The co-transfer of plasmid-borne colistin-resistant genes mcr-1 and mcr-3.5, the carbapenemase gene bla\textsubscript{NDM-5} and the 16S methylase gene rmtB from Escherichia coli

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We found an unusual Escherichia coli strain with resistance to colistin, carbapenem and amikacin from sewage. We therefore characterized the strain and determined the co-transfer of the resistance determinants. Whole genome sequencing was performed using both Illumina HiSeq X10 and MinION sequencers. Short and long reads were subjected to de novo hybrid assembly. Sequence type, antimicrobial resistance genes and plasmid replicons were identified from the genome sequences. Phylogenetic analysis of all IncHI2 plasmids carrying mcr-1 available in GenBank was performed based on core genes. Conjugation experiments were performed. mcr-3.5 was cloned into E. coli DH5\(\alpha\). The strain belonged to ST410, a type with a global distribution. Two colistin-resistant genes, mcr-1 and mcr-3.5, a carbapenemase gene bla\textsubscript{NDM-5}, and a 16S methylase gene rmtB were identified on different plasmids of IncHI2(ST3)/IncN, IncP, IncX3 and IncFII, respectively. All of the four plasmids were self-transmissible and mcr-1, mcr-3.5, bla\textsubscript{NDM-5} and rmtB were transferred together. mcr-1-carrying IncHI2 plasmids belonged to several sequence types with ST3 and ST4 being predominant. In conclusion, carbapenem resistance, colistin resistance and high-level aminoglycoside resistance can be transferred together even when their encoding genes are not located on the same plasmid. The co-transfer of multiple clinically-important antimicrobial resistance represents a particular challenge for clinical treatment and infection control in healthcare settings. Isolates with resistance to both carbapenem and colistin are not restricted to a given sequence type but rather are diverse in clonal background, which warrants further surveillance. The amino acid substitutions of MCR-3.5 have not altered its activity against colistin.

Colistin is the last resort antimicrobial agent to treat infections caused by most Gram-negative bacteria commonly seen in clinical settings, including Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii and Pseudomonas aeruginosa\textsuperscript{1,2}. Bacterial strains that have acquired resistance to colistin have emerged worldwide. In addition to mutations or interruptions in certain chromosomal genes, acquired resistance to colistin has occurred due to plasmid-borne genes\textsuperscript{1}. Eight plasmid-borne colistin resistance genes, i.e. mcr-1\textsuperscript{3}, mcr-2\textsuperscript{4}, mcr-3\textsuperscript{5}, mcr-4\textsuperscript{6}, mcr-5\textsuperscript{7}, mcr-6\textsuperscript{8}, mcr-7\textsuperscript{9} and mcr-8\textsuperscript{10}, have been reported. The co-existence of two plasmid-borne colistin-resistant genes in bacterial isolates is uncommon, but recently, we reported the co-existence of mcr-1 and mcr-3 plus the carbapenemase gene bla\textsubscript{NDM-5} in an E. coli clinical strain, WCHEC020123, of phylogenetic group A and sequence type 206 (ST206)\textsuperscript{11}. Here we report a second independent occurrence of the co-existence of mcr-1, mcr-3 and

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Materials and Methods

Recovery of the strain and in vitro antimicrobial susceptibility testing. E. coli strain WCHEC025943 was recovered from the influx mainstream of hospital sewage at West China Hospital, Chengdu, western China, in April 2017. The sewage sample was mixed with 100 ml brain heart infusion broth (Oxoid, Hampshire, UK) in a 500 ml flask. After overnight incubation at 37 °C, the culture suspension was diluted to a McFarland standard and an 100 μl aliquot was plated onto a CHROMAagar Orientation agar plate (CHROMagar, Paris, France) containing 4 μg/ml colistin and 16 μg/ml meropenem. The plate was then incubated at 37 °C overnight. The pink colony that represents E. coli was screened for mcr-1 as described previously3. Species identification was established by Vitek II (bioMérieux, Marcy-l’Étoile, France) and by MALDI-TOF MS (Bruker, Billerica, MA, USA).

