Transferable Plasmid-Borne mcr-1 in a Colistin-Resistant Shigella flexneri Isolate

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ABSTRACT Since the initial discovery of mcr-1 in an Escherichia coli isolate from China, the gene has also been detected in Klebsiella pneumoniae and Salmonella enterica but is rarely reported in other Enterobacteriaceae. Here, we report the isolation and identification of a Shigella flexneri strain harboring mcr-1 from stool samples in a pig farm in China from 2009. The MIC of colistin for the isolate is 4 μg/ml. Conjugation assays showed that the donor S. flexneri strain has functional and transferable colistin resistance. Sequencing revealed that mcr-1 was present on a putative composite transposon flanked by inverted repeats of IS_Apl1.

IMPORTANCE There are four species of Shigella, and Shigella flexneri is the most frequently isolated species in low- and middle-income countries (LMICs). In this study, we report a functional, transferable, plasmid-mediated mcr-1 gene in S. flexneri. We have shown that mcr-1 is located on a novel composite transposon which is flanked by inverted repeats of IS_Apl1. The host strain is multidrug resistant, and this multidrug resistance is also transferable. The finding of a functional mcr-1 gene in S. flexneri, a human-associated Enterobacteriaceae family member, is a cause for concern as infections due to S. flexneri are the main Shigella infections in most low- and middle-income countries.

KEYWORDS IS_Apl1, Tn6390, multidrug resistance, plasmid transfer, composite transposon

Antimicrobial resistance is a major global health issue and is on the national and international agendas of all United Nations member states and many organizations, including the World Health Organization (1). Decreased susceptibility to the most widely used antibiotics, including ampicillin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline, for enteric pathogens has become a major concern, especially in low- and middle-income countries (LMICs) (2, 3). As a result of the emergence of metallo-beta-lactamases, including NDM-1, and extended-spectrum beta-lactamases such as the CTX-M group in the Enterobacteriaceae, carbapenems and third-generation cephalosporins can no longer be relied upon as treatments for infections caused by multidrug-resistant Enterobacteriaceae (4, 5). For this reason, the polymyxins (colistin and polymyxin B) have become last-resort antibiotics (6) and were reclassified as critically important for human medicine by the WHO in 2011 (7).

Since the first report of transferable, plasmid-mediated colistin resistance conferred by mcr-1 (8), researchers in different countries have found that many Enterobacteriaceae carry mcr-1 (9–13). The origins of mcr-1-positive strains are varied. Agricultural
lishments, retail meat, and patients with infections are three major sources of colistin-resistant bacteria. Since the initial discovery of mcr-1 in an Escherichia coli isolate from China, the gene has been detected in Southeast Asia, Europe, America, and Africa (14–17). Most of the mcr-1-positive strains belong to E. coli, Klebsiella pneumoniae, and Salmonella enterica, while the gene is rarely reported in other Enterobacteriaceae. A recent report described the presence of an mcr-1-positive Shigella sonnei strain from Vietnam; however, a colistin resistance phenotype was observed only following transfer to E. coli (18).

Shigella spp. are recognized as etiological agents of diarrhea and have been responsible for serious worldwide epidemics (19). Shigella flexneri is the most frequently isolated species in many countries and is responsible for approximately 10% of all diarrheal episodes in children younger than 5 years (20). S. flexneri 3a is also commonly isolated in male homosexuals in the United States (21) and the United Kingdom (22). Between 2004 and 2015, S. flexneri strains were isolated and collected in China. By screening available isolate collections via PCR, we identified a single mcr-1-positive strain of S. flexneri.

RESULTS

Bacterial strains and mcr-1 screening. A total of 2,127 S. flexneri strains were isolated from samples collected from 13 different areas in China; these were Beijing, Shenyang, Shandong, Henan, Anhui, Hubei, Xinjiang, Gansu, Sichuan, Guizhou, Yunnan, Guangxi, and Guangdong provinces. There are 15 different serotypes among the S. flexneri strains. Most of the strains were isolated from stool samples of patients who were suffering from clinically diagnosed gastroenteritis; a small number of strains (<1%) was isolated from farm and urban environments. Through PCR screening for the presence of mcr-1 among all the S. flexneri strains, only one mcr-1-positive isolate, named C960, was found. The serotype of the positive isolate is Y, and it was isolated from pig stool samples from a pig farm in Guangxi province in 2009.

