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

Chanika Pungpian [1], Scarlett Lee [2], Suthathip Trongjit [1], Nuananong Sinwat [3], Sunpetch Angkititrakul [4], Rangsiya Prathan [1], Songsak Srisanga [1], Rungtip Chuanchuen [1,*]

[1]Research Unit in Microbial Food Safety and Antimicrobial Resistance, Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14850, United States
[3]Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine Kasetsart University, Kamphangsaen Nakornpathom 73140, Thailand
[4]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

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***Corresponding author:**
Rungtip Chuanchuen
Faculty of Veterinary Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand.
E-mail: chuanchuen.r@gmail.com

ORCID iDs
Chanika Pungpian
https://orcid.org/0000-0002-0335-6761
Scarlett Lee
https://orcid.org/0000-0002-0486-5621
Suthathip Trongjit
https://orcid.org/0000-0003-3346-6299
Nuananong Sinwat
https://orcid.org/0000-0003-3445-4206

ABSTRACT

Background: Colistin and carbapenem-resistant bacteria have emerged and become a serious public health concern, but their epidemiological data is still limited.
Objectives: This study examined colistin and carbapenem resistance in *Escherichia coli* and *Salmonella* from pigs, pig carcasses, and pork in Thailand, Lao PDR, and Cambodia border provinces.
Methods: The phenotypic and genotypic resistance to colistin and meropenem was determined in *E. coli* and *Salmonella* obtained from pigs, pig carcasses, and pork (n = 1,619). A conjugative experiment was performed in all isolates carrying the *mcr* gene (s) (n = 68). The plasmid replicon type was determined in the isolates carrying a conjugative plasmid with *mcr* by PCR-based replicon typing (n = 7). The genetic relatedness of *mcr*-positive *Salmonella* (n = 11) was investigated by multi-locus sequence typing.
Results: Colistin resistance was more common in *E. coli* (8%) than *Salmonella* (1%). The highest resistance rate was found in *E. coli* (17.8%) and *Salmonella* (1.7%) from Cambodia. Colistin-resistance genes, *mcr-1*, *mcr-3*, and *mcr-5*, were identified, of which *mcr-1* and *mcr-3* were predominant in *E. coli* (5.8%) and *Salmonella* (1.7%), respectively. The *mcr-5* gene was observed in *E. coli* from pork in Cambodia. Two colistin-susceptible pig isolates from Thailand carried both *mcr-1* and *mcr-3*. Seven *E. coli* and *Salmonella* isolates contained *mcr-1* or *mcr-3* associated with the IncF and IncI plasmids. The *mcr*-positive *Salmonella* from Thailand and Cambodia were categorized into two clusters with 94%–97% similarity. None of these clusters was meropenem resistant.
Conclusions: Colistin-resistant *E. coli* and *Salmonella* were distributed in pigs, pig carcasses, and pork in the border areas. Undivided-One Health collaboration is needed to address the issue.

Keywords: Drug resistance; swine; Thailand; Laos; Cambodia

Sunpetch Angkititrakul
https://orcid.org/0000-0002-1701-907X
Rangsiya Prathan
https://orcid.org/0000-0003-1242-7493
Songsak Srisanga
https://orcid.org/0000-0002-1710-7424
Rungtip Chuanchuen
https://orcid.org/0000-0002-9714-9199

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Chuanchuen R, Angkititrakul S, Pungpian C; Data curation: Pungpian C, Lee S, Trongjit S, Sinwat N; Formal analysis: Chuanchuen R, Pungpian C; Funding acquisition: Chuanchuen R; Investigation: Chuanchuen R, Angkititrakul S, Pungpian C, Lee S, Trongjit S, Sinwat N, Prathan R, Srisanga S; Methodology: Chuanchuen R, Angkititrakul S, Pungpian C, Lee S, Trongjit S, Sinwat N, Prathan R, Srisanga S; Project administration: Chuanchuen R; Resources: Chuanchuen R, Angkititrakul S; Software: Chuanchuen R, Pungpian C; Supervision: Chuanchuen R; Validation: Chuanchuen R, Angkititrakul S, Pungpian C, Lee S, Trongjit S, Sinwat N, Prathan R, Srisanga S; Visualization: Chuanchuen R; Writing - original draft: Chuanchuen R, Pungpian C; Writing - review & editing: Chuanchuen R, Angkititrakul S, Pungpian C, Lee S, Trongjit S, Sinwat N, Prathan R, Srisanga S.