MICs of amikacin, aztreonam, aztreonam-avibactam, ceftazidime, ceftazidime-avibactam, ciprofloxacin, colistin, imipenem, meropenem, tigecycline and trimethoprim-sulfamethoxazole were determined using the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI)32. For ceftazidime-avibactam, colistin and tigecycline, the breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) were used, while the breakpoints of aztreonam were applied for aztreonam-avibactam.

Whole genome sequencing and analysis. Genomic DNA of strain WCHEC025943 was prepared using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) and was subjected to whole genome sequencing using both the HiSeq X10 platform (Illumina, San Diego, CA, USA) and the long-read MinION Sequencer (Nanopore, Oxford, UK). The de novo hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler13 under conservative mode for increased accuracy. Complete circular contigs generated were then corrected using PlasmidFinder 19 with default settings, resulting in a sum of 56 genes representing the E. coli multi-locus sequence typing database (http://enterobase.warwick.ac.uk/species/index/ecoli). Antimicrobial resistance genes were identified from genome sequences using ResFinder at https://cge.cbs.dtu.dk/services/ResFinder/. Plasmid replicon types and sequence types of IncHI2 and IncF plasmids were determined using PlasmidFinder and pMLST tools at https://cge.cbs.dtu.dk/services/PlasmidFinder/ and https://cge.cbs.dtu.dk/services/pMLST/. Single nucleotide polymorphisms (SNPs) between strain WCHEC025943 and strain WCHEC14828 (also called WCHEC005828, GenBank accession no. RIAW00000000), a bla<sub>NDM-5</sub>-carrying ST410 E. coli identified in the same hospital in 201433, was determined from a two-way whole genome alignment in HarvestTools16.

Nucleotide sequence accession numbers. Complete sequences of the chromosome and plasmids of strain WCHEC025943 have been deposited into GenBank under the accession no. CP027199 to CP027205.

Phylogenetic group typing. E. coli phylogenetic group of strain WCHEC025943 was determined using PCR as described previously37.

Cloning of mcr-3.5. The complete coding sequence of mcr-3.5 was amplified with primers mcr3.5-up (CTGGTCGGAGATATGGGTGT) and mcr3.5-dw (GGCATTCAACATCAGAGCAA) using PrimeSTAR Max DNA Polymerase (Takara, Dalian, China). The primers were designed to amplify the gene with 222-bp upstream and 540-bp downstream sequences of mcr-3.5. Amplicons were ligated to the pMD20-T vector using the Mightyl TA-cloning kit (Takara). The ligated fragments were transformed into E. coli DH5α, mcr-3.5-containing transformants were selected on LB agar plates containing 2 μg/mL colistin. The presence of mcr-3.5 in transformants was confirmed by PCR. MIC of colistin was determined for transformants carrying mcr-3.5 using the broth microdilution method13.

Phylogenetic analysis of IncHI2 plasmids. Complete sequences of all IncHI2 plasmids carrying mcr-1 (n = 25 in addition to pMCR1_025943 and pMCR1_020123) were retrieved from GenBank. Plasmid replicon types and sequence types of these plasmids were determined using PlasmidFinder and pMLST. Annotation was performed using Prokka18 and antimicrobial resistance genes were identified using ResFinder. Orthologues of these plasmids were identified using OrthoFinder19 with default settings, resulting in a sum of 56 genes representing the core genome of these 27 plasmids. The alleles of orthogonal genes were aligned using MAFFT20 and concatenated into a single sequence containing 56 aligned genes for each plasmid. The maximum-likelihood phylogenetic tree was inferred based on the core genome using RAxML21 with a 1000-bootstrap test.

