RESEARCH ARTICLE

Whole genome sequencing and characteristics of *Escherichia coli* with co-existence of ESBL and *mcr* genes from pigs

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Abstract

This study aimed to analyze three ESBL-producing *E. coli* co-harboring *mcr* and ESBL genes from a healthy fattening pig (E. 431) and two sick pigs (ECP.81 and ECP.82) in Thailand using Whole Genome Sequencing (WGS) using either Illumina MiSeq or HiSeq PE150 platforms to determine their genome and transmissible plasmids. E. 431 carrying *mcr-2*.1 and *mcr-3*.1 belonged to serotype O142:H31 with ST29 sequence type. ECP.81 and ECP.82 from sick pigs harboring *mcr-1*.1 and *mcr-3*.1 were serotype O9:H9 with ST10. Two *mcr-1*.1 gene cassettes from ECP.81 and ECP.82 were located on IncI2 plasmid with 98% identity to plasmid pHNSHP45. The *mcr-2*.1-carrying contig in E. 431 showed 100% identity to plasmid pKP37-BE with the upstream flanking sequence of IS1595. All three *mcr-3*.1-carrying contigs contained the ΔTnAs2-mcr-3.1-dgkA core segment and had high nucleotide similarity (85–100%) to *mcr-3*.1-carrying plasmid, pWJ1. The mobile elements i.e. IS4321, ΔTnAs2, ISKpn40 and IS3 were identified in the flanking regions of *mcr-3*. Several genes conferring resistance to aminoglycosides (aac(3)-IIa, aadA1, aadA2b, aph(3")-Ib, aph(3')-Ila and aph(6')-Id), macrolides (mdf(A)), phenicols (cmlA1), sulphonamide (sul3) and tetracycline (tet(A) and tet(M)) were located on plasmids, of which their presence was well corresponded to the host’s resistance phenotype. Amino acid substitutions S83L and D87G in GyrA and S80I and E62K in ParC were observed. The *blaCTX-M-14* and *blaCTX-M-55* genes were identified among these isolates additionally harbored *blaTEM-1B*. Co-transfer of *mcr-1.1/blaTEM-1B* and *mcr-3.1/blaCTX-M-55* was observed in ECP.81 and ECP.82 but not located on the same plasmid. The results highlighted that application of advanced innovation technology of WGS in AMR monitoring and surveillance provided comprehensive information of AMR genotype that could yield invaluable benefits to development of control and prevention strategic actions plan for AMR.

Introduction

Antimicrobial resistance (AMR) in bacteria is one of the serious public health threats worldwide and has been complicated by emerging and spread of bacterial pathogens that are resistant to
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multiple drugs as well as clinically important antimicrobials (CIA) for human medicine. In the past decades, resistance to last-resort antibiotics such as carbapenems and polymyxins has been increasingly reported in Gram-negative bacteria, especially, emergence of carbapenem resistant Enterobacteriaceae (CRE) [1]. This has raised a particular concern of limited treatment antibiotic for bacterial infection and the need for discovering novel powerful antibiotics for future treatment [2].

Colistin is a last-resort antibiotic, especially for the treatment of infections with CRE in humans [3] and classified into the Highest Priority Critically Important Antimicrobials (HPCIA) for human medicine by WHO [4]. However, it has been widely used in livestock production, especially in pigs, for a long time. The global survey of colistin usage varied in different countries [5]. For example, the US government has prohibited colistin use in animal production and human medicine due to its nephrotoxicity and neurotoxicity [6]. China is considered to be the world’s highest user of colistin in agriculture [5], however presently colistin has been banded for using as a feed additive for animals since 2016 [7]. In EU, the use of colistin in Germany, Portugal, Italy and Estonia was more extensive in than other European countries [5]. In Thailand, approximately 40 tons of colistin was used per year as medicated feed mills for prevention and/or treatment of post-weaning diarrhea (PWD) [8].

Resistance to colistin is attributed to chromosomal mutations in the PhoPQ two-component regulatory system and pmrCAB operon, resulting in the modification of the lipid A of lipopolysaccharides [9]. In 2015, plasmid-borne gene, mcr-1, encoding a phosphoethanola-mine transferase was found to be common in Enterobacteriaceae isolated from animals, food stuffs and patients in China [10]. The report has attracted global concern of its high efficiency of horizontal transfer that places mcr-1 as a potential public health threat. Up to date, nine different mcr variants (mcr-1 to mcr-10) have been reported and were isolated from bacteria of different origins, including humans, livestock, wildlife, and environmental samples [10–14]. A previous study indicated that the spread of mcr genes mainly associated with epidemic plasmid replicon types of various size (e.g. IncI2, IncHI1, IncHI2, IncP, IncFIB and IncX4) [15].

Escherichia coli plays an important role in spread of AMR due to its capacity to accumulate AMR genes that can be horizontally transmitted to other bacterial species. Previous studies indicated that the E. coli isolates, particularly the sequence types ST10 and ST155, from food animal served as an important reservoir of mcr-1 [11, 16]. Previously, E. coli with coexistence of mcr-1 and blaVIM-1 [17] or blaCTX-M-55 [18] on a single plasmid was isolated. This could be a serious public health threat potentially leading to pan-drug resistant infection. Moreover, the ISApII insertion sequence has been considered a key element mediating translocation of mcr-1 into diverse types of plasmid and was localized on the chromosome by forming circular intermediates [19]. A previous study confirmed that the presence of mobile elements, including IS4321, ΔTnAs2 or ISKpn40, in the flanking regions of mcr-3 in E. coli [20]. Genetic characterization of the plasmid backbones of mcr genes and other mobile genetic elements (i.e IS and Tn) is required with the expectation to improve the understanding of the molecular mechanisms underlying dissemination of mcr variants.

Next generation sequencing (NGS) appears to be a very useful tool for epidemiological surveillance and characterization of AMR [21]. Due to its ability to provide comprehensive genomic data in a single time, NGS has been increasingly used for genomic characterization of foodborne bacterial pathogens, identification of clonal groups in bacteria of public health importance and molecular characterization of epidemic plasmids harboring AMR or/and virulence genes [22]. We previously isolated E. coli with coexistence of ESBL and mcr genes from healthy and sick pigs [23]. In this study, three E. coli co-harboring ESBL and mcr genes were characterized by using WGS approach.
Materials and methods

Epidemiological background of E. coli

In this study, three E. coli isolates co-expressing ESBL and mcr genes (i.e. E.431, ECP.81 and ECP.82) were obtained from our bacterial collection (Trongjit et al, 2020) [23]. E.431 was collected in 2007 from a healthy fattening pig and carries mcr-2, mcr-3 and blaCTX-M14. The other two E. coli isolates carrying mcr-1 and mcr-3 and simultaneously containing blaCTX-M-14/blaCTX-M-53/blaTEM-1B (ECP.81) and blaCTX-M-14/blaTEM-1B (ECP.82) were isolated from two different clinically sick pigs in 2013.

These isolates were collected as part of the molecular study of colistin resistant and ESBL-producing E. coli obtained from healthy (n = 354) and clinically sick pigs (n = 100) in Thailand [23]. Antimicrobial susceptibilities were determined using two-fold agar dilution method [24] and ESBL production was examined using disk diffusion method [24]. The results for all antimicrobials were interpreted using CLSI breakpoints [24], except EUCAST breakpoints was used for colistin [25]. All were PCR screened for colistin-resistance genes including mcr-1 [10], mcr-2 [26], mcr-3 [11] and mcr-4 [27] and β-lactamase-encoding genes including blaTEM, blAEShV, blaCTX-M, blACMY-1 and blACMY-2 [28–30]. All PCR primers used in this study are listed in S1 Table.

