Characterization of a transferable plasmid-borne *mcr-1* in a colistin-resistant *Shigella flexneri* isolate

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Abstract

Since its initial discovery in an *Escherichia coli* isolate from China, *mcr-1* has also been detected in *Klebsiella pneumoniae* and *Salmonella enterica*, and 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 minimum inhibitory concentration (MIC) to colistin of the isolate is 4 μg/mL. Conjugation assays showed 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*, *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* in *S. flexneri*. We have shown *mcr-1* is located on a novel composite transposon which is flanked by inverted repeats of IS*Apl1*. The host strain is multi-drug resistant and this multidrug resistance is also transferable. The finding of functional *mcr-1* in *S. flexneri*; a human associated Enterobacteriaceae 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*, Tn*6390*, multidrug resistance, plasmid transfer, composite transposon

Introduction

Antimicrobial resistance is a major global health issue and is on the national and international agenda 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). Following 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 a last resort antibiotic (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 origin of *mcr-1* positive strains are varied. Agricultural establishments, retail meat and patients with infections are three major

58 sources of colistin resistant bacteria. Since its initial discovery in an *Escherichia coli*
59 isolate from China, *mcr-1* has been detected in Southeast Asia, Europe, America and
60 Africa (14-17). Most of the *mcr-1* positive strains are *E. coli*, *Klebsiella pneumoniae*
61 and *Salmonella enterica*, whilst the gene is rarely reported in other
62 Enterobacteriaceae. A recent report described the presence of a *mcr-1*-positive
63 *Shigella sonnei* from Vietnam, however a colistin resistance phenotype was only
64 observed following transfer to *E. coli* (18).

65 *Shigella* spp. are recognized as etiological agents of diarrhea and have been
66 responsible for serious worldwide epidemics (19). *Shigella flexneri* is the most
67 frequently isolated species in many countries and is responsible for approximately 10%
68 of all diarrheal episodes in children younger than five years (20). *S. flexneri* 3a is also
69 commonly isolated in male homosexuals in the US (21) and the UK (22). Between
70 2004 and 2015, *S. flexneri* strains were isolated and collected in China. By screening
71 available isolate collections via PCR, we identified a single *mcr-1* positive strain of *S.*
72 *flexneri*.

73

74 **Results**

75 **Bacterial strains and *mcr-1* screening**

76 A total of 2127 *S. flexneri* were isolated from the samples collected from thirteen

77 different areas in China; these were Beijing, Shenyang, Shandong, Henan, Anhui,
78 Hubei, Xinjiang, Gansu, Sichuan, Guizhou, Yunnan, Guangxi and Guangdong
79 province. There are fifteen different serotypes among the *S. flexneri* strains. Most of
80 the strains were isolated from stool samples of patients who were suffering from
81 clinically diagnosed gastroenteritis, a small number of strains (< 10%) were isolated
82 from farm and urban environments. Through the PCR screening for the presence of
83 *mcr-1* amongst all the *S. flexneri* strains, only one *mcr-1* positive isolate, named C960
84 was found. The serotype of the positive isolate is y and it was isolated from pig stool
85 samples in a pig farm in Guangxi province in 2009.

86 **Antimicrobial susceptibility and PCR amplification of resistance genes**
87 Antimicrobial susceptibility tests showed that, besides colistin, *S. flexneri* C960 was
88 resistant to tetracycline, ticarcillin, ampicillin, trimethoprim/ sulfamethoxazole,
89 sulfafurazole and streptomycin (Table 1). Through PCR we found that strain C960
90 carried other acquired resistance genes including *qnrS1*, $bla_{\text{TEM-1}}$, *dfrA14* and *strB*,
91 which could confer decreased susceptibility to quinolones, β-lactam antibiotics,
92 trimethoprim and streptomycin respectively.

