A mcr-1-Carrying Conjugative IncX4 Plasmid in Colistin-Resistant Escherichia coli ST278 Strain Isolated From Dairy Cow Feces in Shanghai, China

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Enterobacteriaceae, including Escherichia coli, has been shown to acquire the colistin resistance gene mcr-1. A strain of E. coli, EC11, which is resistant to colistin, polymyxin B and trimethoprim-sulfamethoxazole, was isolated in 2016 from the feces of a dairy cow in Shanghai, China. Strain EC11 identifies with sequence type ST278 and is susceptible to 19 frequently used antibiotics. Whole genome sequencing of strain EC11 showed that this strain contains a 31-kb resistance plasmid, pEC11b, which belongs to the IncX4 group. The mcr-1 gene was shown to be inserted into a 2.6-kb mcr-1-pap2 cassette of pEC11b. Plasmid pEC11b also contained putative conjugal transfer components, including an oriT-like region, relaxase, type IV coupling protein, and type IV secretion system. We were successful in transferring pEC11b to E. coli C600 with an average transconjugation efficiency of $4.6 \times 10^{-5}$. Additionally, a MLST-based analysis comparing EC11 and other reported mcr-positive E. coli populations showed high genotypic diversity. The discovery of the E. coli strain EC11 with resistance to colistin in Shanghai emphasizes the importance of vigilance in detecting new threats like mcr genes to public health. Detection of mcr genes helps in tracking, slowing, and responding to the emergence of antibiotic resistance in Chinese livestock farming.

Keywords: colistin resistance, mcr-1, Escherichia coli, IncX4 plasmid, whole genome sequence

Abbreviations: CC, clonal complexes; CLSI, Clinical and Laboratory Standards Institute; CRE, carbapenem-resistant Enterobacteriaceae; E. coli, Escherichia coli; ESBL, extended spectrum β-lactamase; EUCAST, European Committee on Antimicrobial Susceptibility Testing; HGT, horizontal gene transfer; IS, inverted repeats; IRs, insertion sequences; MDR, multidrug-resistant; MIC, Minimum Inhibitory Concentration; MLST, Multilocus Sequence Typing; NJ, Neighbor-joining; ORFs, open reading frames; PCR, polymerase chain reaction; PEA, phosphoethanolamine; SEM, scanning electron microscope; ST, sequence type; T4CP, type IV coupling protein; T4SS, type IV secretion system; TEM, transmission electron microscope; WGS, whole-genome sequencing; XDR, extensively drug-resistant.
INTRODUCTION

Antimicrobial resistance is becoming a great challenge to public health worldwide (Laxminarayan et al., 2014). The rapid evolution of MDR Gram-negative bacteria is pushing humankind to the cusp of a post-antibiotic era. Colistin (polymyxins E) is a family of cationic polypeptide antibiotics which acts as the last line of defense in the treatment of severe bacterial infections by MDR or XDR bacteria. In particular, colistin is used to treat ESBL-producing and CRE infections (Li et al., 2006; Paterson and Harris, 2016).

Colistin resistance was assumed to be chromosomally mediated, non-transmissible and an intrinsic property of the bacteria (Olaitan et al., 2014). However, the recent discovery of the Escherichia coli harboring plasmid-borne colistin resistance gene mcr-1 confirms transmission of colistin resistance by HGT (Liu et al., 2016). The MCR-1 encodes a PEA transferase that adds PEA to the lipid A of the lipopolysaccharide, leading to Gram-negative bacteria resistant to colistin (Anandan et al., 2017). This HGT mechanism of colistin resistance has alarmed the medical, media, academic and public health communities.

The global spread of the mcr-1 gene is now evident and being documented. Currently, researchers have discovered five mcr-like genes, ranging from mcr-1 to mcr-5, with a series of mcr genetic variants such as mcr-1.2, mcr-1.3 . . . mcr-1.12. These mcr genes have spread to 40 countries across 5 of 7 continents in multiple ecosystems, including the environment, food, animals (e.g., pig, poultry, and cattle) and humans, and in over 11 species of Enterobacteriaceae (Schwarz and Johnson, 2016; Chen et al., 2017; Feng, 2018). Retrospective studies have shown that an isolate harboring the mcr-1 gene had already existed in three chicken E. coli isolates in China from the 1980s (Shen et al., 2016). The presence of mcr-1 in livestock is indicative of the route of mcr-1 dissemination through the food chain and it is gravely concerning that animal-to-human transmission of MCR-1 colistin resistance has already been found in many countries.

