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MCR-1.6, a New MCR Variant Carried by an IncP Plasmid in a Colistin-Resistant *Salmonella enterica* Serovar Typhimurium Isolate from a Healthy Individual

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ABSTRACT In this study, we report a novel *mcr-1* gene variant, named *mcr-1.6*, carried by an IncP plasmid in a colistin-resistant *Salmonella enterica* serovar Typhimurium isolate from a healthy person. Compared with *mcr-1*, the *mcr-1.6* gene contains two single-nucleotide polymorphisms, one of which results in an arginine to histidine variation (Arg536→His). The plasmid carrying the *mcr-1.6* gene was designated pMCR1.6_P053 and is similar to a recently discovered *mcr-1*-bearing plasmid found in *Klebsiella pneumoniae*.

KEYWORDS colistin resistance, MCR-1.6, IncP plasmid, *Salmonella enterica* serovar Typhimurium

Colistin is one of the last-resort antibacterial drugs and is increasingly used to treat carbapenem-resistant *Enterobacteriaceae* (CRE). Since the first report of plasmid pHNSHP45 carrying the *mcr-1* gene (1), numerous retrospective studies have been performed worldwide to investigate the presence of this specific gene in strains isolated from environmental samples, food animals, food, and humans (2–11), and the earliest evidence for its presence dates back to the 1980s (12). The *mcr-1* gene was recently found to be carried by different plasmid replicon types, such as IncI2, IncHI2, IncP, IncFIP, and IncX4 (4, 13–15). Moreover, the *mcr-1* gene variant *mcr-1.2* and the novel colistin resistance gene *mcr-2* were discovered in isolates in Italy and Belgium, respectively (16, 17).

The *mcr-1* gene product, MCR-1, is predicted to be an integral membrane protein with the catalytic activity of phosphoethanolamine transferases (18). The MCR-1 enzyme modifies the chemical structure of lipid A moiety on bacterial lipopolysaccharide by the addition of phosphoethanolamine, which in turn reduces the binding affinity to colistin (i.e., producing the colistin resistance) (1, 18, 19). To date, only one functional variant of MCR-1, MCR-1.2, has been reported (17). This recent variant had a Gln-to-Leu change in the N-terminal protein region (at position 3) compared with MCR-1.

Here, we report a new *mcr-1* gene variant (named *mcr-1.6*) carried by an IncP plasmid in the colistin-resistant *Salmonella enterica* serovar Typhimurium strain YL14P053, which was isolated in 2014 from a rectal swab sample from a 46-year-old healthy woman who received a medical examination in the Yulin Center for Disease Control and Prevention.

The strain was subjected to 250-bp paired-end whole-genome sequencing with 150× coverage using the MiSeq sequencer (Illumina). A total of 3,051,328 reads and

