Potential transferability of mcr-3 via IS26-mediated homologous recombination in Escherichia coli

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Worldwide dissemination of the mobile colistin-resistance gene mcr-1 is of great concern to public health. Recently, a novel colistin-resistance gene, mcr-3, was reported to share 45.0% nucleotide sequence identity with mcr-1¹. To date, mcr-3-positive isolates have been described in several regions (China, Denmark, and Spain) and species (Escherichia coli, Salmonella Typhimurium, and Aeromonas veronii)²–⁴. Although the plasmid-mediated mcr-3 gene has been identified in most Enterobacteriaceae, with some horizontal mobile elements, such as TnAs2 and ISKpn40⁵–⁶, no evidence exists of mcr-3 gene transmission mediated by these transposons or insertion sequences. Here, we describe an MCR-3-producing E. coli isolate and, for the first time, identify the intermediate circle of the mcr-3-carrying fragment formed by a truncated insertion sequence, IS26, and an intact IS630, which was similar to the transfer of mcr-1 via Tn6330⁷, suggesting this mcr-3-containing segment could be looped out via IS-mediated homologous recombination.

E. coli HN8 was obtained from a fecal sample of an apparently healthy pig at a conventional farm in Henan Province, China, during a routine surveillance study in 2017. PCR and sequencing analyses revealed that mcr-3 was present in E. coli HN8. The mcr-3 in HN8 shared 100% nucleotide sequence identity to the original mcr-3 gene reported by Yin et al.¹. Susceptibility was tested by the broth microdilution method following the CLSI guidelines, and the minimal inhibitory concentrations were interpreted from the CLSI breakpoints⁷. E. coli HN8 was resistant to colistin (4 mg/L), gentamicin (64 mg/L), tetracycline (64 mg/L), ampicillin (64 mg/L), florfenicol (> 128 mg/L), and trimethoprim/sulfamethoxazole (32/608 mg/L), but susceptible to ceftriaxone (0.03 mg/L), imipenem (0.5 mg/L), aztreonam (0.03 mg/L), ciprofloxacin (0.5 mg/L), and amoxicillin–clavulanic acid (16/8 mg/L).

To further analyze this mcr-3-positive HN8 isolate, whole-cell DNA was extracted using a Wizard genomic DNA purification kit (Promega, Beijing, China) and used for whole-genome sequencing (WGS) on an Illumina Hiseq 2500 platform (Berry Genomics Company, Beijing, China). From the WGS data, sequences types were extracted and assigned to ST3634, and various resistance genes were detected in the WGS data, including aminoglycoside-resistance genes aaph(3’)-Ia, adaA5, and aac(3)-IId; rifampicin-resistance gene arr-3; macrolide-resistance gene mphi(A); tetracycline-resistance genes tet(A) and tet(M); florfenicol-resistance gene floR, quinolone-resistance genes qnrS1, qnrS2, qoxAB, and aac(6’)-Ib-cr; trimethoprim-resistance gene dfrA17, β-lactamase-encoding genes blaOXA-1, and blaTEM-1B, tunicamycin-resistance gene tmmB, and bleomycin-resistance gene ble. Four replicons, IncX1, IncR, IncFII, and IncFIB, were detected using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

S1-PFGE and Southern blotting with an mcr-3 probe labeled with digoxigenin revealed that the mcr-3 gene in the HN8 isolate was located on a ~70-kb plasmid, designated pHN8 (data not shown). Although the conjugation failed by filter mating using HN8 as donors and E. coli J53 AzR as the recipient, we obtained the transformant E. coli DH5α/pHN8 by electro-transformation. S1-PFGE and PCR-based plasmid replicon typing analysis
The intermediate sequence obtained by inverse PCR

*Escherichia coli* plasmid pHN8, pig feces, China, MG780294

*Escherichia coli* plasmid pWJ1, pig feces, China, KY924928

Fig. 1 (See legend on next page.)
indicated the DH5α/pHN8 harbored only the IncR-type plasmid, pHN8. The IncR complex replicons were first described in the *K. pneumoniae* *qnrS1*-plasmid, pK245, and carry various resistance genes, including the metallo-β-lactamase genes, *bla*<sub>VIM-1</sub> and *bla*<sub>NDM-1</sub>, in clinical Enterobacteriaceae strains<sup>8</sup>,<sup>9</sup>. However, the plasmids belonging to this complex are nontransferable due to the lack of transfer elements<sup>10</sup>, which was consistent with our failed conjugation experiment. The transformant DH5α/ pHN8 exhibited a 4- to 16-fold increase in the colistin (1 mg/L), gentamicin (32 mg/L), ampicillin (64 mg/L), and ciprofloxacin (0.5 mg/L) MIC values, compared with the recipient DH5α.