Mobile genetic elements such as conjugative plasmids, transposons, integrons and IS are important vehicles of HGT of MDR or XDR bacteria. In particular, colistin-resistant E. coli EC11 strain was isolated from cow feces collected from a commercial dairy farm. We will use WGS to outline the mechanism for acquiring and transferring colistin resistance in this strain.

MATERIALS AND METHODS

Bacterial Strains and Identification

In May 2016, we cultured E. coli strains from fecal samples collected from a commercial dairy farm in Shanghai, China. Samples (25 g) were dispensed in sterile plastic bags containing 225 ml of Mueller–Hinton broth and incubated at 37°C for 24 h. All samples were seeded on MacConkey agar plates with 2 μg/mL colistin and incubated at 37°C for 18 h. One putative positive E. coli colony per sample was selected on the basis of morphology, size, and color (peachblow), then inoculated overnight on eosin-methylene blue agar. Species were further confirmed by the amplification and sequencing of 16S rRNA, while SEM and TEM analyses were conducted. All bacterial isolates were stored in the Luria-Bertani medium (Land Bridge, Beijing, China) with 30% glycerol at −80°C.

mcr-1 and β-Lactamase Gene Screening

Screening for the mcr-1 gene was performed using PCR amplification and sequencing. The specific primers used to produce the 309 bp amplicon were as previously described: CLR5-F (5′- CGGTCACTCGTTTGTTC-3′) and CLR5-R (5′-CTTGTCGGTGCTGTAGG-3′) (Liu et al., 2016). Further screening for the presence of the mcr-2, mcr-3 and the main β-lactamase gene groups (blaTEM, blaSHV, blaCTX-M, blaKPC, and blaNDM) was performed by previously reported primers. In this study, all primers used are presented in Supplementary Table S1. Each PCR reaction system was performed in 25 μL, containing 12.5 μL of PCR Mix (Sangon Biotech, Shanghai, China), 9.5 μL of dd H2O, 1 μL of forward and reverse primers, and 1 μL of DNA template. Finally, one E. coli isolate designated as E. coli EC11 was determined to harbor the mcr-1 gene, and this isolate was selected to perform the follow-up experiments.

Antibiotic Susceptibility Testing

The MIC for 22 common antibiotics was determined for 22 tested isolates from fecal samples collected from a commercial dairy farm. We will use WGS to outline the mechanism for acquiring and transferring colistin resistance in this strain.

https://clsi.org/
2http://www.eucast.org/
### TABLE 1 | Minimum inhibitory concentration (µg/mL) for Escherichia coli EC11, transconjugant EC11-T and recipient E. coli C600.