INTRODUCTION

Antimicrobial resistance (AMR) is an ongoing One Health problem of global dimensions. The emergence and rapid spread of multidrug resistance (MDR) have complicated the AMR situation. Last-line antibiotics, e.g., polymyxins and carbapenems, have been used to treat infections involved in MDR Gram-negative bacterial pathogens. On the other hand, resistance to last-resort treatments has been increasingly reported [1], which has raised the alarm over the potentially limited treatment options in the near future.

Carbapenems are critically essential antimicrobials used to treat infections caused by MDR, including ESBL-producing Enterobacteriaceae [1]. Carbapenem-resistant *Enterobacteriaceae* (CRE) infections with high mortality have increased rapidly in hospitals [2], and CRE has also been observed in food animals [1]. Carbapenem resistance is commonly mediated by carbapenemase genes (e.g., bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA}), which are located on large conjugative plasmids carrying other resistance genes [3]. The coexistence of carbapenem and colistin resistance genes was reported in *E. coli* in patients and animals [1,4].

Colistin or polymyxin B is a last-resort antibiotic against MDR infections, especially CRE in humans [4]. The drug is used widely in pigs to treat intestinal infections caused by *Escherichia coli*, particularly post-weaning diarrhea [5]. Colistin resistance has been identified in pathogens from various sources, e.g., *E. coli* from pigs in China [5]; poultry in Argentina [6]; patients, pigs, and poultry in Cambodia [2]; and *Salmonella* from patients in Denmark [7]. Since the report of plasmid-mediated *mcr-1* in *E. coli* and *Klebsiella pneumoniae* from animals and patients in China in 2016 [8], several *mcr* variants have been discovered [9]. Currently, the detection of plasmid-mediated colistin resistance is included in various national AMR monitoring and surveillance programs globally [10].

The coexistence of bla_{NDM-1} and *mcr-1* was detected in *E. coli* from patients in China and Taiwan and CRE from broilers and pigs in China [1]. The cotransfer of carbapenem and colistin resistance genes located on different plasmids has been demonstrated [3]. These could contribute to the emergence and spread of superbugs no longer susceptible to any commercially available antibiotic.

Pig and pork production is one of the largest markets worldwide, and several antimicrobials are used to improve the quantity and quality of products [11]. This phenomenon also occurs in Southeast Asia border trade areas, where imports and exports of live animals and products are an important contributor to increased cross-border trade. As a borderless One Health threat, AMR in one region may pose a risk to public health, animal health, and environmental health within or outside the region. AMR monitoring and surveillance is the foundation for assessing the burden of AMR and providing information for strategic action to control and prevent threats and evaluate the strategic interventions. Many developing countries still lack national AMR surveillance. Epidemiological data on AMR and data on last-line antibiotics are insufficient, particularly in these areas. Therefore, this study examined the prevalence and genetics underlying the resistance to carbapenems and colistin in *E. coli* and *Salmonella* isolated from pigs, pig carcasses, and pork in Thailand, Lao PDR, and Cambodia border provinces.

MATERIALS AND METHODS

Sample collection and bacterial isolation

A total of 988 *E. coli* and 631 *Salmonella* isolates from pigs (n = 377 and 139), pig carcasses (n = 328 and 174), and pork (n = 283 and 318) were included. They were isolated previously as part of AMR epidemiological studies in food animals and products in Southeast Asia between 2014 and 2018 [12-15]. The *E. coli* and *Salmonella* isolates were obtained from Thailand (n = 508 and 276), Lao PDR (n = 351 and 237), and Cambodia (n = 129 and 118) (**Table 1**). The sampling sites were located in the provinces with the two largest border-crossing points between Thailand-Lao PDR (i.e., Nong Khai-Vientiane and Mukdahan-Savannakhet) and Thailand-Cambodia (i.e., Sa Kaeo-Banteay Meanchey).

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

The rectal swabs were collected from the dead pigs immediately after bleeding but before the scalding process at the slaughterhouses. The pig carcass samples were obtained by swabbing the inner side of a half pig carcass from the neck to the bottom after the first whole carcass was cut lengthwise and divided into two halves. The pork samples were collected by swabbing at least 50 cm^2 of raw meat at the retail fresh markets. Sample collection at the slaughterhouses and retail markets was performed by the authors' sample collection team consisting of laboratory staff and veterinarians trained in the same sampling protocol [16]. All samples collected were stored in an icebox and transported to the laboratory within 24 h after sampling.

