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ORIGINAL RESEARCH

# Co-Occurrence of the *mcr-1.1* and *mcr-3.7* Genes in a Multidrug-Resistant *Escherichia coli* Isolate from China

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**Objective:** A colistin-resistant *Escherichia coli* strain isolated from dog feces was characterized in this study.

**Methods and Results:** A multiplex PCR assay was used to detect the presence of colistin-resistant *mcr* genes; it was found that *E. coli* QDFD216 co-harbored the *mcr-1* and *mcr-3* genes. Whole-genome sequencing and further bioinformatics analysis revealed that *E. coli* QDFD216 belonged to serotype O176:H11, fimH1311 type and ST132. The resistance genes $bla_{\text{CTX-M-14}}$, *mdfA, dfrA3, acrA, acrB, tolc*, and *sul3* were present in the chromosome. The *mcr-1.1* and *mcr-3.7* genes were located in two plasmids of different incompatibility groups. *mcr-1.1* was carried by a IncX4-type plasmid within an typical IS*26-parA-mcr-1.1-pap2* cassette, while *mcr-3.7* was encoded by an IncP1-type plasmid with a genetic structure of Tn*As2-mcr-3.7-dgkA*-IS*26*. No additional antibiotic resistance genes were carried by either plasmid.

**Conclusion:** This is the first report of an *E. coli* isolate co-harboring a *mcr-1.1*-carrying IncX4 plasmid and a *mcr-3.7*-carrying IncP1 plasmid. The evolution and mechanism of *mcr* gene co-existence need further study to assess its impact on public health.

**Keywords:** colistin resistance, whole-genome sequencing, *mcr* genes, *mcr-1*, *mcr-3*

## Introduction

Colistin (polymyxin E) is a cyclic polypeptide antibiotic produced by *Bacillus polymyxa*.[1] This antibiotic is lethal to Gram-negative bacteria by targeting lipopolysaccharides (LPSs) and phospholipids in the cell membrane, inducing alterations in the cell permeability and thus leading to leakage of cellular contents and cell death.[2] Colistin has been considered as one of the last-resort options for the treatment of severe human infections caused by multidrug-resistant Gram-negative organisms.[3] It was thought that chromosomal mutation was the only mechanism to acquire resistance to colistin in bacteria.[4] However, the plasmid-mediated transferable colistin-resistance determinant MCR-1 was first reported in 2016 in *Escherichia coli* and *Klebsiella pneumonia* strains isolated from China.[5] The *mcr-1* gene has been detected worldwide in recent years in dozens of bacterial species from multiple origins, which raises great clinical concerns.[6–9] MCR proteins function as phosphoethanolamine (PEA) transferases that catalyze the addition of a PEA moiety from phosphatidylethanolamine to the head group of lipid A through a ping-pong mechanism.[10,11] Modification of lipid A with PEA is thought to reduce the affinity of colistin to LPS, and thus confers

bacterial resistance to the antibiotic.[11] To date, 10 *mcr* genes (*mcr-1* to *mcr-10*) carried by plasmids with varied replicon types have been identified in various bacterial species.[5,12–20] Each *mcr* gene has multiple variants. For example, 27 *mcr-1* and 30 *mcr-3* variant sequences have been reported in the NCBI database.

It is common for one bacterial isolate to harbor a single *mcr* determinant. However, recent studies have revealed the co-existence of the *mcr-1* and *mcr-3* genes in *E. coli* and *K. pneumonia* strains isolated from different countries.[21–23] These observations depict the complexities of colistin resistance dissemination mediated by MCR determinants. Here we characterized one *E. coli* strain isolated from dog feces that co-harbors *mcr-1.1* and *mcr-3.7* in different plasmids.

# Materials and Methods

## Bacterial Isolates and Antimicrobial Susceptibility Testing

We collected fecal samples from dogs in pet hospitals during the period from June to August 2019 in Jilin Province, China. The feces were resuspended in brain-heart infusion broth (Hopebio, Qingdao, China) followed by centrifugation at 300 rpm for 5 min at 4°C. The supernatants were plated on the MacConkey agar (Hopebio, Qingdao, China) plates containing 2 mg/L of colistin (Sigma-Aldrich, St Louis, MO, USA). Strain identification was confirmed using 16S rRNA gene sequencing. The presence of *mcr* genes in the bacterial isolates was tested by an established multiplex-PCR assay.[24] The susceptibility of the isolates to 30 antibiotics (Table S1) was measured by the Kirby-Bauer disk diffusion method. The data were analyzed according to the protocol of Clinical and Laboratory Standards Institute (CLSI) guidelines. The MICs of colistin, tigecycline and meropenem were analyzed according to the standard of the European Committee on Antibiotic Susceptibility Testing.[25] Multidrug resistances were defined as resistance to at least three antibiotic classes. The *E. coli* strain ATCC 25922 was used as the control.

## Whole-Genome Sequencing, Assembly and Bioinformatics Analysis

Total genomic DNA of the isolate was prepared using Qiagen's DNA Mini Kit with the manufacturer's instructions (Qiagen, Hilden, Germany). The genomic DNA extracted from the isolate was qualified and quantified with a Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Whole-genome sequencing was performed using PacBio single-molecule real-time (SMRT) and Illumina Novaseq sequencing technology. PacBio *RS* II and Illumina Novaseq 6000 sequencer were used, respectively. The PacBio library (10 kb) and Illumina PE150 library (350 bp) were constructed. The low-quality and short reads were filtered out with the SMRT portal (version 5.0.1). The filtered high-quality reads were assembled *de novo* using SOAPdenovo to generate the complete chromosome and plasmids without gaps. Genome annotation was performed using GeneMarkS version 4.17 to predict the related genes. Serotypes, sequence types, FimH types and resistance genes were identified using SerotypeFinder 2.0, Multi-Locus Sequence Typing (MLST) v.2.0, FimTyper 1.0 and ResFinder v.3.2 databases from the Center for Genomic Epidemiology (CGE). In addition, antimicrobial resistance genes were confirmed using the Comprehensive Antibiotic Resistance Database (CARD) and the Antibiotic Resistance Genes Database (ARDB).

## Plasmid Analysis

The plasmid replicon genotype, resistance genes and insertion sequence (IS) elements of the plasmid were identified using PlasmidFinder 2.1, ResFinder 3.2 and ISfinder. Comparative analysis of plasmids was performed and visualized using Easyfig version 2.2.3 and BLAST Ring Image Generator (BRIG).

## Nucleotide Sequence Accession Numbers

The sequences of the chromosome and plasmids have been submitted in the NCBI database under the accession numbers: CP053211, CP053212 and CP053213.

## Plasmid Conjugation

Conjugation experiment was performed between the colistin-resistant *E. coli* QDFD216 (donor) and the rifampin-resistant *E. coli* strain C600 (recipient) as described previously.[26] Transconjugants were selected on MacConkey agar plates containing 2 mg/L of colistin and 512 mg/L of rifampin. The presence of the *mcr-1* and *mcr-3* in the transconjugants was analysed by PCR with specific primers.

# Results and Discussion

## Antibiotic Resistance Profile of the *E. coli* Strain QDFD216 That Co-Harbors *mcr-1* and *mcr-3*

A total of 513 colistin-resistant isolates were obtained from 91 samples of dog feces in Jilin Province, China, in 2019.

All colistin-resistant isolates were screened by a multiplex PCR assay to detect the presence of *mcr* genes. Interestingly, PCR and sequencing showed that one isolate (designated *E. coli* QDFD216, isolated from one sample collected at the Veterinary Teaching Hospital of Jilin University) co-harbored the *mcr-1* and *mcr-3* genes. Antimicrobial susceptibility testing against a panel of antibiotics revealed that *E. coli* QDFD216 was resistant to penicillin, oxacillin, ampicillin, carboxypenicillin, piperacillin, cefazolin, cephalexin, cefradine, ceftriaxone, cefuroxime, cefoperazone, ceftazidime, amikacin, gentamicin, kanamycin, maddie mycin, clindamycin and colistin (MIC=16 mg/L), but remained susceptible to minocycline, norfloxacin, ofloxacin, ciprofloxacin, chloramphenicol, tigecycline, meropenem and rifampin (Table S1).

## Genome Analysis of the *E. coli* Strain QDFD216

Next, the *E. coli* strain QDFD216 was subjected to whole-genome sequencing by the PacBio and Illumina platforms. The *de novo* assembly resulted in a circular chromosome and two circular plasmids (designated pQDFD216.1 and pQDFD216.2). The chromosome of *E. coli* QDFD216 was 4,577,885 bp in length and had a GC content of 50.91%. Genome annotation by GeneMarkS resulted in the identification of 4543 coding sequences. MLST, SerotypeFinder and FimTyper analyses showed that *E. coli* strain QDFD216 belonged to ST132, serotype O176:H11and fimH1311 (Table S2). Resistance determinants were determined by the CARD and ARDB; $bla_{\text{CTX-M-14}}$, *mdfA, dfrA3, acrA, acrB, tolc*, and *sul3* were present in the chromosome. *mcr-1.1* and *mcr-3.7* were found to be located on two different plasmids (pQDFD216.1 and pQDFD216.2, respectively) (Table S2).

## The Genetic Context of *mcr-1.1* on pQDFD216.1

The *mcr-1.1* was located on pQDFD216.1 which was 33,308 bp in length and had an average GC content of 41.83%. PlasmidFinder analysis indicated that pQDFD216.1 belonged to the IncX4 incompatibility group (Table S2). pQDFD216.1 had a typical IncX4 plasmid structure, including plasmid replication (*pir*-type replicon), maintenance (*H-NS* and *topB*), toxin-antitoxin genes (*hicA-hicB*), insertion sequence elements (IS*26*), a partition gene (*parA*) and the conjugative transfer system (*TraG, VirB1, VirB2, VirB4, VirB5, VirB6, VirB8-11, VirD2* and *VirD4*) (Figures 1A and 2A).

BLASTn analysis of pQDFD216.1 with the current database showed that this plasmid was highly similar to pLD93-1-MCR1 (GenBank accession: CP047664.1), p2HS-C-1 (GenBank accession: CP038181.1), p787-MCR (GenBank accession: MG825367.1), pE13-43-mcr -1 (GenBank accession: LT838201.1) and pWI2-mcr (GenBank accession: LT838201.1) (Figure 1A). These *mcr-1*-carrying plasmids showed 99.91–99.95% nucleotide sequence identity with pQDFD216.1. The *mcr-1.1* gene in pQDFD216.1 is carried by the genetic structure IS*26-parA -mcr-1.1-pap2* (Figures 1A and 2A), which is nearly identical to the *mcr-1.1*-carrying IncX4 plasmids pmcr1_IncX4 (GenBank accession: KU761327.1) and pCSZ4 (GenBank accession: KX711706.1) (Figure 2A).

Similar to other *mcr-1.1*-carrying IncX4-type plasmids, the typical IS*26-parA-mcr-1.1-pap2* cassette was identified in pQDFD216.1 (Figure 2A).[27] The IS*Apl1* insertion sequence (IS*30* family) was lost in the *mcr-1* gene cassette.[28] Generally, IS*Apl1* is a highly active insertion element and a key component required for the mobilization of the gene-cassette containing the *mcr-1* gene.[29,30] The absence of IS*Apl1* along *mcr-1* genes could reduce further chromosomal integration.[28] Previous studies have shown that IncX4-type plasmids play a vital role in increasing the spread of plasmid-mediated *mcr* genes.[31,32] Remarkably, in all the *mcr-1*-carrying IncX4-type plasmids, IS*Apl1* in front of *mcr-1* was lost.[33] Therefore, the loss of the composite transposon IS*Apl1* might increase the stability of the *mcr* gene in IncX4 plasmids, and promote the widespread dissemination of the *mcr* gene.

## The Genetic Contexts of *mcr-3.7* on pQDFD216.2

The *mcr-3.7*-bearing plasmid pQDFD216.2 was 50,638 bp in size and had a GC content of 47.08%. PlasmidFinder analysis revealed that pQDFD216.2 belonged to the IncP1 type plasmid (Table S2). The IncP1 plasmids have a broad host range and are widespread in Gram-negative organisms, including *E. coli, K. pneumonia* and *S. enteric*.[34–36] The association of *mcr-3.7* with the IncP plasmid is worrisome because it may have the potential to promote the dissemination of *mcr-3* in Gram-negative pathogens.

The plasmid pQDFD216.2 did not carry any other resistance gene besides *mcr-3.7*. The pQDFD216.2 plasmid has the conjugative transfer/type IV secretion system (*tra* and *trb*), replication initiator (*trfA*), replication and partition genes (*parA* and *parB*), toxin-antitoxin genes

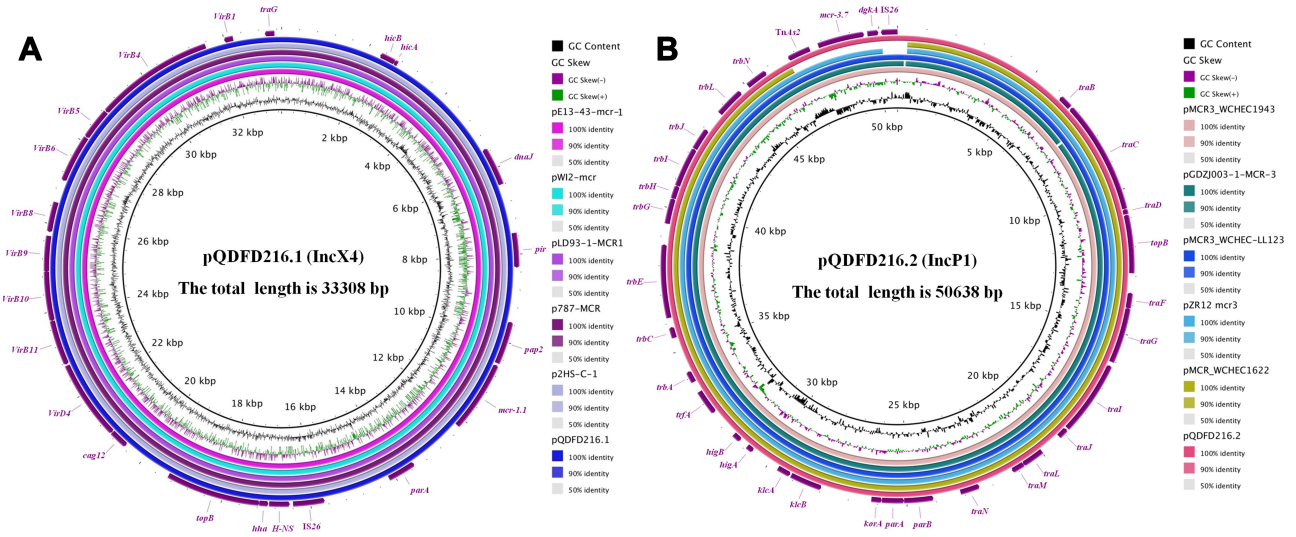


**Figure 1** Comparison of *mcr*-carrying plasmids and other closely related plasmids. (**A**) Comparative analysis of pQDFD216.1 with five closely related *mcr-1.1*-carrying plasmids including pLD93-1-MCR1 (GenBank accession: CP047664.1), p2HS-C-1 (GenBank accession: CP038181.1), p787-MCR (GenBank accession: MG825367.1), pE13-43-mcr-1 (GenBank accession: LT838201.1) and pWI2-mcr (GenBank accession: LT838201.1). pQDFD216.1 was used as the reference plasmid for BRIG. (**B**) Comparative analysis of pQDFD216.2 with five closely related *mcr-3*-carrying plasmids, pMCR3_WCHEC1943 (GenBank accession: MF678351.1), pGDZJ003-1-MCR-3 (GenBank accession: MH043625.1), pMCR3_WCHEC-LL123 (GenBank accession: MF489760.1), pZR12 (GenBank accession: MF455227.1) and pMCR_WCHEC1622 (GenBank accession: KY463452.1). The plasmid pQDFD216.2 was used as the reference plasmid for BRIG.

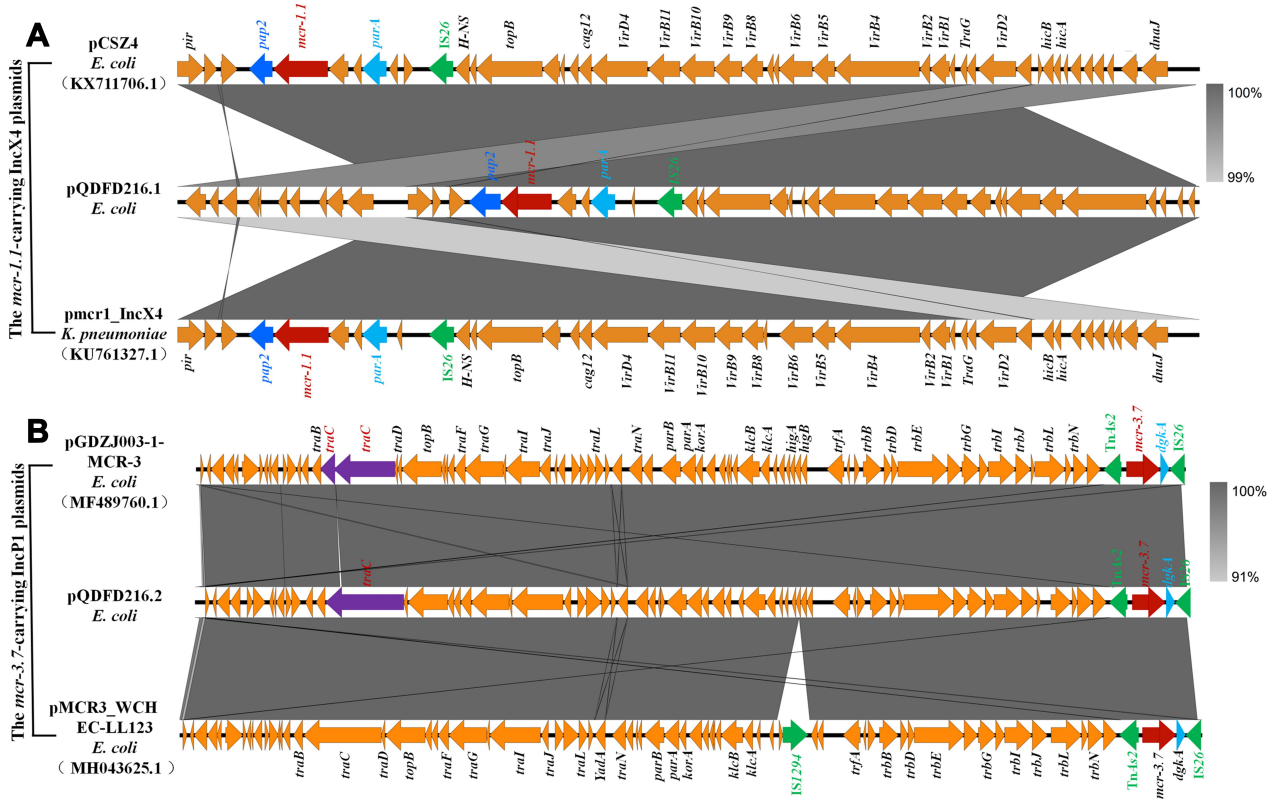


**Figure 2** A linear depiction of the genetic configuration of *mcr*-carrying plasmids compared to other closely related plasmids. (**A**) Structure comparisons of pQDFD216.1, pmcr1_IncX4 (GenBank accession: KU761327.1) and pCSZ4 (GenBank accession: KX711706.1). (**B**) Structure comparisons of pQDFD216.2, pGDZJ003-1-MCR-3 (GenBank accession: MH043625.1) and pMCR3_WCHEC-LL123 (GenBank accession: MF489760.1). The map was generated with Easyfig.