Conjugation experiment

Biparental filter mating experiment was conducted to confirm the localization of mcr and ESBL genes on conjugative plasmid [31] in E. 431, ECP.81 and ECP.82. The plasmid-free SE12RifR, spontaneous rifampicin-resistant Salmonella Enteritidis (rifampicin MIC = 256 μg/ml), was used as recipient [31]. Transconjugants were confirmed to be Salmonella on Xylose Lysine Deoxycholate agar (Difco, MD, USA) containing 32 μg/mL rifampicin and an appropriate antibiotic (i.e. 100 μg/mL ampicillin and/or 2 μg/mL colistin). The presence of mcr (n = 3) and ESBL (n = 3) genes in transconjugants was determined by PCR using specific primers as described above.

DNA preparation and Whole Genome Sequencing (WGS)

Genomic DNA of E. 431, ECP.81 and ECP.82 were extracted by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Plasmid DNA of ECP.81T and ECP.82T, the transconjugants obtained from ECP. 81 and ECP.82, were extracted by using Qiagen Plasmid Maxi Kit (Qiagen). The amount of all DNA samples was quantified according to Illumina sequencing sample requirements. The DNA samples were dissolved in 10mM Tris buffer to obtained at least 10 nM in 10 μl of minimum volume. The purity of DNA was determined at A260/280 and A260/230 using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA).

The quantified DNA were subjected to Whole Genome Sequencing (WGS) by using Illumina platform MiSeq or HiSeq PE150 (Illumina, San Diego, CA, US). The libraries were prepared by using Nextera XT sample preparation kit and sequenced with 2×250 or 350 paired-end reads protocol on an Illumina platform (MiSeq or HiSeq PE150) at Omics Sciences and Bioinformatics Center (OSBC), Faculty of Science, Chulalongkorn University and Singapore Joint Venture & Sequencing Center Novogen AIT.

Analysis of DNA sequence data

Trimming of raw sequence reads was performed using the CLC Genomics Workbench software version 11.0.0 (CLC bio, Aarhus, Denmark) with default settings. De novo assembly of
the trimmed reads was conducted using CLC Genomics Workbench or SPAdes 3.5.0 software [32]. Identification of Open Reading Frames (ORFs) and genome annotation of the assembled genetic elements was performed by using Prokka [33] and/or PATRIC 3.6.5 [34] with default settings.

WGS data sets were analyzed using Open-access bioinformatic webtool available at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). In silico typing based on WGS of assembled genomes/contigs in FASTA format was carried out by using Serotype Finder 2.0 [35] with selected threshold of 90% identity and 60% total serotype gene length. Multi-Locus Sequence Typing (MLST 2.0) was applied for molecular typing of *E. coli* [36]. The MLST allele sequences and profile data used in MLST 2.0 were obtained from https://www.PubMLST.org and determined at 90% identity and 60% minimum length. The core genome MLST (cgMLST) profile of these isolates were investigated using cgMLSTFinder 1.1 from CGE databases [37]. Res Finder 4.1 [38] and Point Finder [39] were used to predict AMR genes and chromosomal point mutations from genomic sequences based on 90% identity. Plasmid replicon sequence analysis and identification of virulence genes were performed using Plasmid Finder 2.1 [40] and Virulence Finder 2.0 [41], respectively with the threshold of 90% identity and 60% minimum length. Genome assemblies of three *E. coli* isolates were further analyzed for genomic relatedness using the *E. coli* cgMLST scheme available at the BacWGSTdb 2.0 (http://bacdb.cn/BacWGSTdb/) [42].

**Plasmid and phylogenetic analysis**

Plasmid reconstruction from WGS data was conducted by using highly homologous complete plasmid sequence references available in NCBI. The alignment and assembly of sequences was performed using CLC Genomics Workbench or PATRIC 3.6.5 [34]. Plasmid sequences were annotated by Prokka [33] or PATRIC 3.6.5 [34] and manually edited. Then, annotated plasmid sequences were analyzed. Circular comparison between IncI2 plasmid carrying *mcr-1* and most identical reference plasmids available at NCBI database was generated by CG view server [43]. All reference plasmids used for comparison in this study and obtained from NCBI database are listed in (S1 and S2 Tables).

The phylogenetic three of the *mcr-1.1*-carrying IncI2 plasmids was analyzed by comparing the two *mcr-1.1*-carrying IncI2 plasmids in ECP81 and ECP82 with 20 of *mcr-1*-carrying IncI2 plasmids from *E. coli* deposited in the GenBank database. The sequences were aligned using MUSCLE and phylogenetic interferences were obtained using the neighbor-joining method within the MEGA 10 software at set up of 1000 times bootstrap values to generate a majority consensus tree. The phylogenetic relationship of the core genome segment of *mcr-3* carrying contigs was conducted by MEGA 10.0 program with maximum likelihood method (1,000 bootstrap replicates). All *mcr-3* harboring contig sequences used to produce the phylogenetic trees were generated in this study and the *mcr-3* reference genome sequences were obtained from the GenBank database (S2 Table).

**Nucleotide sequence accession number**

The complete sequences of *mcr-1* carrying IncI2 plasmids from ECP81 and ECP82 in this study have been deposited at in GenBank under the accession numbers OK323956 and OK323955, respectively. Partial sequences of *mcr-3*-bearing plasmids from E431, ECP81 and ECP82 and partial *mcr-2* sequence from E431 were deposited in GenBank database with the accession of OK323954, OK323952, OK323953 and OK323951, respectively.
**Results**

**Antimicrobial susceptibilities and molecular characteristics**

E.431 from healthy pig and ECP.81 and ECP.82 from sick pigs exhibited multidrug resistance (MDR) phenotype (resistance to at least three different antimicrobial classes). E. 431 from healthy pig was resistant to all antibiotics tested, except trimethoprim (Table 1). ECP. 81 was resistant to all antimicrobials tested while the other EC.P. 82 remained susceptible to trimethoprim and ceftazidime.

Genome size of E431 was 5,863,412 bp with GC content of 50.07%. The genome size of ECP81 and ECP82 was 5,402,991bp and 5,579,866 bp, respectively, with 50.2% GC content. Based on in silico typing and MLST, E. 431 belonged to serotype O142:H32 with sequence type ST29. The other two *E. coli* isolates, ECP.81 and ECP.82 were serotype O9:H9 belonging to ST10.