Conjugation. Conjugation experiments were carried out in brain heart infusion broth at 30 °C using azide-resistant E. coli strain J53 as the recipient. Transconjugants were selected on LB agar plates containing 150 μg/ml sodium azide plus 2 μg/ml colistin for mcr-1.1 and mcr-3.5, plus 1 μg/ml meropenem for bla<sub>NDM-5</sub>, or plus 64 μg/ml amikacin for rmtB. Transconjugants were also selected on LB agar plates containing 150 μg/ml sodium azide plus 2 μg/ml colistin, 1 μg/ml meropenem and 64 μg/ml amikacin to examine whether mcr, bla<sub>NDM-5</sub> and rmtB could be transferred together. The presence of mcr-1.1, mcr-3.5, bla<sub>NDM-5</sub> and/or rmtB in transconjugants was screened using PCR and Sanger sequencing. Conjugation frequency was calculated as the number of transconjugants per recipient cell.
plasmids. Like strain WCHEC020123, in strain WCHEC025943, \( pMCR1 \) was carried on a 50.5-kb IncP plasmid, designated \( pMCR3_025943 \), in strain WCHEC025943. \( pMCR3_025943 \) is identical to \( pMCR3_020123 \), the mcr-3-carrying IncP plasmid in strain WCHEC020123, except that an insertion sequence, IS1294, is absent from \( pMCR3_025943 \) but is inserted in a spacer region in \( pMCR3_020123 \). mcr-1 was carried on a 265.5-kb plasmid (designated \( pMCR1_025943 \)) containing both \( \text{IncHI2} \) (ST3) and \( \text{IncX3} \) replicons in strain WCHEC020123, which was larger than the 223.7-kb \( mcr-1 \)-sequence, IS1294, is absent from \( pMCR3_025943 \) but is inserted in a spacer region in \( pMCR3_020123 \).

### Results and Discussion

Strain WCHEC025943 was recovered from the sewage sample and grew on the agar plate containing 4 μg/ml colistin and 16 μg/ml meropenem. The complete genome sequence of strain WCHEC025943 was obtained, which was 5.1 Mb and contained a 4.82 Mb circular chromosome and six plasmids of different replicon types (Table 1).

The strain was resistant to amikacin (>512 μg/ml), aztreonam (>512 μg/ml), ceftazidime (512 μg/ml), ceftazidime-avibactam (>512/4 μg/ml), ciprofloxacin (8 μg/ml), colistin (8 μg/ml), imipenem (128 μg/ml), meropenem (128 μg/ml) and trimethoprim-sulfamethoxazole (128/2432 μg/ml), but was susceptible to aztreonam-avibactam (1/4 μg/ml) and tigecycline (0.5 μg/ml). Strain WCHEC025943 had 31 known acquired antimicrobial resistance genes mediating resistance to aminoglycosides \((\text{aac}(3)-\text{Ia}, \text{aac}(3)-\text{IIa}, \text{aac}(6')-\text{Ib-cr}, \text{aadA16}, \text{aph}(3')-\text{Ia}, \text{aph}(3')-\text{Ib}, \text{aph}(6)-\text{Ib})\), ampicillin \((\text{bla}_{\text{TEM-1}}, \text{bla}_{\text{SHV-1}}, \text{bla}_{\text{OXA-23}}, \text{bla}_{\text{CTX-M-32}}, \text{bla}_{\text{OXA-40}}, \text{bla}_{\text{OXA-41}}\), fosfomycin \((\text{fosA})\), chloramphenicol \((\text{cmr}-1.1, \text{cmr}-1.5\)) and macrolide-lincosamide-streptogramin B \((\text{b谁知道}(F), \text{mph}(A), \text{mph}(B), \text{mph}(C), \text{mph}(D))\), rifampicin \((\text{rrmT}, \text{rrmB})\), 