93 **Plasmid DNA sequencing and analysis**

94 After sequencing and assembly of the plasmids of *S. flexneri* C960, analysis showed
95 that *mcr-1* in C960 was located on a 65538 bp plasmid designated pRC960-2. The
96 plasmid has a GC content of 43.2%, contains 92 predicted open reading frames

(ORFs) and has a typical IncI2 plasmid backbone (57756 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 that 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 SNP in the region upstream of *mcr-1* (Fig 2 A). Additionally, there are inverted copies of IS*Apl1* flanking m*cr-1* and some other ISs in plasmid pRC960-2 compared with pHNSHP45 and the other two homologous plasmids (Fig 2 B). Except for the inverted repeat of IS*Apl1*, 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 *qnrS*1, $bla_{\text{TEM-1}}$, *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). Through BLAST, the plasmid which contains other resistance

118 genes aligned closely with *E. coli* strain PGRT46 plasmid pPGRT46 found in Nigeria
119 (Fig S1).

120 **Conjugation assays**

121 In order to determine if the plasmids could be transferred, we performed conjugation
122 experiments of *S. flexneri* C960 with *E. coli* J53 as the recipient strain. The *E. coli*
123 J53 transconjugant, was found to be resistant to colistin (MIC 4 mg/L) the same as the
124 donor. The MIC to other antimicrobials also increased and the *E. coli* J53 recipient
125 had almost the same antimicrobial susceptibilities as the donor (Table 1). We
126 detected *mcr-1*, *qnrS1*, $bla_{\text{TEM-1}}$, *dfrA14* and *strB* gene in the transconjugant by PCR.
127 This suggests that both plasmids from the *S. flexneri* C960 transferred into *E. coli* J53
128 recipient and explains the increase in resistance phenotypes observed.

129 **Excision of *Tn6390***

130 We found there is an inverted copy of IS*Apl1* flanking *mcr-1*, which is unusual
131 as copies of IS*Apl1* are usually directly repeated as in Tn*6330* (23, 24). This putative
132 composite transposon was re-amplified by PCR and the PCR products were
133 sequenced to ensure that it was not an artifact due to sequence mis-assembly of the
134 plasmid reads. The putative composite transposon (>IS*Apl1-mcr-1-orf*-IS*Apl1*<) was
135 given the designation Tn*6390* by the Transposon Registry (25). We used primers
136 MCR1-RC-F and MCR1-R (19) to test the ability of Tn*6390* to generate a circular
137 intermediate molecule. Through this pair of reverse primers we got a 1598bp

fragment which contains an intact PAP2 and parts of *mcr-1*. The putative structure of Tn*6390* showed in Fig 4 B. Then we used primers IS-2 and IS-6 in order to detect the structure formed by two IS*Apl1*. The 1293 bp PCR product (Fig 4 C and D) amplified by IS-2 and IS-6 was the intact IS*Apl1* and a part of *nik*B (located in the 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 different from *Salmonella* spp. and *E. coli*, which are more widely distributed in the environment. Isolation of plasmid-mediated colistin resistance in *S. flexneri* from animal faeces on a farm suggests it is circulating via the faecal – oral route at least amongst the animals on that farm and possibly further afield via the food distribution network. In addition, it suggests that farm environments may be an unrecognized reservoir of *S. flexneri*.

The use of colistin in Chinese agriculture has been enormous and sustained. Between 2470 – 2875 metric tons have been used in the growth of food-producing animals annually in last 5 years (27). Because of the varied and uncontrolled drug administration techniques (injection, added to feed and water) in food-animal rearing, the selective pressures are high enough to suggest that a large proportion of drug resistance emerged from the agriculture sector. This use has allowed for the selection,

158 transfer and maintenance of plasmid mediated colistin resistance into clinical strains
159 of *E. coli*, *K. pneumoniae* and *Salmonella* spp. and rarely into other
160 Enterobacteriaceae. With such a sustained selective pressure and transferable
161 resistance circulating amongst these strains it is unlikely that this will be the only
162 *Shigella flexneri* strain containing transferable colistin resistance in a farm
163 environment. Also, as only a small number of strains (< 10%) were isolated from
164 farm and urban environments, we were surprised to find 1 with *mcr-1* on a
165 transferable plasmid which is a relatively high frequency of detection compared to the
166 clinical strains.