The cgMLST scheme based on the presence or absence of 2,513 genes in E431, ECP81 and ECP82 were assigned into cg13958, cg40289 and cg 136327, respectively. Based on the cgMLST

| Table 1. Antimicrobial susceptibilities of ESBL-producing *E. coli* carrying *mcr* from pigs in Thailand (n = 3). |
|---------------------------------------------------------------|
| **Antimicrobial agent** | **Healthy pig** | **Clinically ill pig** |
| | **E. 431** | **ECP. 81** | **ECP. 82** |
| | **MIC (μg/mL)** | **S/R** | **MIC (μg/mL)** | **S/R** | **MIC (μg/mL)** | **S/R** |
| **Polymyxin** | | | | | | |
| Colistin | 8 | R | 8 | R | 8 | R |
| **Beta-lactams** | | | | | | |
| Ampicillin | >512 | R | >512 | R | >512 | R |
| Cefotaxime | 32 | R | 32 | R | 64 | R |
| Cefotaxime/clavulanic acid | 0.12 | ESBL<sup>a</sup> | 0.12 | ESBL<sup>a</sup> | 0.12 | ESBL<sup>a</sup> |
| Ceftazidime | 2 | R | 1 | S | 1 | S |
| Ceftazidime/clavulanic acid | ≤0.12 | ESBL<sup>a</sup> | 0.25 | ESBL<sup>a</sup> | 0.25 | ESBL<sup>a</sup> |
| Cefepime | 8 | I | 8 | I | 4 | I |
| Cefoxitin | 2 | S | 8 | S | 8 | S |
| Ertapenem | <0.015 | S | 0.03 | S | 0.03 | S |
| Imipenem | <0.12 | S | <0.12 | S | ≤0.12 | S |
| Meropenem | ≤0.03 | S | ≤0.03 | S | ≤0.03 | S |
| **Aminoglycosides** | | | | | | |
| Streptomycin | 32 | R | 256 | R | 256 | R |
| Gentamicin | 1024 | R | 256 | R | 256 | R |
| **Fluoroquinolones** | | | | | | |
| Ciprofloxacin | 128 | R | 32 | R | 32 | R |
| **Phenicol** | | | | | | |
| Chloramphenicol | 64 | R | 256 | R | 128 | R |
| **Tetracycline** | | | | | | |
| Tetracycline | 1 | S | 256 | R | 256 | R |
| **Folate pathway inhibitors** | | | | | | |
| Sulfamethoxazole | 256 | S | >2048 | R | >2048 | R |
| Trimethoprim | 64 | R | 1024 | R | 1 | S |

<sup>a</sup> = indicate ESBL production.

S, Susceptible.

I, Intermediate.

R, Resistant.

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Table 2. Molecular characteristics of ESBL-producing E. coli carrying mcr from pigs in Thailand (n = 3).

| Strain | Source     | Serotype | MLST       | Plasmid content                                                                 | Virulence profile                      | Resistance genes | Others by WGS | Amino acid change in GyRA and/or ParC |
|--------|------------|----------|------------|--------------------------------------------------------------------------------|----------------------------------------|------------------|---------------|-------------------------------------|
| E. 431 | Healthy pig| O142: H31| 29         | Col156, Col440II, IncFIB, IncFII, IncHI2, P0111                            | astA, cebA, cif, eae, efa1, espA, espB, espF, espI, gad, hra, iss, iucC, iutA, katP, ipfA, nleA, nleC, ompT, sepA, terC, toxB, traT, tsh | mcr 2.1, mcr 3.1 | blaCTX-M-14 | aac(3)-Ila, aadA1, aadA2b, aph (3’)-Ib, aph(3’)-Ila, aph(6)-Id, mdf(A), cmlA1, oxaA, oxaB, sul3, tet(A), tet(M) | GyRA (S83L, D87G), ParC (S80I) |
| ECP. 81 | Sick pig   | O9:H9    | 10         | Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-1 (γ), IncI2               | astA, fedA, fedF, gad, hra, sta1, terC, traT | mcr 1.1, mcr 3.1 | blaCTX-M-14, blaCTX-M-55, blatem1b | aac(3)-Ila, aadA1, aadA2b, aph (3’)-Ib, aph(3’)-Ila, aph(6)-Id, cfr, linA(F), mdf(A), catA2, cmlA1, oxaA, oxaB, qnrS1, sul2, sul3, tet(A), tet(M) | GyRA (S83L), ParC (S80I) |
| ECP. 82 | Sick pig   | O9:H9    | 10         | Col440I, ColpVC, IncFIB, IncFII, IncHI2, IncI1-1 (γ), IncI2               | astA, fedA, fedF, gad, hra, terC        | mcr 1.1, mcr 3.1 | blaCTX-M-14, blaCTX-M-1B | aac(3)-Ila, aadA1, aadA2b, aph (3’)-Ib, aph(3’)-Ila, aph(6)-Id, mdf(A), cmlA1, oxaA, oxaB, qnrS1, sul2, sul3, tet(A), tet(M) | GyRA (S83L), ParC (S80I, E62K) |

Whole genome sequencing and characteristic of E. coli with co-existence of ESBL and mcr genes

scheme, ECP81 and ECP82 had high genomic similarity to E. coli strain TZ7_Sa from a pig in Australia (Accession no. NZ_SAIB01) with distance of 324 and 287 different alleles, respectively and E. coli strain STEC_545 isolated from a diarrhea patient in the Netherlands (Accession no. LODB01) with different alleles of 349 and 309, respectively. Both the TZ7_Sa and STEC_545 were ST10 E. coli strains and carried similar AMR genes detected in ECP81 and ECP82 (i.e. aadA1, aadA2, blatem1b, cmlA1, dfrA12, mdf(A), sul3, in TZ7_Sa and mdf(A) in STEC_545). However, neither mcr nor ESBL genes were detected in the TZ7_Sa. No closest-genomic similarity in the database was observed for E431.

Plasmid finder showed that all the isolates harbored four plasmid replications, including IncFIB, IncFII, IncHI2 and Col440I. E. 431 additionally carried Col156 and P0111 replication types, while ECP81 and ECP81 additionally contained ColpVC, IncI1-1(γ) and IncI2 replication types (Table 2).

Identification of virulence profiles revealed that E. 431, ECP81 and ECP82 harbored similar virulence genes including astA encoding heat stable toxin, fedA/fedF encoding fimbria adhesin, gad encoding glutamate decarboxylase, hra encoding heat resistance agglutinin, terC encoding tellurium ion resistance protein and traT encoding outer membrane protein resistance. The presence of certain virulence genes including cebA, cif, eae, efa1, espA, iss, iucC, iutA, katP, ipfA, nleA, nleB, nleC, ompT, sepA, toxB and tsh varied among the three isolates (Table 2).

**Presence and horizontal transfer of AMR determinants**

WGS data analysis using Resfinder revealed the presence of AMR genes that were consistent with their resistance phenotype (Table 2). E.431 carried mcr2.1, mcr3.1 and blaCTX-M-14. Both ECP.81 and ECP.82 carried mcr1.1 and mcr3.1 genes. ECP.81 additionally harbored blaCTX-M-14, blaCTX-M-55 and blatem1b while ECP.82 simultaneously carried blaCTX-M-14 and blatem1b. In addition to ESBL and mcr genes, all three isolates concurrently contained various AMR genes conferring (but not limited) resistance to aminoglycoside (aac(3)-Ila, aadA1, aadA2b, aph(3’)-Ib, aph(3’)-Ila and aph(6)-Id), macrolide (mdf(A)), phenicol (cmlA1), sulphonamide (sul3) and tetracycline (tet(A) and tet(M)) that were consistent with the AMR phenotypes. The single point mutations in quinolone resistant-determining regions (QRDR) of gyrA and parC resulting in amino acid substitution in GyRA and ParC were identified including S83L and
Table 3. Antimicrobial resistance genes and plasmid contents in donors and transconjugants revealed by NGS.

| Strain | Role          | AMR gene                               | Plasmid replicon type                                                                 |
|--------|---------------|----------------------------------------|---------------------------------------------------------------------------------------|
| ECP.81 | Donor         | mcr 1.1, blaCTX-M-14, blaCTX-M-55, blatem-1B, aac(3)-IIa, aadA1, aadA2b, aph(3’)-Ib, aph (3’)-IIa, aph(6)-Id, cfr, IncFII, catA2, cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M) | Col440I, ColpVC, IncFIB, IncFII, IncH2, IncI1-1(y), IncI2                        |
| ECP.81T| Transconjugant| mcr-3.1, blaCTX-M-55 aac(3)-IIa          | Col440I, ColpVC, IncFIB, IncFII                                                        |
| ECP.82 | Donor         | mcr 1.1, mcr 3.1, blaCTX-M-14, blatem-1B, aac(3)-IIa, aadA1, aadA2b, aph(3’)-Ib, aph (3’)-IIa, aph(6)-Id, mdf(A), cmlA1, oqxA, oqxB, qnrS13, sul2, sul3, tet(A), tet(M) | Col440I, ColpVC, IncFIB, IncFII, IncH2, IncI1-1(y), IncI2                        |
| ECP.82T| Transconjugant| mcr 1.1, blatem-1B aac(3)-IIa, aadA2b, aph(3’)-Ib, aph(6)-Id, qnrS13, sul2 | Col440I, ColpVC, IncFIB, IncFII, IncI1-1(y), IncI2 |
entire length exhibited 90% identity to the 26687 to 28611 bp region of the published sequence of plasmid pKP37-BE in *E. coli* strain KP37 (Accession No. LT598652). No plasmid replicon and other AMR genes were detected on the same contig.