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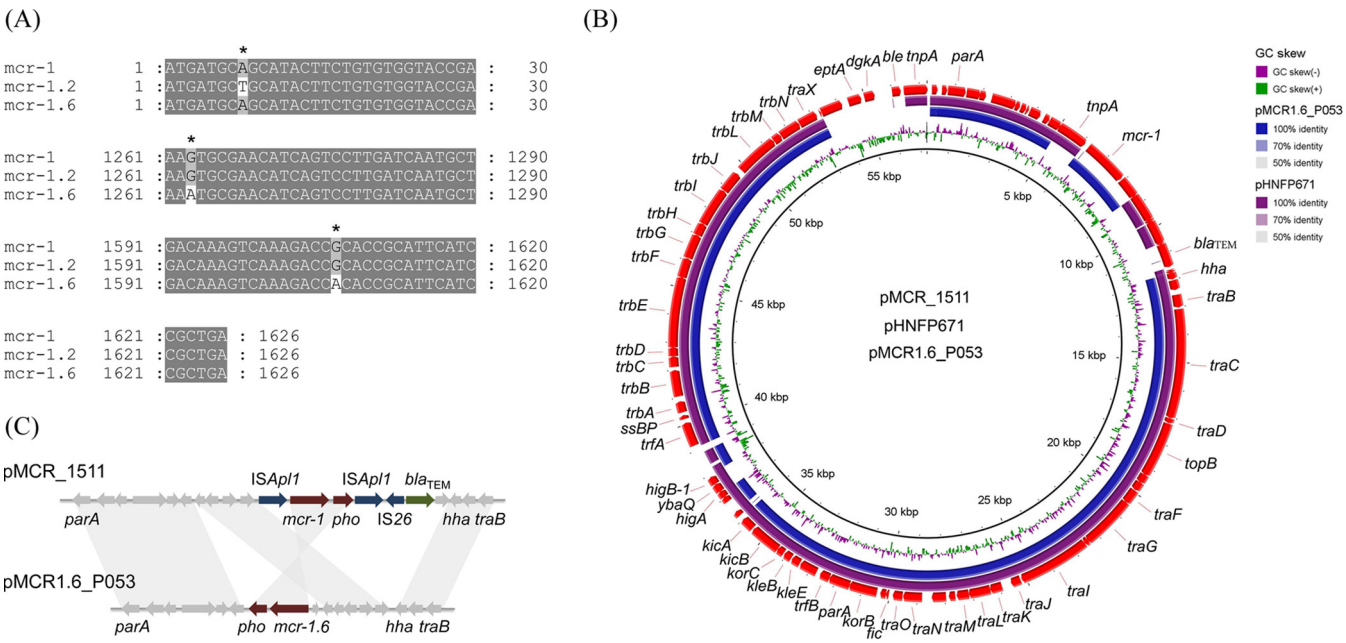

FIG 1 Alignment of the *mcr-1* gene with its variants and comparison of pMCR1.6_P053 with its closely related plasmids. (A) Nucleotide alignment of *mcr-1*, *mcr-1.2*, and *mcr-1.6* genes. Only the alignment block containing the mutations is shown. Asterisks (*) indicate the positions of mutations. *mcr-1.2*: in nucleotide position 8, A was mutated to T, leading to a Gln to Leu change in amino acid position 3; *mcr-1.6*: in nucleotide position 1263, G was mutated to A, which is a synonymous mutation; in nucleotide position 1607, G was mutated to A, leading to an Arg to His change in amino acid position 536. (B) Circular comparison of plasmids pMCR_1511, pHNFP671, and pMCR1.6_P053. Plasmid pMCR_1511 was used as the reference genome sequence. Individual rings range from 1 (inner ring) to 4 (outer ring): ring 1, GC skew of plasmid pMCR_1511 reference genome; ring 2, plasmid pMCR1.6_P053 conservation plot; ring 3, plasmid pHNFP671 conservation plot; ring 4, gene arrangement in plasmid pMCR_1511. (C) Comparison of the genetic environments of *mcr-1* and *mcr-1.6* in plasmids pMCR_1511 and pMCR1.6_P053, respectively. Gray shading shows >99% nucleotide identity.

762,832,000 clean bases were generated and were assembled into 311 contigs (171contigs of ≥1,000 bp) with an N_{50} length of 110,446 bp using the SOAPdenovo program (20). Plasmid finishing was achieved by a PCR-based strategy and Sanger sequencing. A 47,824-bp-long *mcr-1.6*-carrying plasmid, named pMCR1.6_P053, was obtained. Antimicrobial susceptibility was determined by reference broth microdilution using custom plates (PRCDCN2, Thermo Fisher) (21). EUCAST defines colistin resistance as >2 μg/ml for *Enterobacteriaceae* (22). Determinations of the multilocus sequence type (MLST), plasmid replicons, and resistance gene content were performed *in silico* using online tools (http://www.genomicepidemiology.org/).

