (DMAP)

*Correspondence:

Nuvee Prapasarakul

nuvee.p@chula.ac.th

Keywords: colistin resistance, *Escherichia coli, mcr* genes, pigs, virulent factor

Status: Published 10 November 2020 in Frontiers in Veterinary Science;

doi: 10.3389/fvets.2020.582899

Abstract

The presence of the plasmid-mediated colistin-resistance encoding *mcr* gene family in the *Enterobacteriaceae* is one of the crucial global concerns. The use of colistin in livestock rearing is believed to be the cause of *mcr* gene spreading and is of impact to public health. The objectives of this research were to detect the frequency and virulent genes of *mcr*-positive *Escherichia coli* (MCRPE) in fecal samples from healthy pigs in a contract farming system across Thailand. A total of 696 pooled samples were derived from 80 farms, located in 49 provinces across six regions of Thailand. The colistin-resistant *E. coli* were identified by MALDI-TOF mass spectrometry and antimicrobial susceptibility testing by broth microdilution. The antibiogram was determined using an automated susceptibility machine and the genetic characteristics were investigated for *mcr*-1-5 genes, phylogenetic group, replicon types, and virulent genes. In total, 31 of 696 samples were positive, with *E. coli* containing *mcr-1* or combination of *mcr-1* and *mcr-3* with incidence of 4.45% and 0.43%. Phylogenetic groups A and B1 and the IncF and IncFIB replicon types were predominantly found in the MCRPE located in the central area, with multidrug resistant traits against 3–14 types of antimicrobials. Additionally, 19 of 31 isolates identified as enterotoxigenic *E. coli*, were with the *staP* and *stb* (enterotoxin-encoding genes). In conclusion, a low carriage rate of *mcr*-positive *E. coli* was detected in the largescale farming of healthy pigs. The

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

association between of multidrug-resistance MCRPE and their pathogenic potential should be of concern.

Introduction

Antimicrobial resistance (AMR) is an emerging concern for both the human and animal sectors of the world. The inappropriate use of antimicrobials in clinical settings and, most importantly, in livestock farming imposes social and economic burdens on society (Organization, 2001). The diminishing number of active (effective) antimicrobial agents to treat sick farm animals is accompanied by the downfall in food production, and the likelihood of exposure of farmers to resistant bacteria. *Escherichia coli*, a commensal microbe and can accumulate resistance genes. It is widely used as a representative example for monitoring resistance genes, especially for horizontal gene transfer (Kaspar, 2006). Therefore, the assessment of mobile genetic elements from commensal *E. coli*, could highlight the AMR transmission between hosts (E.F.S, Authority, Prevention, & journal, 2016).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Colistin is a cationic antibiotic that has long been regarded as a last resort antibiotic for *Enterobacteriaceae* infections. However, the widespread use of colistin in animal production acts as a selective pressure for the spread of plasmid-mediated colistin resistance genes, which are in the *mcr* family. The first discovery of plasmid-mediated colistin resistance (*mcr*-1 gene) in *E. coli* from China raised an enormous attention globally, and was followed by the subsequent discovery of other *mcr*

resistance genes, including *mcr*-2, *mcr*-3, *mcr*-4, and *mcr*-5, in different geographical areas (Duggett et al., 2018). Recently, another four colistin resistance genes (*mcr*-6, *mcr*-7, *mcr*-8, and *mcr*-9) were identified mainly from members in the *Enterobacteriaceae* family (AbuOun et al., 2018; L. M. Carroll et al., 2019; X. Wang et al., 2018; Y. Q. Yang et al., 2018). Among them, *mcr*-1 being the most frequently detected in farmed animals, and from *Enterobacteriaceae* infections in humans (Skov & Monnet, 2016). These reports raised awareness upon colistin usage, especially in livestock animals.

In Thailand, over 80% of pig farming systems are contract farming between the primary producers and the agribusiness companies, for the latter to procure a certain pre-agreed quality and quantity of products at an economical price and is lesser from the primary producers. Antimicrobials including colistin are feed additives or as prophylactic agents, including colistin, against bacterial infections in pig farms under veterinary prescription (K. Lugsomya, J. Yindee, et al., 2018). Although there have been a few reports regarding a high prevalence (60–90%) of multidrug resistant (MDR) *E. coli* in pigs in Thailand, the antimicrobials used on the farms have not always been clearly defined (Lay, Koowattananukul, Chansong, & Chuanchuen, 2012). Since the colistin-resistance is the crucial epidemiological data of public health concern, monitoring the prevalence of colistin-resistant *E. coli* and their characteristics are of high priorities. The objectives of this study were to characterize the antibiogram and virulent traits of *mcr*-

CHULALONGKORN UNIVERSITY

positive *E. coli* (MCRPE) from the fecal samples of healthy pigs derived from the contract farming system across Thailand.

Materials and methods

Study area and animal selection

Samples were collected from 80 farms, in 49 provinces across six regions of Thailand; comprised of fifteen, five, twelve, seven, four, and six provinces from Central, Northern, Northeastern, Eastern, Western, and Southern Thailand, respectively. Farms were selected based on the available management data, including the antimicrobial usage, housing, vaccination, feed type, and production cycle. However, all historical data was allowed as inclusion criteria for farm selection only but not allowed to be included in the analysis. A total of 696 pooled fecal samples (5–10 samples per farm) were collected from individual 18- to 20-week-old fattening pigs with a normal clinical appearance and no recent history of enteric disease or therapeutic antimicrobial treatment.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Sample collection and bacterial identification

At least 5 g of feces per pig were collected into a sterile container and kept at 4 ^{o}C until processed. Then fecal samples were homogenized and mixed to get pooled fecal samples of total mass 25 g. Then picked up 5 g of well mixed feces and diluted ten-fold using sterile 0.85% (w/v) NaCl. Dilutions of 10^{-7}–10^{-8} were spread on Eosin Methylene blue agar (Oxoid, UK) plates containing 2 µg/mL colistin sulfate (Sigma-Aldrich, USA) to select for the presumptive colistin-resistant *E. coli.* The biohazard

execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 1731021). One representative colony with typical *E. coli* morphology was picked and sub-cultured to get pure culture. The *E. coli* species was confirmed using Matrix Assisted Laser Desorption Ionization combined with timeof-flight analysis (MALDI Biotyper, Bruker, USA). The principle behind MALDI-TOF is based on mass spectrometry and "soft" ionization technique. Depending on the time of flight of each pathogen, the characteristic spectrum will be analyzed and displayed via the inbuilt software. Briefly, the bacterial colony sample was smeared as a thin film directly on a target plate. Then coated with 1μl polymeric matrix (a saturated solution of α- cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried at room temperature. This matrix could penetrate the cell wall of microorganisms and able to extract proteins. The target plate was placed into the mass-spectrometer and irradiated by a laser. Afterwards, the molecules vaporized and ionized at the same time into the vacuum and transported to the detection device. Lastly, the computerized database results compared with reference library database were generated with interpretations (Singhal, Kumar, Kanaujia, & Virdi, 2015).

Antimicrobial susceptibility determination and *mcr* gene detection

For colistin, the broth microdilution procedure was performed according to the CLSI recommendation (Clinical & M100, 2017). The plasmid-mediated colistin

CHULALONGKORN UNIVERSITY

resistance genes (*mcr*-1–5) were detected by multiplex (m)PCR using GoTaq® green mastermix (Promega, USA) and the previously reported primers and PCR conditions (Rebelo et al., 2018). The *E. coli* strain CUP13 (Lugsomya K, 2016) , which is positive for *mcr*-1 and *mcr*-3 (confirmed by Sanger sequencing), and ATCC25922 were used as positive and negative controls, respectively. Briefly, the thermocycling conditions were performed at 94 °C for 15 min, followed by 25 cycles of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 1 min, and then followed by 72 °C for 10 min.

The minimal inhibitory concentration (MIC) of antimicrobial agents against the *E. coli* isolates were determined using the AST-GN 38 test kit in a Vitek2 compact automated susceptibility level detection apparatus (BioMérieux, France). The antimicrobial groups comprised were synchronized with veterinary guidelines (Plumb, 2015). The justification of antibiotic chosen are for AMR monitoring and for the purpose of public health awareness such as the 2nd generation of cephalosporin, aminoglycoside, fluoroquinolone and carbapenem. *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25913 were used as the control strains. The antimicrobials comprised were amikacin (AK), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), cefalexin (CEX), cefpodoxime (CPD), cefovecin (INN), ceftiofur (XNL), chloramphenicol (C), enrofloxacin (ENR), gentamicin (GEN), imipenem (IMP), marbofloxacin (MBR), nitrofurantoin (NIT), piperacillin (PIP), tetracycline (TET), tobramycin (TOB) and

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

trimethoprim/sulfamethoxazole (SXT). The MIC interpretations will be reported according to Food and Drug Administration (FDA) (FDA, 2018), CLSI (CLSI, 2017) and EUCAST values (EUCAST, 2018). The isolates that presented an extended-spectrum beta-lactamase (ESBL) phenotype were confirmed with a double disc synergy test and phenotypic disc confirmatory test as previously reported (Dhara et al., 2012).

Phylogenetic grouping

The MCRPE isolates were determined using an approved mPCR identification of their phylogenetic groups and subgroups (A, B1, B2, C, D, E, and F) as reported (Clermont, Christenson, Denamur, & Gordon, 2013). Each reaction was performed in a 25-μL mixture containing 12.5 μL of GoTaq® green mastermix (supplied with Taq polymerase), 20 pmol of each primer, and 200 ng of genomic DNA. The *E. coli* ATCC 25922 and *E. fergusonii* CUVET427 (Kittitat Lugsomya et al., 2018) strains were used as the controls.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Plasmid replicon typing

The *Enterobacteriaceae* plasmid replicons IncF (IncFIA, IncFIB, IncFIC, and IncFrep), IncI1-Ig, IncN, IncP, IncW, IncHI1, IncHI2, IncL/M, IncT, IncA/C, IncK, IncB/O, IncX, and IncY were detected using five mPCR and three simplex PCR tests. The primers, PCR conditions, and thermal cycles were applied as previously reported (Carattoli et al., 2005). Briefly, PCR amplifications, except the F-simplex, were thermal cycled at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for 30 s, and

72 °C for 1 min, and then followed by 72 °C for 5 min. The F-simplex PCR was performed with the same amplification program except at an annealing temperature of 52 °C. Positive control samples were provided and used as reported (Kittitat Lugsomya et al., 2018).

Detection of virulence genes

The sets of mPCR and simplex PCRs were performed as previously reported (Casey & Bosworth, 2009), with the positive control strains taken from the previously sequenced enterotoxigenic *E coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) strains (Prapasarakul, Tummaruk, Niyomtum, Tripipat, & Serichantalergs, 2010). Primers specific for the *StaP* (heat stable toxin a subdivide p), *Stb* (heat stable toxin b), *Stx2e* (Shiga toxin), *K88* (Fimbriae), *F4* (Fimbriae), and *Ltb* (heat-labile enterotoxin b subunit) genes were used. The PCR assays were prepared with GoTaq® green mastermix (Promega, USA) and thermocycled at 94 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1.5 min increasing by 3 sec each cycle, and then followed by 72 °C for 10 min.

Data analysis

The colistin-resistance rates are presented as percentages divided by region and province in comparison of the rate of with and without *mcr* genes, and the antimicrobial resistance profiles ae reported as the antibiogram patterns of *mcr*-positive *E. coli.* The patterns of virulence gene profiles among MCRPE isolates are presented in

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

percentages. To define MDR and pathogenic traits among the colistin-resistant *E. coli*, the relation between AMR phenotypes and pathotype characteristics was analyzed using the Fischer's exact test ($p \leq 0.05$).

Results

Distribution of colistin-resistant *E. coli* containing *mcr* genes

A total of 105 colistin-resistant *E. coli* from the 696 samples were isolated using the EMB (eosin methylene blue) media. From the broth microdilution method, the MCRPE isolates had MIC values of 4 (n= 17) or 8 (n=14) µg/mL. From the PCR detection, the *mcr-1* gene were found in 31 of these 105 colistin-resistant *E. coli* isolates and among them three isolates were found to also express *mcr*-3. The distributions of colistin-resistant *E. coli* were from Central (5.4%) (Phetchabun, Nakhon Pathom, Ang-Thong and Lopburi), Western (0.4%) (Ratchaburi), and Eastern (1.4%) (Chonburi) Thailand. The geographical distributions of *E. coli* with or without *mcr* genes were shown in Figure 1.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

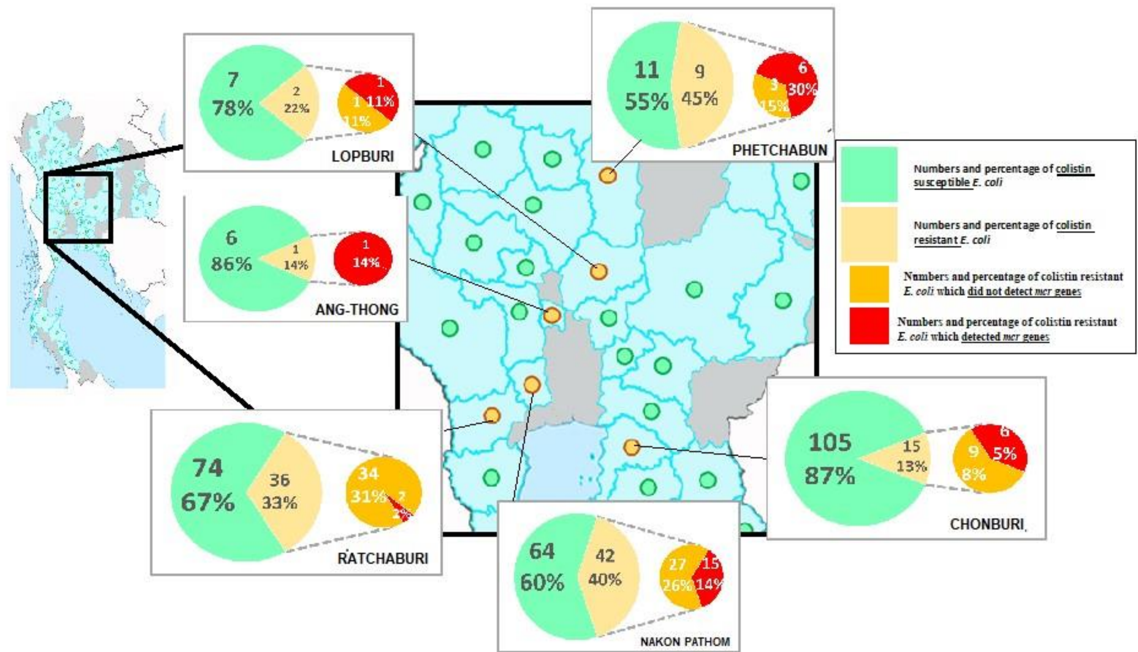


Figure 1. Geographical distribution of either colistin resistant or susceptible *E. coli* from the surveyed contracted pig farms in Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

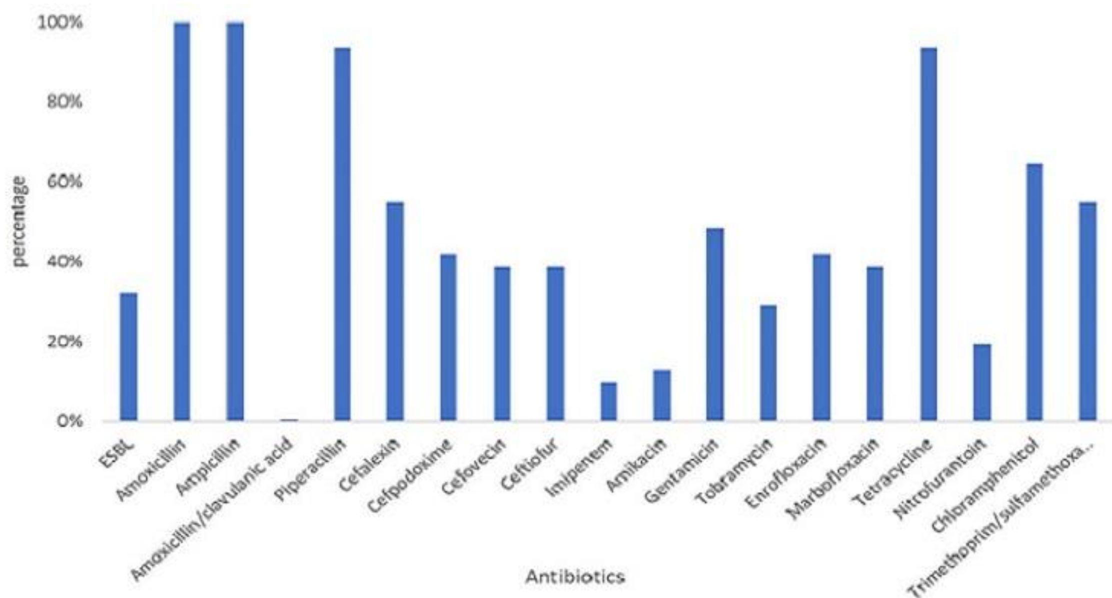


Figure 2. Distribution of resistant rates against 18 antimicrobials and presence of extended- spectrum beta-lactamase (ESBL) characteristic among 105 colistin-resistant *E. coli* isolated from contracted pig farms in Thailand

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Antimicrobial susceptibility testing

All 31 MCRPE were multi-drug resistant (Figure 2), with all being resistant to AMX, AMP, PIP, and TET and over 50% were resistant to CEX, INN, XNL, GEN, ENR, C, and the SXT combination. No pan-drug resistance was detected among the MCRPE isolates. ESBL were found in 32.3% (10/31) *mcr*-1 positive isolates. A total of 26 antibiogram patterns were recorded for 31 MCRPE isolates. Forty eight percent (15/31) of these isolates were MDR with resistance to six antimicrobial groups (Table 1).

Table 1. Antibiograms of the 31 MCRPE isolates distributing in 26 pattern types

Pattern	Profile	Number of ABOs resist	Isolate(s)
A	AMX-AMP-PIP-CEX-CPD-INN-XNL-GEN-ENR-MBR-TET-C-NIT-SXT*	14	1
B	AMX-AMP-PIP-CEX-CPD-INN-XNL-IMP-AK-GEN-ENR-TET-C-SXT*	14	1
C	AMX-AMP-PIP-CEX-CPD-INN-XNL-GEN-TOB-ENR-MBR-TET-C*	13	3
D	AMX-AMP-PIP-CEX-GEN-TOB-ENR-MBR-TET-NIT-C-SXT	12	1
E	AMX-AMP-PIP-CEX-CPD-INN-XNL-IMP-AK-C-SXT*	11	2
F	AMX-AMP-PIP-CEX-CPD-INN-XNL-GEN-TOB-TET-NIT*	11	1
G	AMX-AMP-PIP-CEX-CPD-INN-XNL-TET-C-NIT-SXT*	11	1
H	AMX-AMP-PIP-CEX-CPD-INN-XNL-GEN-TOB-TET-C*	11	1
I	AMX-AMP-PIP-GEN-TOB-ENR-MBR-TET-C-SXT	10	2
J	AMX-AMP-PIP-CEX-CPD-INN-XNL-TET-C-SXT	10	1
K	AMX-AMP-PIP-CEX-CPD-INN-XNL-GEN-TET	9	1
L	AMX-AMP-PIP-CEX-ENR-MBR-TET-C-SXT	9	1
M	AMX-AMP-PIP-ENR-MBR-TET-NIT-C-SXT	9	1
N	AMX-AMP-PIP-CEX-ENR-MBR-TET-SXT	8	1
O	AMX-AMP-PIP-ENR-MBR-TET-C-SXT	8	1
P	AMX-AMP-PIP-GEN-ENR-MBR-TET-SXT	8	1
Q	AMX-AMP-PIP-GEN-TOB-TET-C-SXT	8	1
R	AMX-AMP-PIP-CEX-CPD-AK-TET	7	1
S	AMX-AMP-PIP-CEX-TET-C-SXT	7	1
T	AMX-AMP-PIP-TET-C-SXT	6	1
U	AMX-AMP-PIP-GEN-TET-NIT	6	1
V	AMX-AMP-PIP-TET-NIT	5	1

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

W	AMX-AMP-PIP-TET-C	5	2
X	AMX-AMP-GEN-TET	4	1
Y	AMX-AMP-PIP-TET	4	1
Z	AMX-AMP-TET	3	1

AMC, amoxicillin–clavulanic acid; AMP, ampicillin; AMX, amoxicillin; C, chloramphenicol; CEX, cephalexin; CPD, cefpodoxime; ENR, enrofloxacin; GEN, gentamicin; MBR, marbofloxacin; PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole; INN, cefovecin; AK, amikacin; IMP, imipenem; TET, tetracycline; XNL, ceftiofur; TOB, tobramycin; NIT, nitrofurantoin, * =ESBL

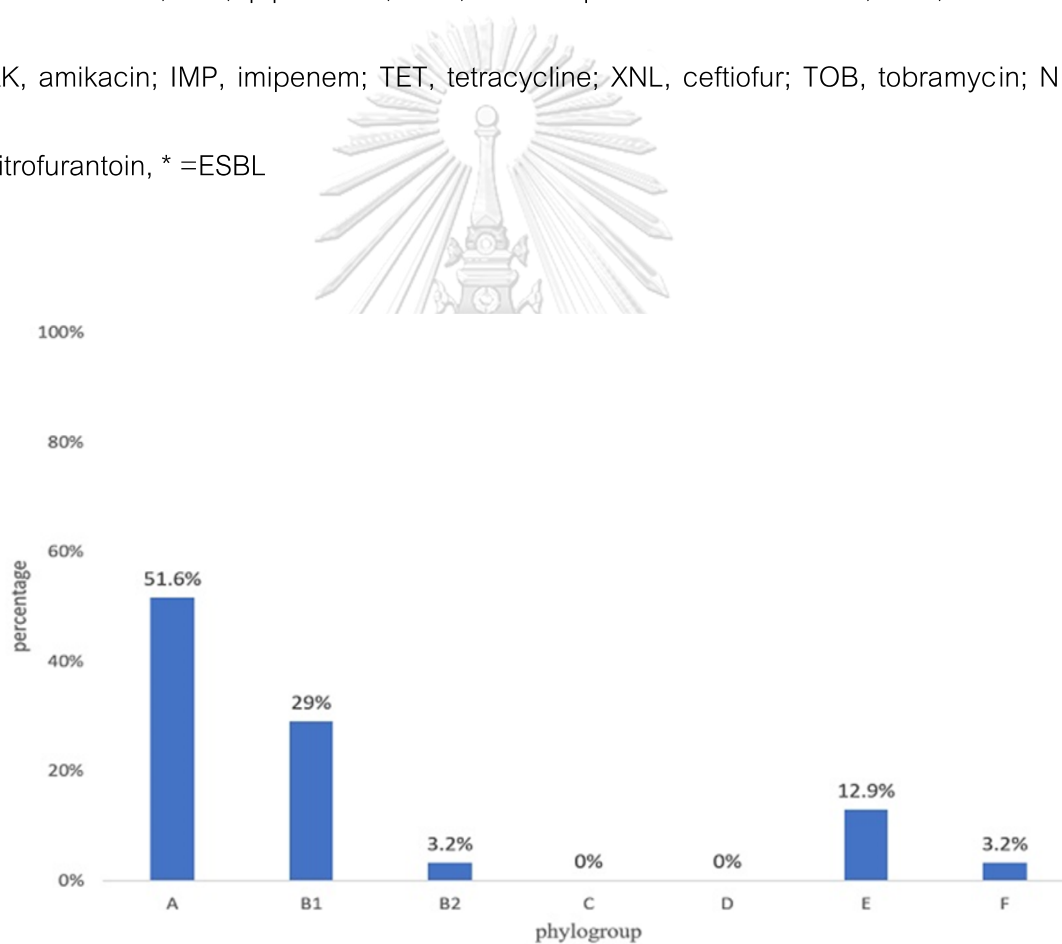


Figure 3. The phylogroups detected among 31 MCRPE isolates in contracted pig farms in Thailand.