All three porcine *E. coli* isolates in this study harbored *mcr-3.1*. The size of assembled contigs ranged from 8,412 to 11,172 bp. The WGS data showed that *mcr-3.1* carrying plasmid in all the isolates had a backbone similar to *mcr-3.1*-carrying plasmid pWJ1 (Accession No. KY924928) (Fig 3). Five insertion sequences (IS) flanking the *mcr-3.1* cassette were identified. The upstream IS were IS4321 (ECP.81 and ECP.82) and ΔTnAs2 (E.431, ECP.81 and ECP.82) and those at downstream were ISKpn40 and IS3 family transposase (ECP.81 and ECP.82) and IS26 (ECP.82). A core segment ΔTnAs2- NimC/NimA- *mcr-3.1*-dgkA and conjugation transfer genes (i.e. *trb* and *traO*, and *traG*) were presented in all three *mcr-3.1*-carrying plasmid. The additional insertion sequence, IS4321, was immediately upstream of the ΔTnAs2- NimC/ NimA- *mcr-3.1*-dgkA segment in *mcr-3* carrying contigs of ECP.81 and ECP.82. None of the contigs contained other AMR genes and plasmid replicon type sequence identified in the PlasmidFinder database.

Phylogenetic tree of the core genome sequences was analyzed in all three *mcr-3.1*-carrying isolates and 12 *mcr-3.1* harboring-plasmids deposited in the GenBank database (Fig 4). The members in this phylogenetic tree can be grouped into three clades. The *mcr-3.1* carrying contigs in ECP. 81 and ECP.82 had a core segment that were similar to pWJ1 (Accession No. KY924928) isolated from in *E. coli* from pigs in China and in *Klebsiella pneumoniae* from pigs in Nakhon Pathom, Thailand (Accession No. CO041095 and CP041104). However, the *mcr-
3.1 carrying contig from fattening pig (E.431) was different from those in sick pigs and reference plasmids.

**Discussion**

The emergence and spread of colistin resistance mediated by plasmid-borne mcr genes has occurred globally, of which the majority was associated with bacterial pathogens including *E. coli*, *Moraxella* spp., *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp. and *Citrobacter* spp [44]. The *mcr* -carrying plasmids frequently co-harbor other resistance determinants e.g. *bla* VIM-1 [17] and *bla* CTX-M-1 [45]. These raise a particular challenge for treatment of MDR Gram-negative bacterial infection due to limited availability of effective antibiotics. *E. coli*, particularly from pig, has been a major species among the *mcr*-carrying bacterial strains [10, 11, 46].

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**Fig 2.** Circular comparison between *mcr*-1.1 -carrying IncI2 plasmids from ECP.81 and ECP.82 to five IncI2 type plasmids carrying *mcr*-1 with the highest similarity obtained from NCBI database. Circular comparison between *mcr*-1.1 -carrying IncI2 plasmids from ECP.81 and ECP.82 to five IncI2 type plasmids carrying *mcr*-1 with the highest similarity obtained from NCBI database (Accession No. KP347127, KX034083, MN232187, AP017622 and AP017614) generated by the Comparative Genomic tool (CG view) freely available at https://CGViewServer.ca. The outer-red circle denotes annotation of *mcr*-1.1 plasmids from the present study. The sequencing alignment indicates the high degree similarity of the *mcr*-1.1 harboring IncI2 plasmids from ECP.81 and ECP.82 to the *mcr*-1-harboring plasmid, pHNSHP45, isolated in China. The insertions element, ISAp1, is conserved in all *mcr*-1-containing plasmids. Gaps indicate regions that were missing in the respective plasmid compared to the reference plasmid.

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In the present study, we performed WGS analysis for molecular characterization of three ESBL producing 
*E. coli* additionally carrying *mcr* originated from pigs.

The *E. coli* isolates from healthy fattening pig, E.431, carrying *mcr-2/mcr-3* belonged to serotype O142:H31 and had sequence type, ST29. The other two isolates originated from clinically sick pigs, ECP.81 and ECP.82, with coexistence of *mcr-1* and *mcr-3* appeared to be serotype O9:H9 with sequence type ST10. This supports previous studies demonstrating that the ST10 *E. coli* served as an important reservoir of *mcr-1* [46, 47]. The clonal group ST10 of *mcr*-carrying *E. coli* was reported previously in the clinical isolates from humans [48] and food-producing animals (i.e. poultry and swine) in Thailand [49] and other Asian countries [50, 51] as well as some European countries [16, 52]. It was shown that the ST10 strains harboring *mcr* commonly carried additional-multiple AMR genes e.g. *bla*\(_{\text{CTX-M1}}\) and *bla*\(_{\text{SHV-12}}\) [16], in agreement with the isolates in this study.

Fig 3. Comparative schematic representation of the flanking regions of the *mcr-3* gene in pWJ1 (Accession no. KY924928) and *mcr-3* carrying contigs in this study. The arrows indicate the positions and directions of the genes. The gray shade indicates homology in the corresponding genetic environment on each contig. AMR genes are indicated in red for *mcr-3.1* and blue for *bleO*, bleomycin resistance gene. The conjugally-transferred proteins are indicated in yellow. The green, pink, orange and brown arrows represent transposon-associated genes (ΔIS4321, ΔTnAs2, ΔISKpm40 and ΔIS26).

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In the present study, we performed WGS analysis for molecular characterization of three ESBL producing *E. coli* additionally carrying *mcr* originated from pigs.

Fig 4. Phylogenetice tree of core genome sequences in E.431, ECP.81 and ECP.82 and other *mcr-3* plasmids deposited in the GenBank database. Sequences were aligned using MUSCLE and phylogenetic inferences were obtained using the maximum likelihood method within the MEGA 10 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis of 1000 times to generate a majority of consensus tree.

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E.431, ECP.81 and ECP.82 were MDR, which exhibited resistance to different antimicrobial classes e.g. colistin, β-lactam, aminoglycosides, fluoroquinolone, tetracycline and sulfathiazole. The AMR phenotype in all these isolates was consistent with AMR genotype revealed by WGS e.g. resistance to colistin (i.e. methoprim). The AMR phenotype in all these isolates was consistent with AMR genotype classes e.g. colistin, β-lactam, aminoglycosides, fluoroquinolone, tetracycline and sulfathiazole. These results agree with previous studies demonstrating that colistin resistant bacteria commonly exhibited MDR phenotype and frequently co-harbored multiple AMR genes [44, 53].