Antimicrobial susceptibility and PCR amplification of resistance genes. Antimicrobial susceptibility tests showed that, in addition to colistin, S. flexneri C960 was resistant to tetracycline, ticarcillin, ampicillin, trimethoprim-sulfamethoxazole, sulfadiazine, and streptomycin (Table 1). Through PCR we found that strain C960 carried other acquired resistance genes, including qnrS1, blaTEM-1, dfrA14, and strB, which could confer decreased susceptibility to quinolones, beta-lactam antibiotics, trimethoprim, and streptomycin, respectively.

Plasmid DNA sequencing and analysis. After plasmids of S. flexneri C960 were sequenced and assembled, analysis showed that mcr-1 in C960 was located on a 65,538-bp plasmid designated pRC960-2. The plasmid has a GC content of 43.2%, contains 92 predicted open reading frames (ORFs), and has a typical IncI2 plasmid backbone (57,756 bp) encoding replication, conjugation apparatus, and stability functions (Fig. 1). The pRC960-2 plasmid sequence (GenBank accession number KY784668) was highly similar (query cover, 95%; identity, 99%) to the sequences of pHNSHP45 (GenBank accession number KP347127) (8) and pABC149-MCR-1 (from E. coli strain ABC149 isolated from the Arabian Peninsula in a clinical blood sample in 2013; GenBank accession number KX013538) (15). Apart from mcr-1, there is no other identifiable resistance gene in pRC960-2 (Fig. 1). Compared with the first described mcr-1 plasmid, pHNSHP45, the region around mcr-1 in plasmid pRC960-2 had one single nucleotide polymorphism (SNP) in the region upstream of mcr-1 (Fig. 2). Additionally, there are inverted copies of ISApI1 flanking mcr-1 and some other insertion elements (ISs) in plasmid pRC960-2 compared with the sequences of pHNSHP45 and the other two homologous plasmids (Fig. 2). Except for the inverted repeat of ISApI1, the other genes around mcr-1 were identical to those in plasmid pABC149-MCR-1, plasmid pEG430-1 (from S. sonnei strain EG430, isolated in a hospital in Vietnam in 2008) (GenBank accession number LT174530) and pHNSHP45 (Fig. 3). Compared with the plasmid pEG430-1, which carries an inactive mcr-1 in Shigella sonnei, there is no 22-bp duplication in mcr-1 (in pRC960-2), which has been previously reported to be responsible for
inactivity (18). Other detected resistance or resistance-associated genes, including qnrS1, *bla*<sub>TEM-1</sub>, *dfrA14*, and *intI1*, were located on a different plasmid without *mcr-1*. This plasmid, pRC960-1, has a length of 75 kb (GenBank accession number KY848295). Based on a BLAST search, plasmid pRC960-1, which contains other resistance genes, aligned closely with the *E. coli* strain PGRT46 plasmid pPGRT46 found in Nigeria (see Fig. S1 in the supplemental material).

**Conjugation assays.** In order to determine if the plasmids could be transferred, we performed conjugation experiments using *S. flexneri* C960 with *E. coli* J53 as the recipient strain. The *E. coli* J53 transconjugant was found to be resistant to colistin (MIC of 4 mg/liter) to the same extent as the donor. The MIC of other antimicrobials also increased, and the *E. coli* J53 recipient had almost the same antimicrobial susceptibilities as the donor (Table 1). We detected the *mcr-1*, *qnrS1*, *bla*<sub>TEM-1</sub>, *dfrA14*, and *strB* genes in the transconjugant by PCR. This suggests that both plasmids from *S. flexneri* C960 transferred into the *E. coli* J53 recipient and explains the increase in resistance phenotypes observed.