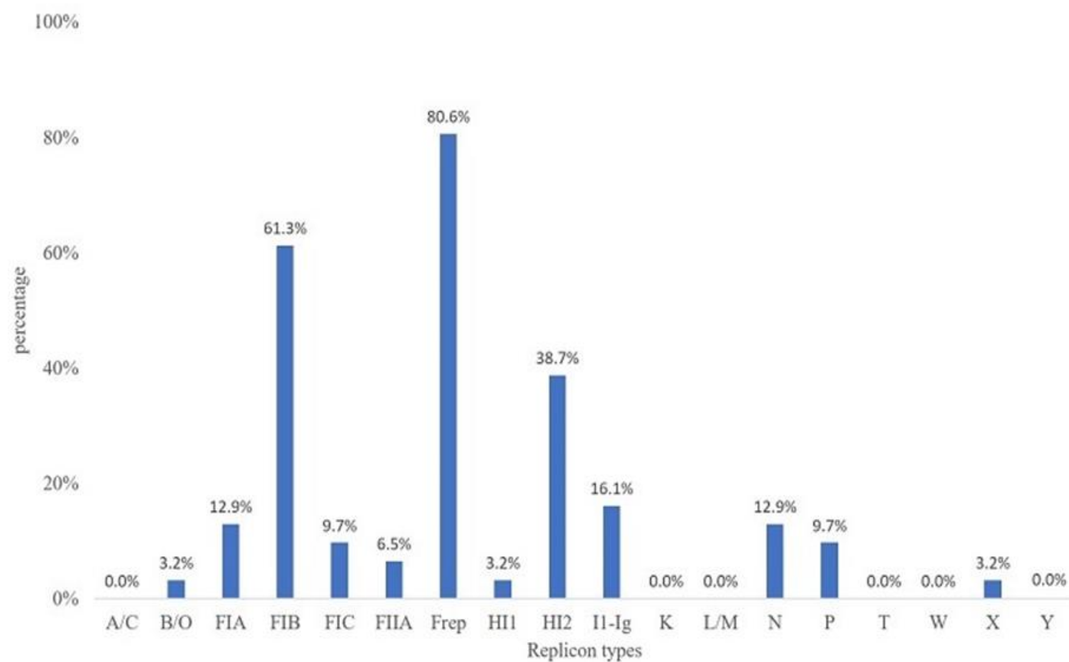


Figure 4. Plasmid replicon types detected among 31 MCRPE isolates in contracted pig farms in Thailand.

Phylogenetic grouping

Most isolates were from phylogenetic group A (51.6%) followed by group B1 (29%), and group E (12.9%), B2 (3.2%) and F (3.2%) (Figure 3).

Chulalongkorn University

Plasmid replicon typing

The predominantly found plasmid replicons were of the IncF and IncFIB replicon types at 80.6% and 61.3% respectively. Plasmid replicon types L/M, W, Y, A/C, T, and K were not detected in this study (Figure 4). The other replicon types were found at low prevalence rates among the MCRPE isolates, with IncX, IncB/O, and IncHI1 being present at the lowest percentages (3.2%).

Characterization of the virulent factors

The virulent genes representing ETEC or EHEC were found in 18 out of 31 (58.1%) MCRPE isolates (Table 2). The ETEC strains possessed the *StaP* and *Stb* enterotoxin-encoding genes as the most frequent pathotype, and one strain (from Phetchabun province) showed a hybrid ETEC-EHEC genotype.

Relation analysis between antimicrobials susceptibility and pathogenicity

The association between the antimicrobial susceptibility and pathogenicity of the 31 MCRPE isolates was analyzed by the Fischer's exact test (Table 3). There was no association between pathogenicity and resistance to six antibiotic groups were found (fluoroquinolones, sulfonamides, tetracyclines, nitrofurazones, phenicols and aminoglycosides) (p = 0.28, 1.00, 1.00, 1.00, 1.00 and 0.15 respectively).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 2. Presence of virulent profiles including toxin and antigenicity of the 31 MCRPEs

Virulence genes	ESBL (%)	Pathotype(s)	Number	%
StaP-Stb-Stx2e	0	ETEC, EHEC	1	3.2
StaP-Stb-K88	3.2	ETEC	1	3.2
StaP-Stb	16.1	ETEC	13	41.9
StaP	3.2	ETEC	3	9.7
Ltb	0	ETEC	1	3.2
Negative	9.7	Non-pathogenic	12	38.7

ETEC; Enterotoxigenic *E. coli*, EHEC; Enterohemorrhagic *E. coli*, ***StaP;*** Heat stable toxin a subdivide p, ***Stb***; Heat stable toxin b, ***Stx2e***; Shiga toxin, ***K88***; Fimbriae, F4, ***Ltb***; Heat-Labile Enterotoxin, b subunit

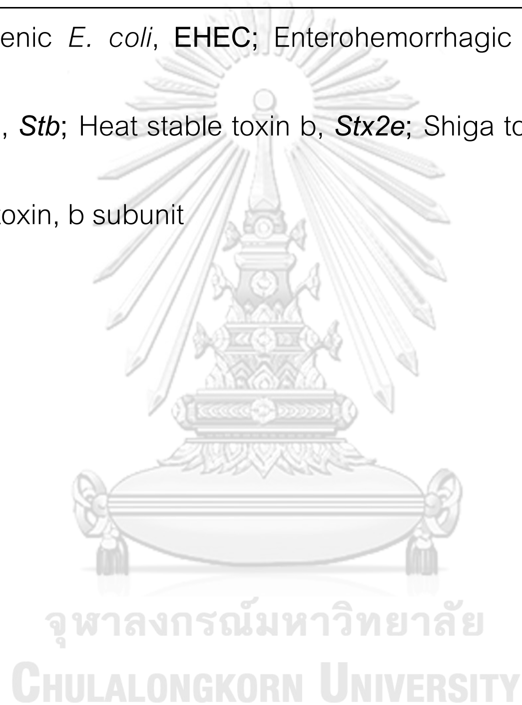

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Table 3. Relation analysis between MCRPE resistance to the other six antimicrobial groups and their pathogenicity

Antimicrobial group	Pathogenicity	Resistant	Susceptible	P value
Aminoglycosides	Non-pathogenic	9	4	0.15
	Pathogenic	7	12	
Fluoroquinolones	Non-pathogenic	7	6	0.28
	Pathogenic	6	13	
Tetracyclines	Non-pathogenic	11	2	1.00
	Pathogenic	17	2	
Nitrofurazones	Non-pathogenic	3	10	1.00
	Pathogenic	4	15	
Phenicols	Non-pathogenic	9	4	1.00
	Pathogenic	12	7	
Sulfonamides	Non-pathogenic	7	6	1.00
	Pathogenic	11	8	

Aminoglycosides; amikacin, gentamicin, and tobramycin, **Fluoroquinolones;** enrofloxacin and marbofloxacin, **Tetracyclines**; tetracycline, **Nitrofurazones**; nitrofurantoin, **Phenicols**; chloramphenicol, **Sulfonamides**; trimetroprim/sulfamethoxazole, **Pathogenic**; ETEC, ETEC-EHEC, **Non-pathogenic;** negative for virulence genes

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Discussion

This national scale study of contract-farmed pigs in Thailand confirmed the existence of colistin-resistant *E. coli* containing *mcr* genes, and that they showed diversity in their phylogenetic group, replicon type, antibiogram, ESBL trait, and pathogenic potential. All recruited contracted pig farms had the strict historical data and management records that can be traced back as an essential inclusion-criteria. The sample collection criteria were set up and executed by the farm workers under the authority of veterinarians. In this study, MALDI-TOF MS was used for identification and confirmation of bacteria strains. This technique has emerged as a powerful technique for identification of microorganisms with an overall 95% accuracy at the species level. The main advantage of MADLI-TOF is being able to identify bacterial species directly from the culture plates as fast as 1 to 15 minutes in a few simple steps (Singhal et al., 2015).

According to mPCR, our results indicated the lower resistant rate of *mcr-1* (4.4% or 31/696) when compared with previous report from healthy pigs in China (21%) Clermont et al., 2013). This study covered all parts of Thailand where high intensity pig farming is done. Unfortunately, all the historical data could not be analyzed due to the company's policy. However, the positive areas were distributed in the western, central, and eastern parts within a radius of about 300 km. The distributions of colistin-resistant *E. coli* were higher (15-30%) in Nakhon Pathom, Ratchaburi, Chonburi, Lopburi and Phetchabun provinces. These provinces reported to have huge number of pig farms and

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

the total number of pigs. Colistin were legal usage in pig feeds for prophylactic purposes in Thailand until March 2018. The high percentage of MCRPE isolates in certain provinces might came from prolonged cumulative selective pressure from their history of colistin usage in pig feeds. To the best of our knowledge, this is the first report of *mcr*-1 gene in *E. coli* isolates from pigs in Thailand. Interestingly, three of the *mcr-1*-positive isolates also co-expressed *mcr-3*. These results could highlight the awareness of the distribution of *mcr* genes and for the national policy of livestock immigration. The *mcr-1* genes have been widely shown to be distributed in Asia, Europe, Africa, and America, and primarily due to the consequence of long-term colistin application in animals (Elbediwi et al., 2019). The *mcr-3* gene was first reported in China in 2017 (Yin et al., 2017) and the prevalence and spread of the *mcr-3* gene in Thailand should be carefully monitored from now on.

CHULALONGKORN UNIVERSITY

According to phylogenetic grouping, the majority of the isolates in our study were in phylogroups A or B1, predominantly related with commensal strains (Yilmaz & Aslantas, 2020). On the other hand, for the virulent *E. coli* groups, phylogroup D was not detected in the current study and there was a low frequency of phylogroup B2. Several studies have reported that phylogroups B2 and D were associated with intestinal and extra-intestinal pathogenic *Escherichia coli* as well as MDR strains. (Sarshar et al., 2017) (Iranpour et al., 2015). Nonetheless, even commensal *E. coli* from various phylogroups have been reported to harbor pathogenicity islands that can serve as integration sites

for virulence and/or AMR determinants (Raimondi et al., 2019) and so may facilitate in converting commensal strains to pathogens.

With respect to plasmid replicon typing, the IncFIB and F plasmids were the mostly commonly found replicon types in this study. They are narrow-host-range type plasmids, which have been reported in worldwide members of the *Enterobacteriaceae* family, associated with various antimicrobial resistant genes (Johnson et al., 2007). The *mcr*-1 and *mcr*-3 genes were previously described on the IncI, IncHI2, and IncX4 plasmids (Kieffer et al., 2018). A variety of replicon types were found in the MCRPE isolates in this study, which suggest that the *mcr* genes can locate and/or transfer to different plasmid types. This is in accordance with a previous report that the *mcr*-1 genes and ESBL could be co-transferred by more than one type of conjugative plasmid, which might alleviate their effective dissemination among bacteria (C. Wu et al., 2018).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

The antibiogram profiles characterized among the MCRPE isolates revealed that MDR was a common phenotype in this study. *E. coli* resistance to beta-lactam and the tetracycline antibiotic groups was very common in Thailand, and aminoglycoside and fluoroquinolones resistance found to be varied upon the farm management such as using antibiotic for prophylactic or treatment purposes (Kittitat Lugsomya et al., 2018). The MDR traits among *mcr*-1-positive *E. coli* have been reported frequently in pigs due to the usage of antibiotics in the production cycle (M. Rhouma et al., 2019). Interestingly,

ESBLs were found at a high prevalence among the MCRPE isolates of this study, which might due to co-selection under selective pressure (C. Wu et al., 2018). Moreover, *E. coli* plasmids that harbor co-localization of *mcr*-1 and $bla_{CTX\text{-}M}$ genes and/or *mcr*-1 and $bla_{NDM\text{-}5}$ genes have been reported previously (Mohamed Rhouma & Letellier, 2017). Genomic characterization should be performed to resolve the reason for this apparent correlation.

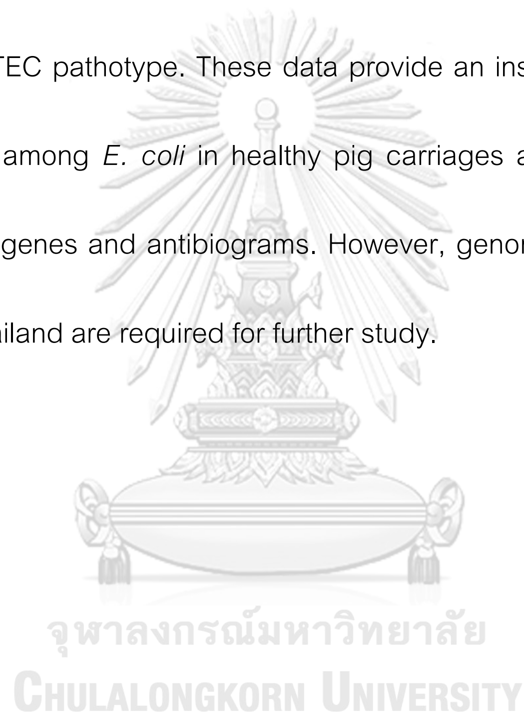
The presence of the *Ltb*, *Stb*, *StaP*, *Stx2e*, and *K88* virulence genes in MCRPE isolates indicated they also had the potential to cause an infection. Thus, healthy pigs could be an important reservoir of colistin-resistant ETEC. Interestingly, one MCRPE isolate was found to be ETEC-EHEC hybrid strain. *E. coli* with highly virulent hybrid pathotype strains had been reported previously both in animals and human diarrhea patients (Leonard, Mammel, Rasko, & Lacher, 2016). Since many of the virulence genes of *E. coli* are carried on mobile genetic elements, the genetic combination of these MGE resulted in the emergence of STEC/ETEC hybrid strains in multiple events (Prager, Fruth, Busch, & Tietze, 2011). The recent finding of a clone of sequence type (ST) 95 showing extreme-drug resistance with a high virulence potential underscores the need to monitor new and emerging trends in antibiotic resistance development in this important global lineage (Forde et al., 2018). On the other hand, aminoglycosides and fluoroquinolones-resistant *E. coli* seemed to have a lower probability to act as an ETEC pathotype in this study. Pathogenic *E. coli* tends to be more susceptible to many antimicrobials (da Silva

CHULALONGKORN UNIVERSITY

& Mendonça, 2012). However, the mechanism is still not elucidated and clonal typing should be included for a more convincing analysis.

Conclusion

In conclusion, a low carriage rate of *mcr*-1 and *mcr*-3 co-positive *E. coli* was detected in large scale contract pig farms in Thailand. The MCRPE isolates showed not only MDR-*E. coli* but also that most of the isolates contained virulence genes representing an ETEC pathotype. These data provide an insight into the occurrence of colistin resistance among *E. coli* in healthy pig carriages and their characteristics, in terms of virulence genes and antibiograms. However, genomic characterization of *mcr* genes found in Thailand are required for further study.


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Chapter III

Longitudinal monitoring reveals persistence of colistin-resistant *Escherichia coli* on a pig farm following cessation of colistin use

Nwai Oo Khine[1,2], Kittitat Lugsomya[3], Tawat Pongpan[4], David J. Hampson[5], Nuvee Prapasarakul[1,6,*]

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,

[2] The International Graduate Program of Veterinary Science and Technology (VST), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,

[3] Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, China,

[4] Swine Veterinarian Service, Charoen Pokphand Foods PCL, Bangkok, Thailand

[5] School of Veterinary Medicine, Murdoch University, Perth, Western Australia

[6] Diagnosis and Monitoring of Animal Pathogens Research Unit (DMAP), Bangkok, Thailand

*Correspondence:

Nuvee Prapasarakul

nuvee.p@chula.ac.th

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Abstract

Colistin-resistant bacteria that contain plasmid-mediated genes of the *mcr* family are of concern as they may be a cause of serious nosocomial infections. It is hypothesized that cessation of colistin use as a feed additive for pigs will reduce the occurrence and distribution of *mcr* genes in farms. The aim of this study was to investigate this hypothesis by longitudinal monitoring and characterizing of *mcr* positive *Escherichia coli* (MCRPE) isolates after colistin was withdrawn on a central Thailand pig farm that previously had a high frequency of MCRPE. Colistin use ceased at the beginning of 2017, and subsequently 170 samples were collected from farrowing sows and suckling piglets (n=70), wastewater (n=50) and farm workers (n=50) over a 3.5-year period. Following selective culture, bacteria were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and minimal inhibitory concentrations of antimicrobials were determined by broth microdilution. The antibiogram of *mcr* positive *E. coli* isolates was determined using the Vitek2 automated susceptibility machine, and multiplex and simplex PCRs were performed for *mcr*-1-8 genes. The clonal relatedness of the 33 *mcr* positive *E. coli* isolates that was analyzed using pulsed-field gel electrophoresis and multi-locus sequence typing. MCRPE containing either *mcr*-1 or *mcr*-3 was isolated from pigs throughout the investigation period, but with a declining trend, whereas MCRPE isolates were recovered from humans only in 2017. MCRPE were still being recovered from wastewater in 2020. Most

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

MCRPE isolates possessed the virulence genes *StaP, Stb*, or *Stx2e*, reflecting pathogenic potential in pigs, and high rates of resistance to ampicillin, gentamicin and tetracycline also were detected. Both typing methods showed that diverse MCRPE clones were distributed on the farm. The study identified declination of pathogenic MCRPE following withdrawal of colistin, with pigs being the primary source, followed by wastewater. However, short-term therapeutic usage of other antibiotics could enhance the re-occurrence of *mcr*-carrying bacteria. Factors including the environment, management, and gene adaptations that allow maintenance of colistin resistance require further investigation, and longer-term studies are needed.

Keywords: colistin resistance, *Escherichia coli*, *mcr* genes, longitudinal monitoring, pigs

Introduction

Colistin (polymyxin E) is one of the World Health Organization's highest priority antimicrobials: it is regarded as a last resort antibiotic, and is the treatment of choice for multidrug-resistant *Enterobacteriaceae* infections (Falagas & Kasiakou, 2005). Unfortunately, the emergence of mobile colistin resistance genes of the *mcr* gene family has jeopardized the efficacy of colistin. In the swine industry, colistin had been applied therapeutically and/or prophylactically in many countries (M. Rhouma, Beaudry, Theriault, & Letellier, 2016). Following the first identification of *mcr*-1 during nation-wide surveillance in Thailand (Khine et al., 2020), from the start of 2017 the Department of Livestock and Development (DLD) has prohibited prophylactic use of colistin sulfate in

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

pig farms. Currently it is still debatable how this withdrawal of colistin usage may have influenced the emergence and spread of *mcr* genes in pigs and in the farm environment (Xia et al., 2019). It is thought to be unlikely that resistance plasmids can be entirely eliminated from bacterial populations (de Toro et al., 2014). Moreover, some studies have shown that even drastic reductions of antibiotic use on farms, antibiotic resistant bacteria could be maintained in the farm by various factors (Brolund et al., 2010; Sundqvist et al., 2010; Yates et al., 2006).

The aim of this study was to determine the occurrence and extent of persistence of MCRPE on a representative pig farm with a history of a high prevalence of MCRPE following the cessation of colistin sulfate use (Khine et al., 2020). Representative MCRPE isolates from pigs, wastewater and farm workers that were obtained over the study period were characterized for *mcr* genes, antimicrobial susceptibility patterns, virulence factors, plasmid replicons, and clonal relationships.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Materials and methods

Study area and farm selection

A typical industrial pig farm with more than 1000 breeder sows located in the central area of Thailand was selected for use in this study. Prior to 2017, colistin sulfate had been administered routinely to all suckling piglets from birth to weaning to prevent and control diarrhoea. It was given via the water at a dose of 10mg/kg body weight. The farm withdrew prophylactic colistin use in piglets from the beginning of 2017, following

the guidance of the DLD. The farm management systems were not otherwise altered, and they continued to follow the recommendations of the Thai standard livestock farm criteria. Piglets with diarrhoea were separated from healthy piglets by placing them in separate pens until they recovered. In cases of diarrhoea in breeding sows and piglets, antibiotic injections including gentamicin, ceftriaxone, and/or penicillin/streptomycin combinations were used for treatment of individual animals.

Sample collection and processing

The number and types of samples (from pigs, wastewater and humans) that were obtained are summarized in (Appendix 1 table 7). Samples were collected at five-time points spanning a 3.5year period from cessation of colistin use: June 2017, September 2018, March 2019, April 2019, and June 2020.

Faecal samples from pigs (n=70) were obtained from both farrowing sows that were of parity 1-6 and between 1 to 3 years old, and randomly selected suckling piglets belonging to the sows that were sampled (sampled at 21-days of age, immediately before weaning). Approximately 25 g of fecal samples were collected from farrowing sows and rectal swab from piglets. Each farrowing sow with their respective litters were kept in farrowing pens, and at each visit one or two sows were sampled from different zones of the farrowing house. The same pens were visited at each sampling time, although the same sows were not necessarily sampled because of animal movements that had occurred. In September 2018, only faecal samples from sows were collected

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

since at the time of sampling the newly-weaned piglets had been moved to another farm.

Wastewater samples (n=50) from under the pens on the farm also were collected, with 10 samples obtained at each visit. Approximately 500 ml volumes were collected from 5 wells in wastewater tanks that were located in close proximity to the sampled pig pens, with the wells being located before and after-biogas treatment. The biogas process involves anaerobic fermentation by fermentative, acetogenic, and methanogenic bacteria to produce methane, carbon dioxide, hydrogen, and hydrogen sulfide gases. In addition, at the request of the company, at each sample collection time the farm submitted rectal swab samples from the same 10 farm workers for routine diagnostic purposes (n=50).

Sampling from the pigs and the wastewater was conducted by an authorized veterinarian from the farm. The biohazard execution control was approved by the Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 2031011). The wastewater sample collection protocol was applied according to HACH water analysis guidelines (HACH, 2013).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Bacterial isolation and identification

All samples were enriched in EC broth (Difco) containing 2 μg/ml colistin sulfate at a 1:9 ratio and incubated at 37°C overnight. The sample suspensions were grown on eosin-methylene blue (EMB) (Oxoid) agar containing 2 μg/ml colistin sulfate, and

incubated overnight. One to three representative colonies with a characteristic metallic sheen on the EMB plates were randomly chosen and sub-cultured on tryptic soy agar (TSA) from the samples from which growth was obtained (Difco). The colonies were identified as *E. coli* using IMViC biochemical tests and Matrix-Assisted Laser Desorption Ionization combined with time of-flight analysis (MALDI Biotyper, Bruker, USA), according to the manufacturer's recommendations (Singhal et al., 2015). For minimal inhibitory concentration (MIC) determinations, antibiotic susceptibility testing, and PCR detection for virulence genes and plasmid replicon types, a single representative isolate from each positive sample was used.

Antimicrobial susceptibility testing

The MIC for colistin was determined using the broth microdilution technique following CLSI guidelines (CLSI, 2020). An MIC value of >2μg/ml was considered to indicate colistin resistance (CLS, 2020). The antibiogram for *E. coli* isolates was determined using the AST-GN 38 test kit in a Vitek2 compact automated susceptibility level detection apparatus (BioMérieux, France). The antimicrobial groups that were included in Vitek2 were synchronized with veterinary guidelines (Plumb, 2015). The 18 antimicrobials comprised amikacin (AK), amoxicillin (AMX), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), cefalexin (CEX), cefpodoxime (CPD), cefovecin (INN), ceftiofur (XNL), chloramphenicol (C), enrofloxacin (ENR), gentamicin (GEN), imipenem (IMP), marbofloxacin (MBR), nitrofurantoin (NIT), piperacillin (PIP), tetracycline (TET),

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

tobramycin (TOB) and trimethoprim/sulfamethoxazole (SXT). The MIC interpretations from the Vitek2 machine system (version-9) were made according to the Food and Drug Administration recommendations (FDA, 2018), CLSI guidelines (CLSI, 2017) and EUCAST values (EUCAST, 2018).

Detection of plasmid-mediated colistin resistance genes

Genomic DNA was extracted from all available MCRPE isolates using the Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Multiplex-PCR was used to detect *mcr*1-5 genes, following a previously published protocol (Rebelo et al., 2018). *E. coli* strain CUP13 (Lugsomya K, 2016) that is positive for *mcr*-1 and *mcr*-3 as confirmed by Sanger sequencing was used for the positive control, and *E. coli* ATCC25922 was the negative control. The PCR conditions for *mcr* 6, 7, and 8 were adjusted and performed according to a previous description (X. Wang et al., 2018).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Plasmid replicon typing

The 18 plasmid replicon types of *Enterobacteriaceae* were investigated by a set of multiplex and simplex PCRs. The primers used and the PCR conditions followed previously described methods (Carattoli et al., 2005). Briefly, PCR amplification, except the F-simplex, were conducted at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min. The amplification was concluded with an extension

program of 1 cycle at 72°C for 5 min. The PCR for F-simplex was performed with duplicate amplifications except for annealing at 52 °C.