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

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

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The *E. coli* strains were isolated and confirmed biochemically [17]. The *E. coli* isolates were selected on MacConkey agar and Eosin Methylene Blue agar and confirmed by an indole test. One *E. coli* colony was collected from each positive sample. The *Salmonella* strains were isolated according to ISO6579:2002 (E) [18] and subjected to serotyping by slide agglutination [19]. A single colony of each serotype was collected from each positive sample. Forty-five serovars were included, of which the serovars Typhimurium and Rissen were most common (**Table 2**).

Table 2. *Salmonella* serovars from pig, pig carcass, and pork samples in Thailand, Lao PDR, and Cambodia border provinces in this study (n = 631)

Salmonella serovars	No. of isolates												Total
	Thailand				Lao PDR				Cambodia				
	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	Pig	Carcass	Pork	Sub total	
Afula					2			2					2
Agona							1	1					1
Anatum	3		15	18	9	25	16	50	1	1		2	70
Braenderup											1	1	1
Chincol											1	1	1
Corvallis			21	21									21
Derby			4	4	4	3	10	17	3	6		9	30
Dessau					2			2					2
Doncaster										1		1	1
Duesseldort			4	4									4
Eindegi					1	6	2	9					9
Enteritidis	1			1						1		1	2
Fufu									1			1	1
Give	1	2	1	4	1		1	2					6
Havana							2	2					2
Hvittingfoss			2	2		1		1					3
Indians											1	1	1
Kedougou	11	4	11	26									26
Kentucky		1		1									1
Lexington									3		2	5	5
London						1		1					1
Mbandaka											1	1	1
Menden	1			1									1
Monschaui			2	2									2
Mowanjum											1	1	1
Newmexico									2	1		3	3
Norwish										1		1	1
Panama		1	2	3	1			1					4
Parathyphi		1	1	2					3	3	13	19	21
Preston							2	2					2
Readind					1			1					1
Rissen	11	20	16	47	7	11	17	35	4		42	46	128
Saintpaul		6	7	13									13
Schleissheim										1	1	2	2
Schwarzengrund			1	1									1
Singapore										2	1	3	3
Stanley	1	6	9	16	11	10	13	34		1	1	2	52
Stuttgart			1	1									1
Tsevie							2	2					2
Typhimurium	27	16	52	95	15	22	19	56	3	8		11	162
Urbana			2	2									2
Wandsworth										1		1	1
Warragul									1	1		2	2
Welterreden	1	6	4	11	5	3	1	9	1			1	21
Worthington	1			1			10	10		1	2	3	14
Total	58	63	154	276	59	82	96	237	22	29	67	118	631

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

The minimum inhibitory concentrations (MICs) of colistin were determined using the agar dilution method [20]. The joint CLSI-EUCAST Polymyxin Breakpoints Working Group recommended the broth microdilution method for determining the colistin MIC. On the other hand, the plastic binding feature of colistin and the synergistic effects of polysorbate 80 with colistin have been observed [21]. The agar dilution method generates a comparable colistin MIC value to the broth microdilution method [21], but is superior in reproducibility, robustness, and user-friendliness. Therefore, the agar dilution method was used to determine the colistin MIC in this study. The colistin concentration range was 0.125–64 μg/mL with an MIC breakpoint of 2 μg/mL [22]. *E. coli* ATCC25922, *Staphylococcus aureus* ATCC29213, and *Pseudomonas aeruginosa* ATCC27853 served as the quality control strains.

Carbapenemase production was screened by the disk diffusion method using meropenem disks (10 μg) [20] and confirmed using the modified Hodge test (MHT) with meropenem (10 μg). *E. coli* ATCC25922 was the control strain.

PCR and DNA sequencing analysis

The total DNA prepared by whole-cell boiling was used as a PCR template [23]. **Table 3** lists all the primers used. The PCR products were purified using Nucleospin Gel and PCR clean up (Macherey-Nagel, Germany) and submitted for DNA sequencing using the PCR primers (First Base Laboratories, Selangor Darul Ehsan, Malaysia). The nucleotide sequences were analyzed by a comparison to the published sequences available at the NCBI website.