167 Worryingly, not only did colistin resistance transfer during the filter mating but
168 also a host of mobile elements including integron, IS and other resistance genes
169 which are present on the other plasmid transferred. This suggests that under the
170 selective pressure of colistin, other plasmids conferring multi-drug resistant
171 phenotypes can be acquired from the *S. flexneri* strain. The integron and IS could also
172 help the strain to obtain other resistance from environment. China have banned
173 colistin as an animal feed additive recently (28), however the phenomenon of other
174 inappropriate prophylactic antimicrobial use in farms could still inadvertently select
175 for multiple resistance phenotypes including co-located colistin resistance.

176 A novel transposon Tn*6390* is found in *S. flexneri* C960 in which two inverted
177 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 1293bp PCR product consisting of intact IS*Apl1* and a part of *nik*B which was presumably the result of a hairpin conformation within the plasmid (Fig 4 B). The consequences on intra and intercellular mobility of the inverted orientation of IS*Apl1* are currently 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 the *mcr-1* strain was from a small number of agriculturally sourced *Shigella* and the changing epidemiology of *Shigella* spp. infections, 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 2127 *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 -80C. Colonies were serologically confirmed by slide agglutination with appropriate group-specific polyvalent antisera, followed by type-specific monovalent antisera (Denka Seikan, Tokyo, Japan). Basic epidemiological data (date and region of isolation, sex and age of patient) were recorded for each isolate. We retrospectively

198 investigated presence of *mcr-1* by PCR screening the historical *S. flexneri* isolates by
199 using the previously published primers (8) CLR5-F
200 (5'-CGGTCAGTCCGTTTGTTC-3') and CLR5-R
201 (5′-CTTGGTCGGTCTGTAGGG-3′).

202 **Antimicrobial susceptibility testing**

203 The susceptibility of 28 antimicrobials (Ceftazidime, Ceftiofur, Ceftriaxone,
204 Cefepime, Cefoperazone, Cefazolin, Cefoxitin, Imipenem, Azithromycin,
205 Nitrofurantoin, Piperacillin, Ampicillin, Amoxicillin/Clavulanic acid, Ticarcillin,
206 Tetracycline, Tobramycin, Gentamicin, Amikacin, Aztreonam, Streptomycin,
207 Chloramphenicol, Timentin, Trimethoprim/ Sulfamethoxazole, Sulfafurazole,
208 Nalidixan, Ciprofloxacin, Levofloxacin, Norfloxacin) of the *S. flexneri* C960,
209 recipient *E. coli* J53 and the *E. coli* J53 transconjugants were determined by the broth
210 microdilution using a 96-well microtiter plate (Sensititre, Trek Diagnostic Systems,
211 Thermo Fisher Scientific Inc). The susceptibility of colistin and polymyxin B were
212 determined by Microbial Viability Assay Kit-WST (Dojindo Molecular Technologies
213 Inc, Japan). A reference strain of *E. coli* (ATCC 25922) was included in the test as a
214 quality control. Interpretation of antimicrobial minimal inhibitory concentrations
215 (MICs) was performed according to the Clinical and Laboratory Standards Institute
216 (CLSI, 2017) criteria.

217 **PCR amplification of resistance genes**

DNA samples were prepared using a TIANamp Bacteria DNA Kit (Tiangen, Beijing) following the manufacturer's recommendations. Reactions were performed with 2.5 U of Taq DNA Polymerase (Takara, Japan) according to the manufacturer's recommendation. The amplification reaction, conducted in a Techne thermo cycler (Bio-Red), consisted of initial denaturation at 94°C for 5 mins 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 ran at 72°C for 10 min. PCR amplicons were fully sequenced. Other antibiotic resistance determinants were detected by PCR using the primers listed in Table 2. The sequences were analyzed using tools located at the NCBI and aligned to the sequences in GenBank.