**Excision of Tn6390.** We found that there is an inverted copy of ISAp11 flanking *mcr-1*, which is unusual as copies of ISAp11 are usually directly repeated as in Tn6330 (23, 24). This putative composite transposon (Tn) was reamplified by PCR, and the PCR products were sequenced to ensure that it was not an artifact due to sequence misassembly of the plasmid reads. The putative composite transposon (ISAp11-*mcr-1*-orf-ISAp11) was given the designation Tn6390 by the Transposon Registry (25). We used primers MCR1-RC-F and MCR1-R (19) to test the ability of Tn6390 to generate a circular intermediate molecule. Through this pair of reverse primers, we obtained a 1,598-bp fragment which contained an intact PAP2 and a part of *mcr-1*. The putative structure of Tn6390 is shown in Fig. 4B. Then we used primers IS-2 and IS-6 in order to detect the

### TABLE 1 Antimicrobial susceptibility results of *S. flexneri* C960, *E. coli* J53, and a transconjugant

| Drug                  | MIC (µg/ml) for the strain<sup>a</sup> |
|-----------------------|----------------------------------------|
|                       | C960 | J53 | Transconjugant |
| Colistin              | 4    | ≤0.2 | 4              |
| Polymyxin B           | 4    | ≤0.2 | 4              |
| Tetracycline          | >8   | ≤4  | >32            |
| Ticarcillin           | >64  | ≥16 | >64            |
| Ampicillin            | >16  | ≥8  | >32            |
| Trimethoprim-sulfamethoxazole | >2 | ≥2  | >4             |
| Sulfamethoxazole      | >256 | ≥16 | >256           |
| Streptomycin          | >64  | ≥2  | >64            |
| Cefazolin             | ≤8   | ≤8  | ≤8             |
| Cefoxitin             | ≤8   | ≤8  | ≤8             |
| Cefazidime            | ≤1   | ≤1  | ≤1             |
| Ceftriaxone           | ≤1   | ≤1  | ≤1             |
| Cefoperazone          | ≤16  | ≥16 | ≤16            |
| Cefotaxime            | ≤0.12| ≤0.5| ≤0.5           |
| Cefepime              | ≤8   | ≤8  | ≤8             |
| Piperacillin          | ≤16  | ≥16 | ≥16            |
| Amoxicillin-clavulanic acid | ≤4 | ≤4  | ≤8             |
| Tetracycline-clavulanic acid | ≤16 | ≥16 | ≥16            |
| Aztreonam             | ≤1   | ≤1  | ≤1             |
| Imipenem              | ≤4   | ≤4  | ≤4             |
| Nalidixic acid        | ≤4   | ≤4  | ≤8             |
| Ciprofloxacin         | ≤0.25| ≤0.015 | ≤0.5         |
| Norfloxacin           | ≤4   | ≤4  | ≤4             |
| Levofloxacin          | ≤2   | ≤2  | ≤2             |
| Tobramycin            | ≤4   | ≤4  | ≤4             |
| Gentamicin            | ≤4   | ≤4  | ≤4             |
| Amikacin              | ≤16  | ≥16 | ≥16            |
| Chloramphenicol       | ≤8   | ≤8  | ≤8             |
| Nitrofurantoin        | ≤32  | ≤32 | ≤32            |
| Azithromycin          | ≤2   | ≤4  | ≤4             |

<sup>a</sup>Values in boldface indicate resistance; all other values indicate susceptibility.
structure formed by two IS\textsubscript{Apl1} elements. The 1,293-bp PCR product (Fig. 4C and D) amplified by IS-2 and IS-6 was the intact IS\textsubscript{Apl1} and a part of nikB (located downstream of mcr-1). The sequences of PCR products were confirmed by Sanger sequencing.

**DISCUSSION**

Among the four species of Shigella, S. flexneri is the most frequently isolated species in LMICs. Humans are the primary reservoir of Shigella spp. (26), which is not the case for Salmonella spp. and E. coli, which are more widely distributed in the environment. Isolation of plasmid-mediated colistin resistance in S. flexneri from animal feces on a farm suggests that it is circulating via the fecal-oral route, at least among the animals

**FIG 1** Structure of plasmid pRC960-2 carrying mcr-1 from Shigella flexneri strain C960. Genes are denoted by arrows and colored based on gene function classification. The innermost circle represents GC content. The second circle presents GC-skew [(G - C)/(G + C)].