The conjugation experiments and plasmid analysis confirmed that mcr-1.1 of ECP.81 and ECP. 82 was located on IncI2 plasmid, in agreement with a previous study [10]. The mcr-1 gene and its variants have been identified to be associated with four major plasmid incompatibility groups i.e. IncX4, IncI2, IncHI2 and ColE10-like [46, 47]. In Thailand, a previous study in clinical CRE isolates carrying mcr-1 revealed that IncX4 was predominant replicon type, followed by IncI2 [54]. Taken together, these results suggest the circulation of IncI2 plasmid harboring mcr-1 among bacterial species of animal and human origins. It was previously shown that IncI2 plasmid can migrate between different bacterial species and E. coli serves as a potential carrier of this plasmid replicon [55].

ISApl1 has been the main driver of mobilized colistin resistance gene, mcr-1, via horizontal gene transfer [56]. Four genetic contexts surrounding mcr-1 cassette were identified including the composite transposon Tn6330 (ISApl1–mcr-1–orf–ISApl1) and a single-ISApl1 located upstream (ISApl1–mcr-1–orf); a single-ISApl1 (mcr-1–orf–ISApl1) located downstream and a structure lacking both copies of ISApl1 (mcr-1–orf) [56]. In present study, the two mcr-1.1 carrying IncI2 plasmids from ECP.81 and ECP. 82 had the structure of ISApl1–mcr-1–orf. The results from the conjugation experiment and plasmid analysis in donor (ECP. 82) and transconjugant (ECP. 82T) confirmed that ISApl1 is associated with the mobilization of mcr-1. This transconjugant, ECP. 82T, carried mcr-1.1 with translocation of ISApl1 to its upstream or ISApl1–mcr-1–orf as observed in its donor strain. A recent study hypothesized that mcr-1 translocation could be mediated through a circular intermediate that mediates the insertion of the mcr-1 gene cassette into other bacterial plasmids or genome [19].

The mcr-2 gene was first described on IncX4 plasmid in E. coli isolated from calves and piglets in Belgium [26]. However, the prevalence of mcr-2 was low and limited to E. coli, Moraxella and K. pneumoniae from pigs, calves, bird, and chicken in some countries e.g. China [57], Belgium [26], USA and Great Britain [58]. Up to date, six mcr-2 variants (Accession no. LT598652, MF176239, NG065452, MT757845, MT757842 and MT757844) were deposited on to GenBank database but only one plasmid sequence, pBK37–BE (Accession no. LT598652), was characterized. In our study, the mcr-2.1 gene in E.431 showed 100% identity to pBK37–BE, with IS1595 located upstream. However, analysis of genetic contexts of the genomic location mcr-2 was limited due to the limited size of the contig and non-transferability in the conjugation experiment.

The mcr-3 gene was first identified on pWJ1 that is a 261 kb IncHI2-type plasmid in E. coli from pigs in China [11]. Up to date, the gene was identified on IncP1, IncFII and IncI1 plasmids in many bacteria species e.g. E. coli, K. pneumoniae, Salmonella, Enterobacter spp. and Aeromonas spp. from pig, chicken, cattle, aquatic environment and human in several countries i.e. China [59], Denmark [60] and Spain [61]. In this study, only mcr-3.1 in ECP. 81 was located on conjugative plasmid. Previous studies described that the dissemination of mcr-3 was involved in mobile elements that can be horizontally transferred such as ΔTnAs2, ISKpn40, IS26 and IS15DI [20]. The mobile elements ΔTnAs2 and ΔISKpn40 were originated from Aeromonas spp and may play an important role in the spread of mcr-3 between
Aeromonas spp. and Enterobacteriaceae [11]. In this study, five mobile elements were identified in the flanking regions of mcr-3 contigs including ΔIS4321, ΔTnA-NimC/NimA-mcr-3-dgkA-ISKpn40-ΔbleO, IS26 and IS3. ECP.81 and ECP.82 contained a core segment of IS4321-ΔTnA-NimC/NimA-mcr-3-dgkA-ISKpn40-ΔbleO, while E. 431 carried ΔTnA-nimC/nimA-mcr-3-dgkA-nimC/nimA-ΔbleO. The core segment ΔTnA-nimC/nimA-mcr-3-dgkA in all three isolates was similar to pWJ1. However, ECP.81 and ECP.82 had IS4321 instead of IS6100. This IS4321 originated from K. aerogenes and was previously identified in mcr-3 carrying plasmids, pZR78 (Accession no. MF455226) and pZR12 (Accession no. MF455227) [20].

ESBL genes are frequently located on conjugative plasmids and has been previously shown to be associated with several plasmid incompatibility groups e.g. IncF, IncI, IncH and IncA/C plasmids [62]. IncF is a commonly described plasmid type identified in humans and animals and E. coli is considered a major reservoir of this plasmid [62]. A previous study in Korea demonstrated the dissemination of bla_{CTX-M14} gene driven by IncF plasmid [63]. A study in China reported that bla_{CTX-M55} in E. coli from pets and food animals were linked to IncI2 plasmid [64]. Both IncH1 and IncH2 plasmids are frequently associated with resistance to multdrugs e.g. sulphonamides, aminoglycosides, tetracyclines and streptomycin in additions to cephalosporins. The bla_{CTX-M2} and bla_{TEM-1B} genes were previously reported to be on a IncH12 plasmid [65]. In this study, many plasmid replicon types were detected in the three E. coli strains, of which the most frequent plasmid replicons were IncFIB, IncFI and IncHI2. The bla_{CTX-M14} gene was detected in all E. coli isolates. The bla_{CTX-M55} gene was found only in ECP.81 and bla_{TEM-1B} was identified in both isolates from ill pigs. Four mobile elements (i.e. Tn3 transposase, Tn903, IS1 and IS26) were identified in flanking regions of ESBL genes. This supports that, in addition to conjugative plasmid, the dissemination of ESBL genes may occur through transposons.

Moreover, the co-transfer of mcr-1.1 and bla_{TEM-1B} was observed in ECP. 82 and the co-transfer of mcr-3.1 and bla_{CTX-M55} was observed in ECP. 81. Using data from WGS analysis of the donor and plasmid analysis of transconjugants, plasmid reconstruction did not show the presence of other AMR genes. This implies that the genes encoding colistin resistance and ESBL production (i.e. mcr-1.1/bla_{TEM-1B} and mcr-3.1/bla_{CTX-M55}) were not colocalized on the same plasmid with other AMR genes. These observations are in agreement with a previous study in China demonstrating the co-transfer of mcr-1.1, mcr-3.5, bla_{NDM-5} and rmtB could occur even though they were not located on the same plasmid [66]. The co-transfer of many clinically important AMR genes at the same time is a big challenge for clinical treatment and disease controlling in both human and veterinary medicine. Such co-transfer of multiple resistance genes could provide the fitness advantage to the host strains [67]. Therefore, using colistin alone may not be the only reason of mcr dissemination. This is additionally supported by the use of ampicillin as selective pressure in conjugative experiment in this study, where colistin resistance gene(s) was co-selected.

In conclusion, the pandemic spread of mcr genes is attributed to several factors e.g. MDR profile in colistin-resistant strains, host fitness adaptation, and co-selection by other antimicrobials. Co-transfer of mcr and ESBL genes has generated a challenging for clinical treatment and a serious concern to global health. Judicious use of antibiotics in livestock and human medicine should be encouraged. Characterization of the coexistence ESBL and mcr genes in three E. coli isolates by using WGS approach provided large database for rapid analysis of genetic context of resistance determinants and supported better understanding on the dissemination of these resistance markers among bacteria from different sources. However, a short-read sequencing platform was used in this study and deemed insufficient to elucidate complete sequence and genetic organization of plasmid. Therefore, long read sequencing approach (e.g. Pacific Biosciences and Oxford Nanopore Technologies) producing long reads with higher-
quality genome assemblies and thus improving de novo assembly is suggested for further study.