| Type of antibiotic | Antibiotic                  | MIC (µg/mL)* Donor E. coli EC11 | MIC (µg/mL)* Transconjugant E11-T | MIC (µg/mL)* Recipient E. coli C600 |
|--------------------|------------------------------|----------------------------------|-----------------------------------|------------------------------------|
| β-lactams          | Amoxicillin-clavulanic       | 2(S)                             | 2(S)                              | 2(S)                               |
|                    | Ampicillin                   | 4(S)                             | 8(S)                              | 8(S)                               |
|                    | Piperacillin                 | 2(S)                             | 4(S)                              | 4(S)                               |
|                    | Cefotaxime                   | <0.125(S)                        | 0.25(S)                           | 0.25(S)                            |
|                    | Cefazidime                   | 0.25(S)                          | 0.5(S)                            | 1(S)                               |
|                    | Cefoxitin                    | 8(S)                             | 4(S)                              | 4(S)                               |
|                    | Cephazolin                   | 2(S)                             | 4(S)                              | 4(S)                               |
|                    | Cefepime                     | <0.125(S)                        | 0.25(S)                           | <0.125(S)                          |
|                    | Imipenem                     | 0.5(S)                           | 1(S)                              | 0.5(S)                             |
|                    | Meropenem                    | <0.125(S)                        | <0.125(S)                         | <0.125(S)                          |
| Aminoglycoside      | Amikacin                     | 4(S)                             | 4(S)                              | 8(S)                               |
|                    | Gentamicin                   | 2(S)                             | 1(S)                              | 1(S)                               |
|                    | Kanamycin                    | 4(S)                             | 4(S)                              | 4(S)                               |
| Tetracycline        | Tetracycline                 | 1(S)                             | 1(S)                              | 1(S)                               |
|                    | Tigecycline                  | <0.125(S)                        | <0.125(S)                         | <0.125(S)                          |
| Quinolone           | Ciprofloxacin                | <0.125(S)                        | 0.125(S)                          | <0.125(S)                          |
|                    | Levofloxacin                 | <0.125(S)                        | 0.25(S)                           | 0.5(S)                             |
|                    | Nalidixic acid               | 4(S)                             | >128(R)                          | >128(R)                            |
| Amino alcohol       | Chloramphenicol              | 16(S)                            | 8(S)                              | 8(S)                               |
| Sulfonyl amide      | Trimethoprim-sulfamethoxazole| 8(R)                             | 8(R)                              | 8(R)                               |
| Cationic polypeptide| Polymyxin B                  | 4(R)                             | 4(R)                              | 1(S)                               |
|                    | Colistin                     | 8(R)                             | 4(R)                              | 1(S)                               |

MIC, minimum inhibitory concentration; R, resistant; I, intermediate; S, susceptible. *In vitro antimicrobial susceptibility was performed by broth microdilution method and the MICs were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria, except for tigecycline, colistin and polymyxin B, which interpretation were performed according to the EUCAST guidelines.

### Conjugation Assay

To determine whether the colistin resistance was carried on a transferable plasmid, a conjugation experiment by filter mating assay (Smith and Guild, 1980) was performed with rifampicin-resistant *E. coli* C600 as the recipient strain. Overnight cultures of the original isolates and recipient *E. coli* C600 in LB broth were adjusted to a 0.5 McFarland standard. A 10 µl aliquot of each culture was individually added to 2 ml of fresh LB broth and then incubated at 37°C for 6 h. The original strains (20 µl) were then separately conjugated with *E. coli* C600 (60 µl) on a microporous membrane. Transconjugants were selected on MacConkey agar plates supplemented with colistin (2 µg/mL) and rifampicin (40 µg/mL), and putative transconjugants were confirmed by both PCR and an antimicrobial susceptibility test (above 22 antibiotics). The mobilization efficiency was calculated as the number of transconjugant colonies divided by the number of donor colonies (Wang et al., 2011).

### Multilocus Sequence Typing

The clonal lineage of the *E. coli* EC11 strain was studied using MLST. MLST was performed as previously described (Tartof et al., 2005). The seven conserved housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA*, and *recA*) were chosen as targets and PCR fragments were sequenced. The alignments of these sequences were determined using DNAMAN software. These sequences were then analyzed using the facility provided by the above-mentioned online tool to assign allele numbers and define the ST and CC.

Furthermore, in order to explore possible genetic relationships between *E. coli* EC11 and other *E. coli* isolates harboring mcr reported worldwide, we performed a systematic review of the literature on mcr published in the NCBI-Pubmed database between November 2015 and March 2018. A phylogenetic tree was constructed using a NJ method by MEGA5.0 software, where the phylogenetic relationships among different strains were analyzed based on nucleotide differences. In addition, we conducted cluster analysis of these strains to understand the relationship between the different ST groups. The eBURST algorithm was used to group strains according to their allelic profiles by employing a user-specified group definition as well as drawing a rough sketch to show the genetic relationship.

### Whole Genome Sequencing

Genomic DNA of *E. coli* strain EC11 was extracted from an overnight culture using the TIANamp Bacteria DNA
RESULTS

Identification of mcr-1-Positive E. coli Isolates

In our study, out of 120 E. coli isolates collected from dairy cow fecal samples in May 2016 in Shanghai, only the E. coli isolate EC11 (Supplementary Figures S1, S2) carried the mcr-Igene, and none of these isolates carried mcr-2/3 determinants or the allelic variants.