Detection of virulence genes

The *mcr* positive *E. coli* were examined for virulence genes that are commonly present in enterotoxigenic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC) by using previously described PCRs (Casey & Bosworth, 2009). Previously sequenced ETEC and EHEC strain were used as positive controls (Prapasarakul et al., 2010) et al., 2010). The PCR assays were performed with GoTaq® green master mix (Promega, USA) with the thermocycler conditions being an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, and annealing at 55 °C for 45 s. Extension was at 72 °C for 1.5 min increased by 3 sec each cycle, followed by a final extension at 72 °C for 10 min.

Conjugation assay

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

To determine whether *mcr* genes were located on transmissible plasmids, and their transferability rate, conjugation assays were performed by the broth mating technique (Gray et al., 2006). All the *mcr* positive strains detected by PCR were designated as donors, and *E. coli* J53, resistant to sodium azide, was used as the recipient strain. Briefly, an overnight culture of bacterial colonies was diluted in Lysogeny broth (LB) and adjusted to OD600 value 1. A 1:1 ratio of donor and recipient then was mixed to obtain a final volume of 2 ml which was incubated overnight. Ten-fold

serial dilutions of the overnight mixture were plated on LB agar (Oxoid) plates containing colistin (2 μg/ml) and sodium azide (100 μg/ml). The plates were incubated at 37 °C for 2 days, and the transconjugant colonies were counted. Finally, PCR detection of *mcr* genes was reperformed on the transconjugants.

Pulsed-field gel electrophoresis (PFGE)

To investigate clonal relatedness, PFGE was performed on all 65 available MCRPE isolates from the 33 positive samples (one to three per sample), following the Centers for Disease Control and Prevention standard protocol (CDC, 2013). Briefly, overnight cultures of *E. coli* isolates were suspended in cell suspension buffer, and the cells were treated with proteinase K and mixed with the agarose gel solution. The gel plugs then were treated with lysis solution, and DNA in the plugs was digested with restriction enzyme *XbaI* (Thermo Scientific). Gel electrophoresis was undertaken using a Bio-Rad CHEF-DRIII system, with a 200V field at an angle of 120° run for 17–20 hours, incorporating *Salmonella* serovar Braenderup H9812 DNA as a standard. Dendrograms were created using the GeneTool program (Syngene, India) and analyzed by the GeneDirectory program (Syngene, India).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Multi-locus sequence typing (MLST)

A representative isolate from each of the 34 PFGE pulsotypes that were identified was randomly selected and included for MLST typing. The simplex PCR was performed for each of the 7 housekeeping genes of *E. coli* used in the Achtman MLST

scheme (Adiri, Gophna, & Ron, 2003). These genes encoded isocitrate/isopropyl malate dehydrogenase (*icd*), ATP/GTP binding motif (*recA*), adenylate kinase (*adk*), DNA gyrase (*gyrB*), malate dehydrogenase (*mdh*), adenyl succinate dehydrogenase (purA) and fumarate hydratase (*fumc*). The sequences were obtained using the Sanger sequencing platform. The *E. coli* MLST database at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli was used to determine allele and sequence types (STs).

Data analysis

The colistin-resistance rates and virulence gene profiles for the representative isolates were described as percentages compared to different sources in each sample collection. The *mcr* positive rates among the samples and the association between each sample collection time were analyzed using Fischer's Exact Test ($p \leq 0.05$). ANOVA was not used for comparisons because of the small numbers in some cells.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Results

Detection of colistin resistant *E. coli*

A total of 33 of the 170 samples (20.6%) yielded colistin-resistant *E. coli*, and their MICs to colistin varied from 4~8 μg/ml. These positive samples were from pigs (n=20/70, 28.6%), wastewater (n=9/50, 18%) and humans (n=4/50, 8%). A comparison of the prevalence of MCRPE isolates for each sample type over the 3.5 years since colistin cessation is shown in Figure 5, and detailed information about the isolates is presented in table 4. In pigs the high prevalence found in 2017 (60%) and 2018 (50%)

was followed by only a single isolate recovered in 2019 (3.3%), and then another increase in 2020 (33.3%). In humans, resistant isolates were only found in 2017 (40%), while a comparatively low rate of positivity in wastewater in 2017 (20%) and 2018 (10%) was followed by none in 2019, and a high prevalence in 2020 (60%). The majority (8/50: 16%) of MCRPE isolates recovered from wastewater were obtained from samples taken before biogas treatment, with only one isolate recovered in 2020 being from a sample taken after the biogas treatment plant (Appendix 1 table 7).

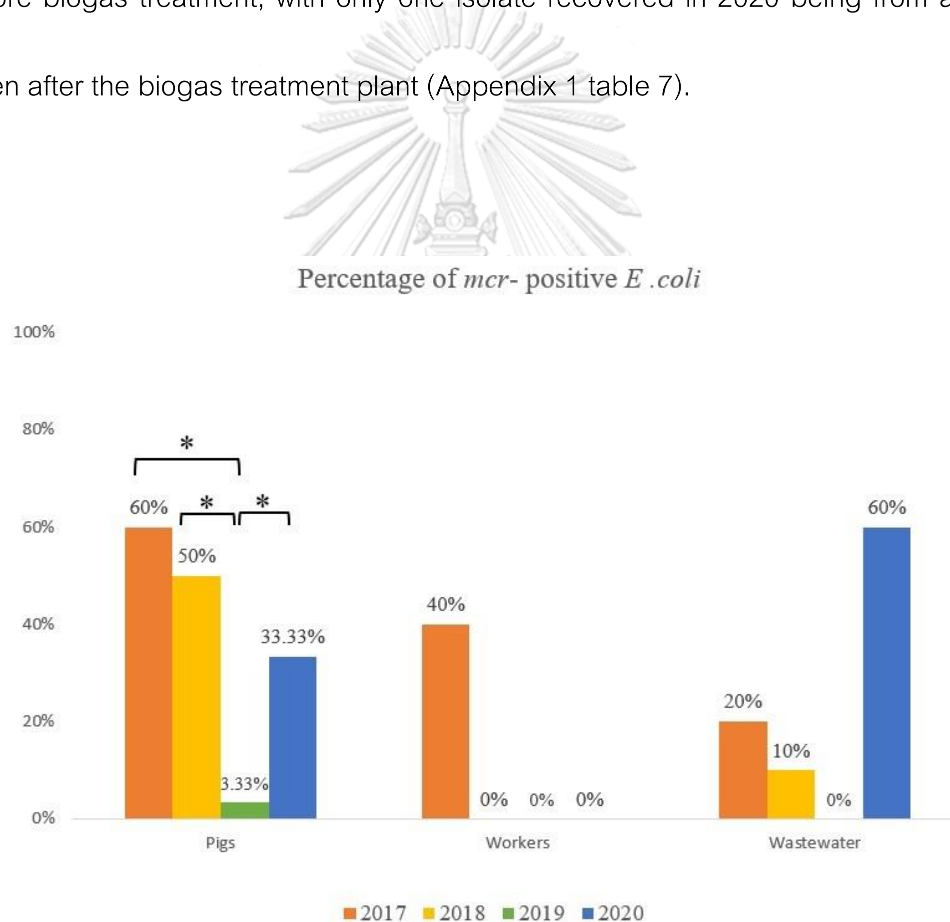


Figure 5. Comparison of the rate of *mcr* positive *E. coli* isolated from pigs, workers and the environment in four sample collection years (*significant difference; $p \leq 0.05$)

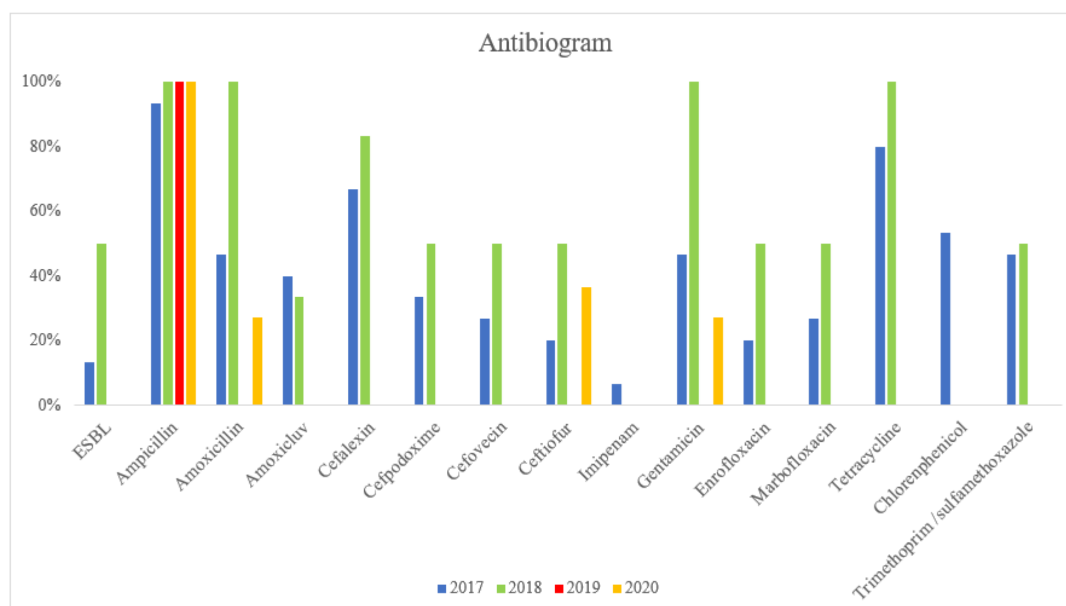


Figure 6. Comparison between resistance rates against 18 antimicrobials and extended-spectrum beta-lactamase (ESBL) in MCRPE isolates at four different sample collection times

CHULALONGKORN UNIVERSITY

Identification of plasmid-mediated colistin resistance genes

Of the colistin resistant isolates obtained in 2017, the *mcr*-1 gene was detected in eight of the pig isolates, while *mcr*-1 and *mcr*-3 were detected together in two of these, and *mcr*-3 alone in one pig isolate. At the same time, *mcr*-1 was detected in all four of the isolates from workers and in both the isolates from wastewater samples (Table 4). In 2018, after colistin withdrawal for one and a half years, *mcr*-1 was detected in three and *mcr*-3 in two of the five resistant isolates from pigs, and *mcr*-1 was found in the single resistant isolate from wastewater. In 2019 the single isolate from a breeder pig contained *mcr*-1. In 2020 *mcr*-1 positive *E. coli* isolates were found in all 5 piglets with mild symptoms of diarrhoea and in wastewater samples (6/10).

Table 4. Characterization of 33 colistin-resistant *mcr* positive *E. coli* isolates from different years and sources

Collection date	Source and number sampled	Number of resistant isolates obtained	*mcr* genes in resistant isolates	Virulence genes in *mcr* positive isolates
2017	Pigs (n=15)	9/15 (60%)	*mcr*-1 (8/15, 53.3%) *mcr*-3 (1/15, 20%)	*StaP*-*Stb* (5/9, 55.6%) *StaP*-*Stb*-*Stx2e* (1/9, 11.1%) Non-pathogenic (3/9, 33.3%)
2017	Humans (n=10)	4/10 (40%)	*mcr*-1 (4/10, 40%)	*StaP*-*Stb* (2/4, 50%) Non-pathogenic (2/4, 50%)
2017	Wastewater (n=10)	2/10 (20%)	*mcr*-1 (2/10, 20%)	*Stb* (2/2, 100%) Non-pathogenic (0%)
2018	Pigs (n=10)	5/10 (50%)	*mcr*-1 (3/10, 30%) *mcr*-3 (2/10, 20%)	*Stb* (2/5, 40%) Non-pathogenic (3/5, 60%)
2018	Wastewater (n=10)	1/10 (10%)	*mcr*-1 (1/10, 10%)	*Stb* (1/1, 100%) Non-pathogenic (0%)
2019	Pigs (n=30)	1/30 (3.33%)	*mcr*-1 (1/30, 3.33%)	Non-pathogenic (100%)
2020	Pigs (n=15)	5/15 (33.3%)	*mcr*-1 (5/15, 33.3%)	*Stb* (4/5, 80%) Non-pathogenic (1/5, 20%)
2020	Wastewater (n=10)	6/10 (60%)	*mcr*-1 (6/10, 60%)	*Stb* (2/6, 33.3%) Non-pathogenic (4/6, 66.7%)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Antimicrobial susceptibility determination

The antimicrobial resistance (AMR) profiles detected from MCRPE isolates are shown in Figure 6. ESBL-producing *E. coli* were identified, and most MCRPE isolates from the first and second samplings were found to demonstrate extreme pan-drug resistance. Interestingly, besides colistin, the isolate from the pig sample in 2019 was phenotypically resistant only to ampicillin. On the other hand, the MCRPE isolated from the last sample collection in 2020 were resistant to aminoglycosides, ampicillin, and ceftiofur, and those antibiotics were used for individual treatments on the farm. The antibiogram results comparing isolates between the 4 sampling years are presented in (Appendix 2 table 8). High rates of resistance to ampicillin, gentamicin, and tetracycline were detected in almost all MCRPE isolates at each sampling time.

Various plasmid replicon types were detected among the MCRPE isolates (Table 5). All the *mcr* positive isolates from different sources contained more than one replicon type. The incompatibility group IncFIB and IncI type plasmids were most commonly found. Although a variety of plasmid types were detected in pigs in 2017 and 2018, there was a decrease in varieties of plasmid types in later sample collection years. For the conjugation assay, the donor *E. coli* transferred *mcr*-1 and *mcr*-3 genes (as confirmed by PCR) to recipient J53 strains with a frequency of $1.7 \sim 2 \times 10^{-4}$.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Table 5. Plasmid replicon types detected in 33 colistin-resistant *E. coli* among the three categories of samples in each sample collection times

Trait	Pigs				Workers	Wastewater		
	2017	2018	2019	2020	2017	2017	2018	2020
	n=9	n=5	n=1	n=5	n=4	n=2	n=1	n=6
I1-Ir	+	+	-	+	-	-	-	+
HI1	+	+	-	-	-	-	-	-
HI2	+	-	-	+	-	-	-	-
N	+	+	-	-	-	-	-	-
X	+	-	-	-	+	-	+	-
FIB	+	+	+	+	+	+	-	+
FIA	+	+	+	-	-	-	-	-
FIC	-	+	+	-	+	-	+	-
P	+	+	-	-	-	-	-	-
Y	+	+	-	+	+	+	-	+
A/C	+	-	-	+	+	-	-	-
I	-	+	+	+	-	-	+	-

+: detected,-: not detected

Virulence gene detection

Virulence gene detection was performed on all the 33 *mcr* positive *E. coli* isolates. Most of the isolates from pigs contained genes associated with ETEC strains (enterotoxin genes), with *StaP* and *Stb* being the most frequent pathotype found in 2017 (Table 4). One strain from a pig in 2017 showed a hybrid ETEC–EHEC genotype. Two of the four colistin-resistant *E. coli* recovered from farm workers in 2017 contained a combination of *StaP* and *Stb* genes. In contrast, the wastewater samples and the piglets' samples obtained after 2017 only contained the *Stb* enterotoxin gene.

Molecular genotypic characterizations

Thirty-four diverse PFGE patterns were obtained for the 65 MCRPE isolates from different sources (Figure 7). No dominant pulsotypes were responsible for *mcr* gene clonal carriage. Moreover, most of the strains from each sample collection time were dispersed on different branches of the dendrogram and were not closely related genetically. The pulsotypes of the MCRPE from humans were not clonally related to any of those from pigs or wastewater. Strains with high similarity (>80%) occurred rarely and were found mainly in the same set of pig or human samples from the same sampling year. Only the MCRPE strains from piglets and wastewater samples in 2020 showed high clonal relatedness, suggesting that MCRPE strains from the piglets with diarrhoea had contaminated the wastewater.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

MLST gave similar results to PFGE, with most isolates belonging to different STs (Appendix 3 table 9). Isolates of the common *E. coli* clonal complex ST10 were detected in 2 pigs and one human sample on the first sampling, and in one wastewater sample on the last sample collection. Isolates belonging to ST 641 were detected in 2 pigs and one wastewater sample in 2018. In 2020, MCRPE isolates belonging to ST3345 (n=3 in wastewater, n=2 in pigs) and ST 5218 (n=2 in pigs) were commonly detected.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

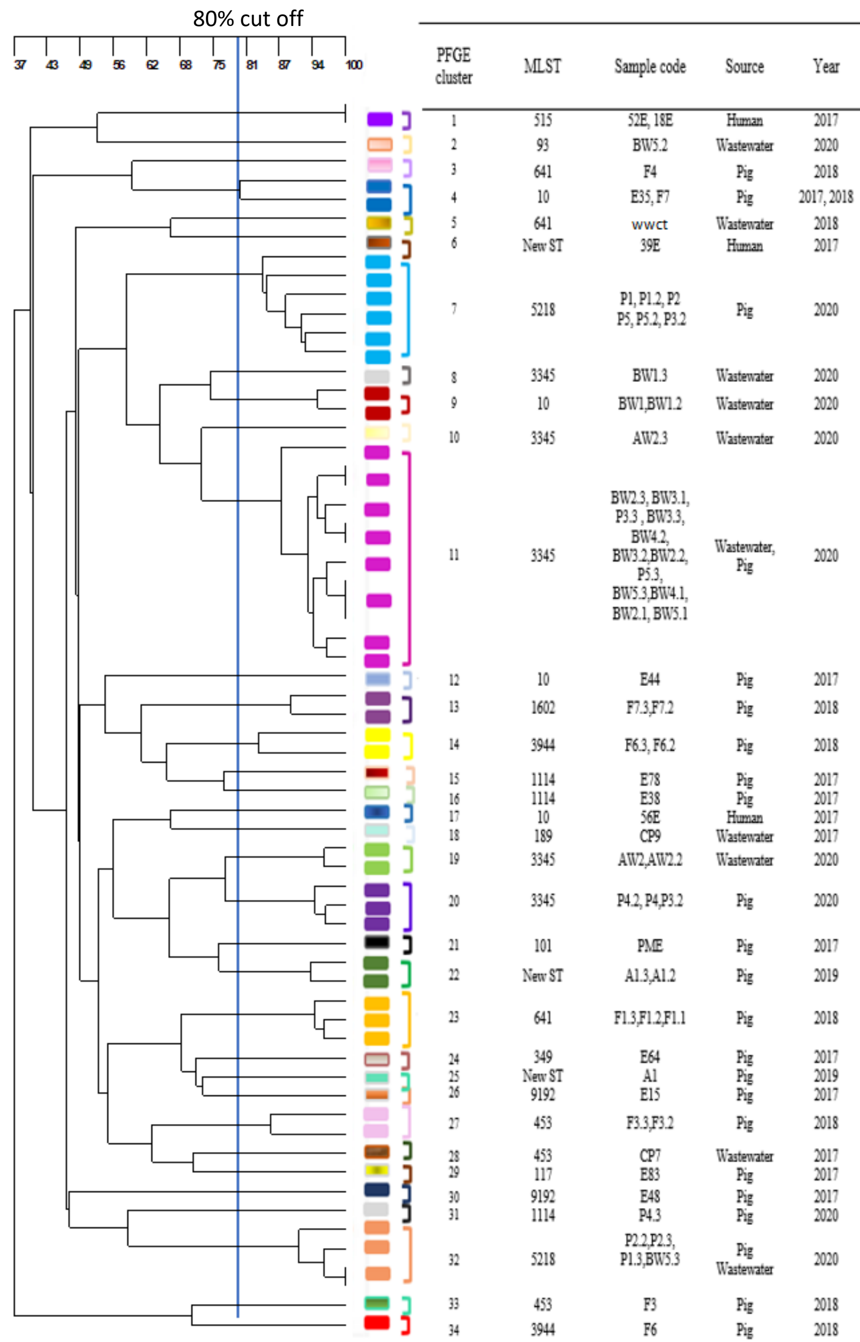


Figure 7. Dendrogram generated from pulsed field gel electrophoresis analysis demonstrating the genetic relatedness among 65 *mcr* positive *Escherichia coli* strains (one to three isolates per positive sample) that were obtained from different sources at each sampling time.

Discussion

The geographical distribution and characterization of colistin-resistant *E. coli* on large-scale pig farms across Thailand has been reported previously (Khine et al., 2020). The current longitudinal study investigated the persistence and diversity of *mcr* positive *E. coli* on a selected commercial Thai pig farm following withdrawal of prophylactic colistin usage. According to the farm history, batches of piglets previously were consistently prescribed colistin up until the time that it was withdrawn from use at the start of 2017. In searching for potential changes in resistance to colistin after its withdrawal, this study focused on examining colistin resistance in *E. coli* from young sows and their suckling pigs, as well as from wastewater. Prior to 2017 colistin was mainly used for controlling *E. coli* in suckling pigs in their first three weeks of life, so it seemed logical to target this bacterial species and this age group when looking for ongoing resistance. In addition, sows were examined since piglets become colonized by oral exposure from the faecal microbiota of their mothers. The sows were exposed to colistin prior to 2017 and might be persistently colonized and hence transfer resistant bacteria to their piglets. The piglets themselves were destined for slaughter by around 5-6 months of age, and so by definition could not be involved in direct transmission in following years. Accordingly, more sows than piglets were sampled to determine whether they still represented a potential long-term reservoir of MCRPE infection. Wastewater also was sampled, as wastewater tanks on pig farms serve as hotspots for

accumulating resistant bacteria since they are composed of pooled faecal discharge from a large number of pigs housed in the same area. Inclusion of this material in the study increased the likelihood of detecting MCRPE. Although relatively small numbers of samples were examined at each sampling time, they were sufficient to confirm the presence of MCRPE throughout the study.

Even following colistin withdrawal for 21 months, MCRPE that were carrying *mcr* genes were still quite commonly found in pigs, indicating that this period is insufficient to have a significant effect on reducing the presence of colistin resistant bacteria after the drug's withdrawal. The presence of *mcr* positive *E. coli* in the faeces of farm workers in mid-2017 is a matter of considerable concern. Bacteria from animals can be transmitted to humans either directly or through food or the environment, and then may transfer resistance genes to pathogenic bacteria that infect humans (Archawakulathep et al., 2014). Farm and food chain workers are likely to be exposed to resistant bacteria throughout the pig production cycle (You et al., 2016). Moreover, *Stb* and *StaP* virulence genes were found in MCRPE isolates from pigs and in two of the workers. These enterotoxin genes are linked to neonatal or postweaning diarrhoea in pigs, but bacteria carrying the genes also can be shed in faeces from healthy animals (Moredo et al., 2015). The *Stb* enterotoxin is commonly found in *E. coli* strains from pigs but is rarely found in humans and is not associated with diarrhoea in humans (Casey, Herring, Schneider, Bosworth, & Whipp, 1998; Echeverria et al., 1984). Therefore, these findings

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

suggested that subclinical ETEC carriers can be found at various stages in the pig production cycle, and may represent a source of transmission to humans. Even though isolates of identical genetic types were not found in humans and pigs, our results highlight possible transmission of *mcr* genes from bacteria infecting livestock to isolates that are present in humans and in the environment. The failure to recover MCRPE from human samples after 2017 may be associated with reduced exposure to colistin and/or to MRCPE from pigs and the environment, despite contamination of the latter still being detected 3.5 years after cessation of colistin usage. One possibility is that following identification of MCRPE in the workers in 2017 these individuals took greater care of their hygiene to reduce their exposure to MCRPE of pig origin.