Plasmid DNA sequencing and analysis

Plasmid DNA of the *S. flexneri* C960 was extracted using a Qiagen Plasmid Midi kit (Qiagen, Germany). The DNA was used to construct a 600-bp insert library using NEBNext Ultra II DNA Library Prep Kit (NEB, Singapore). Then sequenced by MiSeq Reagent Kit v3 using MiSeq platform (Illumina, CA, USA). Raw reads were first assembled into contigs using Newbler version 3.0, followed by gap filling by local assembly. S1-PFGE and Southern blot were used to determine the length of the plasmids. To ensure accuracy, the raw reads were mapped onto the assembled complete genomes to detect the mis-assembly and low quality regions. In order to get complete plasmid sequences, the gaps were filled through combinatorial PCR and

238 Sanger sequencing on an ABI 3730 Sequencer (LifeTechn query ologies, CA, USA).
239 The detection and typing of the plasmids were found using PlasmidFinder
240 (https://cge.cbs.dtu.dk/services/PlasmidFinder/). Each assembled genome was
241 annotated with the Rapid Annotations using Subsystems Technology (RAST) server
242 and verified with the Basic Local Alignment Search Tool (BLAST) against the
243 non-redundant NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Annotation of
244 resistance genes, mobile elements and other genetic structures was based on the
245 relevant databases including CARD, BacMet, β-lactamases Database and ISfinder.
246 Plasmids pHNSHP45 (GenBank accession number KP347127), pABC149-MCR-1
247 (GenBank accession number KX013538), pEG430-1(GenBank accession number
248 LT174530), and pPGRT46 (GenBank accession number KM023153) were used as the
249 reference plasmids for annotation. Plasmid maps were prepared using DNAplotter
250 and Circos. The Tn number was designated by the Transposon Registry (25). The
251 complete sequences of pRC960-1 and pRC960-2 was submitted to GenBank under
252 accession number KY848295 and KY784668, respectively. Raw data of *S. flexneri*
253 C960 has been uploaded to NCBI Sequence Read Archive (SRA) under accession
254 number SRP130733.

255 **Conjugation assays**

256 The ability of *mcr-1* to undergo horizontal gene transfer was assessed by broth
257 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/L sodium azide and 2 mg/L colistin was used to select for *E. coli* J53 transconjugants. Both SS agar and XLD media (BD Difco, USA) with 2 mg/L 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 carried *mcr-1*

To test the stability of the Tn*6330*-like structure, primers were designed to detect the circular structure consisted of IS*Apl1*-*mcr-1*-*orf*-IS*Apl1* (Table 3). The locations of the primers are shown in Fig S2. The PCR amplicons were fully sequenced.

Accession number(s)

The sequences determined in this study have been deposited in GenBank under the accession numbers KY784668 and KY848295. All sequencing data from this study is available through the NCBI Sequence Read Archive (SRA) under accession number SRP130733.

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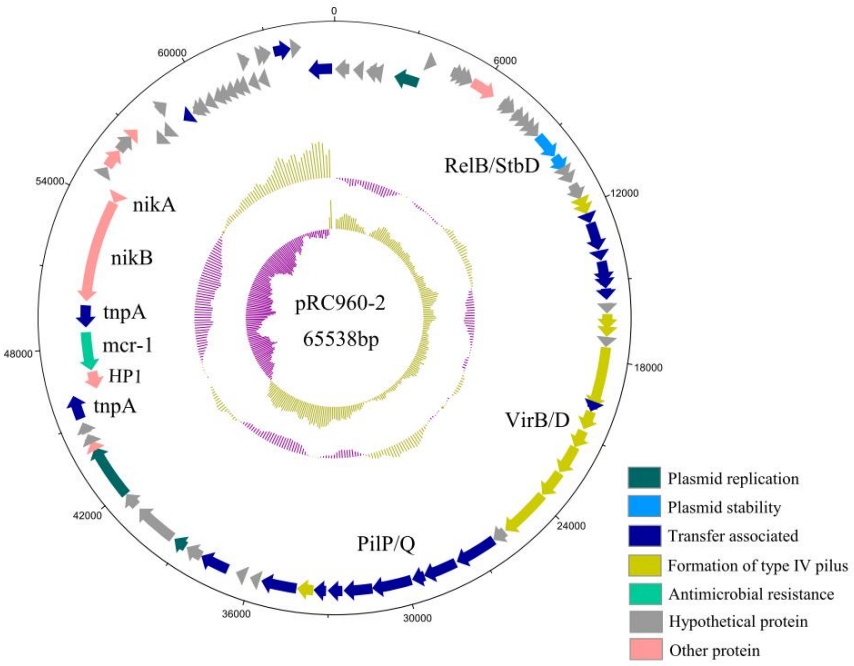


Figure 1.