Susceptibility to Antimicrobial and Conjugative Compounds

According to EUCAST standards, the resistance cutoff of E. coli to colistin is 2 mg/L and the E. coli EC11 strain exhibited the lower level of colistin resistance (8 µg/mL) (Table 1). E. coli EC11 also showed resistance to polymyxin B, and trimethoprim-sulfamethoxazole; but it was susceptible to other 19 common antibiotics, including amoxicillin-clavulanic, ampicillin, piperacillin, cefotaxime, cefazidime, ceftoxitin, cephaloxin, cepfime, imipenem, meropenem, amikacin, gentamicin, kanamycin, tetracycline, tigecycline, ciprofloxacin, levofloxacin, nalidixic acid, chloramphenicol (Table 1). PCR results showed that E. coli EC11 didn’t carry the β-lactamase genes, including blaTEM, blaSHV, blaCTX–M, blaKPC, and blaNDM. Furthermore, the double disk test suggested that E. coli EC11 was a non-ESBL producing isolate (Supplementary Figure S3).
In addition, the filter mating assays indicated that the mcr-1-carrying plasmid could be successfully transferred from the donor (E. coli EC11) to the recipient (E. coli C600) with an average efficiency of 4.6 × 10^{-5}. The MIC value of the transconjugant EC11-T to colistin was 8 µg/mL, which showed an eightfold increase when compared with the recipient E. coli C600 (1 µg/mL). The transconjugant E. coli EC11-T was also found to have resistance to nalidixic acid, trimethoprim-sulfamethoxazole and polymyxin B.

A Diversity of the mcr-1 Positive E. coli Isolates
Multilocus sequence typing (MLST) showed that E. coli EC11 belonged to the ST278 lineage. Based on the literature review, details of the E. coli strains harboring mcr genes, including the source and year of isolation, the presence of the MDR phenotype, ST, and allelic profile, are presented in Supplementary Table S2. A total of 245 STs were identified among the 616 E. coli isolates, indicating a high degree of genotypic diversity.

The application of eBURST resolved the 245 STs into 10 clonal complexes (CC10, CC206, CC46, CC1114, CC648, CC101, CC642, CC6866, CC55, and CC23). CC10 remained the most populated clonal complex and ST10 was defined as the ancestral type of CC10 (Figure 1). The geographical distribution of the different STs is shown in Supplementary Table S3. These ST types were distributed in more than 35 cities across six continents. ST10 was isolated on five continents and China was the country where the most mcr-positive E. coli strains were found, with as many as 162 different STs being discovered.

A NJ tree representing the concatenated sequences of the seven housekeeping gene fragments in 245 mcr-positive E. coli isolates of different ST types is shown in Figure 2. The phylogenetic analyses revealed that E. coli isolates harboring mcr genes were distributed in different lineages, and the isolated E. coli EC11 was located on a single branch rather than belonging to one of the ST10 branches.

Genome Features of E. coli EC11 Harboring mcr-1
Whole gene sequencing (WGS) revealed that the serotype of the E. coli EC11 strain was H7. E. coli EC11 consisted of a chromosome and four circular plasmids (pEC11a, pEC11b, pEC11c, and pEC11d) (Table 2). The chromosome genome size presented 4,933,784 bp, with a G+C content of 47.6%. With an exception of the mcr-1, unexpectedly, any other resistance genes were not defective in EC11. WGS results revealed the mcr-1 gene, which showed 100% BLASTn identities to the known mcr-1 gene of the reference plasmid pHNSHP45 of E. coli SHP45 (Liu et al., 2016). The mcr-1 gene was only located on plasmid pEC11b, which was 31,229 bp in length and had an average G+C content of 41.40%, encoding 38 ORFs (Figure 3). Using PlasmidFinder, the plasmid pEC11b had a typical IncX4 plasmid backbone encoding replication, conjugation apparatus and stability functions, and was probably responsible for the movement of the plasmid between different bacterial hosts. The type II toxin–antitoxin module hicA/hicB was also identified.
TABLE 2 | General features of *E. coli* EC11 genomes.