**Supporting information**

S1 Table. Primers used in this study.

(DOCX)

S2 Table. Information of referent sequences used in this study.

(DOCX)

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**References**

1. Ellaby N, Doumith M, Hopkins KL, Woodford N, Ellington MJ. Emergence of diversity in carbapenemase-producing *Escherichia coli* ST131, England, January 2014 to June 2016. Euro Surveill. 2019; 24(37). Epub 2019/09/19. https://doi.org/10.2807/1560-7917.ES.2019.24.37.1800627 PMID: 31530344; PubMed Central PMCID: PMC6749775.

2. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. Colistin: an update on the antibiotic of the 21st century. Expert Rev Anti Infect Ther. 2012; 10(8):917–34. Epub 2012/10/04. https://doi.org/10.1586/era.12.78 PMID: 23030331.

3. Poirel L, Jayol A, Nordmann P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin Microbiol Rev. 2017; 30(2):557–96. Epub 2017/03/10. https://doi.org/10.1128/CMR.00064-16 PMID: 28275006; PubMed Central PMCID: PMC5355641.

4. WHO. Critically important antimicrobials for human medicine: Ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use. WHO CIA list. 2017;(5th revision); pp. 41.
5. Gharaibeh MH, Shatnawi SQ. An overview of colistin resistance, mobilized colistin resistance genes dissemination, global responses, and the alternatives to colistin: A review. Vet World. 2019; 12 (11):1735–46. Epub 2020/02/06. https://doi.org/10.14202/vetworld.2019.1735-1746 PMID: 32009752. PubMed Central PMCID: PMC6925059.

6. Olaitan AO, Li J. Emergence of polymyxin resistance in Gram-negative bacteria. Int J Antimicrob Agents. 2016; 48(6):581–2. Epub 2016/12/13. https://doi.org/10.1016/j.ijantimicag.2016.11.003 PMID: 27939183.

7. Walsh TR, Wu Y, China bans colistin as a feed additive for animals. The Lancet infectious diseases. 2016; 16(10):1102. https://doi.org/10.1016/S1473-3099(16)30329-2 PMID: 27676338.

8. Lekagul A, Tangcharoensathien V, Mills A, Rushton J, Yeung S. How antibiotics are used in pig farming: a mixed-methods study of pig farmers, feed mills and veterinarians in Thailand. BMJ Global Health. 2020; 5(2):e001918. https://doi.org/10.1136/bmjgh-2019-001918 PMID: 32189998.

9. Quesada A, Porrero MC, Teillez S, Palomo G, Garcia M, Dominguez L. Polymorphism of genes encoding PmrAB in colistin-resistant strains of Escherichia coli and Salmonella enterica isolated from poultry and swine. J Antimicrob Chemother. 2015; 70(1):71–4. Epub 2014/08/26. https://doi.org/10.1093/jac/dku320 PMID: 25150146.

10. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, 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(2):161–8. Epub 2015/11/26. https://doi.org/10.1016/S1473-3099(15)00424-7 PMID: 26603172.

11. Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, et al. Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in Escherichia coli. mBio. 2017; 8(3). Epub 2017/06/29. https://doi.org/10.1128/mBio.00543-17 PMID: 2865818; PubMed Central PMCID: PMC5487729.

12. Wang X, Yang Y, Zhou Y, Li J, Yin W, Wang S, et al. Emergence of a novel mobile colistin resistance gene, mcr-8, in NDM-producing Klebsiella pneumoniae. Emerg Microbes Infect. 2018; 7(1):122. Epub 2018/07/05. https://doi.org/10.1038/s41426-018-0124-z PMID: 29970891; PubMed Central PMCID: PMC6509194.

13. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmann M. Identification of Novel Mobilized Colistin Resistance Gene mcr-9 in a Multidrug-Resistant, Colistin-Susceptible Salmonella enterica Serotype Typhimurium Isolate. mBio. 2019; 10(3). Epub 2019/05/09. https://doi.org/10.1128/mBio.00853-19 PMID: 31064835; PubMed Central PMCID: PMC6509194.

14. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of Escherichia coli. Lancet Infect Dis. 2016; 16(3):281. Epub 2016/01/18. https://doi.org/10.1016/S1473-3099(16)00006-2 PMID: 26774246.

15. Madec JY, Haenni M. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid. 2018; 99:72–81. Epub 2018/09/09. https://doi.org/10.1016/j.plasmid.2018.09.001 PMID: 30194944.

16. Garcia V, Garcia-Menino I, Mora A, Flament-Simon SC, Diaz-Jimenez D, Blanco JE, et al. Co-occurrence of mcr-1, mcr-4 and mcr-5 genes in multidrug-resistant ST10 Enterotoxigenic and Shiga toxin-producing Escherichia coli in Spain (2006–2017). Int J Antimicrob Agents. 2018; 52(1):104–8. Epub 2018/04/11. https://doi.org/10.1016/j.ijantimicag.2018.03.022 PMID: 29635007.

17. Wang C, Feng Y, Liu L, Wei L, Kang M, Zong Z. Identification of novel mobile colistin resistance gene mcr-10. Emerg Microbes Infect. 2020; 9(1):508–16. Epub 2020/03/03. https://doi.org/10.1098/22221751.2020.173231 PMID: 32116151; PubMed Central PMCID: PMC7067168.

18. Madec JY, Haenni M. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid. 2018; 99:72–81. Epub 2018/09/09. https://doi.org/10.1016/j.plasmid.2018.09.001 PMID: 30194944.

19. Garcia V, Garcia-Menino I, Mora A, Flament-Simon SC, Diaz-Jimenez D, Blanco JE, et al. Co-occurrence of mcr-1, mcr-4 and mcr-5 genes in multidrug-resistant ST10 Enterotoxigenic and Shiga toxin-producing Escherichia coli in Spain (2006–2017). Int J Antimicrob Agents. 2018; 52(1):104–8. Epub 2018/04/11. https://doi.org/10.1016/j.ijantimicag.2018.03.022 PMID: 29635007.

20. Poirel L, Kieffer N, Liassine N, Thanh D, Nordmann P. Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of Escherichia coli. Lancet Infect Dis. 2016; 16(3):281. Epub 2016/01/18. https://doi.org/10.1016/S1473-3099(16)00006-2 PMID: 26774246.

21. Wang YQ, Zhang AY, Ma SZ, Kong LH, Li YX, Liu JX, et al. Co-occurrence of mcr-1 and ESBL on a single plasmid in Salmonella enterica. J Antimicrob Chemother. 2016; 71(8):2336–8. Epub 2016/06/23. https://doi.org/10.1093/jac/dkw243 PMID: 27330065.

22. Li R, Xie M, Zhang J, Yang Z, Liu L, Liu X, 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(2):393–401. https://doi.org/10.1093/jac/dkw411 PMID: 28073961.

23. Wang Z, Fu Y, Schwarz S, Yin W, Walsh TR, Zhou Y, et al. Genetic environment of colistin resistance genes mcr-1 and mcr-3 in Escherichia coli from one pig farm in China. Vet Microbiol. 2019; 230:56–61. Epub 2019/03/05. https://doi.org/10.1016/j.vetmic.2019.01.011 PMID: 30827405.