Most pigs were still colonized with colistin-resistant *E. coli* when sampled 21 months after colistin withdrawal; however, by the third year there was a sharp decline in carriage by pigs and neither workers nor wastewater samples were positive for MCRPE. Therefore, the ban on the use of colistin as a prophylactic usage was highly likely beneficial for controlling the emergence and dissemination of *mcr*-1 on pig farms. Pigs reared for meat production are only kept for around 5-6 months before slaughter, although breeder pigs are retained for up to 3-4 years. Presumably transmission cycles of MCRPE between batches of pigs that are selected for meat production or breeding, and/or their exposure to contaminated environments allow them to remain as potential reservoirs for at least 3.5 years. This contrasted with a previous report from Britain,

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

where *mcr*-1 was undetectable in isolates from pigs after the cessation of colistin use for approximately 20 months (Duggett et al., 2018). The re-occurrence and increase in numbers of pigs shedding MCRPE and in isolates recovered from wastewater in the last year of the current study was noteworthy. These colonized pigs had suffered from diarrhoea and had been given therapeutic antimicrobial treatments, unlike the situation in previous batches sampled in earlier years. A possible explanation for the re-occurrence without selective pressure applied from colistin exposure may be the existence of cross-resistance between colistin and other therapeutic antibiotics used in the piglets. A similar phenomenon was reported in previous studies where colistin resistance was found when other antimicrobials such as quinolones or cephalosporins were used in livestock farms (Cao et al., 2020; N. T. Nguyen et al., 2016). However, more complete genomic characterization of the MCRPE isolates involved is required to investigate possible reason for this correlation. Nevertheless, these results are of concern because short-term β-lactam (ceftiofur) or gentamicin use in animals may select for *mcr*-1 in *E. coli* and maintain persistence on farms.

CHULALONGKORN UNIVERSITY

From the antibiotic susceptibility testing, some of the *mcr* positive *E. coli* isolates were found to be ESBL producers and showed extreme pan-drug resistance. A larger number of *E. coli* isolates with ESBL were observed in the samples from 2018 compared to the first sampling time. In Thailand, the application of antimicrobials in pig farms varies according to the management system and geographical area. In the central area

of Thailand, the antimicrobials that are mainly used are colistin, cephalosporins, tiamulin, amoxicillin, tilmicosin, aminoglycosides (gentamycin), and oxytetracycline (Pokhrel et al., 2019). The use of other antimicrobials during the production cycle of pigs could co-select for colistin resistance (Migura-Garcia et al., 2020; Vines et al., 2021). Resistances to other potential agents like heavy metals or biocides that may be linked with antibiotics resistance genes also are a matter for concern.

In the conjugation experiment, MCRPE recovered from pigs without selective pressures from colistin use showed a high transfer frequency. Moreover, various replicon types were found in the colistin-resistant *E. coli* isolates. According to previous reports, *mcr*-1 and *mcr*-3 genes have been found on IncI, IncHI2, and IncX4 plasmids (Kieffer et al., 2018). Likewise, *mcr*-1 was predominantly harbored on the IncX4 plasmid in isolates from healthy human beings in China (Y. Shen, Zhou, et al., 2018). Different AMR genes can be located on the same plasmid or on different plasmids within the same bacterial host, and represent multidrug resistant clones. Plasmids encoding the *mcr* genes, which co-exist with other antimicrobial resistance genes, are a problem for public health. To date, the majority of *mcr* genes have been identified in various plasmid types and are able to locate and/or transfer with other resistance genes by conjugation (M. Rhouma et al., 2019).

For the DNA fingerprinting results, a large number of pulsotypes were observed among the *mcr* positive isolates. Therefore, no epidemic strains were dominant on the

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

farm over time, and the *mcr* genes found in *E. coli* isolates were mainly plasmid-borne. A high diversity of MCRPE isolates from different hosts also was observed in a study from China (C. Shen et al., 2020). Similarly, in a Dutch study where ESBL positive *E. coli* from animals and humans were examined, ESBL transmission did not involve strain transfer but rather plasmid transfer by identical plasmids of the IncI and IncK types (de Been et al., 2014a). Nevertheless, in our study some clonal relatedness was found in MCRPE from piglets and wastewater samples at the last sampling. In this case the resistant bacteria from pigs were likely to be the primary source of *mcr* genes contaminating wastewater. Thus, despite only moderate persistence of *mcr* genes in pigs and low-level environmental dissemination in tested wastewater, the distribution of diverse strains with virulence potential from different niches across years is worrisome. Genes from these *mcr*-1and *mcr*-3 positive isolates might be transferred to other sources and/or other pathogens.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Conclusion

In this study, *E. coli* carrying *mcr* genes were recovered but in gradual declination for 3.5 years after cessation of colistin use. Hence, banning colistin for prophylaxis use was efficient for emergence and dissemination of *mcr*-1 on this pig farm. However, even in the absence of selective pressure exerted by colistin use, the application of other antimicrobials during the production cycle might co-select indirectly for the *mcr* genes and favour their spread. This study provides an initial insight into the

reduction in dissemination of colistin resistant *E. coli* from pigs and the farm environment. Further long-term genomic investigations are necessary to improve understanding and control of MCRPE and colistin-resistance in the pig industry.

Chapter IV

Genomic characterization of *mcr* positive *E. coli* isolates from Pig, Human and Wastewater along 3 investigation years after colistin withdraw from the farm

Nwai Oo Khine[1,2], Thidathip Wongsurawat[3], Piroon Jenjaroenpun[3], Nuvee Prapasarakul[1,4,*]

[1] Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,

[2] The International Graduate Program of Veterinary Science and Technology (VST), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand,

[3] Division of Bioinformatics and Data Management for Research, Research Group and Research Network Division, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

[4] Diagnosis and Monitoring of Animal Pathogens Research Unit (DMAP), Bangkok, Thailand

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

*Correspondence:

Nuvee Prapasarakul

nuvee.p@chula.ac.th

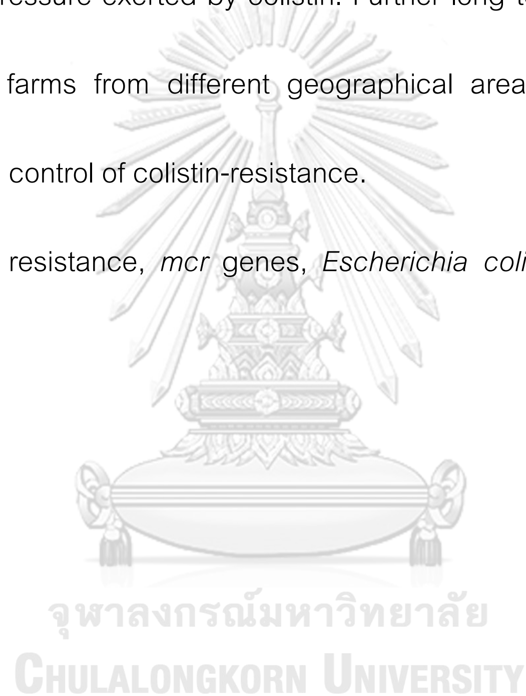
Abstract

The massive use of colistin in swine farms for prophylaxis purposes is believed to be the key factors for the development of *mcr* harboring bacteria. Even after colistin cessation, the *mcr* positive *E. coli* (MCRPE) were persisted in pig carriages over 3.5 years had been assessed. The aim of this study was to identify the underlying factors which enhance the maintenance of *mcr* genes in the pig farm. Here, whole genome sequencing (Illumina for short read and Nanopore for long read) was carried out on six MCRPE isolates selected from pigs, farm workers and wastewater under the same farm environment. The *mcr*-1.1 genes were carried by the epidemic and high stability IncI2 and IncX4 plasmid types. Whereas, one porcine isolate contained two *mcr*-3 variants (*mcr*-3.2 and *mcr*-3.5) on IncFII and IncHI2 plasmids respectively. The *mcr*-3.5-IncHI2 plasmid possessed multidrug resistant (MDR) region with various mobile genetic elements. The genetic environments encompassing *mcr*-1 genes showed variability, but conserved structures were found within the same plasmid family. Although these 6 MCRPE isolates belonged to different *E. coli* lineages, identical *mcr* bearing plasmids were detected between pigs and environment from different investigation years. The MCRPE isolates showed both phenotypic and genotypic MDR traits. Moreover, heavy metals resistant genes were discovered in the genomes of MCRPE isolates with copper and silver resistant genes being the most dominantly found. Quaternary ammonium compounds resistant (*qacC*) gene was detected on *mcr*-3 harboring IncHI2 plasmid of

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

pig and all the isolates from wastewater (n=2) contained antiseptic resistant genes. Additionally, virulence factors of different pathotypes such as ETEC, hemolysin and type III secretion factors related genes were also observed in the MCRPE isolates. This study identified that the plasmid stability nature and co-selection by other antimicrobials as well as antiseptics could be the source for maintenance of *mcr*-genes in pig carriages without selective pressure exerted by colistin. Further long-term genomic investigations approach on pig farms from different geographical areas are needed to improve understanding and control of colistin-resistance.

Keywords: colistin resistance, *mcr* genes, *Escherichia coli*, plasmids stability, hybrid sequencing


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Introduction

The emergence and rapid spread of multidrug-resistant (MDR) bacteria have been a serious global public health threat over the past decade. Colistin being the last-line antibiotics in combating carbapenem resistant infections and the discovery of colistin resistant *mcr* genes by horizontal transmission has threatened the medical community (Kieffer et al., 2018). The *mcr*-1 gene being the most predominant among *mcr* genes family and has now been reported in numerous countries in different ecological niches (C. Shen et al., 2020). Moreover, *mcr*-1 & *mcr*-3 genes have been detected in diverse plasmid types such as IncX4, IncI2, IncHI2 plasmids (Q. Wang et al., 2017; Yin et al., 2017). Moreover, those *mcr* bearing plasmids showed ability to locate and/or transfer with other resistant genes by conjugation (M. Rhouma et al., 2019). Conjugative plasmids are indeed the most important vehicles in transmission and evolution of resistant genes in bacteria (A. San Millan, 2018). Bacteria harboring plasmids which contained the *mcr* genes together with other antimicrobial resistance (AMR) genes is burdensome for clinical practice. Moreover, resistance genes located in the transposable genetic elements facilitate in intracellular DNA mobility (Partridge, Kwong, Firth, & Jensen, 2018). In case of *mcr*-1 gene, the insertion sequence (IS) ISApl1 (IS 30 family) is reported to be responsible for mobilization (Snesrud, McGann, & Chandler, 2018). The *mcr*-3 gene has been reported to be associated with transposon

TnAs2 and ISKpn40, although the proof of these mobile genetic elements responsible for *mcr*-3 transmission is variable (Wang, Fu, Du, Jiang, & Wang, 2018; Yin et al., 2017).

The rapid dissemination of *mcr* bearing bacteria in livestock animals attributed by the extensive use of colistin as prophylaxis and enhancing horizontal transfer of *mcr* genes. Several reports have been strongly implied that farmed animals as the source of *mcr*-1 spreading to humans. Moreover, the co-selection of *mcr* genes and/or the cross-resistance of colistin by usage of biocides or other antibiotics had been reported (Wand et al., 2017; F. Xu et al., 2018). Numerous cases of *mcr* positive bacteria especially *E. coli* isolated from humans, animals and the environment globally are concerning. Although there are several cases of colistin resistant *Enterobacteriaceae* from livestock and human cases have been reported, the detail genomic characterization of *mcr*-positive *E. coli* especially of livestock isolates is scarce in Thailand. This study follows the longitudinal monitoring of *mcr*- positive *E. coli* (MCRPE) on a pig farm following colistin cessation by genomic investigations of MCRPE isolates and their associated plasmids on-farm persistence. The aim of this study was to investigate the genomic comparison and characterization of *mcr*-1 and *mcr*-3 positive plasmids from pigs, humans and environment in response to the colistin ban, using whole genome sequencing (WGS).

CHULALONGKORN UNIVERSITY

Materials and methods

Sampling and Identification

This study followed our previous study on the longitudinal monitoring of the MCRPE isolates on the colistin withdraw farm from 2017-2019. The pig feces, wastewater and human cotton swab samples were collected from the industrial pig farm located in the central area of Thailand. This farm withdrew prophylactic colistin use in piglets since 2017, following the guidance of the DLD. The isolates were identified as *E. coli* using IMViC biochemical tests and Matrix-Assisted Laser Desorption Ionization combined with time of-flight analysis (MALDI Biotyper, Bruker, USA), according to the manufacturer's recommendations (Singhal et al., 2015). The *mcr*-1-5 genes were detected by multiplex PCR according to (Rebelo et al., 2018).

Strain selection and antimicrobial susceptibility testing

Total of 6 MCRPE isolates; n=3 from pigs, n=2 from wastewater and n=1 from human between 2017-2019 were selected for whole genome sequencing. These six *mcr*-bearing strains having different pulsed-field gel electrophoresis (PFGE) profiles meaning these strains descended from various separate ancestors. The MIC for colistin was carried out by using the broth microdilution technique following CLSI guidelines (CLSI, 2020). An MIC value of >2µg/ml was considered as colistin resistance (CLS, 2020). The antimicrobial susceptibility testing for *mcr*-1 positive *E. coli* isolates was performed by using the AST-GN 38 test kit in a Vitek2 compact automated susceptibility level detection apparatus (BioMérieux, France) (Khine et al., 2020).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Whole Genome Sequencing

The genomic DNA of *E. coli* isolates from pigs, workers and wastewater samples was extracted by using ZymoBIOMICS DNA Miniprep Kit according to manufacturer instructions. The extracted DNA was undergone quantity check by Qubit Fluorometer. The quality check was performed by nanodrop according to recommendation (A260/280 1.8~2). Then, the samples were sent for Illumina NovaSeq PE150 platform (2 x150 bp) (short read sequencing) and MinION (Oxford Nanopore Technologies for long read sequencing).

Sequence analysis

The paired-end reads were quality filtered to remove adapters and, low-quality sequences with quality scores <30 by using Trimmomatic v.0.36.5 (Bolger et al., 2014). The related bioinformatic analyses was performed on European Galaxy server (https://usegalaxy.eu). The clean raw reads were assembled and analyzed using the Unicycler hybrid assembly (Galaxy Version 0.4.8.0) with default settings (Wick, Judd, Gorrie, & Holt, 2017). Sequences were analyzed for the species identification (KmerFinder 2.1), Multilocus Sequence Typing (MLST 1.6), identification of virulence (VirulenceFinder 1.2), antimicrobial resistance genes (ResFinder 2.1), plasmid incompatibility groups (PlasmidFinder 1.2) and mobile element finders using the Center for Genomic Epidemiology (CGE) pipeline (Alba et al., 2018). Acquired antimicrobial resistance genes (ARGs), and *E. coli* virulence factors were also identified using

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

ABRicate (Galaxy Version 1.0.1). The databases used for the identification of AMR genes on ABRicate were: CARD Resistance Gene Identifier (McArthur et al., 2013), and ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation) (Gupta et al., 2014) databases and for virulence genes by VFDB databases (Chen, Zheng, Liu, Yang, & Jin, 2016). The genomes of MCRPE isolates were annotated by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) and Prokka (Prokaryotic genome annotation) (Galaxy Version 1.14.6) (Seemann, 2014). Screening of biocides resistant genes were carried out on BacAnt server (Hua et al., 2021) by using ResDB and BacMet: antibacterial biocide and metal resistance genes databases (Pal, Bengtsson-Palme, Rensing, Kristiansson, & Larsson, 2014).

The contigs of *mcr* variants were compared with reference sequences using BLAST. The plasmids carrying *mcr* genes were compared with references belonging to the same Inc groups using BLAST ring image generator (BRIG) (Alikhan, Petty, Ben Zakour, & Beatson, 2011) and the genetic context of *mcr*-1 and *mcr*-3 contigs were visualized by using Easyfig (http://mjsull.github.io/Easyfig/) (Sullivan, Petty, & Beatson, 2011). All reference plasmids and sequences used in this study were recovered from NCBI database.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Nucleotide Sequence Accession Numbers

The complete nucleotide sequences of CP52E, CPE35, CPWW7, CPF6, CPWWCT and CPA1200 were deposited in GenBank under the accession numbers

CP075731, CP075722, CP075716, CP075737, CP076575 and JAHKSR000000000, respectively.

Results

Genomic Characterization of *mcr* positive *E. coli* isolates

Total of 6 MCRPE from three investigation years (2017-2019) were submitted for Whole genome sequencing. These isolates comprised colistin MIC value of 4-8 mg/L and belonged to different PFGE profiles and ST types. Of 6 isolates, *mcr*-1.1 were detected in 3 samples and all showed 100% identity to KP347127; the first identified *mcr*-1 gene from pig in China. The *mcr*-3.5 gene in one sample and *mcr*-3.2 in 2 samples. The *mcr*-1 gene was not found in the *E. coli* of wastewater origin from 2018 even though it had tested positive by PCR. The genome sizes of *mcr* positive *E. coli* isolates range from 4~4.8 Mb and each isolates contained various plasmid replicon types. Among the two porcine *mcr*-3 positive *E. coli* isolates, one pig contained *mcr*-3.2 on IncFII plasmid and the other contained two *mcr*-3 variants (*mcr*-3.2 and *mcr*-3.5) on two different plasmids of IncHI2 and IncFII plasmids respectively. None of the MCRPE isolates resistant to colistin by chromosomal mutation and resistance was solely due to plasmid mediated *mcr* genes. The *mcr*-1.1 genes were located on IncI2 and IncX4 plasmids. Detailed information about the colistin resistant *E. coli* isolates detected by WGS including serotypes of respective strains detected by Serotype finder 2.0 from each source was presented in table 6. Several plasmids both phenotypically known and

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

unnamed plasmids were detected from all MCRPE isolates. IncF and IncI group plasmids were highly detected among all samples. The pigs and wastewater samples contained relatively higher number of replicon types than human isolate (Figure 8). Moreover, all the plasmid replicon types detected in human isolate found to be contained in both pig and environmental samples.

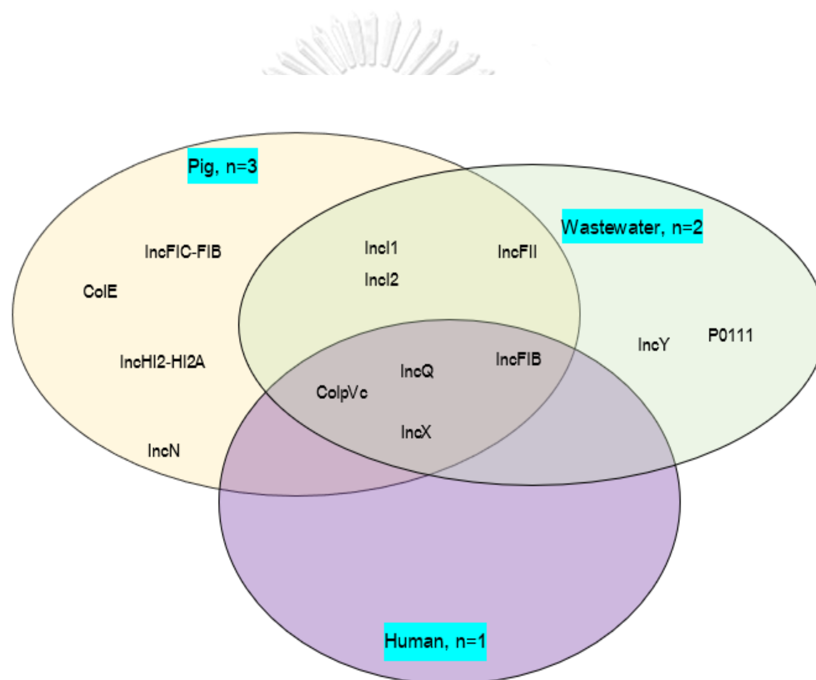


Figure 8. The Venn diagram of the various plasmid replicon types detected from 6 MCRPE isolates

Antibiotic Resistance genes and Virulent genes

A total number of 31 ARGs were identified using Resfinder 4.1 (CGE) and Abricate (Galaxy Version 1.0.1) which encoded resistance to different antibiotic classes such as aminoglycosides, beta-lactams, cephalosporins, fluoroquinolones, trimethoprim, macrolides, chloramphenicols, sulfonamides, tetracyclines and macrolides (Figure 9). The disinfectants: quaternary ammonium compounds and hydrogen peroxide resistant genes were detected in 2 pigs' collected from 2018 and 2019 and in all wastewater samples. Interestingly, most of the plasmids except pCPF6-IncHI2 that harboured *mcr* genes were found to be mono-resistant to colistin only. Moreover, the genomes of 4 MCRPE isolates (except CPE35 and CPWW7) contained resistant genes to heavy metals such as copper (*pcoA, pcoB, pcoC, pcoD, pcoR, pcoS, pcoE*), silver (*silE, silS, silC, silF, silB, silA, silP*) and Zinc (*zntA*) (Appendix 6 table 10). Whereas, the CPWW7 from wastewater carried mercury resistant genes (*merR_Ps, merT, merC*) on the plasmid which harbored various aminoglycosides resistant genes on the same plasmid. Genes encoding a multidrug resistance efflux pump such as *emrD, mdtM mdfA* were also detected on the chromosome of MCRPE isolates.

Moreover, all MCRPE isolates carried various virulence factors; (Appendix 4 figure 16) with majority of virulence genes were presented on the chromosomes of all isolates. The virulent genes of different pathotypes such as adherence factors, flagellar associated proteins, fimbrial adhesin proteins, *hlyE, hlyF* (hemolysin), type III secretion

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

system related genes, toxins *astA* (enteroaggregative heat-stable toxin, EAST-1), *fyuA* siderophore receptor genes were detected. The type III secretion systems, adhesion related and hemolysin genes (*hlyE*) were the most dominant one.