| Replicons | Accession number | Size(bp) | MLST | Plasmid typ | Antibiotic resistance | GC (%) | ORF numbers | tRNA genes | rRNA genes |
|-----------|------------------|---------|------|-------------|-----------------------|--------|-------------|------------|------------|
| Chromosome | CP027255         | 4,933,784 | ST278 | –           | –                     | 50.77  | 4,648       | 85         | 22         |
| pEC11a     | CP027256         | 103,336  | –    | IncFIB      | –                     | 48.08  | 119         | 0          | 0          |
| pEC11b     | CP027257         | 31,229   | –    | IncX4       | mcr-1                 | 41.74  | 38          | 0          | 0          |
| pEC11c     | CP027258         | 31,467   | –    | –           | –                     | 48.41  | 46          | 0          | 0          |
| pEC11d     | CP027259         | 6,812    | –    | CoI RNAI    | –                     | 47.69  | 9           | 0          | 0          |

**Genome Features of mcr-1-Carried Plasmid**

BLASTn analysis showed that the backbone of the plasmid pEC11b (GenBank accession number CP027257.1) was strikingly similar with (the query cover of 100% and the identities 99%) other previously sequenced *mcr-1*-carrying IncX4 plasmids, such as pICBEC72H of *E. coli* (isolated in Brazil; the GenBank accession no. CP015977.1), pMCR1-IncX4 of *Klebsiella pneumoniae* (China; KU761327.1), and pNG14043 of *Salmonella* (China; KY120364) (Figure 4). In all, these IncX4 plasmids bearing *mcr-1* showed very high architectural conservation.

An approximately 2.6 kb *mcr-1-pap2* element was identified in the above-mentioned plasmids pEC11b, PICBEC72H, pMCR1-IncX4, and PNG14043. In addition, an IS6 element was identified in pEC11b, IS26 was identified in PICBEC72H and PNG14043, and trpA was identified in pMCR1-IncX4 (Figure 4). The promoter sequences of *mcr-1* in the aforementioned sequences were similar to that of pAf23 and pAf48 reported by Poirel et al (Poirel et al., 2016) as well as pMCR1_IncI2 and BJ10 by Zhang et al (Zhang et al., 2017) (Supplementary Figure S4).

The putative conjugal transfer components of pEC11b were also detected by using *oriT*finder. A *tra* gene cluster encoding a T4SS belonging to Type P was predicted on pEC11b. It encoded a relaxase (C6C13_26300) belonging to the MOB subfamily. It also encoded a T4CP (C6C13_26225) belonging to the VirD4 subfamily. The *oriT*-like region (coordinate: 27,146-27,223 bp) contained a pair of 14-bp IRs (GCAGGTGAGCAAAG...CTTTGTTCACCTGC). This evidence confirms that the plasmid pEC11b is a conjugative plasmid.
DISCUSSION

Colistin has been widely used as a veterinary drug for the treatment of enterobacterial infections and as an in-feed additive to promote healthy development in food-producing animals, especially in swine and poultry production (Kempf et al., 2013, 2016). Transfer of colistin resistance among bacteria in the gastrointestinal tract of livestock animals is a probable route for the dissemination of these bacteria (Fernandes et al., 2016b; Guenther et al., 2017). These routes can be via the food chain or direct human contact with animals as well as through contamination of fresh and seawater systems (Zhang et al., 2016; Zurfuh et al., 2016). In addition, the persistence of mcr-1 in the human gastrointestinal microflora provides another route for dissemination of these bacteria (Chen et al., 2017). In this study, the mcr-1-carrying plasmid could be conjugated into E. coli isolates in vitro. The mcr-1 gene, if present in gut microbiota, can therefore be horizontally transmitted between different species in the microbiota.