24. Kwong JC, McCallum N, Sintchenko V, Howden BP. Whole genome sequencing in clinical and public health microbiology. Pathology. 2015; 47(3):199–210. Epub 2015/03/03. https://doi.org/10.1097/PAT.0000000000000235 PMID: 25730631; PubMed Central PMCID: PMC4389909.

25. Kovac J, den Bakker H, Carroll LM, Wiedmann M. Precision food safety: a systems approach to food safety facilitated by genomics tools. TrAC Trends in Analytical Chemistry. 2017; 96:52–61.

26. Trongjit S, Assavacheep P, Sammangmnin S, Tran HM, Vo TTA, Simjee S, et al. Plasmid-mediated colistin resistance and ESBL production in Escherichia coli from clinically healthy and sick pigs
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24. CLSI I. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. CLSI document VET01-A4. 2013.

25. EUCAST. Breakpoint tables for interpretation of MICs and zone diameters, version 11.0, valid from 2021-01-01. European Committee on Antimicrobial Susceptibility Testing, Basel, Switzerland. 2021.

26. Xavier BB, Lammens C, Ruhal R, Kumar-Singh S, Butaye P, Goossens H, et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr*-2, in *Escherichia coli*, Belgium, June 2016. Euro Surveill. 2016; 21(27). Epub 2016/07/16. https://doi.org/10.2807/1560-7917.ES.2016.21.27.30280 PMID: 27416987.

27. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, et al. Novel plasmid-mediated colistin resistance *mcr*-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, to 2016. Euro Surveill. 2017; 22(31). Epub 2017/08/12. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30589 PMID: 28797329; PubMed Central PMCID: PMC5553062.

28. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM. Beta-Lactamases among extended-spectrum beta-lactamase (ESBL)-resistant *Salmonella* from poultry, poultry products and human patients in The Netherlands. The Journal of antimicrobial chemotherapy. 2005; 56(1):115–21. https://doi.org/10.1093/jac/dki190 PMID: 15941775.

29. Batchelor M, Hopkins K, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, et al. bla(CTX-M) genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. Antimicrobial agents and chemotherapy. 2005; 49(4):1319–22. https://doi.org/10.1128/AAC.49.4.1319-1322.2005 PMID: 15793104; PubMed Central PMCID: PMC1068621.

30. Li R, Lai J, Wang Y, Liu S, Li Y, Liu K, et al. Prevalence and characterization of *Salmonella* species isolated from pigs, ducks and chickens in Sichuan Province, China. International journal of food microbiology. 2013; 163(1):14–8. https://doi.org/10.1016/j.ijfoodmicro.2013.01.020 PMID: 23474653.

31. Chen S, Zhao S, White DG, Schroeder CM, Lu R, Yang H, et al. Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. Appl Environ Microbiol. 2004; 70(1):1–7. Epub 2004/01/08. https://doi.org/10.1128/AEM.70.1.1-7.2004 PMID: 14716169; PubMed Central PMCID: PMC3212339.

32. Nurk S, Bankevich A, Antipov D, Gurevich A, Korobeynikov A, Lupidus A, et al., editors. Assembling genomes and mini-metagenomes from highly chimeric reads. Annual International Conference on Research in Computational Molecular Biology; 2013: Springer.

33. Seemann T. Prokka: Rapid prokaryotic genome annotation. Bioinformatics. 2014; 30(14):2068–9. Epub 2014/03/20. https://doi.org/10.1093/bioinformatics/btu153 PMID: 24642063.

34. Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, et al. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep. 2015; 5:8365. Epub 2015/02/11. https://doi.org/10.1038/srep08365 PMID: 25666585; PubMed Central PMCID: PMC4322359.

35. Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. J Clin Microbiol. 2015; 53(6):2410–26. https://doi.org/10.1128/JCM.06094-11 PMID: 23972421.

36. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol. 2012; 50(4):1355–61. Epub 2012/01/13. https://doi.org/10.1128/JCM.00694-11 PMID: 22238442; PubMed Central PMCID: PMC3318499.

37. Zhou Z, Alikhan NF, Mohamed K, Fan Y, Agama Study G, Achman M. The EnteroBase user’s guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny, and *Escherichia* core genomic diversity. Genome Res. 2020; 30(1):138–52. Epub 2019/12/07. https://doi.org/10.1101/gr.251678.119 PMID: 31892957; PubMed Central PMCID: PMC6961584.

38. Bortolzia V, Kaas RS, Rupke E, Roberts MC, Schwarz S, Cattoir V, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother. 2020; 75(12):3491–500. https://doi.org/10.1093/jac/dkaa345 PMID: 32780112.

39. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012; 67(11):2640–4. Epub 2012/07/12. https://doi.org/10.1093/jac/dks261 PMID: 22782487; PubMed Central PMCID: PMC3468078.

40. Carattoli A, Zankari E, Garcia-Fernandez A, Larsen MV, Lund O, Villa L, et al. PlasmidFinder and pMLST: in silico detection and typing of plasmids. Antimicrobial Agents Chemother. 2014. https://doi.org/10.1128/AAC.02412-14 PMID: 24777092.

41. Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, et al. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. J
42. Feng Y, Zou S, Chen H, Yu Y, Ruan Z. BacWGSTdb 2.0: a one-stop repository for bacterial whole-genome sequence typing and source tracking. Nucleic Acids Res. 2021; 49(D1):D644–D650. Epub 2020/10/04. https://doi.org/10.1093/nar/gkaa821; PubMed Central PMCID: PMC7778894.

43. Stothard P, Wishart DS. Circular genome visualization and exploration using CGView. Bioinformatics. 2005; 21(4):537–9. https://doi.org/10.1093/bioinformatics/bti054; PMID: 15479716.

44. Flament-Simon SC, de Toro M, Mora A, Garcia-V, Garcia-Menino I, Diaz-Jimenez D, et al. Whole Genome Sequencing and Characteristics of mcr-1-Harboring Plasmids of Porcine Escherichia coli Isolates Belonging to the High-Risk Clone O25b:H4-ST131 Clade B. Front Microbiol. 2020; 11:387. Epub 2020/04/09. https://doi.org/10.3389/fmicb.2020.00387; PubMed Central PMCID: PMC7105644.

45. Haenni M, Poirel L, Kieffer N, Chatre P, Saras E, Metayer V, et al. Co-occurrence of extended spectrum beta-lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis. 2016; 16(3):281–2. Epub 2016/01/18. https://doi.org/10.1016/S1473-3099(16)00007-4; PMID: 26774244.

46. Garcia-Menino I, Diaz-Jimenez D, Garcia V, de Toro M, Flament-Simon SC, Blanco J, et al. Genomic Characterization of Prevalent mcr-1, mcr-4, and mcr-5 Escherichia coli Within Swine Enteric Colibacillosis in Spain. Front Microbiol. 2019; 10:2469. Epub 2019/11/19. https://doi.org/10.3389/fmicb.2019.02469; PMID: 31736909; PubMed Central PMCID: PMC6838222.

47. Mateamoros S, Van Hattem JM, Arcilla MS, Willems NC, Melles DC, Penders J, 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(1):1–10. https://doi.org/10.1038/s41598-016-0028-x; PMID: 28127051.

48. Luo Q, Yu W, Zhou K, Guo L, Shen P, Lu H, et al. Molecular Epidemiology and Colistin Resistance Mechanism of mcr-Positive and mcr-Negative Clinical Isolated Escherichia coli. Front Microbiol. 2017; 8:2282. Epub 2017/12/19. https://doi.org/10.3389/fmicb.2017.02282; PubMed Central PMCID: PMC5751374.