All bacterial isolates (n = 1,619) were screened for colistin-resistance encoding genes, including *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* using multiplex PCR with the specific primers [24]. Representatives of PCR amplicons were purified and submitted for DNA sequencing to confirm the specificity of PCR amplification. The DNA sequences obtained were deposited

Table 3. Primers used in this study

Target	Primer	Primer sequenc (5′-3′)	Product size (bp)	Reference
MCR				
Multiplex	*mcr1*-F	AGTCCGTTTGTTCTTGTGGC	320	[24]
	mcr1-R	AGATCCTTGGTCTCGGCTTG		
	mcr2-F	CAAGTGTGTTGGTCGCAGTT	715	[24]
	mcr2-R	TCTAGCCCGACAAGCATACC		
	mcr3-F	AAATAAAAATTGTTCCGCTTATG	929	[24]
	mcr3-R	AATGGAGATCCCCGTTTTT		
	mcr4-F	TCACTTTCATCACTGCGTTG	1116	[24]
	mcr4-R	TTGGTCCATGACTACCAATG		
	mcr5-F	ATGCGGTTGTCTGCATTTATC	1644	[24]
	mcr5-R	TCATTGTGGTTGTCCTTTTCTG		
Carbapenemase genes				
Multiplex	bla_{IMP}-F	GGAATAGAGTGGCTTAAYTCTC	232	[25]
	bla_{IMP}-R	GGTTTAAYAAAACAACCACC		
	bla_{VIM}-F	GATGGTGTTTGGTCGCATA	390	[25]
	bla_{VIM}-R	CGAATGCGCAGCACCAG		
	bla_{OXA}-F	GCGTGGTTAAGGATGAACAC	477	[25]
	bla_{OXA}-R	CATCAAGTTCAACCCAACCG		
	bla_{NDM}-F	GGTTTGGCGATCTGGTTTTC	621	[25]
	bla_{NDM}-R	CGGAATGGCTCATCACGATC		
	bla_{KPC}-F	CGTCTAGTTCTGCTGTCTTG	798	[25]
	bla_{KPC}-R	CTTGTCATCCTTGTTAGGCG		

(continued to the next page)

Table 3. (Continued) Primers used in this study

Target	Primer	Primer sequenc (5′-3′)	Product size (bp)	Reference
PBRT				
Multiplex 1	HI1-F	GGAGCGATGGATTACTTCAGTAC	471	[27]
	HI1-R	TGCCGTTTCACCTCGTGAGTA		
	HI2-F	TTTCTCCTGAGTCACCTGTTAACAC	644	[27]
	HI2-R	GGCTCACTACCGTTGTCATCCT		
	I1-F	CGAAAGCCGGACGGCAGAA	139	[27]
	I1-V	TCGTCGTTCCGCCAAGTTCGT		
Multiplex 2	X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	[27]
	X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC		
	L/M-F	GGATGAAAACTATCAGCATCTGAAG	785	[27]
	L/M-R	CTGCAGGGGCGATTCTTTAGG		
	N-F	GTCTAACGAGCTTACCGAAG	559	[27]
	N-R	GTTTCAACTCTGCCAAGTTC		
Multiplex 3	FIA-F	CCATGCTGGTTCTAGAGAAGGTG	462	[27]
	FIA-R	GTATATCCTTACTGGCTTCCGCAG		
	FIB-F	GGAGTTCTGACACACGATTTTCTG	702	[27]
	FIB-R	CTCCCGTCGCTTCAGGGCATT		
	W-F	CCTAAGAACAACAAAGCCCCCG	242	[27]
	W-R	GGTGCGCGGCATAGAACCGT		
Multiplex 4	Y-F	AATTCAAACAACACTGTGCAGCCTG	765	[27]
	Y-R	GCGAGAATGGACGATTACAAAACTTT		
	P-F	CTATGGCCCTGCAAACGCGCCAGAAA	534	[27]
	P-R	TCACGCGCCAGGGCGCAGCC		
	FIC-F	GTGAACTGGCAGATGAGGAAGG	262	[27]
	FIC-R	TTCTCCTCGTCGCCAAACTAGAT		
Multiplex 5	A/C-F	GAGAACCAAAGACAAAGACCTGGA	465	[27]
	A/C-R	ACGACAAACCTGAATTGCCTCCTT		
	T-F	TTGGCCTGTTTGTGCCTAAACCAT	750	[27]
	T-R	CGTTGATTACACTTAGCTTTGGAC		
	FIIS-F	CTGTCGTAAGCTGATGGC	270	[27]
	FIIS-R	CTCTGCCACAAACTTCAGC		
Simplex 1	FrepB-F	TGATCGTTTAAGGAATTTTG	270	[27]
	FrepB-R	GAAGATCAGTCACACCATCC		
Simplex 2	K-F	GCGGTCCGGAAAGCCAGAAAAC	160	[27]
	K-R	TCTTTCACGAGCCCGCCAAA		
Simplex 3	B/O-F	GCGGTCCGGAAAGCCAGAAAAC	159	[27]
	B/O-R	TCTGCGTTCCGCCAAGTTCGA		
MLST	*fimA*-F	TCAGGGGAGAAACAGAAAACTAAT	760	[28]
	fimA-R	TCCCCGATAGCCTCTTCC		
	manB-F	CCGGCACCGAAGAGA	893	[28]
	manB-R	CGCCGCCATCCGGTC		
	mdh-F	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	849	[28]
	mdh-R	ATATCTTTYTTCAGCGTATCCAGCAT		