Table 6. Genomic features including MLST, serotypes, resistance, virulence profiles of the six *E. coli* strains tested by whole genome sequencing

Strain	Year	Source	ST	Contig	Location	Serotype	Resistance genes	Virulence genes
CP 52E	2017	Human	515	CP52E-Chromosome	Chromosome	O128: H12	*dfrA12, mdf(A)*, *tet(A)*, *sul3*, *bla*$_{TEM-1B}$ *bla*$_{CTX-M-14}$, *cmlA1* *aph(3'')-Ib*, *aadA2* *aph(6)-Id*, *aph(3')-Ia*, *aac(3)-IId*, *aadA1,sul1*	*fyuA*, *gad*, *irp2*, *terC*, *hlyE*, T3SS
				pCP52E-IncFIB	Plasmid		*bla*$_{TEM-1B}$	
				pCP52E-IncX4	Plasmid		*mcr*-1.1	
				pCP52E-ColpVC	Plasmid			
CP E35	2017	Pig	10	CPE35-chromosome	Chromosome	O101:H9	*tet(A)*	*gad, iss*, *terC*, *hlyE*
				pCPE35-IncFIB	Plasmid		*bla*$_{CMY-2}$	
				pCPE35-IncX1	Plasmid		*aadA1, aadA2* *aph(3')-Ia*, *aph(6)-Id*, *bla*$_{TEM-1B}$, *cmlA1*	

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

							dfrA12, strA, sul3	
				pCPE35-IncFII	Plasmid		*erm(B), mcr*-3.20	
				pCPE35-ColE10	Plasmid			
				pCPE35-ColPVc	Plasmid			
CPWW7	2017	Waste water	453	CPWW7-chromosome		O23:H16	*mdf(A), sitABCD*	*gad, iss, fyuA lpfA, astA, hlyE, hlyF, terC,* T3SS
				pCPWW7-IncFII	Plasmid		*erm(B), qnrS(1)*	
				pCPWW7-IncI2	Plasmid		*mcr*-1.1	
				pCPWW7-IncY	Plasmid		*tet(A), aadA2, sul1, sul3, qacE, dfrA12*	
				pCPWW7-unnamed plasmid	Plasmid		bla_{TEM-1B}, *aph(6)-Id, aph(3')-Ia, aph(3'')-Ib aadA22, aac(3)-IId, sul2, catA2*	
CPF6	2018	Pig	3944	CPF6-chromosome	Chromosome	O8:H2		*fyuA, gad, terC traT, hlyE* T3SS

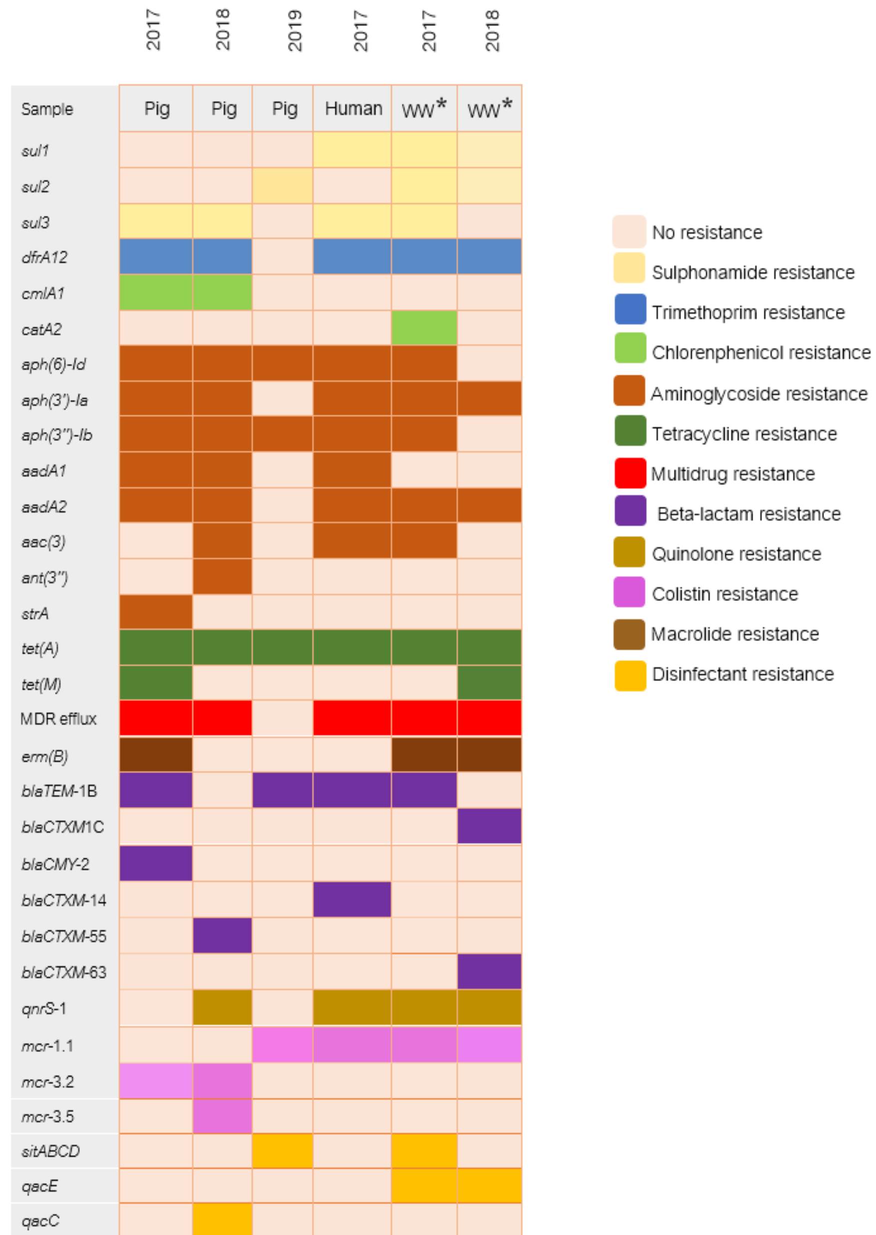
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

				CPF6-IncHI2	Plasmid		*aac(3)-IId, aadA1 aadA2, aph(3'')-Ib aph(3')-Ia, aph(6)-Id* bla$_{CTX-M-55}$, *cmlA1 dfrA12, mcr-*3.2, *qacC, qnrS1, sul3, tet(A)*	
				CPF6-IncFII	Plasmid		*mcr*-3.5	
				pCPF6-IncFIB	Plasmid			
				pCPF6-IncI1-I	Plasmid			
				pCPF6-IncX1	Plasmid			
CPWWCT	2018	Waste water	453	CPWWCT-chromosome	Chromosome	O70:H10	*sul2, sul1, mdf(A), bla*$_{CTX-M-63}$, *dfrA12, aadA2, qnrS1, qacE*	*gad, iss, terC, hlyE,* T3SS
				pCPWWCT-unnamed plasmid	Plasmid		*sul1, aph(3')-Ia, aadA2, qacE*	
				pCPWWCTp0111	Plasmid		*bla*$_{CTXM-1C}$	
				pCPWWCT-IncI1	Plasmid			
				pCPWWCT-IncQ1	Plasmid			
				pCPWWCT-ColpVC	Plasmid			
				pCPWWCT-	Plasmid		*tet(M), tet(A),*	

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

				IncX1			*erm(B), qnrS1*	
CP A1200	2019	Pig	New ST	CPA1200-chromosome	Chromosome	08:H2	*mdf(A)*	*iss, ompT, terC, hlyE, hlyF*
				pCPA1200-IncFIB-FIC	Plasmid		*aph(6)-Id, aph(3'')-Ib,* bla_{TEM-1B}*, tet(A), sul2, sitABCD*	*iss, iucC, iutA, ompT, sitA, traT tsh*
				pCPA1200-IncI2	Plasmid		*mcr*-1.1	
				pCPA1200-IncI1	Plasmid			
				pCPA1200-IncQ1	Plasmid			
				pCPA1200-IncFII(pCoo)	Plasmid			

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY



*WW= wastewater

Figure 9. Heatmap showing the presence or absence of antimicrobial resistance genes of different antibiotic classes in 6 MCRPE from pig, human and environment

Genomic insights into the *mcr*-1 *and mcr*-3 positive *E. coli* strains

Of the 6 MCRPE isolates investigated by WGS, 5 carried the *mcr*-genes in plasmids (IncX4; n=1, IncHI2; n=1, IncI2; n=2 and IncFII; n=2). The ~33kb pCP52E-IncX4 plasmid harbored by *E. coli* of human origin from 2017 did not contain any resistance genes except *mcr*-1.1 on the same plasmid and no ISApl1 elements were found to flank the *mcr*-1.1 gene. The typical plasmid backbone features such as type IV secretion system (T4SS) proteins, toxin-antitoxin system (HicA-HicB) were present. A structural comparison of pCP52E-IncX4 against other reference IncX4 plasmids that contained *mcr*-1 is shown in (Figure 10). The IncX4-*mcr*-1 plasmid in this study showed 95% in coverage and 99.95% in identity to pCSZ4 (GenBank no. KX711706) from *E. coli* of pork origin in China.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

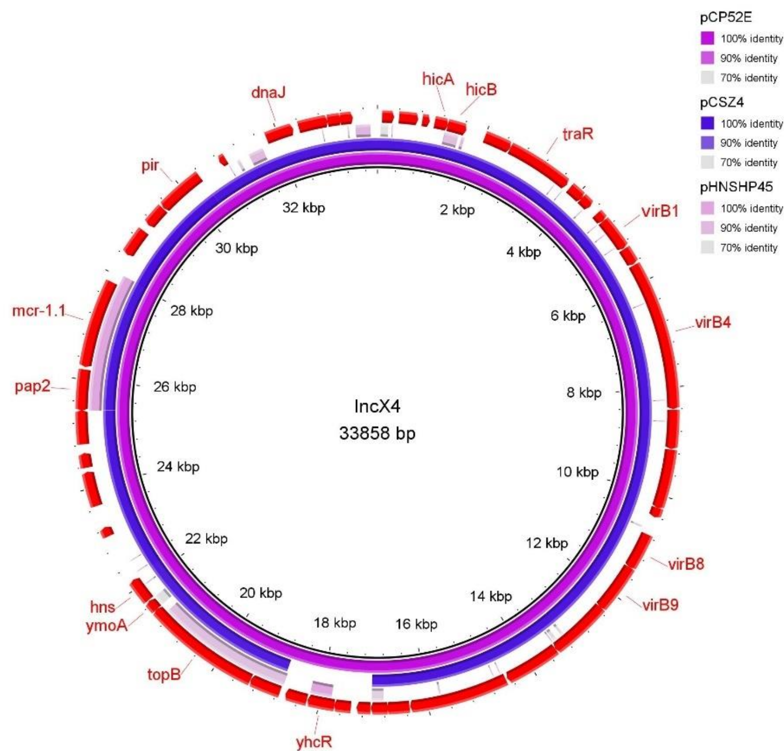
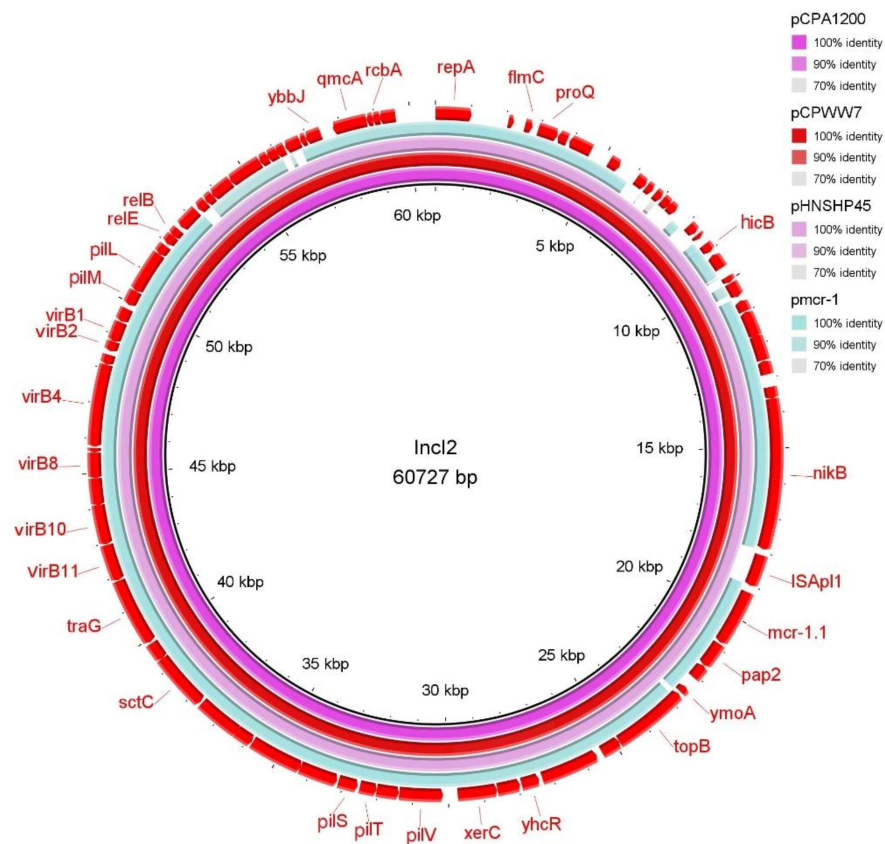


Figure 10. Sequence alignment of pCP52E-IncX4 *mcr*-1 plasmid with pCSZ4 (GenBank no KX711706) and pHNSHP45 (GenBank no KP347127). The outer circle with red arrows denotes annotation of the plasmid pCP52E-IncX4.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

The *mcr*-1.1 positive IncI2 plasmids of pCPA1200 (pig, 2019) and pCPWW7 (wastewater, 2017), were found to be identical showing 99% in coverage with 100% in identity. The structural comparison of the pCPA1200 with pCPWW7 and pHNSHP45 (IncI2 plasmid of first *mcr*-1 report from China) was shown in Figure 11. Moreover, these IncI2 plasmids contained numerous conjugation related genes such as T4SS, pilus modification and conjugative transfer system proteins. The ~60kb IncI2 plasmids did not carry other antimicrobial resistance (AMR) genes except *mcr*-1.1 and showed the same genetic structure ISApl1-*mcr*-1-pap2 with loss of downstream ISApl1. The comparison of

genetic environment of the *mcr*-1.1 cassette from IncI2 and IncX4 plasmids from this study and the references plasmids were demonstrated in figure 12.



CHULALONGKORN UNIVERSITY

Figure 11. Sequence alignment of pCPA1200-IncI2 *mcr*-1 plasmid with pCPWW7-IncI2 (This study) and pHNSHP45 (GenBank no. KP347127) and p*mcr*1_IncI2 (GenBank no. KU761326). The outer circle with red arrows denotes annotation of the plasmid pCPA1200-IncI2.

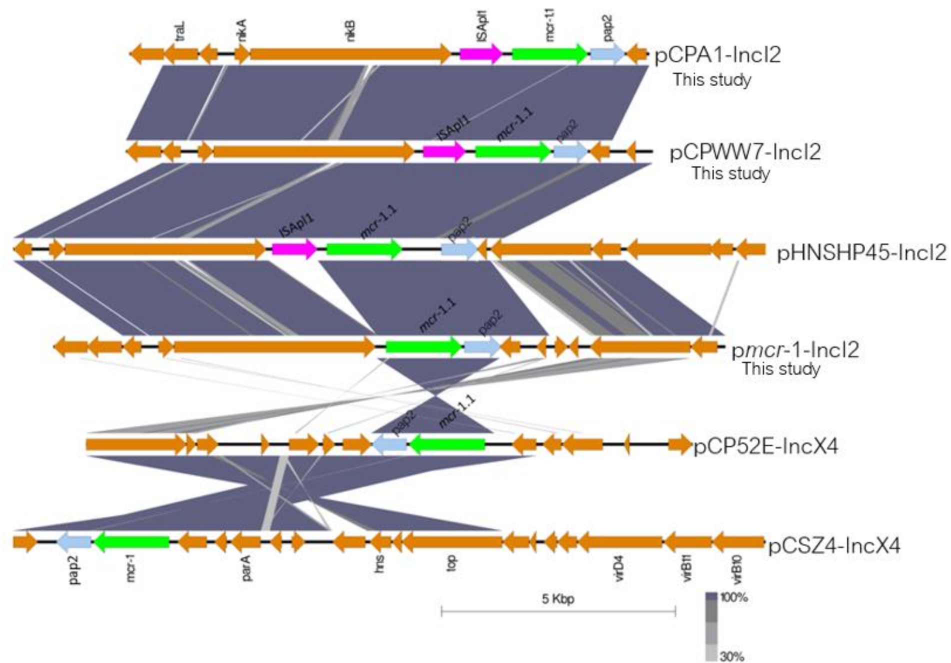


Figure 12. Comparison of the genetic environment of *mcr*-1.1 gene from MCRPE of this study with references plasmids. The gray area indicates the blast identities, the percentage of identity is indicated in the legend. Open arrows represent coding sequences (green for *mcr*-1.1, blue for PAP2, purple for ISApl1 and yellow for other genes. The arrow size is proportional to the gene length. The image was generated using EasyFig with default parameters.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

The ~83kb IncFII plasmids which carried *mcr*-3.2 from pCPE35 (pig origin from 2017) and *mcr*-3.5 from pCPF6 (pig origin from 2018) showed 92% coverage with 99% identity. Notably, the above two IncFII plasmids also showed highly similar to pECQ4552 (GenBank no. CP077064.1) that belonged to *mcr*-1 and *mcr*-3 producing *E. coli* of pig in France (figure 13). Both IncFII plasmids harbored conjugation related transfer proteins (tra). Moreover, pCPE35-IncFII plasmid consisted of microcin producing protein (McmM) while pCPF6-IncFII consisted of MDR efflux pump (Tap). The *mcr*-3.2 variants from pCPF6 located on IncHI2 plasmid also shared high identity with the IncHI2 plasmid pWJ1 (GenBank no. KY924928) of porcine *Escherichia coli* from China (Figure 14). The genetic arrangements in the vicinity of *mcr*-3.2 showed TnAs2–*mcr*-3.2–dgkA–ISKpn40. In contrast, *mcr*-3.5 on pCPF6-IncFII plasmid was flanked by TnAs2-*mcr*-3.5-dgkA-IS26 (Figure 14). The pCPF6-IncHI2 consisted multiple resistant genes of aminoglycosides, tetracycline and ESBL. Moreover, disinfectant resistant gene (*qacC*) and integron (*IntI*1) were also detected downstream of *mcr*-3.2 gene on the same plasmid. The comparison of genetic environment surrounding *mcr*-3 cassette from IncFII and IncHI2 plasmids from this study and the references plasmids were demonstrated in Figure 15.

CHULALONGKORN UNIVERSITY

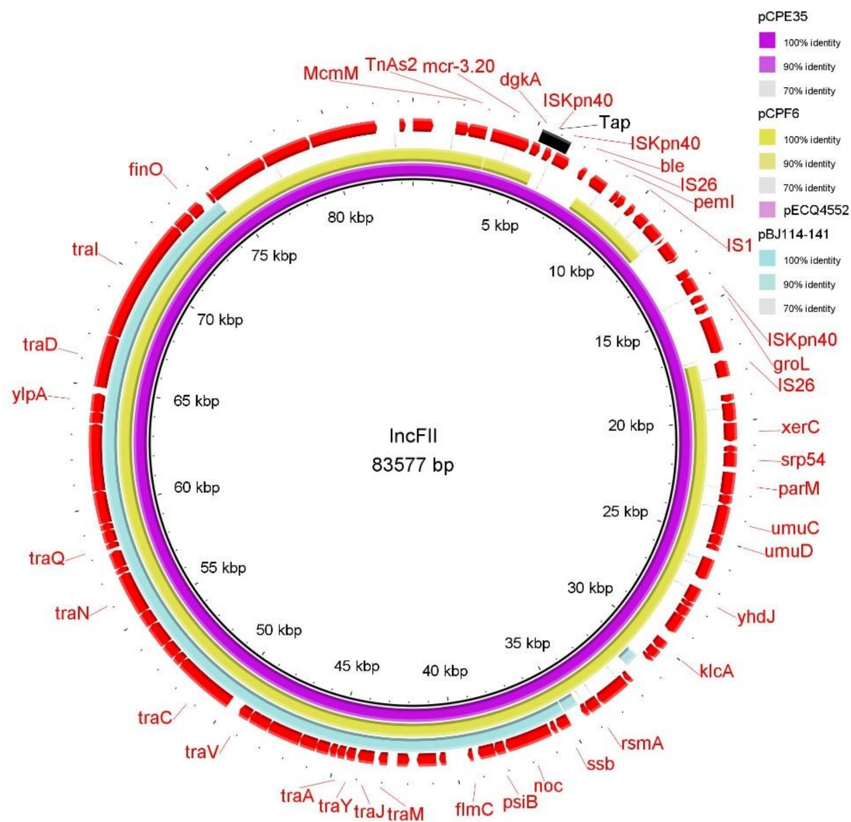


Figure 13. Sequence alignment of pCPE35-IncFII *mcr*-3.2 plasmid with pCPF6-IncFII *mcr*-3.5 plasmid (This study) and the reference plasmids pECQ4552 (GenBank no. CP077064.1), pBJ114-141 (GenBank no. MF679146). The outer circle with red arrows denotes annotation of the plasmid pCPE35-IncFII. The black bar represents multidrug efflux pump (Tap) from pCPF6.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

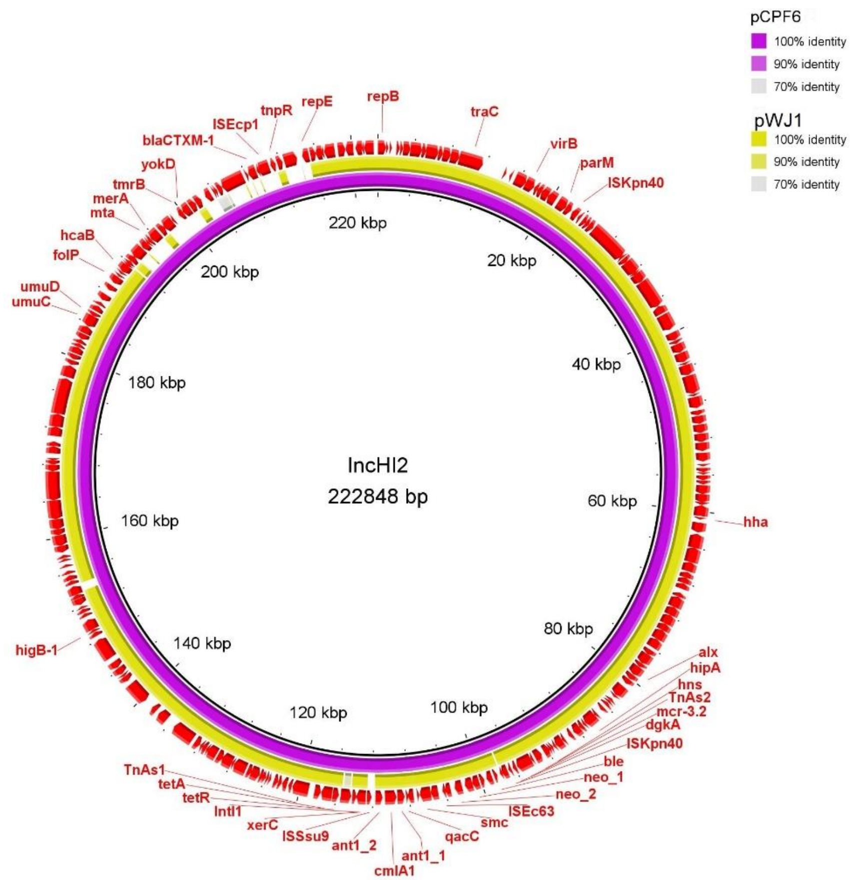


Figure 14. Sequence alignment of pCPF6-IncHI2 *mcr*-3.2 plasmid with pWJ1 (GenBank no KY924928). The outer circle with red arrows denotes annotation of the plasmid pCPF6-IncHI2.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 15. Comparison of the genetic environment of *mcr*-3 genes from MCRPE isolates of this study with references plasmids. The gray area indicates the blast identities, the percentage of identity is indicated in the legend. Open arrows represent coding sequences (green for antimicrobial resistance genes, blue for dgkA (orf), purple for mobile genetic elements and yellow for other genes. The arrow size is proportional to the gene length. The image was generated using EasyFig with default parameters.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

The *E. coli* isolate pCPWWCT origin of wastewater sample of 2018 didn't find *mcr*-1 after in silico analysis by WGS, even though previous PCR tested was positive. Seemingly, the isolate lost the *mcr*-1 plasmid during sub-culturing, resulting in a false-negative result. When we analyzed the plasmids that contained in this *E. coli* strain and found that the IncX1 plasmid harbored various mobile genetic elements together with various AMR genes (Appendix 5 figure 17).

Discussions

The longitudinal monitoring on the *mcr*-1 *and mcr*-3 positive *E. coli* after withdrawal of colistin led to a declining trend of the prevalence in pigs and environment with complete elimination in farm workers had been carried out. However, besides declination, the potentially pathogenic MCRPE were found to be persisted over 3.5 years of observation and pigs being the primary source of dissemination. Therefore, identify the underlying factors which allowed the maintenance of *mcr* genes and characterization of mobile genetic elements that might enhance in stabilization was essential for AMR control. To the best of our knowledge, this is the first report on genomic analysis and comparison upon persistence of *mcr* genes in *E. coli* from pigs, human and environment under the same farm after colistin withdraw by WGS approach in Thailand.

Based on the screening of colistin resistant isolates by PCR from our previous work, the *mcr*-1 genes were persisted in pigs for more than 2 years after colistin

withdraw. On the other hand, *mcr*-3 genes were found to be eliminated once the selective pressure was withdrawn. According to *E. coli* clonal typing techniques, the diverse MCRPE clones were distributed on the farm between different sources along investigation years. Moreover, all the MCRPE isolates were multidrug resistant clones according to phenotypic antimicrobial susceptibility testing by Vitek2 automated susceptibility machine. In accordance with these results, the 6 isolates that selected for WGS also showed resistant genes of various antibiotic classes both on chromosomes and plasmids. However, no other resistant genes were located on the same plasmids that carrying *mcr* genes except in pCPF6-IncHI2 plasmid. Moreover, antiseptic resistant genes as well as biocides resistant genes were also detected in MCRPE isolates. Prolonged exposure of *E. coli* strains to sub-lethal doses of antiseptic or biocides led to the development of resistant bacteria. Since biocides were often composed in agricultural products and feed additives, and their stability nature in environment act as the prolong exposure and selective pressure on bacteria (Seiler & Berendonk, 2012). Notably, bacteria resistant to above compounds could co-select and co-expression of various antibiotic resistant genes (McNeilly, Mann, Hamidian, & Gunawan, 2021).