Self-transmissible IncX4-type plasmids are now accepted as key vehicles responsible for the dissemination of the mcr-1 gene among Enterobacteriaceae worldwide (Fernandes et al., 2016a; Sun J. et al., 2017; Wang et al., 2017). In this study, we identified an IncX4-type plasmid carrying mcr-1 in E. coli, which was nearly identical to the other IncX4 plasmids bearing mcr-1 in GenBank. IncX4 plasmid architecture is highly conserved and studies have shown similar IncX4 plasmids bearing mcr-1 from different species. These species were isolated from different geographic locations and belonged to different STs (Sun J. et al., 2017; Wang et al., 2017). Plasmid pEC11b has four typical conjugal modules: an origin of transfer (oriT-like) region, a T4CP gene, a relaxase gene, and a gene cluster for the bacterial T4SS apparatus. The T4SS can act as a conjugative machine in conjugative plasmids (Cascales and Christie, 2003). These gene clusters are vital to the HGT of intra- and inter-species bacterial resistance genes (Frost et al., 2005). Also, the plasmid pEC11b contains the mcr-1-pap2 cassette which has proven that it could be horizontally transferred into diverse plasmid replicon types (Li et al., 2016).

Multilocus sequence typing (MLST) is a powerful genetic fingerprinting technique for molecular epidemiology and population genetic studies of bacterial pathogens (Maiden et al., 1998; Urwin and Maiden, 2003; Maiden, 2006). In this study, we reported the first recorded instance of an mcr-1 producing E. coli EC11 belonging to the ST278 lineage. We performed a MLST-based analysis of the mcr-positive E. coli population structure among 616 isolates collected in different laboratories in over 35 countries since 2016. The 245 STs among the 616 isolates indicate that the mcr-positive E. coli population is extremely diverse. Applying eBURST and NJ tree analyses simultaneously in this global dataset allows for better resolution in discerning the epidemiology and genetic population structure of mcr-positive isolates. Combined with previous studies (Matamoros et al., 2017), we speculate that the diversity in ST types of these E. coli strains may be related to highly promiscuous plasmids disseminating mcr genes. It also indicates that mcr-1 has a huge risk of vertical transmission and may become more widespread and prevalent in the future. A ST which is highly disseminated in food, environment, animals, and human intestinal samples is ST10 (Matamoros et al., 2017; Sun P. et al., 2017). The epidemic clone ST131 (Ortiz de la Tabla et al., 2017), ST648 (Yang et al., 2016), and ST206 (Zheng et al., 2018) were reported to be the most common STs associated with various β-lactamases, including ESBLs, NDM, and KPCs, etc. Many reports indicated that bacteria carrying mcr-1 were often
associated with ESBLs (Sun et al., 2016). In this study, *E. coli* EC11 only conferred resistance to polymyxin B, colistin, and trimethoprim-sulfamethoxazole, which are antibiotics that are extensively prescribed in veterinary medicine (Catry et al., 2015).

Currently, a number of countries have already restricted the use of colistin in animal production. China has now stopped the use of colistin as an antibiotic growth promoter (Walsh and Wu, 2016). South Africa has responded to the threat of losing colistin as an antibiotic for human health through a program to advance national stewardship of colistin across the ‘One Health’ platform (Mendelson et al., 2018). The discovery of the *E. coli* strain EC11 with resistance to colistin in Shanghai emphasizes the importance of vigilance in detecting new threats like *mcr* genes to public health.

**CONCLUSION**

In this work, we report the first case of colistin-resistant *mcr-1* gene in *E. coli* strain EC11 isolated from dairy cow feces in Shanghai, China. We show that this *E. coli* strain carrying the *mcr-1* gene can transfer resistance through HGT. This study confirms the need to monitor and survey the use of colistin and other types of antibiotics to enable proactive and effective strategies (e.g., risk assessment and risk management) for preserving the efficacy of antibiotics in the future.

**Nucleotide Sequence Accession Number**

The genome sequences of the chromosome and four plasmids of the *E. coli* strain EC11 were deposited as GenBank accession no. CP027255-CP027259.

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**AUTHOR CONTRIBUTIONS**

YZ, YP, and HL conceived and supervised the study. FB designed the experiments. FB and ZZ performed the experiments. FB and XL analyzed the data. BN and XL revised the paper. PM edited the paper. FB wrote the paper.

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**SUPPLEMENTARY MATERIAL**

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