PBRT, polymerase chain reaction-based replicon typing; MLST, multi-locus sequence typing.

in the GenBank database, of which the accession numbers are as follows: *mcr-1*, MW314264; *mcr-3*, MW314265; *mcr-5*, MW314266.

Carbapenem-resistance encoding genes were detected in all bacterial isolates using multiplex PCR with the specific primers [25], including bla_{IMP}, bla_{VIM}, bla_{OXA}, bla_{NDM-1}, and bla_{KPC}.

Conjugation experiments and plasmid replicon typing

A biparental mating experiment was performed in all *mcr*-positive *E. coli* and *Salmonella* isolates. All *mcr*-carrying *E. coli* (n = 57) were used as donors and spontaneous rifampicin-resistant *Salmonella* Enteritidis SE12 (SE12rif^r, MIC = 256 μg/mL) was used as the recipient [26]. The transconjugants were selected on LB agar containing rifampicin (32 μg/mL)

with colistin (2 μg/mL) and confirmed to be *Salmonella* on brilliant green agar and xylose lysine deoxycholate agar (Difco, MD, USA) containing 2 μg/mL colistin. For all *mcr*-positive *Salmonella* isolates (n=11), the spontaneous rifampicin-resistant *E. coli* K12 strain MG1655 (MG1655rif^r) was used as the recipient [26]. The transconjugants were selected on LB agar containing rifampicin (32 μg/mL) with colistin (2 μg/mL) and confirmed to be *E. coli* on MacConkey agar and Eosin Methylene Blue agar (Difco, MD) containing 2 μg/mL colistin. The presence of the corresponding genes in all transconjugants was confirmed by PCR using specific primers as described above.

The plasmid Inc groups were determined in all the isolates containing the conjugative plasmid by a PCR-based replicon typing (PBRT) [27]. Eighteen plasmid Inc groups were detected using five multiplex PCR (i.e., HI1/HI2/I1-Iγ; X/L-M/N; FIA/FIB/W; Y/P/FIC and A-C/T/FIIA) and three simplex PCR (i.e., F, K, and B/O).

Salmonella phylogenetic analysis

All *mcr*-positive *Salmonella* (n = 11) were characterized for the phylogenetic groups by multi-locus sequence typing (MLST). Three housekeeping genes, *fimA*, *manB*, and *mdh* were PCR amplified as described previously [28]. All PCR products were purified and submitted for nucleotide sequencing. The obtained sequences were assembled and proofread using the DNASTAR program [29] and aligned using the MUSCLE options in MEGA-X software [30]. A phylogenetic tree with 1000 bootstrap replicates was constructed based on the concatenated alignment of *fimA*, *manB,* and *mdh* sequences using the Neighbor-Joining (NJ) method [28]. The Maximum Composite Likelihood method was used to calculate the evolutionary distances [30]. All positions containing gaps and missing data were removed for each sequence pair. The final data set contained 18,129 positions. Evolutionary analyses were conducted using MEGA X [30].