(*higA-higB*), host-lethal genes (*klc*) and their regulators (*kor*) (Figures 1B and 2B). BLASTn analysis showed that plasmids pMCR3_WCHEC1943 (GenBank accession: MF678351.1), pGDZJ003-1-MCR-3 (GenBank accession: MH043625.1), pMCR3_WCHEC-LL123 (GenBank accession: MF489760.1), pZR12 (GenBank accession: MF455227.1) and pMCR_WCHEC1622 (GenBank accession: KY463452.1) were most similar to pQDFD216.2 (Figure 1B). These plasmids showed 99.96–99.98% nucleotide sequence identity with pQDFD216.2. The *mcr-3.7* gene was located in pQDFD216.2 with the structure Tn*As2-mcr-3.7-dgkA*-IS*26*, which was also found in the *mcr-3.7*-carrying IncP1 plasmids pMCR3_WCHEC-LL123 and pGDZJ003-1-MCR-3 (Figure 2B). However, unlike pQDFD216.2, pGDZJ003-1-MCR-3 partially lacked the conjugal transfer gene *TraC* of the IncP-type plasmid (Figure 2B). Furthermore, when compared with pMCR3_WCHEC-LL123, the mobile genetic element IS*1294* was absent in pQDFD216.2 (Figure 2B).

## Plasmid Conjugation

Conjugation experiment was performed to determine the transferability of the pQDFD216.1 and pQDFD216.2. The *mcr*-harboring plasmids in the *E. coli* strain QDFD216 can be transferred to the recipient *E. coli* strain C600 at a frequency of $1.2x10^{-3}$ per donor cell. One hundred transconjugants were selected for PCR to characterize the presence of *mcr* genes. Thirty-two transconjugants contained only one *mcr* gene (19 for *mcr-1* and 13 for *mcr-3*). Strikingly, 68 out of the 100 transconjugants were found to harbor both *mcr-1* and *mcr-3*, indicating the co-transfer of pQDFD216.1 and pQDFD216.2.

The rapid dissemination of *mcr* genes in bacteria has raised great public concerns. Recent publications described the co-occurrence of different *mcr* alleles within a single bacterial strain of diverse species isolated from various regions,[21,22,37,38] adding complexity and flexibility to the spread of colistin resistance. Based on the available literatures, *mcr-1*/*mcr-3* are the most frequently detected combination of co-existing *mcr* genes,[21–23,39] while at a lower detection frequency, the co-occurrence of *mcr-3*/*mcr-8* as well as *mcr-1*/*mcr-5* has also been described.[37,38] Further molecular surveillance of colistin resistance may reveal more combinations of co-existing *mcr* genes, and their influences on public health require further assessment. The presence of *mcr* genes in companion animals has been reported previously, which may cause possible transmission of *mcr*-harboring bacterial strains between companion animals and humans.[40,41] To the best of our knowledge, our study reports the first bacterial strain isolated from companion animals that co-harbors *mcr-1* and *mcr-3*, adding another layer of threat of colistin-resistant bacteria from companion animals to human health.

# Conclusions

In this study, we identified the co-occurrence of a *mcr-1.1*-carrying IncX4 plasmid and a *mcr-3.7*-carrying IncP1 plasmid in *E. coli* strain QDFD216 isolated from dog feces in China. Compared to other studies worldwide, our data provide insight into the dissemination routes of plasmid-mediated colistin resistance. Furthermore, the co-existence of different *mcr* genes in the same isolate presents a great challenge for infection control in multidrug-resistant Gram-negative organisms.

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# Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

# Disclosure

The authors declare no conflicts of interest.

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