**FIG 2** Comparison of the surrounding structure of mcr-1 in four similar plasmids: pHNSHP45, pABC149-MCR-1, pEG430-1, and pRC960-2. Compared with the sequences of the other three plasmids, an additional, inverted repeat of IS\textsubscript{Apl1} is present downstream of mcr-1 in plasmid pRC960-2. A single SNP upstream of mcr-1 (−36) changes from T to C in plasmid pRC960-2.
on that farm and possibly further afield via the food distribution network. In addition, it suggests that farm environments may be unrecognized reservoirs of *S. flexneri*.

The use of colistin in Chinese agriculture has been enormous and sustained. Between 2,470 and 2,875 metric tons have been used in the growth of food-producing animals annually in the last 5 years (27). Because of varied and uncontrolled drug administration techniques (injection or addition to feed and water) in food-animal rearing, the selective pressures are high enough to suggest that a large proportion of drug-resistant bacteria emerged from the agricultural sector. This use has allowed for
the selection, transfer, and maintenance of plasmid-mediated colistin resistance into clinical strains of *E. coli*, *K. pneumoniae*, and *Salmonella* spp. and rarely into other *Enterobacteriaceae*. With such sustained selective pressure and transferable resistance circulating among these strains, it is unlikely that this will be the only *Shigella flexneri* strain found to contain transferable colistin resistance in a farm environment. Also, as only a small number of strains (<10%) were isolated from farm and urban environments, we were surprised to find one with *mcr-1* on a transferable plasmid, which is a relatively high frequency of detection compared to that for clinical strains.

It is of concern that not only colistin resistance was transferred during the filter mating but also a host of mobile elements, including integron, IS, and other resistance genes which are present on the other plasmid were transferred. This suggests that under the selective pressure of colistin, other plasmids conferring multidrug resistance phenotypes can be acquired from the *S. flexneri* strain. The integron and IS could also help the strain to obtain other resistance elements from the environment. China banned colistin as an animal feed additive recently (28); however, the phenomenon of other inappropriate prophylactic antimicrobial use in farms could still inadvertently select for multiple resistance phenotypes, including colocated colistin resistance.

A novel transposon, Tn6390, is found in *S. flexneri* C960 in which two inverted copies of IS*Apl1* flank *mcr-1*. IS*Apl1* plays a pivotal role in the transposition of *mcr-1* (24, 29); however, almost all other reported structures formed by IS*Apl1*-mcr-1-orf-IS*Apl1* have two direct repeats of IS*Apl1* (23, 24, 30). There is a 1,293-bp PCR product consisting of intact IS*Apl1* and a part of *nikB*, which was presumably the result of a hairpin conformation within the plasmid (Fig. 4B). The consequences for intra- and intercellular mobility of the inverted orientation of IS*Apl1* are under investigation.

Overall, our research shows that a functional and transferable *mcr-1* exists in a multidrug-resistant *S. flexneri* strain isolated from an agricultural environment. Considering that the *mcr-1* strain was from a small number of agriculturally sourced *Shigella* strains and that the epidemiology of *Shigella* sp. infections changes, surveillance of *mcr-1* in both environmental and clinical isolates would be advised.

**MATERIALS AND METHODS**

**Strains and mcr-1 screening.** During the period of 2004 to 2015, a total of 2,127 *S. flexneri* strains were isolated as part of the national pathogen monitoring system in China. These strains were identified by standard microbiological techniques and then stored in glycerol stocks at −80°C. Colonies were serologically confirmed by slide agglutination with appropriate group-specific polyvalent antiserum,
followed by type-specific monovalent antiserum (Denka Seikan, Tokyo, Japan). Basic epidemiological data (date and region of isolation and sex and age of patient) were recorded for each isolate. We retrospectively investigated the presence of \textit{mcr-1} by PCR screening of the historical \textit{S. flexneri} isolates by using the previously published primers (8) CLR5-F (5\textsuperscript{-}CACGTTTTGACCA 3\textsuperscript{-}) and CLR5-R (5\textsuperscript{-}CTTGGTCGGTCTGTAGGG-3\textsuperscript{-}).