Statistical analysis

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

RESULTS

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

Table 4 lists the colistin resistance rates of *E. coli* and *Salmonella* from Thailand, Lao PDR, and Cambodia. The prevalence of colistin-resistant *E. coli* was higher than that of *Salmonella* in all countries ($p < 0.05$). Colistin resistance was commonly observed in either *E. coli* (17.8%;

Table 4. Colistin resistance rates of *E. coli* and *Salmonella* from pigs, pig carcasses, and pork in Thailand, Lao PDR, and Cambodia (n = 1,619)

Bcateria	Resistance rate*												Grand total
	Thailand				Lao PDR				Cambodia				
	Pig	Carcass	Pork	Total	Pig	Carcass	Pork	Total	Pig	Carcass	Pork	Total	
E. coli	9.4% (17/180)	5.4% (10/185)	7.7% (11/143)	7.5%†,§ (38/508)	7.8% (9/115)	6.1% (8/132)	1.9% (2/104)	5.4%†,§ (19/351)	22% (18/82)	9.1% (1/11)	11.1% (4/36)	17.8%$^{\dagger,\|\|}$ (23/129)	8% (80/988)
Salmonella	3.4% (2/58)	0 (0)	0.6% (1/155)	1.1%‡,§ (3/276)	0 (0)	1.2% (1/83)	0 (0)	0.5%‡,§ (1/218)	4.5% (1/22)	0 (0)	1.5% (1/67)	1.7%$^{\ddagger,\|\|}$ (2/118)	1% (6/631)

*In parenthesis, Number of colistin-resistant isolates/Number of bacterial isolates from each source; †,‡values bearing a different superscript in the same column are significantly different ($p < 0.5$); $^{\S,\|\|}$values bearing a different superscript in the same row are significantly different ($p < 0.5$).

23/129) or *Salmonella* (1.7%; 2/118) from Cambodia ($p < 0.05$). When considering the sample sources, colistin-resistant *E. coli* were most common in pigs in Thailand (9.4%; 17/180), Lao PDR (7.8%; 9/115), and Cambodia (22%; 18/82). Colistin-resistant *Salmonella* was identified most frequently in pigs in Thailand (3.4%; 2/58) and Cambodia (4.5%; 1/22) (**Table 4**). None of the isolates in this collection was meropenem resistant.

Distribution of mcr genes (n = 1,619)

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

Table 5. Presence and transfer of *mcr* genes in *E. coli* and *Salmonella* (n = 1,619)

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

MIC, minimum inhibitory concentration.
*The number indicates the colistin-resistant isolate that can transfer *mcr*; †,‡Values for *E. coli* from different countries bearing a different superscript in the same column are significantly different ($p \leq 0.05$); §,‖Values for *E. coli* and *Salmonella* bearing a different superscript in the same column are statistical different ($p \leq 0.05$).

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Table 6. Characteristics of *E. coli* and *Salmonella* transconjugant (n = 7)

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

MIC, minimum inhibitory concentration.

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

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

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

Horizontal transfer and plasmid replicon typing of mcr gene carrying isolates

Two *E. coli* isolates from pig carcass and pork in Thailand were *mcr-1* transferred conjugally to *Salmonella* recipients. The *mcr-3* gene in two *E. coli* isolates (one from Thai pork and the others from a Laos pig) and three *Salmonella* (two from Thai pigs and one from Cambodian pig) were transferred horizontally. All the isolates were resistant to colistin (colistin MIC = 4–8 μg/mL) and harbored the IncF plasmid. The *mcr*-conjugative plasmids were in the IncF and IncI groups (**Table 6**).

Evolutionary relationship among mcr-carrying Salmonella

The NJ phylogenetic tree showed that *mcr*-carrying *Salmonella* isolates (n = 11) could be classified into two clusters, Clusters A and B (**Table 7**). The percentage similarity within each cluster ranged from 94% to 97%, and the average branch lengths of the *Salmonella* isolates within the same cluster ranged from 0.00 to 0.01 (**Fig. 1**). The phylogenetic cluster A (n = 7) was predominant, including four isolates from Thailand [*S.* Typhimurium from pigs (n = 2), *S.* Kedougou from pork (n = 1), and *S.* Rissen from a pig (n = 1)] and three isolates from Cambodia [*S.* Parathyphi from pigs (n = 2) and *S.* Kentucky from a pig (n = 1)]. Cluster B

Table 7. Cluster and characteristics of selected *Salmonella* in multi-locus sequence typing (n = 11)