| Plasmid     | Replicon type | Size (bp) | Antimicrobial resistance genes |
|-------------|---------------|-----------|--------------------------------|
| p1_025943   | Y             | 95,859    | \( \text{bla}_{\text{CTX-M-45}} \) |
| p2_025943   | FII, FIB      | 75,779    | \( \text{bla}_{\text{TEM-1}}, \text{bla}_{\text{OXA-23}}, \text{bla}_{\text{CTX-M-32}} \) |
| p3_025943   | Col (B5512)   | 2,088     | \( \text{bla}_{\text{TEM-1}} \) |
| pMCR1_025943| H12, N        | 265,538   | \( \text{bla}_{\text{TEM-1}}, \text{bla}_{\text{OXA-23}}, \text{bla}_{\text{CTX-M-32}}, \text{bla}_{\text{OXA-41}} \) |
| pMCR3_025943| P             | 50,520    | \( \text{cmr}-3.5 \) |
| pNDM5_025943| X3            | 45,275    | \( \text{bla}_{\text{NDM-5}} \) |

**Table 1.** Plasmids and antimicrobial resistance genes in strain WCHEC025943. \( \text{bla}_{\text{CMY-2}} \) was located on the chromosome.

Of note, \( \text{cmr}-3.5 \) encodes three amino acid substitutions (M23V, A456E and T488I) compared with the original \( \text{mcr}-3 \). Of note, \( \text{cmr}-3.5 \) encodes three amino acid substitutions (M23V, A456E and T488I) compared with the original \( \text{mcr}-3 \).
genes (traN, traU, traW) encoding conjugation in the former but absent from the latter. ST3-IncHI2 plasmids have been found increasingly as the vector of mcr-1 and are particularly large and complex in structure with the ability to acquire multiple antimicrobial resistance genes and additional plasmid replicons27–29.

mcr-1-carrying IncHI2 plasmids were mostly found in *E. coli* and were also present in several other species of the Enterobacteriaceae (Fig. 1). A few IncHI2 plasmids also contain additional replicons, among which IncN replicon was the most common (Fig. 1). These plasmids were large in size (125,572 to 256,620 bp for plasmids...
containing IncHII2 replicons alone and 238,539 to 369,298bp for those containing additional replicons) and commonly carried multiple antimicrobial resistance genes (Fig. 1). Most of these plasmids belong to ST3 (n = 15) or ST4 (n = 8), while one belongs to ST14 and the sequence type is not assigned to three plasmids due to the absence of an allele for four IncH12 pMLST. This suggests that several types of IncH12 plasmids could mediate the transfer of mcr-1 and ST3-IncH12 is the most common type (Fig. 1). These plasmids were also aligned against pSLK172-1 (GenBank accession no. CP017632), the largest (369,298 bp) mcr-1-carrying IncH12 plasmid, using BRIG. This revealed that mcr-1-carrying IncH12 plasmids are complex and highly variable in structure (Fig. 2).

In strain WCHEC025943, the four plasmids carrying mcr-1-1, mcr-3-5, blaoaNDM-5 or rmtB were all self-transmissible at a $10^{-3}$, $10^{-4}$, $10^{-3}$ and $10^{-4}$ frequency, respectively. Alarmingly, the four plasmids could be transferred together to a single transconjugant at a $10^{-6}$ frequency. This suggests that carbapenem resistance, colistin resistance and aminoglycoside resistance can be transferred together even when their encoding genes are located on separate plasmids.

**Conclusion**

The above findings suggest that carbapenem resistance, colistin resistance and high-level aminoglycoside resistance can be transferred together even when their encoding genes are not located on the same plasmid. The co-transfer of multiple clinically-important antimicrobial resistance represents a particular challenge for clinical treatment and infection control in healthcare settings, which warrant more surveillance and further studies to explore counter measures. Isolates with resistance to both carbapenem and colistin are not restricted to a given sequence type but rather are diverse in clonal background. mcr-1-carrying IncH12 plasmids belonged to several sequence types with ST3 and ST4 being predominant.

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**Author Contributions**

Z.Z. designed the study. H.L., Y.F., K.M. and L.L. collected the data. H.L., A.M. and Z.Z. analyzed and interpreted the data. Z.Z. wrote the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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