**Antimicrobial susceptibility testing.** The susceptibilities to 28 antimicrobials (ceftazidime, ceftiofur, ceftriaxone, ceftazidime, cefoxitin, imipenem, azithromycin, nitrofurantoin, piperacillin, ampicillin, amoxicillin-clavulanic acid, ticarcillin, tetracycline, tobramycin, gentamicin, amikacin, streptomycin, chloramphenicol, ticarcillin-clavulanic acid [Timentin], trimethoprim-sulfamethoxazole, sulfafurazole, nalidixic acid, ciprofloxacin, levofloxacin, and norfloxacin) of the \textit{S. flexneri} C960 strain, recipient \textit{E. coli} J53 strain, and the \textit{E. coli} J53 transconjugants were determined by broth microdilution using a 96-well microtiter plate (Sensititre; Trek Diagnostic Systems, Thermo Fisher Scientific, Inc.). The susceptibilities to colistin and polymyxin B were determined by a microbial viability assay kit using the dye WST (Dojindo Molecular Technologies, Inc., Japan). A reference strain of \textit{E. coli} (ATCC 25922) was included in the test as a quality control. Interpretation of antimicrobial MICs was performed according to the Clinical and Laboratory Standards Institute criteria (31).

### TABLE 2 Primers used in PCR amplification of antibiotic resistance genes

| Primer target group and name | Nucleotide sequence (5' to 3') | Target | Length (bp) | Reference |
|-----------------------------|-------------------------------|--------|-------------|-----------|
| **Beta-lactamases**         |                               |        |             |           |
| bla\textsubscript{CTX-M-1}, group-F | GGTTAAAAATCACTGCGTC | \textit{bla}\textsubscript{CTX-M-1} group | 873 | This study |
| bla\textsubscript{CTX-M-1}, group-R | TTACAAACCGTCGGTGACGA | \textit{bla}\textsubscript{CTX-M-1} group | 873 | This study |
| bla\textsubscript{CTX-M-9}, group-F | AGAGTGCAACGGATGATG | \textit{bla}\textsubscript{CTX-M-9} group | 868 | This study |
| bla\textsubscript{CTX-M-9}, group-R | CGAGGATGGATGATGCGGA | \textit{bla}\textsubscript{CTX-M-9} group | 868 | This study |
| **Integrons**               |                               |        |             |           |
| IntI1-F2                   | ACATGTGATGGCCGCAGCA | \textit{intI1} | 569 | 34 |
| IntI1-R2                   | ATTCCTGTCTGGCAGCGCA | \textit{intI1} | 569 | 34 |
| IntI2-F3                   | CACGGATATGGCAGAAAGGT | \textit{intI2} | 789 | 34 |
| IntI2-R3                   | GTACGAAACGGATGACCAAGT | \textit{intI2} | 789 | 34 |
| hep58                      | TCTACGTCCTGGTTAATGGT | Class 1 integron variable region | Variable | This study |
| hep59                      | GTAGGGCTTATTTGACCGAC | Class 1 integron variable region | Variable | This study |
| hep74                      | CGGAATCCGGGCGACATGCTGGAATTGTA | Class 2 integron variable region | Variable | 19 |
| hep51                      | GTGCGCATGCACATGCGATAC | Class 2 integron variable region | Variable | 19 |
| **Chromosomal mutation-mediated** |                               |        |             |           |
| gyRA-F                     | TACACCGGTCAACATTGAGG | \textit{gyrA} | 648 | 35 |
| gyRA-R                     | TTAATGATGGCCCGCTGGCG | \textit{gyrA} | 648 | 35 |
| gyRB-F                     | TGAATGAGCAGCGGTAAGGG | \textit{gyrB} | 309 | 35 |
| gyRB-R                     | GCTGTAACGCGGTACCGGGG | \textit{gyrB} | 309 | 35 |
| parC-F                     | GTAGGGCTTATTTGACCGAC | \textit{parC} | 531 | 35 |
| parC-R                     | TTCGCCGTGCTGATTAATGGC | \textit{parC} | 531 | 35 |
| parE-F                     | ATTCGCGGCCGCTAAAAGGT | \textit{parE} | 290 | 35 |
| parE-R                     | TCGCTGCGCTGACGATAC | \textit{parE} | 290 | 35 |
| **Plasmid-mediated quinolone resistance** |                               |        |             |           |
| qnrA-F                     | ATTTCGCGCGGAGGATTG | \textit{qnrA} | 516 | 36 |
| qnrA-R                     | GATCGCGAAGAGAGTGTCA | \textit{qnrA} | 516 | 36 |
| qnrB-F                     | GATCGTGTAAGCAGAAAGGG | \textit{qnrB} | 469 | 36 |
| qnrB-R                     | AGCAATCTGATGATGGTCCC | \textit{qnrB} | 469 | 36 |
| qnrD-F                     | CGAGATCGTTCAGTGGGGAATA | \textit{qnrD} | 656 | 32 |
| qnrD-R                     | AACACTGTAAGCGCCTGGT | \textit{qnrD} | 656 | 32 |
| qnr5-F                     | AGCAATCTGTCACCTGCA | \textit{qnrS} | 417 | 36 |
| qnr5-R                     | TAAATGGCGACCTGGTAAGCC | \textit{qnrS} | 417 | 36 |
| aac(6')-ib-cr-F            | GCAAAGCGAAAAACAAGATTAGG | \textit{aac(6')-ib-cr} | 560 | 37 |
| aac(6')-ib-cr-R            | GTGTTTGGACACGTGTA | \textit{aac(6')-ib-cr} | 560 | 37 |
TABLE 3 Primers used in PCR amplification to confirm the arrangement of the transposon ISapl1-mcr-1-orf-ISapl1