The complete pHN8 sequence was obtained by combining the Illumina Hiseq 2500 platform with single-molecule real-time sequencing (SMRT) platforms (Sino-biocore, Beijing, China). pHN8 is a 53,148-bp plasmid, consistent with the size predicted by Southern hybridization within the margin of error, with 66 open reading frames (ORFs) and an average GC content of 49.8%. It contained an 9.14-kb conservative region of the IncR-type plasmid, which shared 98.5% nucleotide sequence identity with that of *Enterobacter cloacae* plasmid pNDM1_SZ2 (GenBank accession number KU302802) and mainly harbored toxin–antitoxin system gene operon *vapB/C*, replication gene *repB*, partition protein-encoding genes *parA/B*, and SOS mutagenesis gene *umuC*<sup>10</sup>. A 32.28-kb MDR region harbored seven of the above-mentioned resistance genes (*mcr-3*, *aac(3)-IId*, *bla*<sub>TEM-1B</sub>, *qnrS1*, *tet* (M), *tmrB*, and *ble*), which were flanked by or interspersed with various insertion sequences, with the IS6 family being highly abundant (IS26, n = 4, IS15DI, n = 2, Fig. 1a).

The 6288-bp *mcr-3*-carrying segment, ranging from △TnAs2 to IS15DI, shared 99.9% nucleotide sequence identity to the corresponding region of the original *mcr-3*-harboring plasmid, pWJ1 (Fig. 1b). A truncated (△) IS26 element, the 428-bp 3′-region of IS26 (393–820 bp), was present immediately upstream of △TnAs2. Notably, IS15DI, which differed from IS26 by only two nucleotides (A614G/A615G), was detected downstream of *mcr-3*. To determine the potential transferability of the pHN8 *mcr-3*-carrying segment, inverse PCR was performed for both HN8 and its transformant, DH5α/pHN8, using the primers located within the *mcr-3* gene (Fig. 1b). A 5990-bp circular intermediate carrying *mcr-3* flanked by △TnAs2, △IS26, and ISKpn40 was identified in both isolates (Fig. 1b). Based on sequence comparison of plasmid pHN8 and the 5.99-kb circular intermediate, we speculated that it was the two nearly identical insertion sequences, △IS26 and the 3′-region of intact IS15DI, simultaneously contributing to the looping progress by homologous recombination (Fig. 1b). This event is consistent with previous reports in which △IS26 and the 3′-region of intact IS26 also formed a circular intermediate<sup>11</sup>,<sup>12</sup>; however, the *mcr-3*-carrying circle mobilization evidenced in this study requires further investigation.

In summary, our study describes an MCR-3-producing *E. coli* ST3634 isolate, and *mcr-3* was located on an IncR plasmid that included a mosaic structure containing multiple IS26 sequences. An *mcr-3*-carrying circular intermediate was mediated by a truncated IS26 and a complete IS15DI, most likely via homologous recombination. Although the pHN8 belongs to an IncR-type non-conjugative plasmid, the *mcr-3* gene is transferable either to other plasmids or to chromosomes by IS-mediated transposition, and the IncR plasmid could act as a resistance gene pool in Enterobacteriaceae strains as per previous papers<sup>8</sup>–<sup>10</sup>.

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Authors’ contributions
Y.W. and H.J designed the study. Z.W. and Y.F. collected the samples and conducted the experiments. Z.W. and Y.W. analyzed and interpreted the data. Z.W., X.-D.D., Y.W. and H.J. drafted the manuscript. All authors reviewed, revised, and approved the final report.

Conflict of interest
The authors declare that they have no conflict of interest.

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