Furthermore, the MCRPE isolates not only displayed MDR profiles, but also contained various virulence factors both on chromosome and on plasmids. Moreover, the isolate CPE35 of pig origin belonged to ST10 with serotype O101: H9, which is reported to be associated with animal and human diseases (He et al., 2021). Moreover,

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

the above serotype had been reported in Shiga toxin-producing *E. coli* (STEC) in humans and from enterotoxigenic *E. coli* (ETEC) of diarrheal calves in Europe (Begaud, Mondet, & Germani, 1993; Contrepois, Bertin, Pohl, Picard, & Girardeau, 1998). The serotype O128: H12 of MCRPE from human; CP52E, had been found in ETEC as well as in enteropathogenic *E. coli* (EPEC) (Contrepois et al., 1998). Therefore, healthy pigs could be carrier for MDR bacteria which also harbored various virulence genes and disseminate to human.

The epidemic plasmids IncI2 and IncX4 were responsible for the acquisition of *mcr*-1.1 genes in this study. IncI2 plasmids were largely detected in various *mcr*-1 cases from different hosts around the world (R. Wu et al., 2018). Meanwhile, IncX4 plasmid type had been reported as the dominant *mcr*-1-carrier in healthy humans in China (C. Shen et al., 2020) and carbapenem and *mcr*-1 co-carrying *Enterobacteriaceae* of clinical patients across Thailand (Paveenkittiporn, Kamjumphol, Ungcharoen, & Kerdsin, 2020). The IncX4 plasmids were reported to be genetically least variable and relatively smaller in size. (R. Li et al., 2021). Meanwhile, IncI2 replicon type plasmids were known to be having strongest competitive and fitness advantage to host when compared to other plasmid types such as IncHI2 or IncX4 plasmids (W. Li et al., 2021; R. Wu et al., 2018). These findings also applied with our study that the *mcr*-1-IncI2 plasmid was still recovered in *E. coli* isolate even after colistin ban in the farm for 3 years. In accordance to our results, the previous study also found the *mcr*-1 bearing

CHULALONGKORN UNIVERSITY

IncI2 plasmids persisted in fattening pigs with lack of colistin exposure (R. Wu et al., 2018). It is noteworthy that the IncI2 plasmids contained high conjugation transfer systems than genes associated in replication which leaded that they are dedicated to spread from host to host than replication (figure. 11). This explained why *mcr*-1-IncI2 plasmids are less diverse and found in wide diversity of bacteria (Meinersmann, 2019).

On the other hand, *mcr*-3 genes were detected on IncFII and IncHI2 plasmids which were reported to be the most divergent and encompassed MDR region with a variable order of AMR genes (Fang et al., 2018). The *mcr*-3 gene was first identified in IncHI2 plasmid in China (Yin et al., 2017) and *mcr*-3 mediated IncP and IncFII plasmids in *E. coli* had been reported in Thailand (Tansawai et al., 2021). The MDR plasmid of pCPF6-IncHI2-*mcr*-3.2 from this study contained resistance genes of tetracycline (*tet*A),(*tet*R), aminoglycosides (*ant*1_1, *ant*1_2, *neo*_1, *neo*_2), chloramphenicol (*cmlA1*), cephalosporin (bla_{CTXM-1}), disinfectant (*qacC*) as well as colistin (*mcr*-3.2). This is worrisome since various antibiotic resistance genes located in the same plasmid could enhance the persistence and co-selection of *mcr* genes even after colistin was withdrawn (Vines et al., 2021). Therefore, aside from colistin withdraw, the continual monitoring of other antimicrobials usage during pig production cycle and farm management are needed for colistin resistant control in pig farms. Moreover, the finding of identical plasmids between MCRPE isolates from different sample collection years implied that these plasmids were disseminated in the farm along the investigation years.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

This finding confirmed our suspicion, that since there was no dominant *E. coli* PFGE pulsotypes was detected, the *mcr* genes distributed in the farm were mainly due to plasmid borne.

According to previous studies, *mcr*-1 is mobilized by a composite transposon called Tn6330 where the *mcr*-1 with a putative open reading frame (PAP2 like protein) were flanked by two ISApl1 insertion sequences (Snesrud et al., 2018). The IS30 family, ISApl1 performs a 'copy-out, paste-in' mechanism and are highly active (Snesrud et al., 2017). However, *mcr*-1 with partial or complete loss of this insertion sequences leading to the stabilization of *mcr*-1 in plasmid background had been detected in several studies (R. Wang, L. van Dorp, et al., 2018). Although the loss of ISApl1 would prevent mobilization of *mcr*-1 gene, remaining of a single copy is sufficient to mobilize and the presence of upstream copy being functionally more important (Snesrud et al., 2016). This explained that *mcr*-1 genes from pig and environment in this study were actively mobilizable and able to disseminate among different sources until 2 years without colistin exposure. Moreover, in agreeing with our results, there were significantly more *mcr*-1 cases with attached insertion sequence ISApl1 in animal isolates rather than in human isolates (Y. Wang et al., 2017). These findings in line with animals being the primary source for *mcr*-1 bearing bacteria transmitted to humans.

In this study, the *mcr*-3 genes were prone to be eliminated from population as early as 1 and half year after colistin withdraw. In opposed to our results, previous vitro

CHULALONGKORN UNIVERSITY

experiment showed that the plasmids carrying *mcr*-3 have higher stability than *mcr*-1 plasmids in the absence of colistin with *mcr*-3 being exposed lower fitness cost (Q. E. Yang et al., 2020). On the other hand, the study by (R. Li et al., 2021) found that certain *E. coli* strains were more likely to eliminate *mcr*-3 genes than *mcr*-1 in vitro with or without colistin. Therefore, the persistence and fitness cost of the *mcr*-1 and *mcr*-3 in bacteria might differ depending upon plasmid as well as host genetic background. In this study, the genetic structure of TnAs2-*mcr*-3.2-dgkA-ISKpn40 was detected on both IncFII and IncHI2 plasmids of pigs from 2017 and 2018 respectively. This similar structure was observed in *mcr*-3.2 positive *E. coli* of bovine (Alba et al., 2018) and *mcr*-3.1 of porcine origin (Yin et al., 2017). In case of *mcr*-3.5 plasmid pCPF6-IncFII, IS26 was presented instead of ISKpn40 downstream of *mcr*-3.5 gene. IS*Kpn40* belongs to the IS*3* family was firstly identified in *E. coli* strain from a swine whereas IS6 family of IS26 was previously reported in mobilization of resistance genes in Gram-negative bacteria (Partridge et al., 2018). Although the genetic environment of *mcr*-3 determinants were variable, the core structure of TnAs2-*mcr*-3-dgkA, accompanied with other mobile elements or resistance genes, was highly conserved (R. Li et al., 2021).

Moreover, unlike *mcr*-1 which can locate both in chromosome and plasmid, the world-wide reports of *mcr*-3 found only in varieties of plasmids rather than in chromosomes except in *Aeromonas* spp. (Y. Shen, Xu, et al., 2018). Therefore, the stability of *mcr*-3 in host genetic background is lower than that of *mcr*-1. These findings

could explain the *mcr*-3 being less prevalence than *mcr*-1 isolated from different sources. Additionally, previous vitro study discovered that under no antibiotic selective pressure, the IncFII plasmids were instable and outcompeted by plasmid free cells (R. Wu et al., 2018). Notably, the cost of the plasmid would be increased according to the metabolic load of the plasmid such as expression of biomolecules or energy rich compounds as well as introduction of efflux pump (A. C. Carroll & Wong, 2018). This is consistent with our results that IncFII plasmids in this study consisted of either bacteriocin producing protein or efflux pump. Meanwhile, the IncHI2 plasmid in this study was MDR plasmid which contained resistance genes of various AMR classes and disinfectants as well as several mobile genetic elements. Such kind of MDR plasmid may constitute a fitness burden and prone to deletion of *mcr*-3 gene or plasmid from the bacteria.

Generally, small sized plasmid presence of only a single antibiotic resistance might less costly than large plasmids comprising several resistance determinants along with numerous plasmid trait genes (Alvaro San Millan et al., 2009; Sandegren, Lindqvist, Kahlmeter, & Andersson, 2008). However, (Vogwill & MacLean, 2015) stated that, the size of the plasmid is not directly related with fitness cost of the respective plasmid. The cost of a plasmid raises relatively with increasing levels of resistance genes on the plasmid and the level of their phenotypic expression (Vogwill & MacLean, 2015). These phenomenon could be applied in our findings that the *mcr*-1 bearing plasmid detected

CHULALONGKORN UNIVERSITY

in this study were colistin mono-resistant plasmid with high stability traits. Moreover, the moderate MICs (4-8mg/L) of colistin found in MCRPE isolates, and the fitness benefit expressed by *mcr*-1-bearing IncI2 and IncX4 plasmids further supported their long-term persistence in the colistin withdraw farm. Hence, not only cessation of colistin selective pressure, but also the characteristic of *mcr* bearing plasmids and co-selection by other antibiotics usage and farm management should pay attention in controlling colistin resistant bacteria in the population.

Conclusion

In this study, the *mcr* bearing *E. coli* isolates of different lineages showed both phenotypic and genotypic multi-drug resistance profiles. The disinfectant resistant genes were also found either with *mcr* gene or on separate plasmids. The presence of AMR genes of various antibiotic families and disinfectant resistance genes enhanced the persistence of colistin resistant bacteria even without selective pressure. Therefore, beside colistin withdrawal, the prudent management for other antimicrobials usages in the farm is also very important. The *mcr*-1 genes located on highly competitive plasmids further assisted in maintenance of MCRPE for longer period in the farm. Thus, the dissemination and persistence of colistin resistance bacteria could also depend upon plasmid dynamics on which *mcr* genes are located. Further genomic investigations approach on pig farms from different ecological background are needed to improve better understanding and control of colistin-resistance in pig farms.

จุฬาลงกรณ์มหาวิทยาลัย

Chapter V

General discussion, conclusion and further recommendations

This study initially conducted the national scale surveillance upon characterization of *mcr*-positive *E. coli* (MCRPE) from fecal samples of healthy pigs derived from the contract farming system. Among 696 pooled fecal samples collected from 80 farms, in 49 provinces across six regions of Thailand, 4.5% were detected as *mcr*-1 and/or *mcr*-3 positive. From this nation-wide study, we confirmed the existence of colistin-resistant *E. coli* comprising *mcr*-1 and *mcr*-3 genes in large scale pig farms across Thailand. The colistin-resistant *E. coli* were relatively higher in central areas of Thailand where the number of pig farms as well as total number of pigs were higher. Moreover, these MCRPE isolates showed MDR profiles from phenotypic testing. ESBL positive *E. coli* were also detected among MCRPE isolates and majority of isolates were resistant to tetracycline and aminoglycosides. The dissemination of *mcr*-1 positive *E. coli* with ESBL producers are serious threat to public threat. The usage of antimicrobials in pig farming depends on the farm management and geographical area. In the central area of Thailand, colistin, cephalosporins, tiamulin, amoxicillin, tilmicosin, aminoglycosides (gentamycin), and oxytetracycline were mainly applied (Pokhrel et al., 2019).

The MCRPE isolates not only showed MDR profiles but also several isolates comprised ETEC traits shown by PCR virulent genes detection. According to phylogroup

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

characterization, these MCRPE isolates belonged to commensal *E. coli* phylogroups A or B1. Therefore, even commensal *E. coli* strains could be an important reservoir for colistin resistant genes with ETEC virulence potential. The *mcr*-1 genes have been widely detected globally with the livestock being the key source owing to the consequence of long-term colistin usage in animals (Elbediwi et al., 2019). Although currently a few studies had already been reported regarding colistin resistant situation in pigs in Thailand, by the time this study was conducted, the nation-wide monitoring study for *mcr* genes in pigs was limited. Moreover, how many *mcr* genes determinants being disseminated in the country was out of data. Therefore, this study raised an awareness of *mcr*-1 and *mcr*-3 genes mediated colistin resistant *E. coli* distributing in high intensity pig farms in Thailand.

Since the rising of plasmid mediated *mcr* genes from various sources around the world, the practice of colistin withdraw as prophylactic uses in livestock had been prioritized in several countries (Walsh & Wu, 2016). In Thailand, this practice has been started from the beginning of 2017 according to the Department of Livestock and Development (DLD). Therefore, our next target was to longitudinal monitoring on the representative pig farm where was reported as *mcr*-1 gene positive in pig carriages, workers, and environment from our initial nation-wide survey. This selected farm was in the central area of Thailand and colistin sulfate usage as feed additives had been banned after the DLD statement on colistin withdraw. In this part, we successfully

CHULALONGKORN UNIVERSITY

identified the persistence of pathogenic MCRPE over 3.5 years of observation following withdrawal of colistin. Among the three sources detected, pigs were found to be the primary source of emergence and dissemination of *mcr* genes. The main objective of this study was to monitor the colistin withdraw effect on the farm, therefore we focused on examining young sows and their suckling pigs, as well as from wastewater. Moreover, relatively more sows were sampled than piglets to find out the rate of colistin long term persistence in the farm since piglets were slaughter or sold to another farm after weaning.

From this longitudinal monitoring study, until 21 months post-withdraw of colistin, *E. coli* carrying *mcr* genes were quite commonly found in pigs. The highest percentage were detected in mid-2017 (approximately 6 months after withdrawal) and the *mcr*-1 positive *E. coli* were presented in the feces of farm workers in this time. This is of worrisome since several reports have been described upon resistant bacteria from animals transmitted to humans especially farm workers are of risk group (Archawakulathep et al., 2014; You et al., 2016). The significant decline of *mcr* genes in pigs and elimination in workers and wastewater samples were observed in third year after colistin banned in the farm. These findings indicated that the period of 1 year is not enough to show significant effect of *mcr* genes decline after removing the selective pressure. Our results contradicted with the previous study, where undetectable *mcr*-1 from pigs was observed after colistin cessation for approximately 20 months (Duggett et

CHULALONGKORN UNIVERSITY

al., 2018). Therefore, the underlying reasons why these *mcr* genes persisted in pigs for longer term was needed for further study. Notably, the increase in MCRPE were recovered again from pigs and wastewater in 2020. At this time, only the piglets that showing diarrhea and had received therapeutic antibiotics were tested positive for *mcr*-1 genes. The cross-resistance between colistin and other therapeutic antibiotics used in the piglets could be the reason for this re-occurrence of these *mcr* genes without selective pressure of colistin. According to the farm management, when breeding sows and piglets got diarrhoea, therapeutic antibiotic injections such as gentamicin, ceftriaxone, and/or penicillin/streptomycin combinations were applied. In accordance with this information, the phenotypic testing by Vitek 2 showed that the MCRPE from the piglets were resistance to aminoglycosides, ampicillin, and ceftiofur.

Moreover, the MCRPE recovered in this study showed a high conjugative transfer frequency without selective pressures from colistin. According to plasmid replicon type detection, the MCRPE isolates presented various plasmid replicon types. By DNA fingerprinting from both MLST and PFGE testing, diverse *E. coli* clonal lineages were observed among the *mcr* positive isolates. Therefore, the *mcr* genes transmission from pigs to environmental sources were mainly due to plasmid-borne. Thus, we suspected that although the reduction in *mcr* positive *E. coli* after withdrawal, the usage of other antimicrobials during the production cycle might co-select the *mcr* genes and

CHULALONGKORN UNIVERSITY

enhance their persistence. However, genomic investigations are necessary to prove these hypothesis for better understanding and control of MCRPE in the pig industry.

Therefore, we continued to conduct the genomic study of these MCRPE isolates by hybrid whole genome sequencing (Illumina and Nanopore). At this time, 6 MCRPE isolates of different PFGE profiles were selected from 2017-2019. Since our primary focus was on the persistence of *mcr* positive *E. coli* on the farm, we didn't perform WGS analysis on piglets' isolates from 2020. Because the piglets were sent to slaughter by around 5-6 months of age, and so by definition could not be associated with persistence on the farm. Among the 6 isolates tested, the *mcr*-1.1 genes were located on IncI2 plasmids of pigs' origins from 2019 and wastewater samples of 2017. Moreover, according to blast analysis, these 2 plasmids were found to be identical. Likewise, the identical IncFII plasmids were also found in both *mcr*-3 positive pig isolates from 2017 and 2018. The finding of identical *mcr* bearing plasmids from different *E. coli* lineages explained our hypothesis that the *mcr* transmission in the farm due to plasmid borne rather than strain dependence.

Notably, the three *mcr*-1.1 plasmids (n=1 in human) (n=2 in pigs) were located in the most abundantly reported *mcr*-1 plasmid types of IncI2 and IncX4, which are relatively smaller size plasmids. These plasmid types were reported to be less costly and high stability in population (W. Li et al., 2021). Moreover, those plasmids comprised the type IV secretion system proteins (T4SS), and conjugal transfer protein (TraG) which

CHULALONGKORN UNIVERSITY

enabled the plasmid to be self-transmissible. Although the genetic contexts surrounding *mcr*-1 genes showed variable, conserved structures were shown within the same plasmid family. In case of *mcr*-1.1 on IncI2 plasmids, the loss of downstream ISApl1 insertion sequence were detected which is similar to the first reported *mcr*-1 plasmid from China (Liu et al., 2016). According to (Snesrud et al., 2016), the presence of upstream ISApl1 is sufficiently mobilizable for *mcr*-1 genes. Therefore, the *mcr*-1.1 detected from our study were still highly transposable to host genome or transfer to other plasmids. Moreover, these IncI2 plasmids contain relatively high amount of conjugation related transfer proteins.

In case of *mcr*-3, the genetic environment varied depending on *mcr*-3 variants counterparts and were found on IncFII and IncHI2 plasmid types. In our study, we found that the *mcr*-3 genes were more prone to eliminate than *mcr*-1 genes after colistin withdraw. This result was contradicted with previous vitro experiments that *mcr*-3 plasmids stably persist, even without colistin (Q. E. Yang et al., 2020). Therefore, to understand this underlying reasons, we traced back the plasmid characteristic of *mcr*-3 bearing plasmids. The *mcr*-3.2 plasmid of pCPF6-IncHI2 was relatively large plasmid comprising MDR region as well as disinfectant resistant genes together with several mobile genetic elements. The cost of plasmid was predicted to be lower in mono-resistant plasmids than those of MDR ones (Vogwill & MacLean, 2015). Notably, the same author also stated that not only the carriage of the number of genes on plasmid,

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

the expression of their phenotypic properties is also important. This phenomenon could be applied to our results that our MCRPE isolates not only carried resistant genes on plasmids, but they also presented phenotypic expression. This could be the possible explanation why *mcr*-3 genes or plasmid were easier to be deleted than *mcr*-1 from the host in the absence of selective pressure.

Moreover, the expression of diverse antibiotic resistant genes and presence of antiseptic resistant genes either in the same plasmid with *mcr* or on separate plasmids was found. This proved our hypothesis on *mcr* genes persistence enhanced by the existence of other antibiotic resistance, heavy metals or biocides genes. Therefore, the usage of other antimicrobials and farm biosecurity is also very important for long term management of colistin resistance control. This study has some limitations since we focused mainly on one representative pig farm and fewer samples were selected for WGS analysis. Thus, variations between different farm management upon *mcr* genes persistence could not be carried out. We suggested that further long-term genomic investigations on pig farms from different geographical areas to improve understanding and control of colistin-resistance.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix

Appendix 1

Table 7. Details of the sample types and numbers collected at five different sampling times between 2017 and 2020, and numbers of samples found positive for MCRPE

Year	Type of sample	Sampling time	Number	Age of pigs at time of sampling	Number of samples positive for MCRPE
2017	Farrowing sows	1	10	1-3 years	9
	Suckling piglets	1	5	21 days	0
	Wastewater (Before-biogas treatment)	1	5	-	2
	Wastewater (After biogas treatment)	1	5	-	0
	Farm workers	1	10	-	4
2018	Farrowing sows	2	10	1-3 years	5
	Suckling piglets	2	0	-	0
	Wastewater (Before-biogas treatment)	2	5	-	1
	Wastewater (After biogas treatment)	2	5	-	0
	Farm workers	2	10	-	0
2019 March	Farrowing sows	3	10	1-3 years	0
	Suckling piglets	3	5	21 days	0
	Wastewater (Before-biogas treatment)	3	5	-	0
	Wastewater (After biogas treatment)	3	5	-	0
	Farm workers	3	10	-	0

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

	Farrowing sows	4	10	1-3 years	1
	Suckling piglets	4	5	21 days	0
2019 April	Wastewater (Before-biogas treatment)	4	5	-	0
	Wastewater (After biogas treatment)	4	5	-	0
	Farm workers	4	10	-	0
	Farrowing sows	5	10	1-3 years	0
	Suckling piglets	5	5	21 days	5
2020	Wastewater (Before-biogas treatment)	5	5	-	5
	Wastewater (After biogas treatment)	5	5	-	1
	Farm workers	5	10	-	0

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

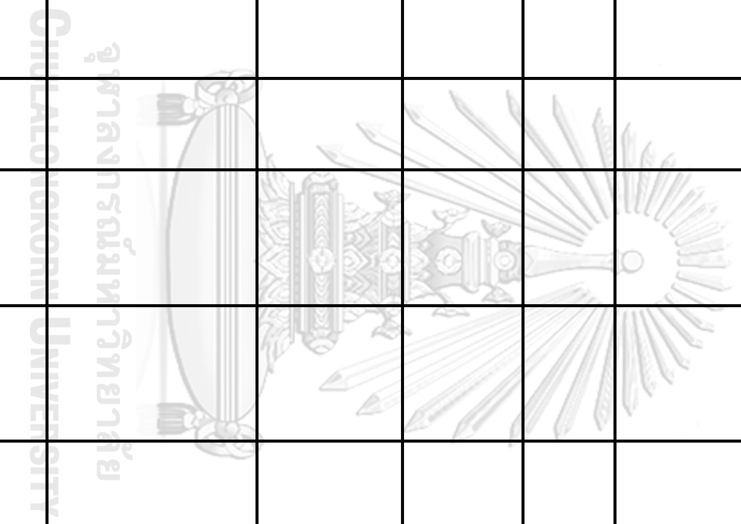
Appendix 2

Table 8. Antibiogram patterns detected from the 33 MCRPE isolates from samples collected in four different years

Year			Year			Year			Year		
2017			2018			2019			2020		
	No. of ABOs resistant	No. of isolates		No. of ABOs resistant	No. of isolates		No. of ABOs resistant	No. of isolates		No. of ABOs resistant	No. of isolates
CEX-GEN-CST	3	1	AMX-AMP-PIP-CEX-GEN-TET-CST*	7	2	AMP-CST	2	1	AMP-TET-CST	3	7
AMX-AMP-TET-CST	4	2	AMX-AMP-AMC-PIP-CEX-GEN-ENR-MBRL-TET-CST	10	1				AMX-AMP-XNL-TET-CST	5	2
AMX-AMP-GEN-TET-CST	5	1	AMX-AMP-PIP-CEX-CPD-INN-CEF-GEN-TET-SXT-CST*	11	1				AMX-AMP-XNL-GEN-TET-CST	6	2
AMX-AMP-PIP-TET-C-CST	6	1	AMX-AMP-PIP-CEX-CPD-INN-CEF-GEN-ENR-MBRL-TET-SXT-CST*	13	1						

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

AMX-AMP-PIP-TET-SXT-C-CST	7	1	AMX-AMP-AMC-PIP-CEX-CPD-INN-CEF-GEN-ENR-MBRL-TET-SXT-CST	14	1						
AMP-AMC-CEX-CEF-C-SXT-CST	7	1									
AMP-CEX-CEF-GEN-TET-CST	7	1									
AMX-AMP-AMC-PIP-CEX-CPD-TET-CST	8	1									
AMP-AMC-CEX-CEF-CPD-INN-TET-C-SXT-CST	10	1									
AMP-CEX-CEF-GEN-ENR-MBRL-TET-C-SXT-CST	10	1									
AMP-AMC-CEX-CEF-GEN-ENR-MBRL-TET-C-SXT-CST*	11	1									
AMP-CEX-CEF-CPD-INN-GEN-	11	1									



MBRL-TET-SXT											
AMX-AMP-AMC-PIP-CEX-CPD-INN-CEF-IMP-C-SXT-CST	12	1									
AMP-AMC-CEX-CEF-CPD-INN-GEN-ENR-MBRL-TET-C-CST*	13	1									