49. Khanawatee A, Kerdsin A, Chopijit P, Boueroy P, Hatrongjit R, Akeda Y, et al. Distribution and Molecular Characterization of Escherichia coli Harboring mcr Genes Isolated from Slaughtered Pigs in Thailand. Microb Drug Resist. 2021; 27(7):971–9. Epub 2020/12/17. https://doi.org/10.1089/mdr.2020.0242; PMID: 33325796.

50. Bai L, Hurley D, Li J, Meng Q, Wang J, Fanning S, et al. Characterization of multidrug-resistant Shiga toxin-producing Escherichia coli cultured from pigs in China: Co-occurrence of extended-spectrum beta-lactamase and mcr-1-encoding genes on plasmids. Int J Antimicrob Agents. 2016; 48(4):445–8. Epub 2016/08/17. https://doi.org/10.1016/j.ijantimicag.2016.06.021; PMID: 27526978.

51. Falgenhauer L, Waezsada S-E, Gwozdzinski K, Ghosh H, Djojja S, Bunk B, et al. Chromosomal locations of mcr-1 and blactxM-15 in fluoroquinolone-resistant Escherichia coli ST410. Emerg Infect Dis. 2016; 22(9):1689. https://doi.org/10.3201/eid2209.160692; PMID: 27322919.

52. El Garch F, Sauget M, Hodquet D, LeChaudue D, Waehrs F, Bertrand X. mcr-1 is borne by highly diverse Escherichia coli isolates since 2004 in food-producing animals in Europe. Clin Microbiol Infect 2017; 23(1):51, e1-. e4. https://doi.org/10.1016/j.cmi.2016.08.033; PMID: 27615718.

53. Alba P, Leekitcharoenphorn P, Franco A, Fellrini F, Ianzano A, Caprioli A, et al. 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. 2018; 9:1217. Epub 2018/06/29. https://doi.org/10.3389/fmicb.2018.01217; PMID: 29951045; PubMed Central PMCID: PMC6008537.

54. Pavvankittiporn W, Kamjumphol W, Uncharoen R, Kerdsin A. Whole-Genome Sequencing of Clinically Isolated Carbapenem-Resistant Enterobacteriaceae Harboring mcr Genes in Thailand, 2016–2019. Front Microbiol. 2020; 11:56836. Epub 2021/01/29. https://doi.org/10.3389/fmicb.2020.568368; PMID: 33505364; PubMed Central PMCID: PMC7829498.

55. Wong MH-y, Liu L, Yan M, Chan EW-c, Chen S. Dissemination of IncI2 plasmids that harbor the blactxM element among clinical Salmonella isolates. Antimicrob Agents Chemother. 2015; 59(8):5026–8. https://doi.org/10.1128/AAC.00775-15; PMID: 26014934.

56. Snesrud E, McGann P, Chandler M. The Birth and Demise of the ISAp1-1-mcr-1-ISAp1 Composite Transposon: The Vehicle for Transferable Colistin Resistance. mBio. 2018; 9(1):16. Epub 2018/02/15. https://doi.org/10.1128/mBio.02381-17; PMID: 29440577; PubMed Central PMCID: PMC5821093.

57. Zhang J, Chen L, Wang J, Yassin AK, Butaye P, Kelly P, et al. Molecular detection of colistin resistance genes (mcr-1, mcr-2 and mcr-3) in nasal/oropharyngeal and anal/cloacal swabs from pigs and poultry. Sci Rep. 2018; 8(1):3705. Epub 2018/03/01. https://doi.org/10.1038/s41598-018-22084-4; PMID: 29487327; PubMed Central PMCID: PMC5829079.
58. AbuOun M, Stubberfield EJ, Duggett NA, Kirchner M, Dormer L, Nunez-Garcia J, et al. *mcr-1* and *mcr-2* variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to 2015. J Antimicrob Chemother. 2017; 72(10):2745–9. Epub 2017/11/02. https://doi.org/10.1093/jac/dkx286 PMID: 29091227; PubMed Central PMCID: PMC5890717.

59. Ling Z, Yin W, Li H, Zhang Q, Wang X, Wang Z, et al. Chromosome-Mediated *mcr-3* Variants in *Aeromonas veronii* from Chicken Meat. Antimicrob Agents Chemother. 2017; 61(11). Epub 2017/08/30. https://doi.org/10.1128/AAC.01272-17 PMID: 28848017; PubMed Central PMCID: PMC5655048.

60. Litrup E, Kill K, Hammerum AM, Roer L, Nielsen EM, Torpdahl M. Plasmid-borne colistin resistance gene *mcr-3* in *Salmonella* isolates from human infections, Denmark, 2009–2017. Euro Surveill. 2017; 22(31). Epub 2017/08/12. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30587 PMID: 28797325; PubMed Central PMCID: PMC5553060.

61. Hernández M, Iglesias MR, Rodríguez-Lázaro D, Gallardo A, Quijada N, Miguela-Villoldo P, et al. Co-occurrence of colistin-resistance genes *mcr-1* and *mcr-3* among multidrug-resistant *Escherichia coli* isolated from cattle, Spain, September 2015. Euro surveillance. 2017; 22(31):30586. https://doi.org/10.2807/1560-7917.ES.2017.22.31.30586 PMID: 28797328.

62. Rozwandowicz M, Brouwer MSM, Fischer J, Wagenaar JA, Gonzalez-Zorn B, Guerra B, et al. Plasmids carrying antimicrobial resistance genes in *Enterobacteriaceae*. J Antimicrob Chemother. 2018; 73 (5):1121–37. Epub 2018/01/26. https://doi.org/10.1093/jac/dky488 PMID: 29370371.

63. Kim J, Bae IK, Jeong SH, Chang CL, Lee CH, Lee K. Characterization of IncF plasmids carrying the *blaCTX-M-14* gene in clinical isolates of *Escherichia coli* from Korea. J Antimicrob Chemother. 2011; 66 (6):1263–8. Epub 2011/03/19. https://doi.org/10.1093/jac/dkr106 PMID: 21415040.

64. Lv L, Partridge SR, He L, Zeng Z, He D, Ye J, et al. Genetic characterization of IncI2 plasmids carrying *blaCTX-M-55* spreading in both pets and food animals in China. Antimicrob Agents Chemother. 2013; 57 (6):2824–7. https://doi.org/10.1128/AAC.02155-12 PMID: 23479683.

65. Egervarn M, Borjesson S, Byfors S, Finn M, Kaipe C, Englund S, et al. *Escherichia coli* with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and *Salmonella* on meat imported into Sweden. Int J Food Microbiol. 2014; 171:8–14. Epub 2013/12/04. https://doi.org/10.1016/j.ijfoodmicro.2013.11.005 PMID: 24296257.

66. Long H, Feng Y, Ma K, Liu L, McNally A, Zong Z. The co-transfer of plasmid-borne colistin-resistant genes *mcr-1* and *mcr-3*, the carbapenemase gene *blaKPC-5* and the 16S methylase gene *mtrB* from *Escherichia coli*. Sci Rep. 2019; 9(1):696. Epub 2019/01/27. https://doi.org/10.1038/s41598-018-37125-1 PMID: 30679636; PubMed Central PMCID: PMC6346057.

67. Wu R, Yi LX, Yu LF, Wang J, Liu Y, Chen X, et al. Fitness Advantage of *mcr-1*-Bearing IncI2 and IncX4 Plasmids in Vitro. Front Microbiol. 2018; 9:331. Epub 2018/03/15. https://doi.org/10.3389/fmicb.2018.00331 PMID: 29535696; PubMed Central PMCID: PMC5835064.