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 present GC contents. The second circle presents GC-Skew [(G − C)/(G + C)].

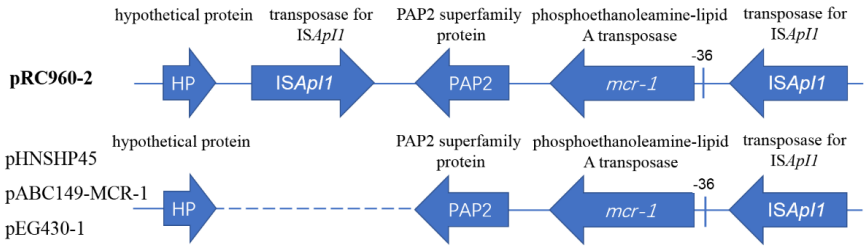


Figure 2.

Comparison of the surrounding structure of *mcr-1* in four similar plasmids pHNSHP45, pABC149-MCR-1, pEG430-1 and pRC960-2. Compared with other three plasmids, an additional, inverted repeat of IS*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.

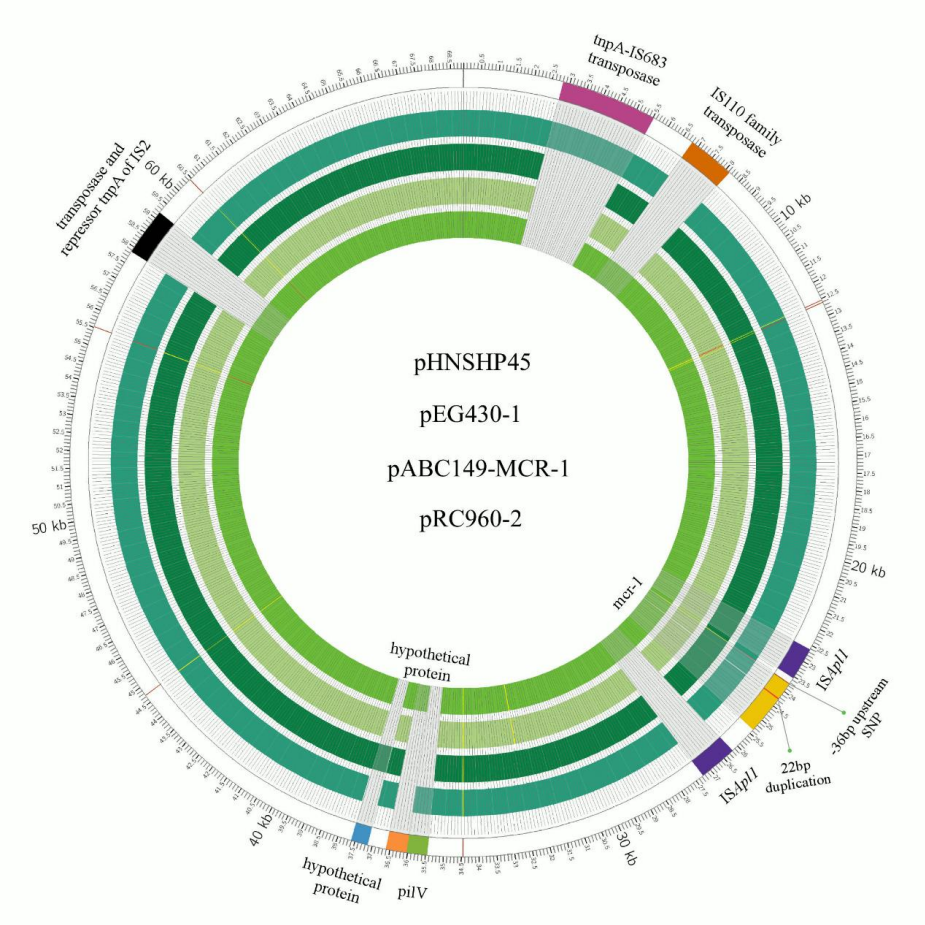


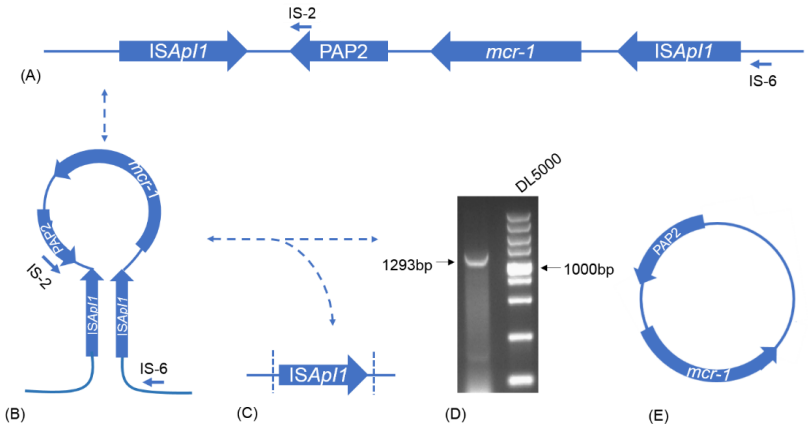
Figure 3.

Comparison of the circular genome map of plasmid sequences of three plasmids genome structures with pHNSHP45 sequence as reference genome. The green circles from inside to outside are pRC960-2, pEG430-1, pABC149-MCR-1 and pHNSHP45 plasmid sequences, respectively, with respect to the reference pHNSHP45 plasmid genome. In pRC960-2, there is an inverted copy of ISA*pl1* flanking *mcr-1*. A 22 bp duplication of bases 503–525 of *mcr-1* ORF is found in plasmid pEG430-1. The single SNP in the upstream of *mcr-1* in pRC960-2,

408 pABC149-MCR-1, pEG430-1 are the same.

409

410



411

412 Figure 4.

413 (A) The linear structure of *mcr-1* surrounding genes, the arrows stand for the locations of the