AMC, amoxicillin–clavulanic acid; AMP, ampicillin; AMX, amoxicillin; C, chloramphenicol; CEX, cephalexin; CPD, cefpodoxime; ENR, enrofloxacin; GEN, gentamicin; MBR, marbofloxacin; PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole; INN, cefovecin; AK, amikacin; IMP, imipenem; TET, tetracycline; XNL, ceftiofur; TOB, tobramycin; NIT, nitrofurantoin, * =ESBL

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix 3

Table 9. MLST sequence types and source and number of the MCRPE isolates that were representatives of the 34 PFGE patterns

Year	Sequence Type	Source	Number of isolates tested
2017	10	Pig	2
	10	Human	1
	101	Pig	1
	349	Pig	1
	9192	Pig	2
	117	Pig	1
	1114	Pig	2
	453	Wastewater	1
	189	Wastewater	1
	515	Human	1
	New ST	Human	1
2018	641	Pig	2
	641	Wastewater	1
	3944	Pig	2
	1602	Pig	1
	453	Pig	2
2019	New ST	Pig	2
2020	3345	Pig	2
	3345	Wastewater	3
	5218	Pig	2
	1114	Pig	1
	10	Wastewater	1
	93	Wastewater	1

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix 4

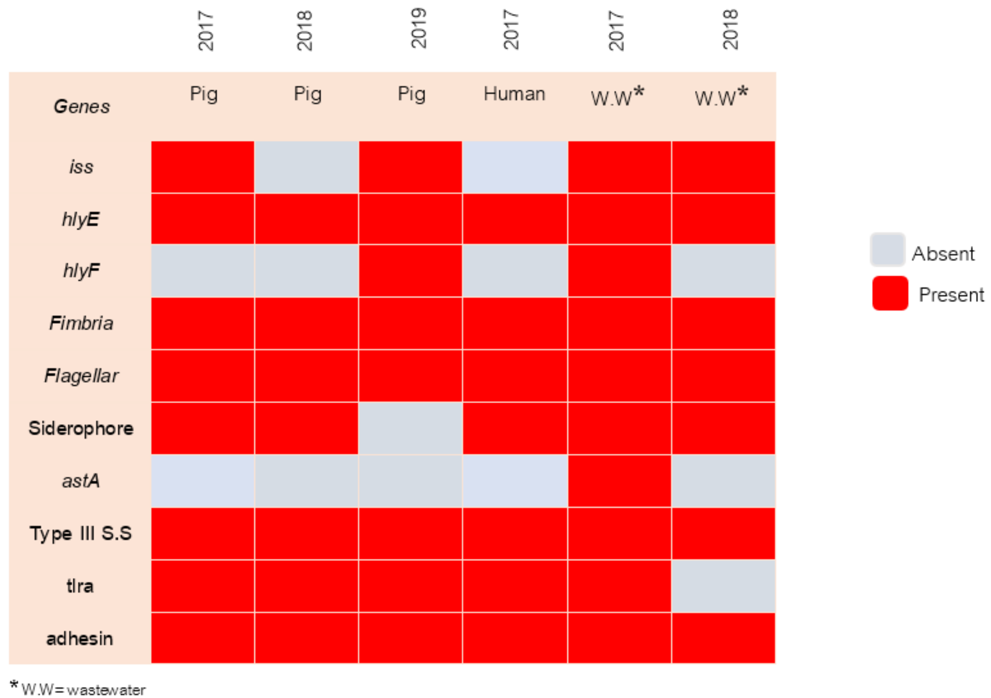


Figure 16. Heatmap showing the presence or absence of various virulence factor genes in 6 MCRPE from pig, human and environment

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Appendix 5

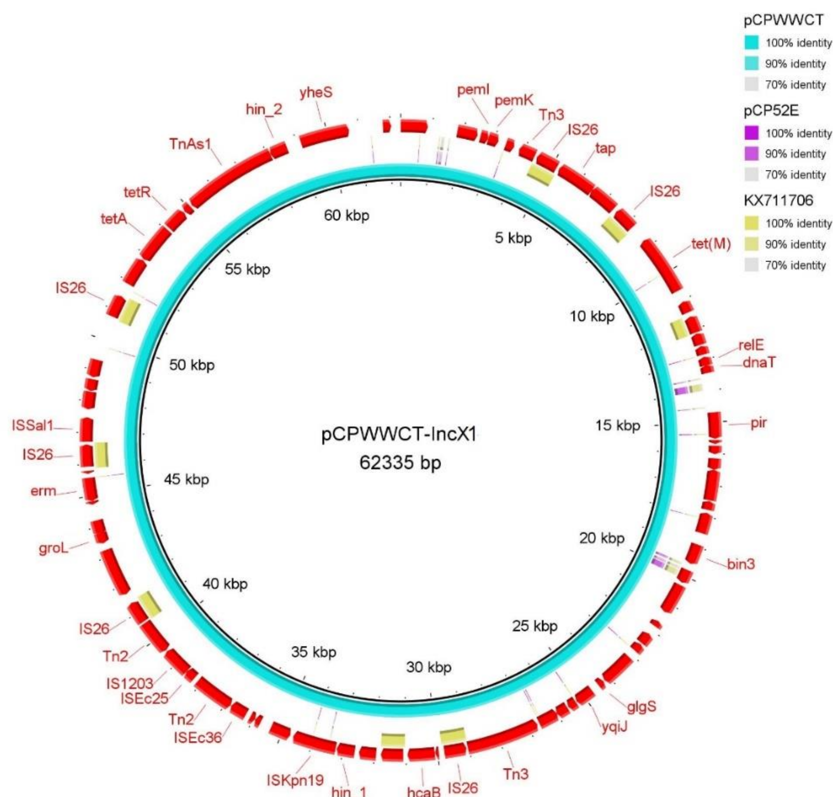
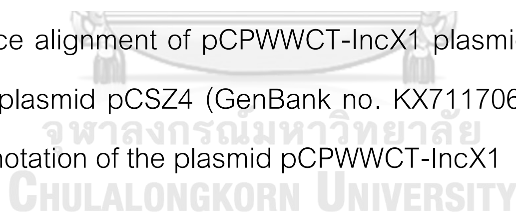


Figure 17. Sequence alignment of pCPWWCT-IncX1 plasmid with pCP52E (This study) and the reference plasmid pCSZ4 (GenBank no. KX711706). The outer circle with red arrows denotes annotation of the plasmid pCPWWCT-IncX1



Appendix 6

Table 10. Heavy metal resistance genes detected among 6 MCRPE isolates

Strains	Year	Source	Location	Heavy metal resistance genes
CP52E	2017	Human	Chromosome	Copper (*pcoA*, *pcoB*, *pcoC*, *pcoD*, *pcoR*, *pcoS*, *pcoE*), Zinc (*zntA)*, Silver (*silE*, *silS*, *silC*, *silF*, *silB*, *silA*, *silP*)
CPE35	2017	Pig	Chromosome	Zinc (*zntA)*
CPWW7	2017	Wastewater	Chromosome	Zinc (*zntA)*
			Plasmid	Mercury (*merR_Ps*, *merT*, *merC*),
CPF6	2018	Pig	Chromosome	Copper (*pcoE*, *pcoS*, *pcoD*, *pcoC*, *pcoB*, *pcoA*), Zinc (*zntA*), Silver (*silP*, *silB*, *silF*, *silC*, *silS*, *silE*)
CPWWCT	2018	Wastewater	Chromosome	Copper (*pcoA*, *pcoB*, *pcoC*, *pcoD*, *pcoS*, *pcoE*), Zinc (*zntA)*, Silver (*silE*, *silS*, *silC*, *silF*, *silB*, *silA*, *silP*)
CPA1200	2019	Pig	Chromosome	Copper (*pcoS*, *pcoD*, *pcoC*, *pcoB*, *pcoA*), Zinc (*zntA)*, Silver (*silP*, *silA*, *silB*, *silF*, *silC*, *silS*, *silE*)

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

REFERENCES

Aarestrup, F. M. (2015). The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. *Philos Trans R Soc Lond B Biol Sci, 370*(1670), 20140085. doi:10.1098/rstb.2014.0085

AbuOun, M., Stubberfield, E. J., Duggett, N. A., Kirchner, M., Dormer, L., Nunez-Garcia, J., . . . Anjum, M. F. (2018). *mcr*-1 and *mcr*-2 (*mcr*-6.1) variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. *J Antimicrob Chemother, 73*(10), 2904. doi:10.1093/jac/dky272.

Adiri, R. S., Gophna, U., & Ron, E. Z. (2003). Multilocus sequence typing (MLST) of *Escherichia coli* O78 strains. *FEMS Microbiol Lett, 222*(2), 199-203. doi:10.1016/S0378-1097(03)00295-7.

administration, F. a. d. (2018). *Microbiology Data for Systemic Antibacterial Drugs — Development, Analysis, and Presentation Guidance for Industry*. Food and Drug Administration Center for Drug Evaluation and Research (CDER).

Alba, P., Leekitcharoenphon, P., Franco, A., Feltrin, F., Ianzano, A., Caprioli, A., . . . Battisti, A. (2018). Molecular epidemiology of *mcr*-encoded colistin resistance in *Enterobacteriaceae* from food-producing animals in Italy revealed through the EU harmonized antimicrobial resistance monitoring. *Front Microbiol, 9*, 1217. doi:10.3389/fmicb.2018.01217.

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., & Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics, 12*, 402. doi:10.1186/1471-2164-12-402.

Andersson, D. I. (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr Opin Microbiol, 9*(5), 461-465. doi:10.1016/j.mib.2006.07.002.

Archawakulathep, A., Kim, C. T. T., Meunsene, D., Handijatno, D., Hassim, H. A., Rovira, H. R., . . . Aung, M. (2014). Perspectives on antimicrobial resistance in livestock

and livestock products in ASEAN countries. *The Thai Journal of Veterinary Medicine, 44*(1), 5-13.

Authority, E. F. S., Prevention, E. C. f. D., & journal, C. J. E. (2016). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. *14*(2), 4380.

Begaud, E., Mondet, D., & Germani, Y. (1993). Molecular characterization of enterotoxigenic *Escherichia coli* (ETEC) isolated in New Caledonia (value of potential protective antigens in oral vaccine candidates). *Res Microbiol, 144*(9), 721-728. doi:10.1016/0923-2508(93)90036-2.

Biswas, S., Brunel, J. M., Dubus, J. C., Reynaud-Gaubert, M., & Rolain, J. M. (2012). Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther, 10*(8), 917-934. doi:10.1586/eri.12.78.

Bok, E., Mazurek, J., Myc, A., Stosik, M., Wojciech, M., & Baldy-Chudzik, K. (2018). Comparison of commensal *Escherichia coli* isolates from adults and young children in Lubuskie province, Poland: virulence potential, phylogeny and antimicrobial resistance. *Int J Environ Res Public Health, 15*(4). doi:10.3390/ijerph15040617.

Brolund, A., Sundqvist, M., Kahlmeter, G., & Grape, M. (2010). Molecular characterisation of trimethoprim resistance in *Escherichia coli* and *Klebsiella pneumoniae* during a two year intervention on trimethoprim use. *PLoS One, 5*(2), e9233. doi:10.1371/journal.pone.0009233.

Cao, Y. P., Lin, Q. Q., He, W. Y., Wang, J., Yi, M. Y., Lv, L. C., . . . Guo, J. Y. (2020). Co-selection may explain the unexpectedly high prevalence of plasmid-mediated colistin resistance gene *mcr*-1 in a Chinese broiler farm. *Zool Res, 41*(5), 569-575. doi:10.24272/j.issn.2095-8137.2020.131.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., & Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods, 63*(3), 219-228. doi:10.1016/j.mimet.2005.03.018.

Carroll, A. C., & Wong, A. (2018). Plasmid persistence: costs, benefits, and the plasmid

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

paradox. *Can J Microbiol, 64*(5), 293-304. doi:10.1139/cjm-2017-0609.

Carroll, L. M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L. O., & Wiedmann, M. (2019). Identification of novel mobilized colistin resistance gene *mcr*-9 in a multidrug-resistant, colistin-susceptible *Salmonella Enterica* serotype Typhimurium isolate. *mBio, 10*(3). doi:10.1128/mBio.00853-19.

Casey, T. A., & Bosworth, B. T. (2009). Design and evaluation of a multiplex polymerase chain reaction assay for the simultaneous identification of genes for nine different virulence factors associated with *Escherichia coli* that cause diarrhea and edema disease in swine. *J Vet Diagn Invest, 21*(1), 25-30. doi:10.1177/104063870902100104.

Casey, T. A., Herring, C. J., Schneider, R. A., Bosworth, B. T., & Whipp, S. C. (1998). Expression of heat-stable enterotoxin *STb* by adherent *Escherichia coli* is not sufficient to cause severe diarrhea in neonatal pigs. *Infect Immun, 66*(3), 1270-1272. doi:10.1128/IAI.66.3.1270-1272.1998.

CDC. (2013). Centers for Disease Control and Prevention, Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, *Escherichia c*oli non-O157 (STEC), *Salmonella serotypes, Shigella sonnei* and *Shigella flexneri*. In. Centers for Disease Control and Prevention.

Chen, L., Zheng, D., Liu, B., Yang, J., & Jin, Q. (2016). VFDB 2016: hierarchical and refined dataset for big data analysis--10 years on. *Nucleic Acids Res, 44*(D1), D694-697. doi:10.1093/nar/gkv1239.

Choi, Y., Lee, J. Y., Lee, H., Park, M., Kang, K., Lim, S. K., . . . Ko, K. S. (2020). Comparison of fitness cost and virulence in chromosome- and plasmid-mediated colistin-resistant *Escherichia coli*. *Front Microbiol, 11*, 798. doi:10.3389/fmicb.2020.00798.

Clermont, O., Christenson, J. K., Denamur, E., & Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep, 5*(1), 58-65. doi:10.1111/1758-2229.12019.

CLSI. (2017). Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. In. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute.

CLSI. (2020). Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. (Vol. M100, 30th edition). Wayne, Pennsylvania.

Contrepois, M., Bertin, Y., Pohl, P., Picard, B., & Girardeau, J.-P. (1998). A study of relationships among F17 a producing enterotoxigenic and non-enterotoxigenic *Escherichia coli* strains isolated from diarrheic calves. *Veterinary microbiology, 64*(1), 75-81.

da Silva, G. J., & Mendonça, N. J. V. (2012). Association between antimicrobial resistance and virulence in *Escherichia coli*. *3*(1), 18-28.

de Been, M., Lanza, V. F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., . . . Tooming-Klunderud, A. J. P. G. (2014b). Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. *PLoS Genet, 10*(12), e1004776. doi: doi:10:1371/journal.pgen.1004776.

de Toro, M., Garcillaon-Barcia, M. P., & De La Cruz, F. (2014). Plasmid Diversity and Adaptation Analyzed by Massive Sequencing of *Escherichia coli* Plasmids. *Microbiol Spectr, 2*(6). doi:10.1128/microbiolspec.PLAS-0031-2014

Dhara, M., Disha, P., Sachin, P., Manisha, J., Seema, B., & Vegad, M. J. N. J. M. R. (2012). Comparison of various methods for the detection of extended spectrum beta-lactamase in *Klebsiella pneumoniae* isolated from neonatal intensive care unit. *2*, 348-353.

Duggett, N. A., Randall, L. P., Horton, R. A., Lemma, F., Kirchner, M., Nunez-Garcia, J., . . . Anjum, M. F. (2018). Molecular epidemiology of isolates with multiple *mcr* plasmids from a pig farm in Great Britain: the effects of colistin withdrawal in the short and long term. *J Antimicrob Chemother, 73*(11), 3025-3033. doi:10.1093/jac/dky292.

Echeverria, P., Seriwatana, J., Patamaroj, U., Moseley, S. L., McFarland, A., Chityothin, O., & Chaicumpa, W. (1984). Prevalence of heat-stable II enterotoxigenic *Escherichia*

coli in pigs, water, and people at farms in Thailand as determined by DNA hybridization. *J Clin Microbiol, 19*(4), 489-491. doi:10.1128/jcm.19.4.489-491.1984.

Eiamphungporn, W., Yainoy, S., Jumderm, C., Tan-Arsuwongkul, R., Tiengrim, S., & Thamlikitkul, V. (2018). Prevalence of the colistin resistance gene *mcr*-1 in colistin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from humans in Thailand. *J Glob Antimicrob Resist, 15*, 32-35. doi:10.1016/j.jgar.2018.06.007.

Elbediwi, M., Li, Y., Paudyal, N., Pan, H., Li, X., Xie, S., . . . Yue, M. (2019). Global burden of colistin-resistant bacteria: mobilized colistin resistance genes study (1980-2018). *Microorganisms, 7*(10). doi:10.3390/microorganisms7100461.

EUCAST. (2018). The European Committee on Antimicrobial Susceptibility Testing: Breakpoint tables for interpretation of MICs.

Falagas, M. E., & Kasiakou, S. K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis, 40*(9), 1333-1341. doi:10.1086/429323.

Fang, L. X., Li, X. P., Deng, G. H., Li, S. M., Yang, R. S., Wu, Z. W., . . . Liu, Y. H. (2018). High genetic plasticity in multidrug-resistant sequence type 3-IncHI2 plasmids revealed by sequence comparison and phylogenetic analysis. *Antimicrob Agents Chemother, 62*(4). doi:10.1128/AAC.02068-17.

FAO. (2016). Drivers, Dynamis and Epidemiology of Antimicrobial Resistance in Animal Production.
CHULALONGKORN UNIVERSITY
FDA. (2018). Microbiology Data for Systemic Antibacterial Drugs — Development, Analysis, and Presentation Guidance for Industry. In: Food and Drug Administration Center for Drug Evaluation and Research (CDER).

Forde, B. M., Zowawi, H. M., Harris, P. N. A., Roberts, L., Ibrahim, E., Shaikh, N., . . . Beatson, S. A. (2018). Discovery of *mcr*-1-mediated colistin resistance in a highly virulent *Escherichia coli* Lineage. *mSphere, 3*(5). doi:10.1128/mSphere.00486-18.

Gama, J. A., Zilhão, R., & Dionisio, F. J. F. i. M. (2020). Plasmid interactions can improve plasmid persistence in bacterial populations. *11*, 2033.

Garcia-Menino, I., Garcia, V., Mora, A., Diaz-Jimenez, D., Flament-Simon, S. C., Alonso, M.

P., . . . Blanco, J. (2018). Swine Enteric Colibacillosis in Spain: Pathogenic Potential of *mcr*-1 ST10 and ST131 *E. coli* Isolates. *Front Microbiol, 9*, 2659. doi:10.3389/fmicb.2018.02659.

Gray, K. J., Wilson, L. K., Phiri, A., Corkill, J. E., French, N., & Hart, C. A. (2006). Identification and characterization of ceftriaxone resistance and extended-spectrum beta-lactamases in Malawian bacteraemic *Enterobacteriaceae*. *J Antimicrob Chemother, 57*(4), 661-665. doi:10.1093/jac/dkl037.

Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L., & Rolain, J. M. (2014). ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother, 58*(1), 212-220. doi:10.1128/AAC.01310-13.

HACH. (2013). Water Analysis Guide. doi:https://DOC316.53.01336.

He, W. Y., Zhang, X. X., Gao, G. L., Gao, M. Y., Zhong, F. G., Lv, L. C., . . . Liu, J. H. (2021). Clonal spread of *Escherichia coli* O101: H9-ST10 and O101: H9-ST167 strains carrying *fosA*3 and *bla* CTX-M-14 among diarrheal calves in a Chinese farm, with Australian Chroicocephalus as the possible origin of *E. coli* O101: H9-ST10. *Zool Res, 42*(4), 461-468. doi:10.24272/j.issn.2095-8137.2021.153.

Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., . . . Piddock, L. J. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet, 387*(10014), 176-187. doi:10.1016/S0140-6736(15)00473-0.

Hua, X., Liang, Q., Deng, M., He, J., Wang, M., Hong, W., . . . Chen, H. (2021). BacAnt: A combination annotation server for bacterial dna sequences to identify antibiotic resistance genes, integrons, and transposable elements. *Front Microbiol, 12*, 649969. doi:10.3389/fmicb.2021.649969.

Humphrey, B., Thomson, N. R., Thomas, C. M., Brooks, K., Sanders, M., Delsol, A. A., . . . Enne, V. I. (2012). Fitness of *Escherichia coli* strains carrying expressed and partially silent IncN and IncP1 plasmids. *BMC Microbiol, 12*, 53. doi:10.1186/1471-2180-12-53.

Iranpour, D., Hassanpour, M., Ansari, H., Tajbakhsh, S., Khamisipour, G., & Najafi, A.

(2015). Phylogenetic groups of *Escherichia coli* strains from patients with urinary tract infection in Iran based on the new Clermont phylotyping method. *Biomed Res Int, 2015*, 846219. doi:10.1155/2015/846219.

Jindal, A. K., Pandya, K., & Khan, I. D. (2015). Antimicrobial resistance: A public health challenge. *Med J Armed Forces India, 71*(2), 178-181. doi:10.1016/j.mjafi.2014.04.011.

Johnson, T. J., Wannemuehler, Y. M., Johnson, S. J., Logue, C. M., White, D. G., Doetkott, C., & Nolan, L. K. (2007). Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Appl Environ Microbiol, 73*(6), 1976-1983. doi:10.1128/AEM.02171-06.

Kaspar, H. (2006). Results of the antimicrobial agent susceptibility study raised in a representative, cross-sectional monitoring study on a national basis. *Int J Med Microbiol, 296 Suppl 41*, 69-79. doi:10.1016/j.ijmm.2006.01.063.

Katsunuma, Y., Hanazumi, M., Fujisaki, H., Minato, H., Hashimoto, Y., & Yonemochi, C. (2007). Associations between the use of antimicrobial agents for growth promotion and the occurrence of antimicrobial-resistant *Escherichia coli* and *Enterococci* in the feces of livestock and livestock farmers in Japan. *J Gen Appl Microbiol, 53*(5), 273-279. doi:10.2323/jgam.53.273.

Kemp, S. A. (2019). Patterns of Antimicrobial Resistant E. coli and Genetic Interplay Between Livestock, Humans and Their Shared Environment in a High-Density Livestock-Human Population in Western Kenya. University of Liverpool.

Kempf, I., Fleury, M. A., Drider, D., Bruneau, M., Sanders, P., Chauvin, C., . . . Jouy, E. (2013). What do we know about resistance to colistin in *Enterobacteriaceae* in avian and pig production in Europe? *Int J Antimicrob Agents, 42*(5), 379-383. doi:10.1016/j.ijantimicag.2013.06.012.

Khine, N. O., Lugsomya, K., Kaewgun, B., Honhanrob, L., Pairojrit, P., Jermprasert, S., & Prapasarakul, N. (2020). Multidrug resistance and virulence factors of *Escherichia coli* harboring plasmid-mediated colistin resistance: *mcr*-1 and *mcr*-3 Genes in Contracted Pig Farms in Thailand. *Front Vet Sci, 7*, 582899.

doi:10.3389/fvets.2020.582899.

Kieffer, N., Nordmann, P., Moreno, A. M., Zanolli Moreno, L., Chaby, R., Breton, A., . . . Poirel, L. (2018). Genetic and functional characterization of an *MCR*-3-Like enzyme-producing *Escherichia coli* Isolate recovered from swine in Brazil. *Antimicrob Agents Chemother, 62*(7). doi:10.1128/AAC.00278-18.

Landers, T. F., Cohen, B., Wittum, T. E., & Larson, E. L. J. P. h. r. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *127*(1), 4-22.

Lanza, V. F., de Toro, M., Garcillan-Barcia, M. P., Mora, A., Blanco, J., Coque, T. M., & de la Cruz, F. (2014). Plasmid flux in *Escherichia coli* ST131 sublineages, analyzed by plasmid constellation network (PLACNET), a new method for plasmid reconstruction from whole genome sequences. *PLoS Genet, 10*(12), e1004766. doi:10.1371/journal.pgen.1004766.

Lay, K. K., Koowattananukul, C., Chansong, N., & Chuanchuen, R. (2012). Antimicrobial resistance, virulence, and phylogenetic characteristics of *Escherichia coli* isolates from clinically healthy swine. *Foodborne Pathog Dis, 9*(11), 992-1001. doi:10.1089/fpd.2012.1175.