MLST analysis indicated that the *S.* Typhimurium YL14P053 strain belonged to sequence type 34 (ST34). It was predicted to carry several resistance genes, e.g., *strA*, *aph(3′)-Ia*, *aph(4)-Ia*, *aac(3)-IVa*, *aac(6′)Ib-cr*, *strB*, bla_{TEM-1B}, bla_{OXA-1}, *oqxA*, *oqxB*, *floR*, *catB3*, *cmlA1*, *arr-3*, *sul1*, *sul2*, *sul3*, *tet(B)*, *dfrA12*, and the *mcr-1* gene variant, able to mediate resistance to aminoglycoside, β-lactam, fluoroquinolone, phenicol, rifampin, sulfonamide, tetracycline, trimethoprim, and colistin. The YL14P053 strain showed a multidrug-resistance phenotype, including resistance to colistin (MIC, 4 μg/ml), ampicillin (MIC, >64 μg/ml), tetracycline (MIC, >32 μg/ml), nalidixic acid (MIC, >64 μg/ml), erythromycin (MIC, >16 μg/ml), chloramphenicol (MIC, >64 μg/ml), and trimethoprim-sulfamethoxazole (MIC, >8/152 μg/ml), but was susceptible to imipenem (MIC, <0.25 μg/ml) and ciprofloxacin (MIC, 1 μg/ml). The absolute MIC values of ceftazidime, cefotaxime, cefazolin, and azithromycin were 1, 1, 1, and 4 μg/ml, respectively.

Interestingly, the *mcr-1* gene variant harbored in this strain carried two single-nucleotide polymorphisms (SNPs), unlike the *mcr-1* gene (confirmed by PCR and Sanger sequencing) (Fig. 1A). The first one was located at position 1263 (G-A), which was a synonymous mutation that did not cause amino acid change. The second one, at position 1607 (G-A), resulted in an arginine to histidine change (Arg536→His). The positions of these two SNPs were different from those in *mcr-1.2*. Therefore, we named

this variant *mcr-1.6*. MCR-1 is organized into two domains: an N-terminal inner-membrane-bound domain predicted to contain 5 transmembrane α-helices and a soluble periplasmic domain containing the putative catalytic center (23). The catalytic domain of MCR-1 (cMCR-1, residues 215 to 541) is a globular protein with an overall hemispherical shape and a centrally located β-sheet composed of seven β-strands sandwiched between α-helical structures (24). Therefore, the amino acid change (Arg536→His) seen in this study was very close to that at the end of cMCR-1. To confirm and evaluate the colistin resistance activity of the *mcr-1.6* gene, recombinant *Escherichia coli* DH5α strains harboring the *mcr-1* and *mcr-1.6* genes, respectively, were constructed by a previously reported method (25). Colistin susceptibility testing showed that the MIC values of strains carrying the recombinant plasmid increased from 0.125 μg/ml to 4 μg/ml, suggesting that the mutation did not obviously impact *mcr-1* gene activity.