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

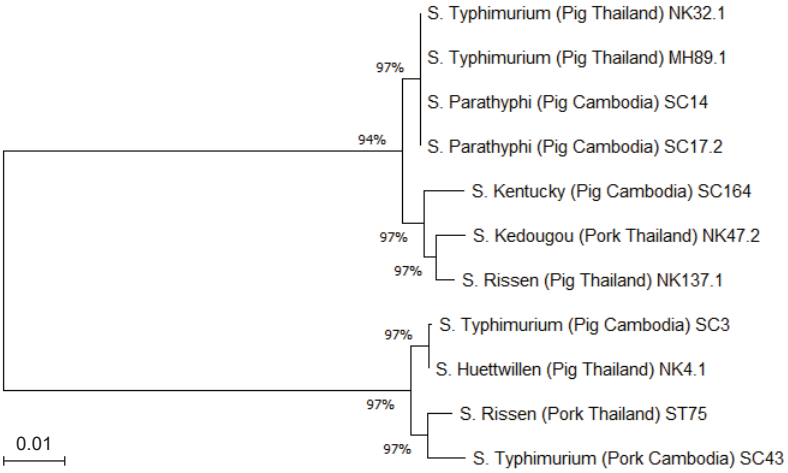

Fig. 1. Phylogenetic tree of the *Salmonella* isolates (n = 11). Phylogenetic analysis was based on the alignment of the three multi-locus sequence typing genes (*manB*, *mdh*, and *fimA*) sequenced. The sequence was aligned by the MUSCLE options in MEGA-X, and a phylogenic tree was constructed using the neighbor-joining method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

contained four *Salmonella* isolates, including three isolates from Thailand [*S.* Typhimurium from a pig (n = 1), *S.* Huettwillen from a pig (n = 1), and *S.* Rissen from pork (n = 1)], and one isolate from Cambodia [*S.* Typhimurium from pork (n = 1)].

DISCUSSION

One of the major findings of this study was the presence of colistin-resistant *E. coli* and *Salmonella* harboring *mcr* in pigs, pig carcasses, and pork, indicating the circulation of colistin-resistant bacteria and genes in the pig production chain in the region. A previous study reported that flattening pigs entering slaughterhouses acted as vehicles for colistin-resistant bacteria distribution to the slaughterhouse environment [31]. This agrees with the observation of a high percentage of colistin-resistant *E. coli* in pigs in this study. Pig carcasses might be contaminated with resistant bacteria during the slaughtering process that is transferred to the pork in the retail meat markets [32]. The resistant bacteria may spread to workers at pig farms or slaughterhouses and butchers at the retail markets by direct contact and the food chain. These results show that the circulation of colistin-resistant bacteria in the pig and pork production chain is a potential route for introducing resistant bacteria to humans and the environment [32].

For Thailand, the colistin-resistant *E. coli* (7.5%) in this study was more common than a previous study reporting the absence of colistin-resistant *E. coli* in pig farms in other provinces of Northeastern Thailand [11]. Although the extent of antimicrobial use is largely unclear, the results may reflect the increase in colistin consumption in pig production in the area [31]. This discrepancy might be associated with diverse sources and different geographical origins of the samples as well. Furthermore, colistin resistance could be the result of co-selection caused by other antimicrobials. A previous study in *E. coli* showed that host cationic antimicrobial peptides (e.g., LL-37 and lysozyme) could promote cross-resistance to polymyxins, possibly explaining the spread of colistin resistance in the limited colistin use [33]. In contrast, the percentage of colistin resistance in Cambodia (17.8%) was

significantly higher than that in Thailand (7.5%) and Lao PDR (5.4%), but was slightly lower than a previous study in Cambodia [34]. This may be because of the higher use of colistin and other antimicrobial drugs in the country, which agrees with a previous study showing that the greater use and longer application of antimicrobials in pig feed on farms may increase the AMR prevalence in Cambodia [34].

The phenotypic colistin resistance and *mcr* in *E. coli* are more common than that in *Salmonella* ($p < 0.05$). A previous study reported that the resistance rates to antimicrobials in different classes in *E. coli* were frequently higher than those in *Salmonella* [35]. *E. coli* are commensals existing in the intestinal tract of all animals and have a longer contact duration to antimicrobials administered to animals than *Salmonella*. Therefore, they have greater effectiveness in the development of resistance to antimicrobials than *Salmonella* [36]. In addition, AMR development in *E. coli* is affected more by the antimicrobials used than in *Salmonella* under the same condition [36].