| Primer name | Nucleotide sequence (5’ to 3’) | Target or function | Length (bp) | Reference |
|-------------|------------------------------|--------------------|-------------|-----------|
| IS-1        | TACCTCCATAGCCATCTTACA        | The whole length of Tn6390 | 4,537       | This study |
| IS-4        | TACCTCCATAGCCATCTTACA        | The whole length of Tn6390 | 4,537       | This study |
| MCR1-RC-F   | CTTGATGTTGATGAG             | To test the ability of Tn6390 to generate circular intermediate IS-1 | 1,598       | 23        |
| MCR1-R      | TGCCACGGTGTAGGCGG          | To test the ability of Tn6390 to generate circular intermediate IS-2 | 1,598       | 23        |
| IS-5        | TTGGTTGATGAG                | ISAp1 and HP1 upstream of mcr-1 | 1,904       | This study |
| IS-7        | AAATCTTGACGCAAGGGCAAGAGA   | ISAp1 and HP1 upstream of mcr-1 | 1,904       | This study |
| IS-2        | GAGCCATACGGTGGTGT          | The intact ISapl1 and a part of mcr-1 | 1,293       | This study |
| IS-6        | CAAATCCATGGTGTGTTT         | The intact ISapl1 and a part of mcr-1 | 1,293       | This study |
| IS-8        | CACGAAAGACAAACGGACTGAC      | ISAp1 downstream of ISAP1 and a part of mcr-1 | 1,293       | This study |
| IS-a        | AACGCTTAATGCTGAGTATGAG      | To sequence Tn6390 | 1,904       | This study |
| IS-b        | GTTCGCAACACGCAAG           | To sequence Tn6390 | 1,904       | This study |
| IS-c        | GTGGCTGTCGGCATGCTT         | To sequence Tn6390 | 1,904       | This study |
| IS-d        | GCTATACCGGGCTGATGATG       | To sequence Tn6390 | 1,904       | This study |
| IS-e        | TGTCGCTGATGTTGTTT          | To sequence Tn6390 | 1,904       | This study |
| IS-f        | GACCCAGCGTGTGCTA           | To sequence Tn6390 | 1,904       | This study |