414 primers used to detect the circular structure. (B) The presumable structure in plasmid pRC960-2

415 of *S. flexneri* strain C960. (C) The sequencing result of the PCR products generated by primers

416 IS-2 and IS-6. (D) Gel picture of the 1293bp PCR products generated by primers IS-2 and IS-6

417 targeting surrounding sequences of Tn*6390*. The amplicon of this pair of primers is consist of

418 parts of *nik*B and a complete IS*Apl1*. (E) Schematic representation of the presumable circular

419 structure including *mcr-1*.

420

421 Table 1. Antimicrobial susceptibility results of *S. flexneri* C960, *E. coli* J53 and transconjugant.

	C960	**J53**	**Transconjugant**
Colistin	4µg/mL R	<=0.2µg/mL S	4µg/mL R
Polymyxin B	4µg/mL R	<=0.2µg/mL S	4µg/mL R
Tetracycline	>8 µg/mL R	<=4µg/mL S	>32µg/mL R
Ticarcillin	>64µg/mL R	<=16µg/mL S	>64µg/mL R
Ampicillin	>16µg/mL R	<=8µg/mL S	>32µg/mL R
Trimethoprim/ sulfamethoxazole	>2µg/mL R	<=2µg/mL S	>4µg/mL R
Sulfafurazole	>256µg/mL R	<=16µg/mL S	>256µg/mL R
Streptomycin	>64µg/mL R	<=2µg/mL S	>64µg/mL R
Cefazolin	<=8µg/mL S	<=8µg/mL S	<=8µg/mL S
Cefoxitin	<=8µg/mL S	<=8µg/mL S	<=8µg/mL S
Ceftazidime	<=1µg/mL S	<=1µg/mL S	<=1µg/mL S
Ceftriaxone	<=1µg/mL S	<=1µg/mL S	<=1µg/mL S
Cefoperazone	<=16µg/mL S	<=16µg/mL S	<=16µg/mL S