The *mcr-1* and *mcr-3* genes were predominant *mcr* variants in this study. The *mcr-1* gene was most common among colistin-resistant *E. coli* (59.6%), which agrees with a previous study in pigs in China [31]. This gene has been detected in *E. coli* and *Salmonella* from humans and livestock in most parts of the world, including Asia (e.g., China) [32], Europe (e.g., Spain) [9], America (e.g., Argentina) [6] and South Africa (e.g., Algeria) [37]. The *mcr-1* gene was located on a conjugative plasmid, supporting its wide presence in *E. coli* and *Salmonella*. On the other hand, it was previously reported that *mcr-1* is stable and can be transferred without selective pressure from colistin [33]. The *mcr-3* gene was found in the *E. coli* and *Salmonella* isolates from pigs, pig carcasses, and pork in Thailand and Cambodia but was predominant among colistin-resistant *Salmonella* (10/11). Previous studies reported *mcr-3* in *Salmonella* in patients in Canada [38] and Denmark [7] who traveled to Thailand and *E. coli* and *Klebsiella pneumoniae* in patients in Thailand [39]. Plasmids carrying *mcr-3* were previously observed in *E. coli* from pigs in China and Malaysia, of which its sequence was similar to that in *K. pneumoniae* patient isolates in Thailand [40]. These data indicate the worldwide spread of *mcr-3* in humans and animals. An *E. coli* isolate from pork in Cambodia carried *mcr-5*. The *mcr-5* gene was previously detected in *E. coli* from pigs and poultry in China and Spain [9], suggesting the wide distribution of *mcr-5*. To the best of the authors' knowledge, this is the first report of *mcr-5* in *E. coli* from pork in Cambodia.

The coexistence of *mcr-1* and *mcr-3* was observed in *E. coli* from pigs in Thailand, which concurs with previous studies in Taiwan and China [41]. Almost all *E. coli* and *Salmonella* isolates carrying individual *mcr-1* or *mcr-3* were resistant to colistin (MIC≥2 μg/mL), suggesting that the presence of *mcr* in these isolates corresponded well to the colistin resistance phenotype. In contrast, the *E. coli* isolates carrying *mcr-1*/*mcr-3* were susceptible to colistin (MIC = 1 μg/mL), which is in agreement with the study in China [41], supporting the premise that the coexistence of *mcr* in a single *E. coli* isolate does not generate a cumulative effect on colistin susceptibility [41]. These results indicate that the contribution of *mcr* to the colistin resistance phenotype varies and that the *mcr* genes should be screened in either colistin-susceptible or resistant isolates.

In this study, transconjugants (n = 7) harbored the *mcr* gene on the IncF (n = 4) or IncI (n = 3) plasmids, which is in agreement with a previous study demonstrating IncF, IncI, IncHI2, and IncX4 plasmids carrying the *mcr-1* and *mcr-3* [42]. The IncF plasmid is associated with several

resistance genes worldwide. Overall, these observations highlight the potential distribution of *mcr* genes globally.

The prevalence of *mcr* genes in *E. coli* and *Salmonella* from Cambodia was significantly higher than in Thailand and Lao PDR, which agrees with the colistin resistance phenotype observed and suggests that the colistin resistance phenotype was being driven by the presence of the *mcr* genes [1]. Such higher prevalence may be associated with the use of colistin and antimicrobial drugs in other classes on-farm, resulting in the preservation and transfer of the genes [11].

Infection with *mcr*-carrying *Salmonella* may be difficult to treat and result in treatment failure. Knowledge of the genetic relatedness of *mcr*-harboring *Salmonella* will increase the understanding of their transmission mode [28]. In this study, all *mcr*-positive *Salmonella* were categorized into two clusters by MLST. Each cluster contained the pig and pork isolates from Thailand and Cambodia. The close similarity within the same cluster suggests the clonal expansion of *mcr*-positive *Salmonella* between the two countries. The *mcr*-positive *Salmonella* in Thailand and Cambodia were categorized into two clusters, possibly due to the multiple and independent acquisitions of *mcr* in bacteria in the same geographical area. This is similar to a study in China where *mcr-1* was harbored by multiple clones of *E. coli* isolates (e.g., ST10, ST101, and ST410) from hospital sewage water [43]. *E. coli* ST624 with *mcr-1* isolates from a patient in South Africa was identified in *E. coli* from poultry in Spain and Japan [44]. Overall, these observations suggest that *mcr-1* has circulated among different bacterial clones within and across countries, which may occur by diverse independent acquisitions of *mcr*-carrying plasmids [43].

CRE has spread globally and was previously reported in patients in the study areas [2]. In contrast, all *E. coli* isolates in this study were susceptible to meropenem and were not positive to any carbapenem resistance gene tested. Carbapenems are not approved for use in livestock in any country, resulting in minimized selective pressure conditions [3]. Simultaneously, carbapenem use is uncommon in livestock production in the region, due mainly to their high cost.

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

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