PCR amplification of resistance genes. DNA samples were prepared using a TIANamp bacterial DNA kit (Tiangen, Beijing) according to the manufacturer’s recommendations. Reactions were performed with 2.5 U of Taq DNA polymerase (TaKaRa, Japan) according to the manufacturer’s recommendation. The protocol for the amplification reaction, conducted in a Techne thermocycler (Bio-Red), consisted of initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A final elongation step was performed at 72°C for 10 min. PCR amplicons were fully sequenced. Other antibiotic resistance determinants were tested by PCR using the primers listed in Table 2. The sequences were analyzed using tools located at the NCBI and aligned to sequences in GenBank.

Plasmid DNA sequencing and analysis. Plasmid DNA of the S. flexneri C960 strain was extracted using a QiaGen Plasmid Midi kit (Qiagen, Germany). The DNA was used to construct a 600-bp insert library using an NEBNext Ultra II DNA Library Prep kit (NEB, Singapore) and then sequenced by a MiSeq reagent kit, version 3, using the MiSeq platform (illumina, CA, USA). Raw reads were first assembled into contigs using Newbler, version 3.0, followed by gap filling by local assembly. Pulsed-field gel electrophoresis using S1 nuclease (S1-PFGE) and Southern blotting were used to determine the length of the plasmids. To ensure accuracy, the raw reads were mapped onto the assembled complete genomes to detect misassembly and low-quality regions. In order to get complete plasmid sequences, the gaps were filled through combinatorial PCR and Sanger sequencing on an ABI 3730 sequencer (Life Technologies, CA, USA). The detection and typing of the plasmids were determined using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Each assembled genome was annotated with the Rapid Annotations using Subsystems Technology (RAST) server and verified with the Basic Local Alignment Search Tool (BLAST) against the nonredundant NCBI database (https://blast.ncbi.nlm.nih.gov/; Blast.cgi). Annotation of resistance genes, mobile elements, and other genetic structures was based on the relevant databases, including CARD, BacMet, the Beta-Lactamase Database (BLDB), and ISfinder. Plasmids pHNSHP45 (GenBank accession number KP347127), pABC149-MCR-1 (GenBank accession number XK013538), pEG430-1 (GenBank accession number LT174530), and pPGRT46 (GenBank accession number KM023153) were used as the reference plasmids for annotation. Plasmid maps were prepared using DNAplotter and Circos. The Tn number was designated by the Transposon Registry (25).

Conjugation assays. The ability of mcr-1 to undergo horizontal gene transfer was assessed by broth and filter mating using a standard E. coli J53 azide-resistant strain as the recipient. The donor/recipient ratio was 10:1, and the temperature was 30°C. MacConkey agar containing 100 mg/liter sodium azide and 2 mg/liter colistin was used to select for E. coli J53 transconjugants. Both Salmonella-Shigella (SS) agar and xylene lysine deoxycholate (XLD) medium (BD Difco, USA) with 2 mg/liter colistin were chosen to select for E. coli J53 transconjugants. Putative transconjugants were confirmed by antimicrobial susceptibility testing and detection of mcr-1 with PCR and sequencing. No spontaneous resistance to azide could be detected in the S. flexneri donor.

Detection of the circular structure carrying mcr-1. To test the stability of the Tn6330-like structure, primers were designed to detect the circular structure consisting of ISapl1-mcr-1-orf-ISapl1 (Table 3). The locations of the primers are shown in Fig. S2 in the supplemental material. The PCR amplicons were fully sequenced.

Accession numbers(s). The complete sequences of pRC960-1 and pRC960-2 determined in this study have been deposited in GenBank under the accession numbers KY848295 and KY784668, respectively. All sequencing data from this study are available through the NCBI Sequence Read Archive (SRA) under accession number SRP130733.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02655-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
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