Lei, C. W., Zhang, Y., Wang, Y. T., & Wang, H. N. (2020). Detection of mobile colistin resistance gene *mcr*-10.1 in a conjugative plasmid from *Enterobacter roggenkampii* of Chicken Origin in China. *Antimicrob Agents Chemother, 64*(10). doi:10.1128/AAC.01191-20.

Leonard, S. R., Mammel, M. K., Rasko, D. A., & Lacher, D. W. (2016). Hybrid Shiga toxin-producing and enterotoxigenic *Escherichia* sp. Cryptic Lineage 1 Strain 7v harbors a hybrid plasmid. *Appl Environ Microbiol, 82*(14), 4309-4319. doi:10.1128/AEM.01129-16.

Li, R., Du, P., Zhang, P., Li, Y., Yang, X., Wang, Z., . . . Bai, L. (2021). Comprehensive genomic investigation of coevolution of *mcr* genes in *Escherichia coli* strains via nanopore sequencing. *Glob Chall, 5*(3), 2000014. doi:10.1002/gch2.202000014.

Li, W., Liu, Z., Yin, W., Yang, L., Qiao, L., Song, S., . . . Shen, J. (2021). *MCR* Expression conferring varied fitness costs on host bacteria and affecting bacteria virulence.

Chulalongkorn University

Antibiotics (Basel), 10(7). doi:10.3390/antibiotics10070872.

Liu, Y. Y., Wang, Y., Walsh, T. R., Yi, L. X., Zhang, R., Spencer, J., . . . Shen, J. (2016). Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis, 16*(2), 161-168. doi:10.1016/S1473-3099(15)00424-7.

Lugsomya, K., Chatsuwan, T., Niyomtham, W., Tummaruk, P., Hampson, D. J., & Prapasarakul, N. (2018). Routine prophylactic antimicrobial use is associated with increased phenotypic and genotypic resistance in commensal *Escherichia coli* isolates recovered from healthy fattening pigs on farms in Thailand. *Microb Drug Resist, 24*(2), 213-223. doi:10.1089/mdr.2017.0042.

Lugsomya K, Chanchaithong P, Tribudharat C,Thanawanh N, Niyomtham W, Prapasarakul N. (2016). Prevalence of plasmid-mediated colistin resistance genes (*mcr*-1) in commensal *Escherichia coli* from fattening pigs in Thailand. *The Thai Journal of Veterinary Medicine*. Retrieved from www.vet.chula.ac.th/cuvc2016.

Lugsomya, K., Yindee, J., Niyomtham, W., Tribuddharat, C., Tummaruk, P., Hampson, D. J., & 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. doi:10.1089/mdr.2018.0154.

Ma, K., Feng, Y., & Zong, Z. J. P. o. (2018). Fitness cost of a *mcr*-1-carrying IncHI2 plasmid. *13*(12), e0209706.

Madoshi, B. P., Kudirkiene, E., Mtambo, M. M., Muhairwa, A. P., Lupindu, A. M., & Olsen, J. E. (2016). Characterisation of commensal *Escherichia coli* isolated from apparently healthy cattle and their attendants in Tanzania. *PLoS One, 11*(12), e0168160. doi:10.1371/journal.pone.0168160.

McArthur, A. G., Waglechner, N., Nizam, F., Yan, A., Azad, M. A., Baylay, A. J., . . . Wright, G. D. (2013). The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother, 57*(7), 3348-3357. doi:10.1128/AAC.00419-13.

McNeilly, O., Mann, R., Hamidian, M., & Gunawan, C. (2021). Emerging concern for silver

nanoparticle resistance in *Acinetobacter baumannii* and other bacteria. *Front Microbiol, 12*, 652863. doi:10.3389/fmicb.2021.652863

Meinersmann, R. J. (2019). The biology of IncI2 plasmids shown by whole-plasmid multi-locus sequence typing. *Plasmid, 106*, 102444.

Mendes, A. C., Novais, A., Campos, J., Rodrigues, C., Santos, C., Antunes, P., . . . Peixe, L. (2018). *mcr*-1 in Carbapenemase-Producing *Klebsiella pneumoniae* with hospitalized patients, Portugal, 2016-2017. *Emerg Infect Dis, 24*(4), 762-766. doi:10.3201/eid2404.171787.

Migura-Garcia, L., González-López, J. J., Martinez-Urtaza, J., Aguirre Sánchez, J., Moreno-Mingorance, A., Perez de Rozas, A., . . . Gonzalez-Escalona, N. (2020). *mcr*-colistin resistance genes mobilized by IncX4, IncHI2, and IncI2 plasmids in *Escherichia coli* of pigs and White Stork in Spain. *Frontiers in microbiology, 10*, 3072.

Moredo, F. A., Pineyro, P. E., Marquez, G. C., Sanz, M., Colello, R., Etcheverria, A., . . . Leotta, G. A. (2015). Enterotoxigenic *Escherichia coli* Subclinical Infection in Pigs: Bacteriological and Genotypic Characterization and Antimicrobial Resistance Profiles. *Foodborne Pathog Dis, 12*(8), 704-711. doi:10.1089/fpd.2015.1959.

Nguyen, N. T., Nguyen, H. M., Nguyen, C. V., Nguyen, T. V., Nguyen, M. T., Thai, H. Q., . . . 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. doi:10.1128/AEM.00337-16.

Nguyen, N. T., Nguyen, H. M., Nguyen, C. V., Nguyen, T. V., Nguyen, M. T., Thai, H. Q., . . . microbiology, e. (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. *82*(13), 3727-3735.

Olaitan, A. O., Morand, S., & Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol, 5*, 643. doi:10.3389/fmicb.2014.00643.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Organization, W. H. (2001). WHO global strategy for containment of antimicrobial resistance.

Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E., & Larsson, D. G. (2014). BacMet: antibacterial biocide and metal resistance genes database. *Nucleic Acids Res, 42*(Database issue), D737-743. doi:10.1093/nar/gkt1252.

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev, 31*(4). doi:10.1128/CMR.00088-17.

Paveenkittiporn, W., Kamjumphol, W., Ungcharoen, R., & Kerdsin, A. (2020). Whole-Genome sequencing of clinically isolated carbapenem-resistant *Enterobacterales* Harboring *mcr* Genes in Thailand, 2016-2019. *Front Microbiol, 11*, 586368. doi:10.3389/fmicb.2020.586368.

Plumb. (2015). *Plumb's Veterinary Drug Handbook* (Vol. 8th ed.). Wiley-Blackwell Press, Indianapolis.

Poirel, L., Jayol, A., & Nordmann, P. (2017). Polymyxins: Antibacterial Activity, Susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev, 30*(2), 557-596. doi:10.1128/CMR.00064-16.

Pokhrel, N., Kasemsuwan, S., Goutard, F., Boonsoongnern, A., Chumsing, S., & Tulayakul, P. (2019). Practices and factors influencing the use of antibiotics in swine farms in Central region of Thailand. *Journal of Kasetsart Veterinarians, 29*(1), 33-55.

Poolperm, P., Tangkoskul, T., Seenama, C., Maknakhon, N., & Thamlikitkul, V. (2020). Association between the use of colistin for short-term treatment of Gram-negative bacterial infections and the emergence of colistin-resistant *Enterobacteriaceae* in swine from selected swine farms in Thailand. *PLoS One, 15*(10), e0238939. doi:10.1371/journal.pone.0238939.

Prager, R., Fruth, A., Busch, U., & Tietze, E. (2011). Comparative analysis of virulence genes, genetic diversity, and phylogeny of Shiga toxin 2g and heat-stable enterotoxin *STIa* encoding *Escherichia coli* isolates from humans, animals, and environmental sources. *Int J Med Microbiol, 301*(3), 181-191.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

doi:10.1016/j.ijmm.2010.06.003.

Prapasarakul, N., Tummaruk, P., Niyomtum, W., Tripipat, T., & Serichantalergs, O. (2010). Virulence genes and antimicrobial susceptibilities of hemolytic and nonhemolytic *Escherichia coli* isolated from post-weaning piglets in central Thailand. *J Vet Med Sci, 72*(12), 1603-1608. doi:10.1292/jvms.10-0124.

Raimondi, S., Righini, L., Candeliere, F., Musmeci, E., Bonvicini, F., Gentilomi, G., . . . Rossi, M. (2019). Antibiotic resistance, virulence factors, phenotyping, and genotyping of *E. coli* Isolated from the feces of healthy subjects. *Microorganisms, 7*(8). doi:10.3390/microorganisms7080251.

Randall, L. P., Horton, R. A., Lemma, F., Martelli, F., Duggett, N. A. D., Smith, R. P., . . . Teale, C. J. (2018). Longitudinal study on the occurrence in pigs of colistin-resistant *Escherichia coli* carrying *mcr*-1 following the cessation of use of colistin. *J Appl Microbiol, 125*(2), 596-608. doi:10.1111/jam.13907.

Rebelo, A. R., Bortolaia, V., Kjeldgaard, J. S., Pedersen, S. K., Leekitcharoenphon, P., Hansen, I. M., . . . Hendriksen, R. S. (2018). Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* for surveillance purposes. *Euro Surveill, 23*(6). doi:10.2807/1560-7917.ES.2018.23.6.17-00672.

Rhouma, M., Beaudry, F., Theriault, W., & Letellier, A. (2016). Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. *Front Microbiol, 7*, 1789. doi:10.3389/fmicb.2016.01789.

Rhouma, M., & Letellier, A. (2017). Extended-spectrum β-lactamases, carbapenemases and the mcr-1 gene: is there a historical link? (2017): 269-271.

Rhouma, M., Theriault, W., Rabhi, N., Duchaine, C., Quessy, S., & Fravalo, P. (2019). First identification of *mcr-1/mcr*-2 genes in the fecal microbiota of Canadian commercial pigs during the growing and finishing period. *Vet Med (Auckl), 10*, 65-67. doi:10.2147/VMRR.S202331.

San Millan, A. (2018). Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends Microbiol, 26*(12), 978-985. doi:10.1016/j.tim.2018.06.007.

San Millan, A., Escudero, J. A., Gutierrez, B., Hidalgo, L., Garcia, N., Llagostera, M., . . . Gonzalez-Zorn, B. (2009). Multiresistance in *Pasteurella multocida* is mediated by coexistence of small plasmids. *Antimicrobial agents and chemotherapy, 53*(8), 3399-3404.

Sandegren, L., Lindqvist, A., Kahlmeter, G., & Andersson, D. I. (2008). Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*. *J Antimicrob Chemother, 62*(3), 495-503. doi:10.1093/jac/dkn222.

Sarshar, M., Scribano, D., Marazzato, M., Ambrosi, C., Aprea, M. R., Aleandri, M., . . . Conte, M. P. (2017). Genetic diversity, phylogroup distribution and virulence gene profile of pks positive *Escherichia coli* colonizing human intestinal polyps. *Microb Pathog, 112*, 274-278. doi:10.1016/j.micpath.2017.10.009.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics, 30*(14), 2068-2069. doi:10.1093/bioinformatics/btu153.

Seiler, C., & Berendonk, T. U. (2012). Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front Microbiol, 3*, 399. doi:10.3389/fmicb.2012.00399.

Shen, C., Zhong, L.-L., Yang, Y., Doi, Y., Paterson, D. L., Stoesser, N., . . . Huang, S. (2020). Dynamics of *mcr*-1 prevalence and *mcr*-1-positive *Escherichia coli* after the cessation of colistin use as a feed additive for animals in China: a prospective cross-sectional and whole genome sequencing-based molecular epidemiological study. *The Lancet Microbe, 1*(1), e34-e43.

Shen, Y., Xu, C., Sun, Q., Schwarz, S., Ou, Y., Yang, L., . . . Shen, J. (2018). Prevalence and genetic analysis of *mcr*-3-positive *Aeromonas* species from humans, retail meat, and environmental water samples. *Antimicrob Agents Chemother, 62*(9). doi:10.1128/AAC.00404-18.

Shen, Y., Zhou, H., Xu, J., Wang, Y., Zhang, Q., Walsh, T. R., . . . Wang, Y. (2018). Anthropogenic and environmental factors associated with high incidence of *mcr*-1 carriage in humans across China. *Nat Microbiol, 3*(9), 1054-1062. doi:10.1038/s41564-018-0205-8.

Singhal, N., Kumar, M., Kanaujia, P. K., & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front Microbiol, 6*, 791. doi:10.3389/fmicb.2015.00791.

Skov, R. L., & Monnet, D. L. (2016). Plasmid-mediated colistin resistance (*mcr*-1 gene): three months later, the story unfolds. *Euro Surveill, 21*(9), 30155. doi:10.2807/1560-7917.ES.2016.21.9.30155.

Snesrud, E., He, S., Chandler, M., Dekker, J. P., Hickman, A. B., McGann, P., & Dyda, F. (2016). A Model for transposition of the colistin resistance gene *mcr*-1 by ISApl1. *Antimicrob Agents Chemother, 60*(11), 6973-6976. doi:10.1128/AAC.01457-16.

Snesrud, E., McGann, P., & Chandler, M. (2018). The birth and demise of the ISApl1-*mcr-1*-ISApl1 composite transposon: the vehicle for transferable colistin resistance. *mBio, 9*(1). doi:10.1128/mBio.02381-17.

Snesrud, E., Ong, A. C., Corey, B., Kwak, Y. I., Clifford, R., Gleeson, T., . . . McGann, P. (2017). Analysis of Serial Isolates of *mcr*-1-positive *Escherichia coli* reveals a highly active ISApl1 transposon. *Antimicrob Agents Chemother, 61*(5). doi:10.1128/AAC.00056-17.

Sullivan, M. J., Petty, N. K., & Beatson, S. A. (2011). Easyfig: a genome comparison visualizer. *Bioinformatics, 27*(7), 1009-1010. doi:10.1093/bioinformatics/btr039.

Sun, J., Zhang, H., Liu, Y. H., & Feng, Y. (2018). Towards understanding *mcr*-like colistin resistance. *Trends Microbiol, 26*(9), 794-808. doi:10.1016/j.tim.2018.02.006.

Sundqvist, M., Geli, P., Andersson, D. I., Sjolund-Karlsson, M., Runehagen, A., Cars, H., . . . Kahlmeter, G. (2010). Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *J Antimicrob Chemother, 65*(2), 350-360. doi:10.1093/jac/dkp387.

Tangcharoensathien, V., Chanvatik, S., & Sommanustweechai, A. J. B. o. t. W. H. O. (2018). Complex determinants of inappropriate use of antibiotics. *96*(2), 141.

Tansawai, U., Yu, Y., Kiddee, A., Assawatheptawee, K., Sands, K., Hassan, B., . . . Niumsup, P. R. (2021). Emergence of *mcr*-3-mediated IncP and IncFII plasmids in Thailand. *J Glob Antimicrob Resist, 24*, 446-447. doi:10.1016/j.jgar.2021.02.006.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *Am J Infect Control, 34*(5 Suppl 1), S3-10; discussion S64-73. doi:10.1016/j.ajic.2006.05.219.

Tietgen, M., Semmler, T., Riedel-Christ, S., Kempf, V. A. J., Molinaro, A., Ewers, C., & Gottig, S. (2018). Impact of the colistin resistance gene *mcr*-1 on bacterial fitness. *Int J Antimicrob Agents, 51*(4), 554-561. doi:10.1016/j.ijantimicag.2017.11.011.

Van den Meersche, T., Rasschaert, G., Haesebrouck, F., Van Coillie, E., Herman, L., Van Weyenberg, S., . . . Heyndrickx, M. (2019). Presence and fate of antibiotic residues, antibiotic resistance genes and zoonotic bacteria during biological swine manure treatment. *Ecotoxicol Environ Saf, 175*, 29-38. doi:10.1016/j.ecoenv.2019.01.127.

Vines, J., Cusco, A., Napp, S., Alvarez, J., Saez-Llorente, J. L., Rosas-Rodoreda, M., . . . Migura-Garcia, L. (2021). Transmission of similar *mcr*-1 carrying plasmids among different *Escherichia coli* lineages isolated from livestock and the farmer. *Antibiotics (Basel), 10*(3). doi:10.3390/antibiotics10030313.

Vogwill, T., & MacLean, R. C. (2015). The genetic basis of the fitness costs of antimicrobial resistance: a meta-analysis approach. *Evol Appl, 8*(3), 284-295. doi:10.1111/eva.12202.

Walsh, T. R., & Wu, Y. J. T. L. i. d. (2016). China bans colistin as a feed additive for animals. *16*(10), 1102.

Wand, M. E., Bock, L. J., Bonney, L. C., & Sutton, J. M. (2017). Mechanisms of Increased resistance to chlorhexidine and cross-resistance to colistin following exposure of *Klebsiella pneumoniae* clinical isolates to chlorhexidine. *Antimicrob Agents Chemother, 61*(1). doi:10.1128/AAC.01162-16.

Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M., & Zong, Z. (2020). Identification of novel mobile colistin resistance gene *mcr*-10. *Emerg Microbes Infect, 9*(1), 508-516. doi:10.1080/22221751.2020.1732231.

Wang, Q., Sun, J., Li, J., Ding, Y., Li, X. P., Lin, J., . . . Feng, Y. (2017). Expanding landscapes of the diversified *mcr*-1-bearing plasmid reservoirs. *Microbiome, 5*(1), 70. doi:10.1186/s40168-017-0288-0.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Wang, R., Liu, Y., Zhang, Q., Jin, L., Wang, Q., Zhang, Y., . . . Wang, H. (2018). The prevalence of colistin resistance in *Escherichia col*i and *Klebsiella pneumoniae* isolated from food animals in China: coexistence of *mcr*-1 and *bla*NDM with low fitness cost. *Int J Antimicrob Agents, 51*(5), 739-744. doi:10.1016/j.ijantimicag.2018.01.023.

Wang, R., van Dorp, L., Shaw, L. P., Bradley, P., Wang, Q., Wang, X., . . . Balloux, F. (2018). The global distribution and spread of the mobilized colistin resistance gene *mcr*-1. *Nat Commun, 9*(1), 1179. doi:10.1038/s41467-018-03205-z.

Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., . . . Wang, Y. (2018). Emergence of a novel mobile colistin resistance gene, *mcr*-8, in NDM-producing *Klebsiella pneumoniae*. *Emerg Microbes Infect, 7*(1), 122. doi:10.1038/s41426-018-0124-z.

Wang, Y., Tian, G. B., Zhang, R., Shen, Y., Tyrrell, J. M., Huang, X., . . . Shen, J. (2017). Prevalence, risk factors, outcomes, and molecular epidemiology of *mcr*-1-positive *Enterobacteriaceae* in patients and healthy adults from China: an epidemiological and clinical study. *Lancet Infect Dis, 17*(4), 390-399. doi:10.1016/S1473-3099(16)30527-8.

Wang, Y., Xu, C., Zhang, R., Chen, Y., Shen, Y., Hu, F., . . . Shen, J. (2020). Changes in colistin resistance and *mcr*-1 abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study. *Lancet Infect Dis, 20*(10), 1161-1171. doi:10.1016/S1473-3099(20)30149-3.

Wang, Z., Fu, Y., Du, X. D., Jiang, H., & Wang, Y. (2018). Potential transferability of *mcr*-3 via IS26-mediated homologous recombination in *Escherichia coli*. *Emerg Microbes Infect, 7*(1), 55. doi:10.1038/s41426-018-0057-6.

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol, 13*(6), e1005595. doi:10.1371/journal.pcbi.1005595.

Wu, C., Wang, Y., Shi, X., Wang, S., Ren, H., Shen, Z., . . . Wang, S. (2018). Rapid rise of the ESBL and *mcr*-1 genes in *Escherichia coli* of chicken origin in China, 2008-

2014. *Emerg Microbes Infect, 7*(1), 30. doi:10.1038/s41426-018-0033-1.

Wu, R., Yi, L. X., Yu, L. F., Wang, J., Liu, Y., Chen, X., . . . Liu, J. H. (2018). Fitness Advantage of *mcr*-1-Bearing IncI2 and IncX4 Plasmids in Vitro. *Front Microbiol, 9*, 331. doi:10.3389/fmicb.2018.00331.

Xia, X., Wang, Z., Fu, Y., Du, X. D., Gao, B., Zhou, Y., . . . Wu, Y. (2019). Association of colistin residues and manure treatment with the abundance of *mcr*-1 gene in swine feedlots. *Environ Int, 127*, 361-370. doi:10.1016/j.envint.2019.03.061.

Xu, F., Zeng, X., Hinenoya, A., & Lin, J. (2018). MCR-1 confers cross-resistance to bacitracin, a widely used in-feed antibiotic. *mSphere, 3*(5). doi:10.1128/mSphere.00411-18.

Xu, Y., Zhong, L. L., Srinivas, S., Sun, J., Huang, M., Paterson, D. L., . . . Feng, Y. (2018). Spread of *mcr*-3 colistin resistance in china: an epidemiological, genomic and mechanistic study. *EBioMedicine, 34*, 139-157. doi:10.1016/j.ebiom.2018.07.027.

Yang, Q. E., MacLean, C., Papkou, A., Pritchard, M., Powell, L., Thomas, D., . . . Walsh, T. R. (2020). Compensatory mutations modulate the competitiveness and dynamics of plasmid-mediated colistin resistance in *Escherichia coli* clones. *ISME J, 14*(3), 861-865. doi:10.1038/s41396-019-0578-6.

Yang, Y. Q., Li, Y. X., Lei, C. W., Zhang, A. Y., & Wang, H. N. (2018). Novel plasmid-mediated colistin resistance gene *mcr*-7.1 in *Klebsiella pneumoniae*. *J Antimicrob Chemother, 73*(7), 1791-1795. doi:10.1093/jac/dky111.

Yates, C. M., Shaw, D. J., Roe, A. J., Woolhouse, M. E., & Amyes, S. G. (2006). Enhancement of bacterial competitive fitness by apramycin resistance plasmids from non-pathogenic *Escherichia coli*. *Biol Lett, 2*(3), 463-465. doi:10.1098/rsbl.2006.0478.

Yilmaz, E. S., & Aslantas, O. (2020). Phylogenetic group/subgroups distributions, virulence factors, and antimicrobial susceptibility of *Escherichia coli* strains from urinary tract infections in Hatay. *Rev Soc Bras Med Trop, 53*, e20190429. doi:10.1590/0037-8682-0429-2019.

Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., . . . Wang, Y. (2017). Novel Plasmid-

Mediated colistin resistance gene *mcr*-3 in *Escherichia coli*. *mBio, 8*(3). doi:10.1128/mBio.00543-17.

You, Y., Leahy, K., Resnick, C., Howard, T., Carroll, K. C., & Silbergeld, E. K. (2016). Exposure to pathogens among workers in a poultry slaughter and processing plant. *American journal of industrial medicine, 59*(6), 453-464.

Zhang, Y., Liao, K., Gao, H., Wang, Q., Wang, X., Li, H., . . . Wang, H. (2017). Decreased fitness and virulence in ST10 *Escherichia coli* harboring *bla*NDM-5 and *mcr*-1 against a st4981 strain with *bla*NDM-5. *Front Cell Infect Microbiol, 7*, 242. doi:10.3389/fcimb.2017.00242.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

VITA

NAME	Nwai Oo Khine
DATE OF BIRTH	21.04.1992
PLACE OF BIRTH	Yangon, Myanmar
INSTITUTIONS ATTENDED	1. University of Veterinary Science, Yezin, NayPyiTaw (B.V.Sc) 2. Kasetsart University, Faculty of Veterinary Medicine, Department of Parasitology (M.Sc) 3. Chulalongkorn University, Faculty of Veterinary Science, Department of Microbiology
HOME ADDRESS	No.488, Shwe Thway Street, North Dagon, Myanmar
PUBLICATION	Multidrug Resistance and Virulence Factors of Escherichia coli Harboring Plasmid-Mediated Colistin Resistance: mcr-1 and mcr-3 Genes in Contracted Pig Farms in Thailand. Front. Vet. Sci., 10 November 2020 | https://doi.org/10.3389/fvets.2020.582899
AWARD RECEIVED	Oral presentation award at CUVC 2019

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY