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Exploring the epidemiology of *mcr* genes, genetic context and plasmids in *Enterobacteriaceae* originating from pigs and humans on farms in Thailand

Thongpan Leangapichart[1], Milan S. Stosic[1]†, Rachel A. Hickman[2,3], Kamonwan Lunha[4], Jatesada Jiwakanon[5], Sunpetch Angkititrakul[5], Ulf Magnusson[4], Thomas P. Van Boeckel[6,7], Josef D. Järhult[3] and Marianne Sunde[1]*

[1]*Section for Food Safety and Animal Health Research, Norwegian Veterinary Institute, Ås, Norway;* [2]*Department of Medical Biochemistry and Microbiology, Zoonosis Science Center, Uppsala University, Uppsala, Sweden;* [3]*Department of Medical Sciences, Zoonosis Science Center, Uppsala University, Uppsala, Sweden;* [4]*Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden;* [5]*Research Group for Animal Health Technology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand;* [6]*Health Geography and Policy Group, ETH Zurich, Zurich, Switzerland;* [7]*Center for Diseases Dynamics Economics & Policy, Washington, DC, USA*

*Corresponding author. E-mail: marianne.sunde@vetinst.no
†Present address: Faculty of Health Sciences, OsloMet—Oslo Metropolitan University, Oslo, Norway.

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Objectives: In veterinary medicine, colistin has been widely used as therapeutic and prophylactic agent, and for growth promotion. However, colistin has been re-introduced into treatment of human MDR bacterial infections. We assessed the characteristics and spread of plasmid-borne colistin resistance among healthy pigs, workers with animal-contact and their household members in Thailand.

Methods: WGS and MIC data of 146 *mcr*-positive isolates from a cross-sectional One Health study were analysed. Long-read sequencing and conjugation were performed for selected isolates.

Results: *mcr*-carrying isolates were detected in 38% of pooled-pig samples and 16% of human faecal samples. Of 143 *Escherichia coli* and three *Escherichia fergusonii*, *mcr-1*, *mcr-3*, and *mcr-9* variants were identified in 96 (65.8%), 61 (41.8%) and one (0.7%) isolate, respectively. Twelve *E. coli* co-harboured two *mcr* variants (*mcr-1* and *mcr-3*). Clonal transmission was detected in five out of 164 farms. *mcr-1* was mostly harboured by epidemic IncX4 and IncHI1 plasmids (89.9%). Conversely, *mcr-3* was harboured by a range of different plasmids. Comparative plasmid studies suggested IncP and IncFII plasmids as possible endemic *mcr-3* plasmids in Asian countries. Moreover, *mcr-3* was associated with different mobile genetic elements including Tn*As2*, IS*Kpn40* and IS*26/15DI*. Detected genetic signatures (DRs) indicated recent *mcr-3* transpositions, underlining the mobilizable nature of the *mcr-3* cassette.

Conclusions: The epidemiology of *mcr* and the possible evolution of successful plasmids and transposition modules should be carefully monitored. Of special concern is the growing number of different horizontal gene transferring pathways encompassing various transposable modules the *mcr* genes can be shared between bacteria.

Introduction

Colistin (polymyxin E), has been re-introduced into the treatment of infections caused by MDR Gram-negative bacteria, particularly carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and other members of Enterobacteriaceae.[1] However, in veterinary medicine, colistin has been widely used both as a therapeutic and prophylactic agent and for growth promotion.[2] Bacteria resistant to colistin have emerged worldwide with mechanisms not only caused by mutations or interruptions in specific chromosomal genes,[1] but also by plasmid-borne genes.[3] Until now, 10 plasmid-borne colistin resistance genes, i.e. *mcr-1* to *mcr-10*, have been reported.[4,5] *mcr-1* has been extensively studied since the first description in 2015. Plasmids belonging to the incompatibility groups IncI2, IncX4 and IncHI2 are considered common dissemination vectors for *mcr-1*.[6,7] Co-occurrence of two plasmids with different variants of *mcr* in a single isolate has scarcely been

reported. However, some studies have recently reported such an event, co-occurrence of *mcr-1* and *mcr-3* genes in *E. coli* from patients in China[8] and in New Zealand,[9] in *E. coli* from pigs in China,[10] and recently in *K. pneumoniae* from a healthy human in Thailand.[11]

The prevalence of *mcr*-positive bacteria in clinical isolates in Thailand have been described in *E. coli* and *K. pneumoniae* with 29.7% and 1.4%, respectively.[12] Furthermore, environmental dissemination of *mcr-1* has been found in 16% of investigated blowflies in a Northern province in Thailand,[13] as well as in 7% of flies collected in animal farms.[14] However, dissemination of plasmids containing *mcr* genes has never been assessed in human- and animal-originating bacteria in Thailand. Here, we investigated the distribution of *mcr* genes and performed an in-depth characterization of *mcr* plasmids in pig and human bacterial isolates collected as a part of a One Health cross-sectional study performed in Northern Thailand. Furthermore, we investigated the geographic distribution of *mcr* plasmids, determined the genetic environment of *mcr* and performed detailed characterization of strains harbouring multiple *mcr* gene variants.

Materials and methods

Ethics

The study was conducted according to the Helsinki Declaration for the human subjects and the EU Directive 2010/63/EU for animal experiments; the protocol involving human participants and animals was approved by the Khon Kaen University Ethics Committee (Project ID: HE612268 and 0514.1.75/66, respectively). Informed consent was obtained from all participants. Pig samples were collected with the permission of the owner of the pig herd.

Study participants

Sample collection, methods and main findings have previously been described.[15–17] Briefly, rectal swabs from pigs and human faecal samples were collected from 164 pig farms between September and December 2018 in Khon Kaen, Thailand. From each farm an attempt was made to collect faecal samples from a farmworker (termed contact, C) and an individual living in the same household, however, without direct contact with pigs (termed non-contact, U). Pig samples (up to 10 individual rectal swabs per farm) were pooled and analysed as one sample per farm (termed pig, P). The samples were investigated selectively for carbapenem, extended-spectrum cephalosporin and colistin-resistant bacteria, and isolates were subjected to WGS and MIC determination as previously described.[16]

WGS and genetic analyses

Illumina WGS data of 146 *Escherichia* spp. isolates containing at least one *mcr* variant were further analysed. The study included one *mcr-1*-positive *K. pneumoniae* (strain 90CP1) from a human contact, from our previous study[16] as this isolate might share *mcr*-plasmid with an *E. coli* isolate from pigs at the same farm (farm no. 90). Fourteen selected isolates were sequenced using Oxford Nanopore technologies sequence platform. This included 10 isolates with multiple *mcr* gene variants, one *mcr-9* carrying isolate, one IncFIA/FIB/*mcr-3.5* carrying isolate and two IncX4/*mcr-1* harboured by different bacterial species (*K. pneumoniae* and *E. coli*) from pig and human hosts at the same farm (farm no. 90). Hybrid genome assembly of both short and long reads was sequentially performed. Due to budget limitations, the two remaining genomes (71PM1 and 98UM1) harbouring two *mcr* gene variants and initially sequenced by Illumina, were mapped to the hybrid assembled genomes (35CM1 and 98CM1) to circularize the plasmid sequences. In 16 isolates, we were able to fully close all plasmids with *mcr* genes. In subsequent steps, resistance genes, plasmid replicons, conjugation modules and insertion sequences were identified in all included isolates. The closed plasmids served as reference for additional analyses and mapping of *mcr* plasmids and contigs from other strains. Geographic distribution of the most prevalent *mcr-1* and *mcr-3* plasmids was created using ArcGIS (https://www.arcgis.com/index.html). The genetic surroundings of *mcr-1* and *mcr-3* genes were investigated and analysed. Conjugation experiments was performed with 16 strains with closed plasmids. Details of sequencing and methodological approaches are described in the Supplementary Information/Material.

Availability of data and materials

Reads (fastq files) from the study have been submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) under study accession number PRJEB38313 and PRJEB39885.

Results

mcr gene diversity, gene location and geographical distribution of *mcr* plasmids

WGS data of the 146 included genomes identified several *mcr* variants. Most of the variants belonged to the *mcr-1* (*mcr-1.1*, *mcr-1.11*, *mcr-1.2*) and *mcr-3* group (*mcr-3.1*, *mcr-3.19*, *mcr-3.2*, *mcr-3.39* and *mcr-3.5*) while only one isolate harboured *mcr-9* (Figure 1, Table S1, available as Supplementary data at *JAC* Online). *mcr-3.39* represents a new variant, available in GenBank under accession no MT872721. In total, 96 strains contained an *mcr-1* gene variant, 61 contained an *mcr-3* gene variant and one contained *mcr-9* (strain 162CM1). Interestingly, 12 isolates co-harboured two *mcr* variants, *mcr-1* and *mcr-3*. In addition, 83 isolates harboured $bla_{\text{CTX-M}}$ variants, $bla_{\text{CTX-M-55 and}}$ $bla_{\text{CTX-M-14}}$. Apart from *mcr* variants, the associated plasmid incompatibility group or chromosomal location could be determined for 117 *E. coli* isolates (Figure 1). In the remaining 29 isolates (*mcr-1*/$n=4$; *mcr-3*/$n=25$), *mcr* location was uncertain due to the short contig lengths (<10 kb) and/or no replicon within the contig. These isolates were omitted from further analysis. The most common IncHI1, IncX4, IncI2 carrying *mcr-1* and IncX1, IncFII, IncP carrying *mcr-3* plasmids (Figure 1) were assessed for their geographical origin. The plasmids were mostly interspersed throughout the sampling area except for IncHI1/*mcr-1* plasmids that clustered on several farms located in the South-West part (Figure 2).

Co-resistance to other classes of antimicrobial agents and colistin minimum inhibitory concentrations

Most isolates were resistant to ampicillin, tetracycline, sulfamethoxazole, trimethoprim, chloramphenicol, ciprofloxacin and cefotaxime (Table 1). A majority ($n=126/146$) were MDR (resistant to three or more antibiotic classes). Colistin MIC values ranged between 1–16 mg/L. Most isolates were resistant to colistin except the *mcr-9* isolate (162CM1) and two *mcr-3.5*-positive isolates (34PM2 and 60CM1, with colistin MICs at 1 mg/L). Colistin MIC of isolates co-harbouring two *mcr* variants ranged between 4 and 8 mg/L. Detailed antimicrobial resistance profiles are presented in Table S1.

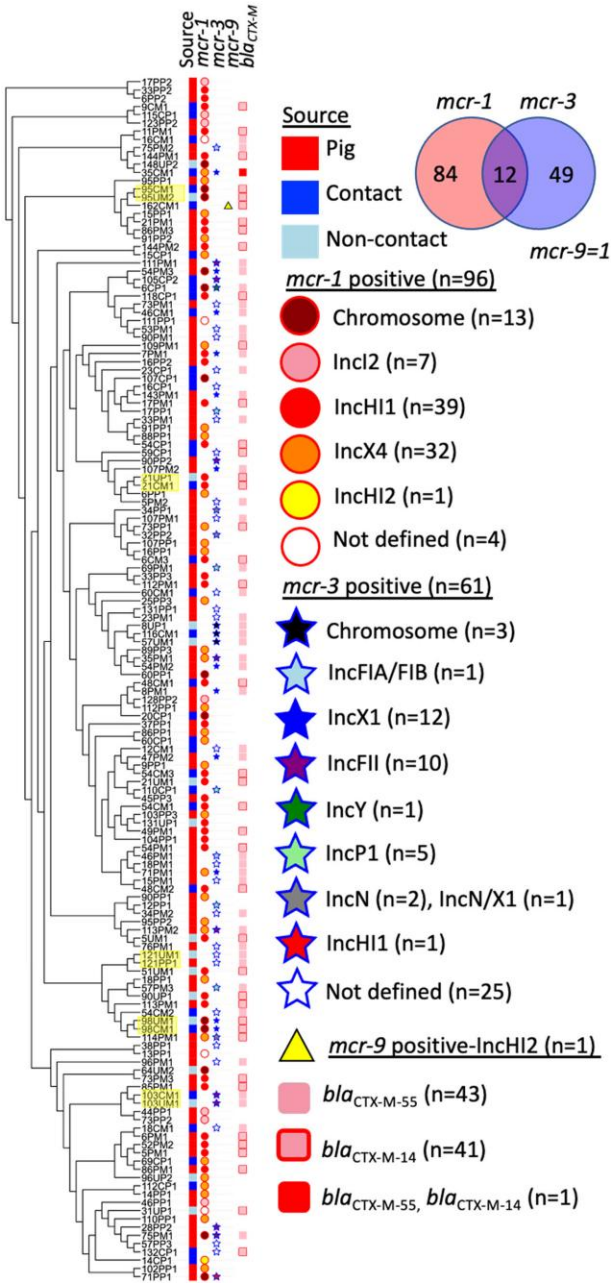


Figure 1. Cladogram of *mcr*-positive *Escherichia coli*/*fergusonnii* isolates. Maximum likelihood tree of isolates from pigs, contacts and non-contacts based on SNPs in the core genome. Isolate pairs from the same farm with zero SNPs differences were highlighted in yellow. Abbreviations: P, pigs; C, contacts; NC, non-contacts; ST, sequence type.

Genetic relatedness among mcr-containing strains

The 143 *E. coli* isolates grouped into 78 different STs, including 17 new STs identified by the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) (Figure 1, Table S1). ST10 clonal complex was the most prevalent ST (49/143) (Figure S1). Three *E. fergusonii* containing *mcr-1*, all from pigs, belonged to ST10568, ST10576 and ST7059. A maximum likelihood tree based on detected SNPs in the core genomes of the 146 included strains, with additional strain characteristics, is shown in Figure 1.

To investigate a possible transfer of *mcr*-containing strains between different hosts on the same farm we inspected the number of SNPs between isolates grouping into the same ST, obtained from the same farm. Between-host clonal transmission within farms was found on five farms, no. 21, 95, 98, 103 and 121 (Figure 1). Each pair of isolates (pig–human and/or human–human) exhibited zero SNP differences (Table S2).

Genetic analysis of mcr-1-carrying plasmids and mcr-1 genetic context

Our data enabled an in-depth analysis of 92 *mcr-1* positive isolates. In 79 isolates, *mcr-1* was present on a plasmid, while the 13 remaining isolates harboured *mcr-1* on the chromosome. In seven *E. coli* and one *K. pneumoniae*, the *mcr-1* plasmids were fully closed, plasmid-related contigs were identified in the remaining 71 isolates. Most *mcr-1* plasmids belonged to incompatibility groups IncHI1 ($n=39/79$) and IncX4 ($n=32/79$) (Figure 1; Table 2). In addition, seven isolates contained IncI2/*mcr-1* plasmids and one contained IncHI2/*mcr-1*. Table 2 shows an overview of *mcr* variants and their genome location. Characteristics of strains co-harbouring two *mcr* variants, *mcr-1* and *mcr-3*, are presented in Figure 3. All detected *mcr-1* plasmids and plasmid-related contigs exhibited high similarity with previously published plasmids as illustrated in Figure S2A–E. Seven complete IncX4/*mcr-1* plasmid sequences were obtained, ranging between 33 309 and 39 574 bp sharing identical backbone structure with p90PP1(33 309 bp) (Figure 4). p114PM1-IncX4/*mcr-1* had an additional insertion of a putative transposon (5716 bp) previously found on other plasmids (Figure 4)[18]. Identical IncX4/*mcr-1* plasmids were shared between strains from human and animal hosts. In addition, this plasmid was present in *K. pneumoniae* and *E. coli* obtained from the same farm, suggesting inter-species plasmid transmission (Figure 4).

We characterized the genetic context of *mcr-1* in all isolates (Figure S3). The most common *mcr-1* context was *mcr-1-pap2*,[19] presumably generated by the initial insertion of Tn*6330*.[20] Truncated or complete Tn*6330* was identified on seven chromosomes (Figure S3). Five of these isolates harboured Tn*6330* encircled with a 2-bp direct repeat, AG/AC, as described previously.[19,21] Moreover, an IS*1294* insertion into a complete Tn*6330* found on two chromosomes generated an IS*Apl1*-*mcr-1*-Δpap2-IS*1294*-Δpap2-IS*Apl1* cassette, a putative circular intermediate indicating plasticity of *mcr-1* mobilization.[22] However, in most *mcr-1*-carrying isolates, we detected a truncated form of Tn*6330* lacking a single copy of IS*Apl1* downstream or upstream of *mcr-1* [Figure S3(A–C)]. Complete loss of both IS*Apl1* was discovered in all IncX4 plasmids (32/32), in three IncHI1, one IncHI2 and two IncI2-related contigs. The genetic surroundings of *mcr-1* genes of included isolates are shown in Figure S3(A–C).

Genetic analysis of mcr-3 carrying plasmids and mcr-3 genetic context

Our data enabled an in-depth analysis of 36 *mcr-3* positive isolates. *mcr-3* was plasmid located in 33, and chromosomally located in three isolates. In contrast to *mcr-1* plasmid types, *mcr-3* plasmids were more diverse including IncFII ($n=10$),

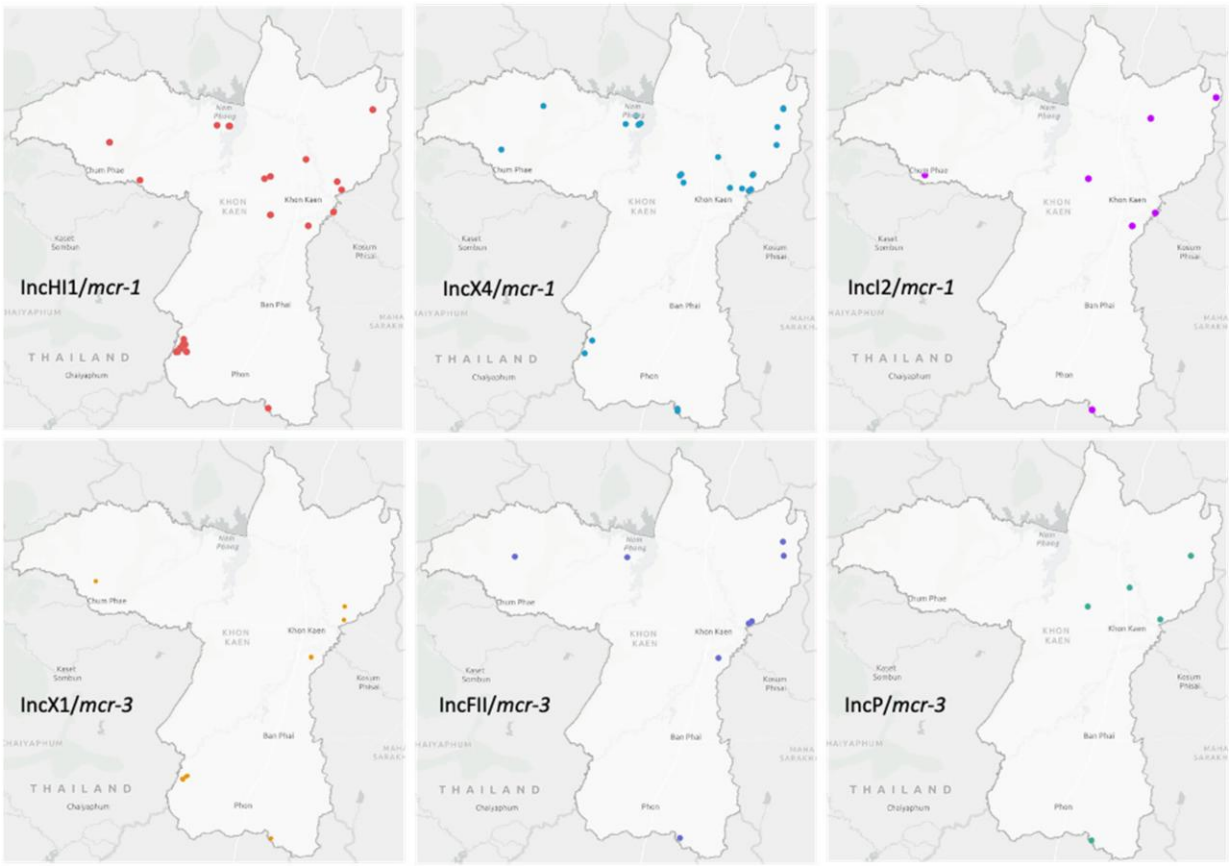


Figure 2. Geographical distribution of the most common *mcr-1* and *mcr-3* plasmids. The map was created using ArcGIS (https://www.arcgis.com/index.html).

Table 1. Antimicrobial susceptibility profiles of *mcr*-positive *E. coli* and *E. fergusonii*

Number (%) of isolates resistant	All isolates (*n* = 146)	*mcr-1* (*n* = 84)	*mcr-3* (*n* = 49)	*mcr-1* and *mcr-3* (*n* = 12)	*mcr-9* (*n* = 1)
Ampicillin	140 (96)	79 (94)	49 (100)	11 (92)	1 (100)
Cefotaxime	72 (49)	23 (27)	37 (75)	11 (92)	1 (100)
Ceftazidime	17 (12)	6 (7)	9 (18)	2 (17)	0 (0)
Colistin	143 (98)	84 (100)	47 (96)	12 (100)	0 (0)
Gentamicin	23 (16)	10 (12)	9 (18)	3 (25)	1 (100)
Tetracycline	118 (81)	67 (80)	39 (80)	11 (92)	1 (100)
Meropenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ciprofloxacin	80 (55)	38 (45)	31 (63)	11 (92)	0 (0)
Chloramphenicol	82 (56)	37 (44)	35 (71)	9 (75)	1 (100)
Tigecycline	3 (2)	2 (2)	1 (2)	0 (0)	0 (0)
Trimethoprim	85 (58)	48 (57)	28 (57)	8 (67)	1 (100)
Sulfamethoxazole	110 (75)	57 (68)	42 (86)	10 (83)	1 (100)
Azitromycin	13 (9)	7 (8)	3 (6)	2 (17)	1 (100)
Nalidixic acid	31 (21)	14 (17)	12 (25)	4 (33)	1 (100)

IncX1 (*n* = 12), IncP1 (*n* = 5), IncN (*n* = 2), IncX1/N (*n* = 1), IncFIA/FIB (*n* = 1), IncHI1 (*n* = 1) and IncY (*n* = 1) incompatibility groups (Figure 1, Table 2).

In total, 13 *mcr-3* plasmids were circularized (Figure 3). BLAST search with these plasmids against NCBI and PLSDB databases did not result in an exact match for IncX1, IncX1/N and IncFIA/

Table 2. Distribution of *mcr* gene and number of closed plasmids

Gene (no. of isolates)	*mcr* location	Number of closed plasmids (size of plasmid)
mcr-1 (*n* = 96)	IncHI1 (*n* = 39)	one (217 496 bp)
	IncX4 (*n* = 32)	seven (33 309–39 574 bp)
	IncI2 (*n* = 7)	—
	IncHI2 (*n* = 1)	—
	chromosome (*n* = 13)	—
	not defined (*n* = 4)	—
mcr-3 (*n* = 61)	IncX1 (*n* = 12)	six (32 893–53 563 bp)
	IncFII (*n* = 10)	three (42 864–89 737 bp)
	IncP1 (*n* = 5)	—
	IncN (*n* = 2)	—
	IncX1/N (*n* = 1)	one (64 994 bp)
	IncFIA/FIB (*n* = 1)	one (70 757 bp)
	IncHI1 (*n* = 1)	one (302 729 bp)
	IncY (*n* = 1)	one (109 103 bp)
	chromosome (*n* = 3)	—
	not defined (*n* = 25)	—
mcr-9 (*n* = 1)	IncHI2 (*n* = 1)	one (263 699 bp)

FIB plasmids. Characteristics of these plasmids and plasmid-related contigs are shown in Table S3, Figure S4A–I. Conversely, p71PP1-IncHI1/*mcr-3.1* (*E. coli* 71PP1; ST48) shared almost identical backbone structure with pCP131-IncHI1/*mcr-3.1* identified in *E. coli* (ST156) isolated from a pig slaughterhouse in China (accession number CP053721.1) as illustrated in Figure S4F. Moreover, p6CP1-IncY/*mcr-3.5* (*E. coli* 6CP1; ST7122) had a similar backbone structure with a non-conjugative phage P7-like plasmid, pHYEC7-*mcr-1*, hosted by an *E. coli* isolated from a pig farm in China (accession number: KX518745.1) (Figure S4G). Additionally, BLASTing of five almost identical *mcr-3* harbouring IncP-1 related contigs (99% similarity, ranging in size from 49 345 to 52 980 bp) resulted in matching, with previously published, IncP1 plasmids hosted by *E. coli* from pigs and humans in Taiwan and China (100% coverage and >99.5% identity) (Figure S4H). Finally, IncFII/*mcr-3* plasmids/plasmid-related contigs shared identical backbone structure with pPN42 identified in *E. coli* from a duck in Thailand (Figure S4I). Our findings suggest that some conserved *mcr-3* containing plasmids, mainly belonging to IncP and IncFII, and to some extent also IncHI1 and IncY may be circulating among human and animal hosts in South-East Asia.

Investigations of the genetic context of *mcr-3* showed a high level of variability regardless of plasmid type. Overall, an *mcr-3-dgkA* was found to be a core structure in all *mcr-3* containing isolates. The structure was found flanked by several combinations of ISs (Figure 5, Figure S5).

On *mcr-3.2* carrying chromosomes of three human-originating ST10 isolates, we identified a structure that might represent a transposition unit (TU)-like structure, *mcr-3.2-dgkA*-IS*Kpn40*. While left and right inverted repeats (IR), IR_L and IR_R flanked exclusively IS*Kpn40*, an alternative IR_{R1} and IR_L flanked the complete structure (Figure 5A). The TU was found inserted into the *nimA/C* gene flanked by the 4 bp DRs, CACC (Figure 5B). As identical DRs are generated by the insertion of the composite transposon IS*Kpn40-mcr-3-dgkA*-IS*Kpn40*, the TU-like structure might originate from the same composite transposon.[23,24] Moreover, a search in GenBank showed that *nimA/C* was targeted by the same TU on nine previously published plasmids of different incompatibility groups originating from various Enterobacteriaceae isolates in Asia (Figure 5B). On all these nine plasmids, the same DR repeat was found indicating a recent insertion event.

For the *mcr-3.1* gene, the identical structure, *mcr-3.1*-dgkA-IS*Kpn40* was found on three plasmids characterized in this study (Figure 5C) hosted by isolates 71PP1, 98CM1 and 98UM1. Isolates 98CM1 and 98UM1 were found to be closely related (ST10562, 0 SNPs), sharing a similar plasmid. However, we were unable to detect DRs in these plasmids. The similar *mcr-3.19*-dgkA-IS*Kpn40* structure was also found on four IncFII/*mcr-3.19* plasmid-related contigs; in isolates 103CM1 (ST278), 103UM1 (ST278), 111PM1 (ST48) and 28PP2 (ST101) (Figure S5). Neither an alternative IR nor DRs were found.

In our study, we identified a ΔTn*As2-mcr-3-dgkA*-IS*26/15DI* structure on nine closed plasmids (IncX1, IncFIA/FIB, IncY, IncFII) and 13 contigs (IncX1, IncN, IncP1) (Figure 6A and Figure S5). Due to the end of contigs only IS*26/15* remnants were detected upstream from *dgkA* (Figure S5). Two similar structures, ΔIS*26/15DI*-Tn*As2-mcr-3.5-dgkA* and ΔIS*26*-ΔTn*As2-mcr-3.1-dgkA*-IS*Kpn40-ble* identified on plasmids and harboured by two *E. coli* isolates (from a pig slaughterhouse, China) have been previously described.[25] These structures have been shown to generate a circularized intermediate, indicating their possible mobilization.[24] Identification of DRs surrounding the structure found in our study further provides evidence of mobilization. A pair of 5 bp DRs was identified on three complete plasmids: p75PM2-IncFIA/FIB (TACCT), p7PM1-IncX1 (TTTAC), p113PM2-IncFII (GAGTT) and on one IncP1/*mcr-3.5*-related contig 110CP1 (ATGTA) (Figure 6B). Moreover, an identical structure was also identified on GenBank deposited plasmids belonging to different Inc types, IncP1, IncFII, IncFIA/FIB and IncX1/R, hosted by various bacterial hosts, from different countries (Figure 6B). The 5 bp DR sequence on these plasmids was unique for each except for IncP1-related contig and GenBank available IncP1 plasmids that shared identical DR sequence (ATGTA) and backbone structure (Figure 6B and Figure S4H).

On 11 of 13 *mcr-3* circular plasmids (*mcr-3.1*, *n* = 2; *mcr-3.39/3.5*, *n* = 9), we found several AMR genes in the vicinity of *mcr-3* indicating a possible hotspot[26] for accumulation of various AMR genes (Figure 5C and Figure 6A). On two IncX1/*mcr-3.1* plasmids (98CM1 and 98UM1), $bla_{CTX\text{-}M\text{-}14}$ was carried by IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$-IS*903* transposon (2874 bp)[27] downstream of the *mcr-3* cassette (Figure 5C). On nine *mcr-3.5* carrying plasmids, we observed an identical pattern of *qnrS1*, which was almost exclusively found associated with IS*3*-like insertion sequence. Whereas $bla_{CTX\text{-}M\text{-}55}$-*orf477* was always found in close proximity to IS*Ecp1* remnants, a key mobilizer of $bla_{CTX\text{-}M}$[29,28] (Figure 6A). Different Inc-type plasmids, p6CP1-IncY/*mcr-3.5* and p75PM1-IncFII/*mcr-3.5* shared almost an identical structure of Tn*As2-mcr-3.5*-dgkA-IS*15DI*-TinR-qnrS1-IS*3like*-Tn*2-orf477*-$bla_{CTX\text{-}M\text{-}55}$. This might indicate the involvement of a putative mobile element on these plasmids.

mcr-9 gene cassette

One *mcr-9.1*-positive isolate was found (162CM1), originating from a human host. The plasmid, belonging to IncHI2 group,

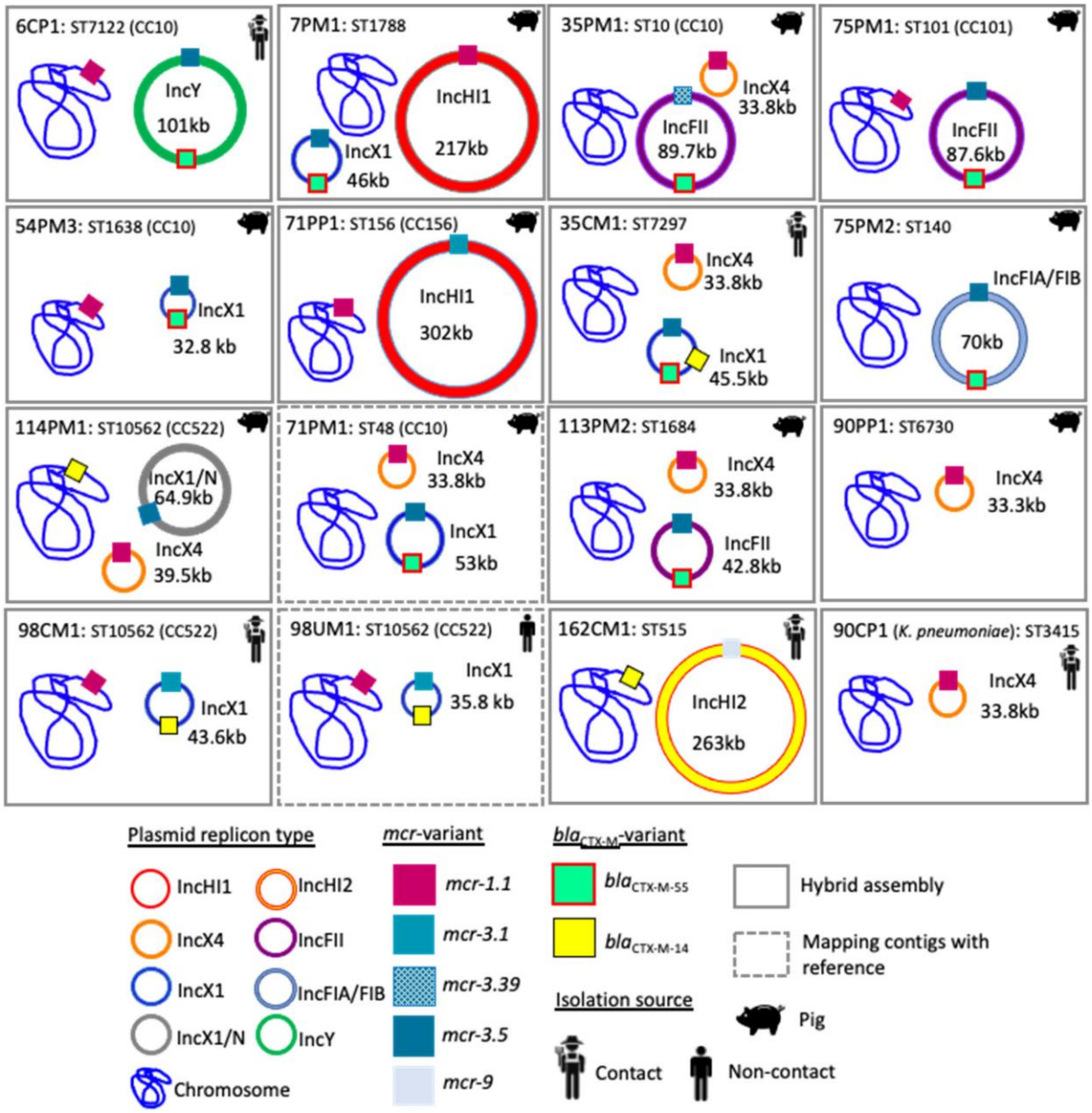


Figure 3. Location of *mcr* and $bla_{CTX\text{-}M}$ variant in 15 *E. coli* and one *K. pneumoniae*.

shared identical backbone with plasmid pD610-IncHI2 (accession number MG288680.1, *K. pneumoniae*, China) containing an '*IS26-wbuC-mcr-9.1*-IS*903B*' genetic structure (Figure S6), which has been reported in several bacterial species.[30]

Co-occurrence and co-transfer of mcr-1 and mcr-3 variants

The isolates co-harbouring *mcr-1* and *mcr-3* were analysed by long-read sequencing ($n=10$) and mapping ($n=2$) to resolve the location of the *mcr* variants within the genomes. Six isolates had chromosomally located *mcr-1*. *mcr-3* was exclusively found on plasmids in these 12 strains. Figure 3 shows the characteristics of strains co-harbouring *mcr-1* and *mcr-3* genes.

We circularized 22 *mcr*-carrying plasmids in this study (Figure 3). Conjugation experiments were performed with all 16 isolates (Table S4 and Table S5) with fully closed plasmids as donors. Seven out of eight *mcr-1* plasmids were successfully transferred including the plasmid in *K. pneumoniae*. Only two *mcr-3* carrying plasmids were found transferable, simultaneously with *mcr-1* plasmids from the pig-originating strains 7PM1 (IncHI1/*mcr-1* and IncX1/*mcr-3.5*) and 114PM1 (IncX4/*mcr-1* and IncX1/N/*mcr-3.5*). Among 14 out of 22 circular plasmids, transfer-related modules such as oriT, relaxase gene, T4CP and type IV secretion system cluster were found (Table S4).

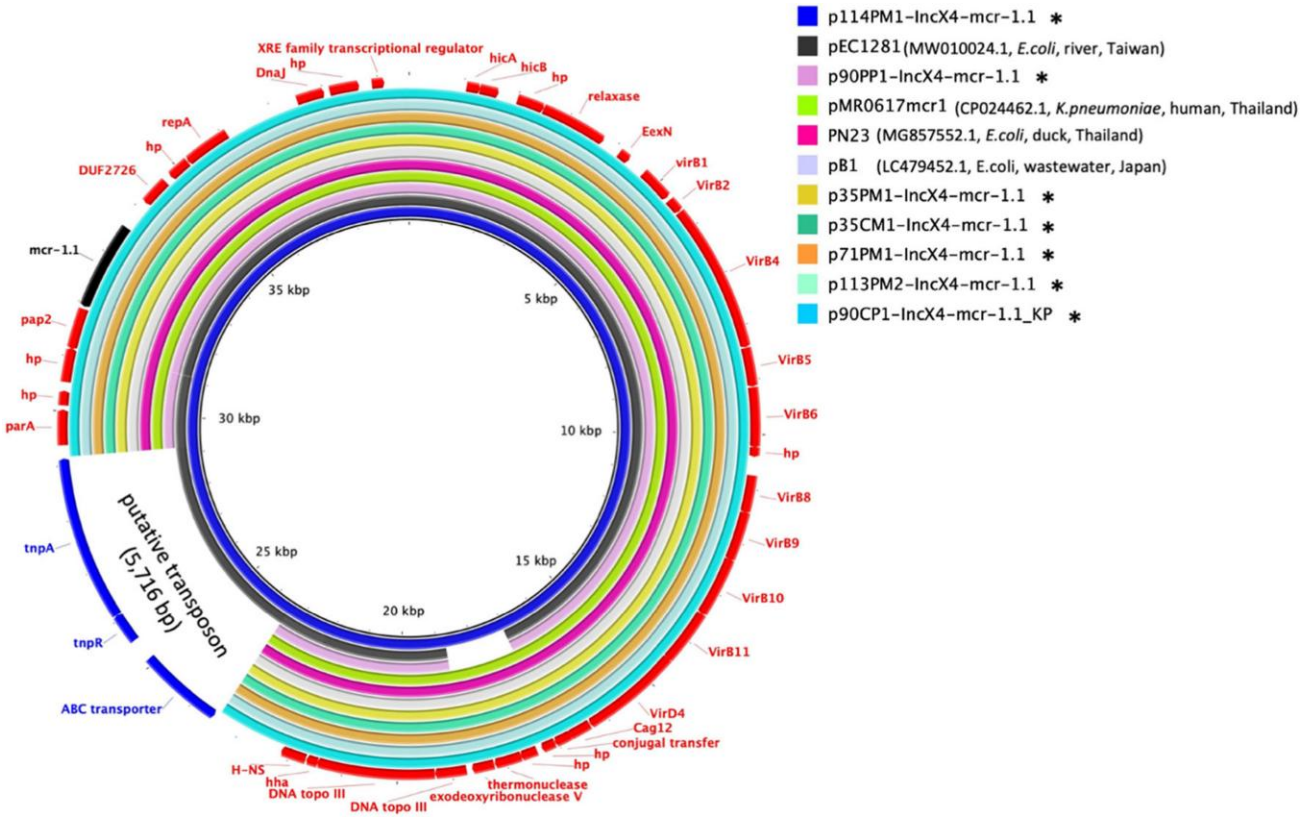
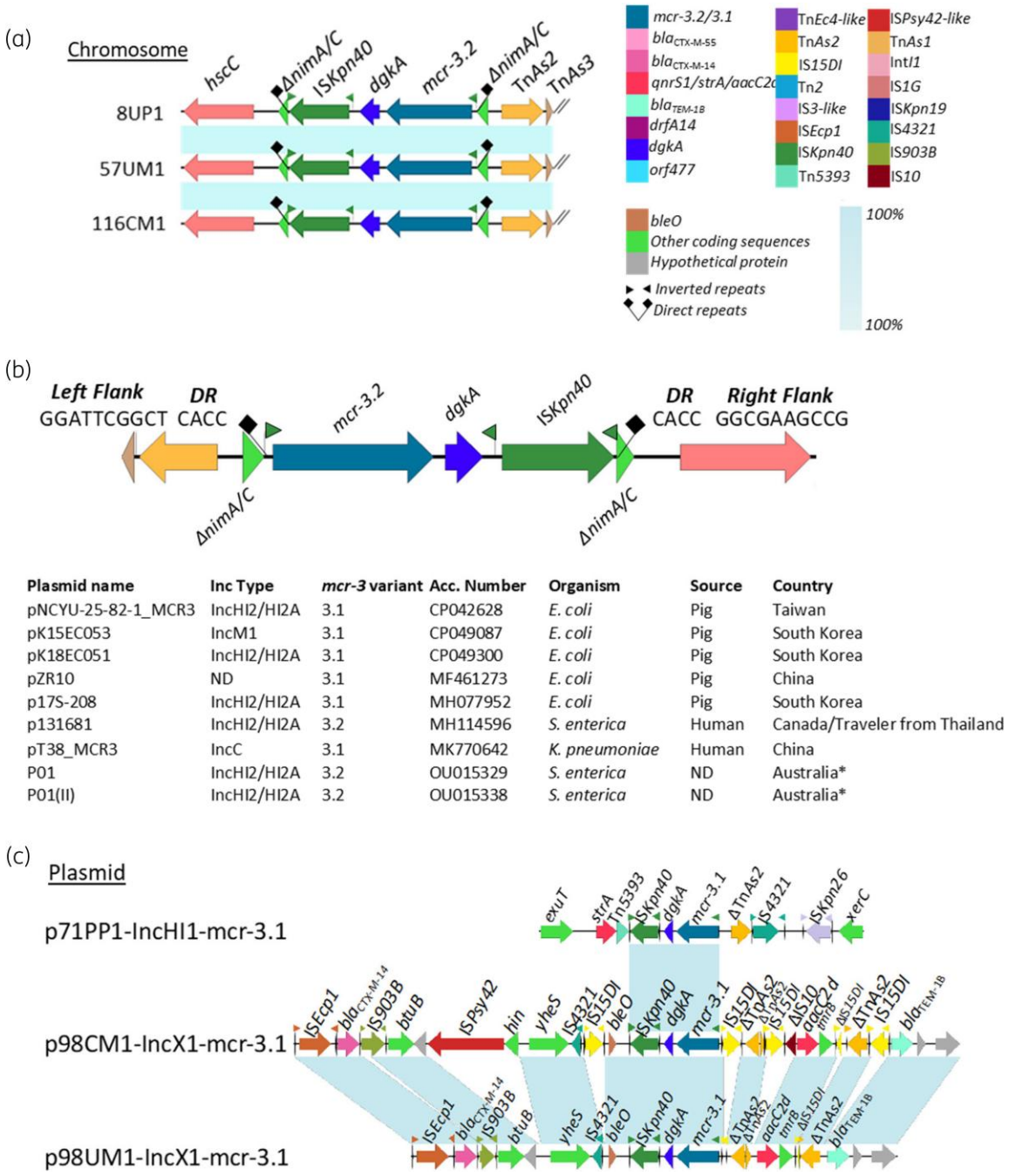


Figure 4. Circular alignments of IncX4/*mcr-1* complete plasmids. Alignment of mcr-1/IncX4 complete plasmids in this study (marked with asterisk*) with plasmid sequences from Genbank: pEC1281 (accession no. MW010024.1), pMR0617mcr1 (accession no. CP024462.1), PN23 (accession no. MG557852.1) and pB1 (accession no. LC479452.1). The innermost circle represents the main reference sequence in the alignment. ARGs and mobile genetic elements are labelled in the outermost circle.

Discussion

In Thailand, colistin was banned as a feed additive in March 2018. However, prolonged cumulative selective pressure of both colistin and other antimicrobial agents might have resulted in a high prevalence of bacteria resistant to these agents.[31,32] Here, we conducted a comprehensive sampling and screening of plasmid-mediated colistin resistance in pig and human samples from farms in Northern Thailand collected in late 2018. A strength of our study was the sampling behind our dataset. We sampled 164 farms in the Khon Kaen Province and from most of the farms ($n=156$), samples from both pigs and humans were obtained. Our sampling design and extensive genome sequencing enabled us to study *mcr* prevalence, transmission of strains, plasmids and *mcr* variants among hosts living on the same farm. To the best of our knowledge, our study includes the highest number of sampled epidemiological units (farms) to study *mcr* transmission between pigs and humans.

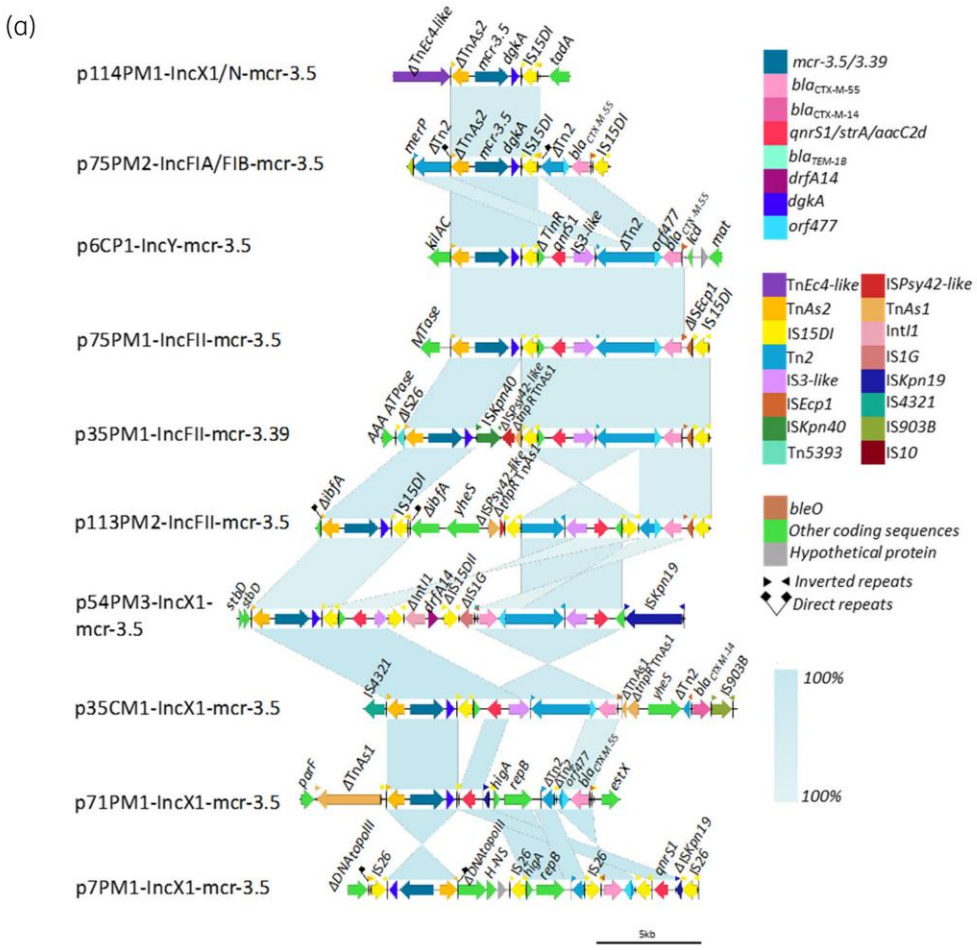
Colistin was reported to be used on only two of the 164 farms.[33] However, *mcr-1*/*mcr-3* carrying *Escherichia* spp. was found in high occurrence; 37.8% (62/164) and 16.1% (44/164) pig and human samples, respectively. This may be explained by the following (i) higher-than-reported use of colistin on the study farms, (ii) prolonged effects of previous colistin consumption and/or (iii) dissemination of colistin-resistant strains in the community. Since *mcr*-carrying isolates were more common among pigs than humans suggest the first two alternative explanations as more important factors. The percentage of *mcr*-positive samples on farms was significantly higher than in previous reports from other provinces, which ranged between 4.45% and 5.8%.[34,35] However, the prevalence of *E. coli* co-harbouring *mcr-1* and *mcr-3* was comparable to a study of flies collected from farms in Thailand.[14] Furthermore, our results showed that 86.3% of *mcr*-carrying *E. coli* were MDR. The predominant ESBL genes accompanying *mcr* were $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-14}}$, the most common ESBL-resistance genes found in Enterobacteriaceae in Thailand.[29,36] *mcr* genes were present in a highly diverse set of host strains. Clonal transmission of *mcr*-positive *E. coli* within a farm was detected on five farms indicating a limited number of human-to-human or pig-to-human whole bacterium transmission events. Conversely, the evidence of plasmid spread was noted, particularly dissemination of epidemic IncX4/*mcr-1* and IncHI1/*mcr-1* plasmids. Similar plasmids have also been described in the Thai community previously.[13,37] This may indicate that horizontal gene transfer/plasmid transfer plays the most important role in the spread of *mcr*. Our result contradicts previous reports of IncI2 as the major *mcr-1* plasmid type in Asia.[6] Furthermore, the dissemination of conserved IncP/*mcr-3* and IncFII/*mcr-3* plasmids over the Asian continent was suggested and should be further investigated.[23,38,39]



Plasmid name	Inc Type	*mcr-3* variant	Acc. Number	Organism	Source	Country
pNCYU-25-82-1_MCR3	IncHI2/HI2A	3.1	CP042628	*E. coli*	Pig	Taiwan
pK15EC053	IncM1	3.1	CP049087	*E. coli*	Pig	South Korea
pK18EC051	IncHI2/HI2A	3.1	CP049300	*E. coli*	Pig	South Korea
pZR10	ND	3.1	MF461273	*E. coli*	Pig	China
p17S-208	IncHI2/HI2A	3.1	MH077952	*E. coli*	Pig	South Korea
p131681	IncHI2/HI2A	3.2	MH114596	*S. enterica*	Human	Canada/Traveler from Thailand
pT38_MCR3	IncC	3.1	MK770642	*K. pneumoniae*	Human	China
P01	IncHI2/HI2A	3.2	OU015329	*S. enterica*	ND	Australia*
P01(II)	IncHI2/HI2A	3.2	OU015338	*S. enterica*	ND	Australia*

Figure 5. Genetic environment of *mcr-3.2/3.1* variants in chromosome and complete plasmids. (a) The genetic environment of *mcr-3.2* on chromosomes; (b) the 4-bp direct repeats surrounding on *mcr-3.2* structure found in this study and on other plasmids in GenBank and (c) the genetic environment of *mcr-3.1* on plasmids.

Assessment of the *mcr* genetic contexts showed that Tn*6330* and its derivatives were common vectors of horizontal *mcr-1* transfer as previously reported.[40] The observation of complete loss of IS*Apl1* from Tn*6330* renders the *mcr-1-pap2* non-mobilizable indicating a stabilization of *mcr-1*[40] on IncX4, IncHI1 and IncI2 plasmids in the study. Due to the shorter assembled sequences, we were unable to retrieve the full length of mobile genetic elements surrounding *mcr-3* gene for all isolates. Despite this limitation, we discovered various plasmid types harbouring *mcr-3* flanked by several combinations of mobile elements including Tn*As2*, IS*26*/IS*15DI* and IS*Kpn40*. These mobile elements have been previously considered key elements of *mcr-3* transposition between different bacterial species.[23–25,41] Additionally, the identified DRs in our study indicated a recent



Plasmid name	Inc Type	Acc. Number	Organism	Source	Country	Left flank	DR	Right flank
p75PM2-IncFIA/FIB-mcr-3.5	IncFIA/FIB	This study	*E. coli*	Pig	Thailand	TGCCTGTGAC	TACCT	CAGCCCGGAG
p7PM1-IncX1-mcr-3.5	IncX1	This study	*E. coli*	Pig	Thailand	ATGCAAGGTC	TTTAC	GAAACTCATA
p113PM2-IncFII-mcr-3.5	IncFII	This study	*E. coli*	Pig	Thailand	AGGGATATCA	GAGTT	GAACTTGATG
110CP1-contig-mcr-3.5	IncP1	This study	*E. coli*	Human	Thailand	CCCGCTGCGC	ATGTA	CTGTCCCGGC
pMCR3_WCHEC-LL123	IncP1	MF489760	*E. coli*	Human	China	CCCGCTGCGC	ATGTA	CTGTCCCGGC
pMCR3_WCHEC1943	IncP1	MF678351	*E. coli*	Sewage	China	CCCGCTGCGC	ATGTA	CTGTCCCGGC
pMCR3_025943	IncP1	CP027203	*E. coli*	Sewage	China	CCCGCTGCGC	ATGTA	CTGTCCCGGC
pNCYU-29-69-3_MCR3	IncP1	CP042602	*E. coli*	Pig	Taiwan	CCCGCTGCGC	ATGTA	CTGTCCCGGC
p2018S07-013-3_MCR3	IncP1	CP044383	*K. pneumoniae*	Human	Taiwan	CCCGCTGCGC	ATGTA	CTGTCCCGGC
pCP66-6-IncFII	IncFII	CP053725	*E. coli*	Pig	China	TAAATCCATG	AAGAC	GGCTTACGCC
pT3	IncFIA/FIB	MK656937	*E. coli*	Crickets	Switzerland	CTGGCCATGC	TTTTT	GACTTTCCAC
pPN156	IncFII	MT449721	*E. coli*	Human	Thailand	TTATTGTGAT	AAATA	GTTGAGTGGA
plasmid unnamed4	IncX1/R	CP041114	*E. coli*	Pig	Thailand	CTGTTTTGAC	AGGTT	CTGCCTCACC

Figure 6. Genetic environment of *mcr-3.5/3.39* variants in complete plasmids. (a) Linear comparison of *mcr-3.5* genetic environment identified in complete plasmids/contigs. (b) The 5-bp direct repeats surrounding on *mcr-3.5/3.39* structure found in this study and on other plasmids in Genbank.

insertion of *mcr-3-dgkA*-IS*Kpn40* and ΔTnAs2-*mcr-3-dgkA*-IS*15DI* structures, and thereby possible mobilization and circulation in Asia.

The presence of bla_{CTX-M} and other AMR genes in the vicinity of *mcr-3* might indicate accumulation of AMR genes as reported earlier in our *K. pneumoniae* study.[26] Evidence of co-transfer of *mcr-1* and *mcr-3* plasmids was confirmed in two isolates. However, the experimental conditions may have been suboptimal for transmission of all collected plasmids. Isolates co-harbouring two *mcr* variants might be considered an exceptional *mcr gene* disseminator.[26]

In conclusion, our One Health approach enabled us to explore the epidemiology of *mcr* genes, *mcr* genetic contexts and plasmids. We discovered that *mcr-1*, was mostly carried by conserved epidemic plasmids. *mcr-3* was located on a more diverse set of plasmid types, some of them not previously reported as *mcr-3* carriers. Our study also discovered possible endemic IncP and IncFII plasmids with *mcr-3*, disseminated in several Asian countries. We found genetic signatures in line with recent *mcr-3* transposition events, underlining the mobilizable nature of the *mcr-3* gene cassette. Of special concern is the growing number of different horizontal gene transferring pathways encompassing various transposable modules the *mcr* genes can be shared between bacteria. Careful monitoring and follow-up studies are needed to get more insight into the epidemiology *mcr* genes and the possible evolution of successful plasmids and transposition modules containing *mcr* and other antimicrobial resistance genes of clinical relevance.

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Transparency declarations

None to declare.

Supplementary data

Methods, Tables S1–S5 and Figures S1–S6 are available as Supplementary data at *JAC* Online.

References

1 Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol* 2014; **5**: 1–18. https://doi.org/10.3389/fmicb.2014.00643

2 Rhouma M, Beaudry F, Thériault W *et al*. Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. *Front Microbiol* 2016; **7**: 1789. https://doi.org/10.3389/fmicb.2016.01789

3 Liu YY, Wang Y, Walsh TR *et al*. 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* 2016; **16**: 161–8. https://doi.org/10.1016/S1473-3099(15)00424-7

4 Ling Z, Yin W, Shen Z *et al*. Epidemiology of mobile colistin resistance genes *mcr-1* to *mcr-9*. *J Antimicrob Chemother* 2020; **75**: 3087–95. https://doi.org/10.1093/jac/dkaa205

5 Wang C, Feng Y, Liu L *et al*. Identification of novel mobile colistin resistance gene *mcr-10*. *Emerging Microbes & Infections* 2020; **9**: 508–16. http://dx.doi.org/10.1080/22221751.2020.1732231

6 Matamoros S, van Hattem JM, Arcilla MS *et al*. Global phylogenetic analysis of *Escherichia coli* and plasmids carrying the *mcr-1* gene indicates bacterial diversity but plasmid restriction. *Sci Rep* 2017; **7**:15364. https://doi.org/10.1038/s41598-017-15539-7

7 Zelendova M, Papagiannitsis CC, Valcek A *et al*. Characterization of the complete nucleotide sequences of *mcr-1*-encoding plasmids from Enterobacterales isolates in retailed raw meat products from the Czech Republic. *Front Microbiol* 2021; **11**: 3552. https://doi.org/10.3389/fmicb.2020.604067

8 Liu L, Feng Y, Zhang X *et al*. New variant of *mcr-3* in an extensively drug-resistant *Escherichia coli* clinical isolate carrying *mcr-1* and bla_{NDM-5}. *Antimicrob Agents Chemother* 2017; **61**: e01757-17. https://doi.org/10.1128/AAC.01757-17

9 Creighton J, Anderson T, Howard J *et al*. Co-occurrence of *mcr-1* and *mcr-3* genes in a single *Escherichia coli* in New Zealand. *J Antimicrob Chemother* 2019; **74**: 3113–6. https://doi.org/10.1093/jac/dkz311

10 Li R, Zhang P, Yang X *et al*. Identification of a novel hybrid plasmid co-producing MCR-1 and MCR-3 variant from an *Escherichia coli* strain. *J Antimicrob Chemother* 2019; **74**: 1517–20. https://doi.org/10.1093/jac/dkz058

11 Yu Y, Andrey DO, Yang R *et al*. A *Klebsiella pneumoniae* strain co-harbouring *mcr-1* and *mcr-3* from a human in Thailand. *J Antimicrob Chemother* 2020; **75**: 2372–4. https://doi.org/10.1093/jac/dkaa133

12 Eiamphungporn W, Yainoy S, Jumderm C *et al*. 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* 2018; **15**: 32–5. https://doi.org/10.1016/j.jgar.2018.06.007

13 Yang QE, Tansawai U, Andrey DO *et al*. Environmental dissemination of *mcr-1* positive Enterobacteriaceae by *Chrysomya spp.* (common blowfly): an increasing public health risk. *Environ Int* 2019; **122**: 281–90. https://doi.org/10.1016/j.envint.2018.11.021

14 Fukuda A, Usui M, Okubo T *et al*. Co-harboring of cephalosporin (*bla*)/colistin (*mcr*) resistance genes among Enterobacteriaceae from flies in Thailand. *FEMS Microbiol Lett* 2018; **365**: fny178. https://doi.org/10.1093/femsle/fny178

15 Lunha K, Leangapichart T, Jiwakanon J *et al*. Antimicrobial resistance in fecal *Escherichia coli* from humans and pigs at farms at different levels of intensification. *Antibiotics* 2020; **9**: 662. https://doi.org/10.3390/antibiotics9100662

16 Hickman RA, Leangapichart T, Lunha K *et al*. Exploring the antibiotic resistance burden in livestock, livestock handlers and their non-livestock handling contacts: a one health perspective. *Front Microbiol* 2021; **12**: 651461. https://doi.org/10.3389/fmicb.2021.651461

17 Huber L, Hallenberg GS, Lunha K *et al*. Geographic drivers of antimicrobial use and resistance in pigs in Khon Kaen Province, Thailand. *Front Vet Sci* 2021; **8**: 659051. https://doi.org/10.3389/fvets.2021.659051

18 Teng CH, Wu PC, Tang SL *et al*. A large spatial survey of colistin-resistant gene *mcr-1*-carrying *E. coli* in rivers across Taiwan.

Microorganisms 2021; **9**: 449–56. https://doi.org/10.3390/microorganisms9040722

19 Kieffer N, Aires-de-Sousa M, Nordmann P *et al*. High rate of MCR-1–producing *Escherichia coli* and *Klebsiella pneumoniae* among pigs, Portugal. *Emerg Infect Dis* 2017; **23**: 2023–9. https://doi.org/10.3201/eid2312.170883

20 Li R, Xie M, Zhang J *et al*. Genetic characterization of *mcr-1*-bearing plasmids to depict molecular mechanisms underlying dissemination of the colistin resistance determinant. *J Antimicrob Chemother* 2017; **72**: 393–401. https://doi.org/10.1093/jac/dkw411

21 Zhao F, Feng Y, Lü X *et al*. Remarkable diversity of *Escherichia coli* carrying *mcr-1* from hospital sewage with the identification of two new *mcr-1* variants. *Front Microbiol* 2017; **8**: 2094. https://doi.org/10.3389/fmicb.2017.02094

22 Sun J, Li XP, Fang LX *et al*. Co-occurrence of *mcr-1* in the chromosome and on an IncHI2 plasmid: persistence of colistin resistance in *Escherichia coli*. *Int J Antimicrob Agents* 2018; **51**: 842–7. https://doi.org/10.1016/j.ijantimicag.2018.01.007

23 Xiang R, Liu BH, Zhang AY *et al*. Colocation of the polymyxin resistance gene *mcr-1* and a variant of *mcr-3* on a plasmid in an *Escherichia coli* isolate from a chicken farm. *Antimicrob Agents Chemother* 2018; **62**: e00501-18. https://doi.org/10.1128/AAC.00501-18

24 He Y-ZZ, Long T-FF, Chen C-PP *et al*. IS*Kpn40*-mediated mobilization of the colistin resistance gene *mcr-3.11* in *Escherichia coli*. *Antimicrob Agents Chemother* 2020; **64**: 1–5. https://doi.org/10.1128/AAC.00851-20

25 Li R, Du P, Zhang P *et al*. Comprehensive genomic investigation of co-evolution of *mcr* genes in *Escherichia coli* strains via nanopore sequencing. *Glob Challenges* 2021; **5**: 2000014. https://doi.org/10.1002/gch2.202000014

26 Stosic MS, Leangapichart T, Lunha K *et al*. Novel *mcr-3.40* variant co-located with *mcr-2.3* and *blaCTX-M-63* on an IncHI1B/IncFIB plasmid found in *Klebsiella pneumoniae* from a healthy carrier in Thailand. *J Antimicrob Chemother* 2021; **76**: 2218–20. https://doi.org/10.1093/jac/dkab147

27 Park J, Shin E, Park AK *et al*. Co-infection with chromosomally-located $bla_{CTX\text{-}M\text{-}14}$ and plasmid-encoding $bla_{CTX\text{-}M\text{-}15}$ in pathogenic *Escherichia coli* in the Republic of Korea. *Front Microbiol* 2020; **11**: 545591. https://doi.org/10.3389/fmicb.2020.545591

28 Zeng S, Luo J, Chen X *et al*. Molecular epidemiology and characteristics of CTX-M-55 extended-spectrum β-Lactamase-producing *Escherichia coli* From Guangzhou, China. *Front Microbiol* 2021; **12**: 730012. http://dx.doi.org/10.3389/fmicb.2021.730012

29 Tansawai U, Walsh TR, Niumsup PR. Extended spectrum ß-lactamase-producing *Escherichia coli* among backyard poultry farms, farmers, and environments in Thailand. *Poult Sci* 2019; **98**: 2622–31. https://doi.org/10.3382/ps/pez009

30 Fuentes-Castillo D, Sellera FP, Goldberg DW *et al*. Colistin-resistant *Enterobacter kobei* carrying *mcr-9.1* and $bla_{CTX\text{-}M\text{-}15}$ infecting a critically endangered Franciscana dolphin (*Pontoporia blainvillei*), Brazil. *Transbound Emerg Dis* 2021; **68**: 3048–54. https://doi.org/10.1111/tbed.13980

31 Lekagul A, Tangcharoensathien V, Mills A *et al*. How antibiotics are used in pig farming: a mixed-methods study of pig farmers, feed mills and veterinarians in Thailand. *BMJ Glob Heal* 2020; **5**: e001918. https://doi.org/10.1136/bmjgh-2019-001918

32 Xu F, Zeng X, Hinenoya A *et al*. MCR-1 confers cross-resistance to bacitracin, a widely used in-feed antibiotic. *mSphere* 2018; **3**: e00411-18. https://doi.org/10.1128/mSphere.00411-18

33 Hallenberg GS, Jiwakanon J, Angkititrakul S *et al*. Antibiotic use in pig farms at different levels of intensification—farmers' practices in Northeastern Thailand. *PLoS One* 2020; **15**: e0243099. https://doi.org/10.1371/journal.pone.0243099

34 Khine NO, Lugsomya K, Kaewgun B *et al*. 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* 2020; **7**: 582899. https://doi.org/10.3389/fvets.2020.582899

35 Pungpian C, Lee S, Trongjit S *et al*. 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. *J Vet Sci* 2021; **22**: e68. https://doi.org/10.4142/jvs.2021.22.e68

36 Lay KK, Jeamsripong S, Sunn KP *et al*. Colistin resistance and ESBL production in *Salmonella* and *Escherichia coli* from pigs and pork in the Thailand, Cambodia, Lao PDR, and Myanmar Border Area. *Antibiotics* 2021; **10**: 657. https://doi.org/10.3390/antibiotics10060657

37 Srijan A, Margulieux KR, Ruekit S *et al*. Genomic characterization of nonclonal *mcr-1*-positive multidrug-resistant *Klebsiella pneumoniae* from clinical samples in Thailand. *Microb Drug Resist* 2018; **24**: 403–10. https://doi.org/10.1089/mdr.2017.0400

38 Tansawai U, Yu Y, Kiddee A *et al*. Emergence of *mcr-3*-mediated IncP and IncFII plasmids in Thailand. *J Glob Antimicrob Resist* 2021; **24**: 446–7. https://doi.org/10.1016/j.jgar.2021.02.006

39 Zhou Y, Farzana R, Sihalath S *et al*. A one-health sampling strategy to explore the dissemination and relationship between colistin resistance in human, animal, and environmental sectors in Laos. *Engineering* 2022; **15**: 45–56. https://doi.org/10.1016/j.eng.2022.01.013

40 Snesrud E, McGann P, Chandler M. The birth and demise of the IS*Apl1*-*mcr-1*-IS*Apl1* composite transposon: the vehicle for transferable colistin resistance. *MBio* 2018; **9**: e02381-17. https://doi.org/10.1128/mBio.02381-17

41 Wang Z, Fu Y, Du X-D *et al*. Potential transferability of *mcr-3* via IS*26*-mediated homologous recombination in *Escherichia coli*. *Emerg Microbes Infect* 2018; **7**: 55. https://doi.org/10.1038/s41426-018-0057-6