The pMCR1.6_P053 plasmid has no known antimicrobial resistance genes other than *mcr-1.6*, and the whole sequence was highly similar (98% coverage and 99% nucleotide identity) to that of the recently reported *mcr-1*-harboring IncP-type plasmid pMCR_1511 (KX377410), which was found in *Klebsiella pneumoniae* isolated from hospital sewage at West China Hospital (11). However, four fragments in pMCR_1511 were missing in pMCR1.6_P053. The first fragment (nucleotide [nt] 5015 to 6091) contained one complete IS*Apl1* mobile element (IRL [inverted repeat left], transposase, and IRR [inverted repeat right]). The second fragment (nt 8688 to 11753) included one IS*Apl1* mobile element interrupted by Tn*3* and IS*26* mobile elements and a bla_{TEM} gene. The third fragment (nt 37198 to 37993) included *higA*, *ybaQ*, *higB-1*, and *trfA* genes. The fourth fragment (nt 53335 to 57278) contained one transposase of the Tn*3* family, *eptA*, *dgkA*, *ble*, and one complete IS*26* mobile element (Fig. 1B). In addition, a 2.6-kb fragment (nt 6091 to 8688 in pMCR1.6_P053) harboring the *mcr-1* gene was inverted (Fig. 1C), making the orientation of the *mcr-1* variant in pMCR1.6_P053 opposite to the orientation of the *mcr-1* gene in pMCR_1511. In addition to plasmid from *K. pneumoniae*, a further BLAST search of the GenBank database showed that the pMCR1.6_P053 plasmid was similar to three plasmids from *E. coli* strains, i.e., pHNFP671 (KP324830), pJJ1886_4 (CP006788), and pHS102707 (KF701335) (80% coverage and 90% nucleotide identity). Among them, the pMCR1.6_P053 plasmid shared 92% coverage and 99% nucleotide identity to plasmid pHNFP671 (KP324830), which did not carry *mcr-1* or its variants. The pHNFP671 plasmid was much longer than the pMCR1.6_P053 plasmid, which mainly lacked a long fragment (nt 48067 to 82807 in pHNFP671) flanked by two IS*26* mobile elements and two ~1,000-bp fragments (nt 21611 to 22775 and nt 37890 to 38859 in pHNFP671) containing one IS*Apl1* mobile element and the *higB* and *higA* genes, respectively. Interestingly, the pHNFP671 plasmid lost the 2,600-bp-long fragment (nt 3298 to 5903 in pMCR1.6_P053) containing the complete *mcr-1* cassette (*mcr-1* gene and *orf723*) (Fig. 1C).

To test the host range and transferability of pMCR1.6_P053, conjugation experiments using *S.* Typhi CT18, *E. coli* J53 Azi^R (*met pro*; azide resistant), and *K. pneumoniae* BJ1988 as recipients were performed. Transfer of the colistin resistance determinant by conjugation was assayed on LB agar plates (Oxoid) with an initial donor/recipient ratio of 1, using *E. coli* J53 as the recipient (18). After incubation at 37°C for 4 h, transconjugants were selected on LB agar supplemented with colistin (2 μg/ml) and sodium azide (100 μg/ml). When using *S.* Typhi CT18 or *K. pneumoniae* BJ1988 as recipients, the transformants were selected on LB agar with colistin (2 μg/ml) and streptomycin (5,000 μg/ml). The transfer frequency was expressed as the number of transconjugants per recipient. Positive transconjugants were confirmed by PCR targeting of the *mcr* gene (26). The results demonstrated that plasmid pMCR1.6_P053 was transferred from *S.* Typhimurium YL14P053 to *E. coli* J53, *S.* Typhi CT18, and *K. pneumoniae* BJ1988 with relatively high frequencies of 6.4×10^{-1}, 6.8×10^{-1}, and 8.2×10^{-2}, respectively. The colistin MIC of the transconjugants showed a 32-fold increase (from 0.125 to 4 μg/ml). These results confirmed that this IncP-type *mcr-1.6*-harboring plasmid pMCR1.6_P053

is a broad-host-range plasmid and bears great potential to disseminate the *mcr-1.6* gene.

In conclusion, a new *mcr-1* gene variant, *mcr-1.6*, bearing two SNPs was found in *S.* Typhimurium isolated from healthy human gut. The mutations in *mcr-1.6* did not impact the gene's colistin resistance activity. The *mcr-1.6* gene was found on a variant of a broad-host-range IncP-type *mcr-1*-harboring plasmid recently discovered in *K. pneumoniae*. To our knowledge, this plasmid and its variants have not been found in *S.* Typhimurium. The antibiotic resistance genes are frequently exchanged among *E. coli*, *K. pneumoniae*, and *S. enterica* serovars (27), and the conjugation experiments in this study showed that the plasmid carrying *mcr-1.6* can transfer from *S.* Typhimurium to *E. coli* and *K. pneumoniae*. Therefore, the presence of *mcr-1.6* in clinical *E. coli* and *K. pneumoniae* strains needs to be a focus in public health surveillance.

Accession number(s). The sequence of pMCR1.6_P053 was deposited into GenBank under accession number KY352406.

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