Ceftiofur	<=0.12µg/mL S	<=0.5µg/mL S	<=0.5µg/mL S
Cefepime	<=8µg/mL S	<=8µg/mL S	<=8µg/mL S
Piperacillin	<=16µg/mL S	<=16µg/mL S	<=16µg/mL S
Amoxicillin/Clavulanic acid	<=4µg/mL S	<=4µg/mL S	<=8µg/mL S
Timentin	<=16µg/mL S	<=16µg/mL S	<=16µg/mL S
Aztreonam	<=1µg/mL S	<=1µg/mL S	<=1µg/mL S
Imipenem	<=4µg/mL S	<=4µg/mL S	<=4µg/mL S
Nalidixan	<=4µg/mL S	<=4µg/mL S	<=8µg/mL S
Ciprofloxacin	<=0.25µg/mL S	<=0.015µg/mL S	<=0.5µg/mL S
Norfloxacin	<=4µg/mL S	<=4µg/mL S	<=4µg/mL S
Levofloxacin	<=2µg/mL S	<=2µg/mL S	<=2µg/mL S
Tobramycin	<=4µg/mL S	<=4µg/mL S	<=4µg/mL S
Gentamicin	<=4µg/mL S	<=4µg/mL S	<=4µg/mL S
Amikacin	<=16µg/mL S	<=16µg/mL S	<=16µg/mL S
Chloramphenicol	<=8µg/mL S	<=8µg/mL S	<=8µg/mL S
Nitrofurantoin	<=32µg/mL S	<=32µg/mL S	<=32µg/mL S

Azithromycin	<=2μg/mL S	<=4μg/mL S	<=4μg/mL S

422 (S: Sensitive, R: Resistance.)

423

424 Table 2. Primers used in PCR amplification of antibiotic resistance genes

Primers	Nucleotide sequence (5' to 3')	Target	Length (bp)	Reference
β-lactamases				
bla$_{CTX-M-1}$ group-F	GGTTAAAAAATCACTGCGTC	*bla*$_{CTX-M\ 1}$ group	873	This study
bla$_{CTX-M-1}$ group-R	TTACAAACCGTCGGTGACGA			
bla$_{CTX-M-9}$ group-F	AGAGTGCAACGGATGATG	*bla*$_{CTX-M\ 9}$ group	868	This study
bla$_{CTX-M-9}$ group-R	CCAGTTACAGCCCTTCGG			
bla$_{CTX-M-2/8/25}$ group-F	ACCGAGCCSACGCTCAA	*bla*$_{CTX-M-2/8/25}$ group	221	This study
bla$_{CTX-M-2/8/25}$ group-R	CCGCTGCCGGTTTTATC			
bla$_{TEM}$-F	ATGAGTATTCAACATTTCCG	*bla*$_{TEM}$	1080	(31)
bla$_{TEM}$-R	CCAATGCTTAATCAGTGAGG			
bla$_{OXA}$-F	ATTAAGCCCTTTACCAAACCA	*bla*$_{OXA}$	890	(19)
bl$_{aOXA}$-R	AAGGGTTGGGCGATTTTGCCA			

bl$_{aVIM}$-F3	AGTGGTGAGTATCCGACAG	*bla*$_{VIM}$	509	(32)
bla$_{VIM}$-R3	ATGAAAGTGCGTGGAGAC			
bla$_{NDM-1}$-F	GTCTGGCAGCACACTTCCTA	*bla*$_{NDM-1}$	515	This study
bla$_{NDM-1}$-R	TAGTGCTCAGTGTCGGCATC			
Integrons				
IntI1-F2	ACATGTGATGGCGACGCACGA	*IntI1*	569	(33)
IntI1-R2	ATTTCTGTCCTGGCTGGCGA			
IntI2-F3	CACGGATATGCGACAAAAAGGT	*IntI2*	789	(33)
IntI2-R3	GTAGCAAACGAGTGACGAAATG			
*hep*58	TCATGGCTTGTTATGACTGT	Class 1 integron	variable	This study
*hep*59	GTAGGGCTTATTATGCACGC	variable region		
hep74	CGGGATCCCGGACGGCATGCACGA TTTGTA	Class 2 integron variable region	variable	(19)
hep51	GATGCCATCGCAAGTACGAG			
Chromosomal mutations-mediated quinolone resistance				
gyrA-F	TACACCGGTCAACATTGAGG	*gyrA*	648	(34)

gyrA-R	TTAATGATTGCCGCCGTCGG			
gyrB-F	TGAAATGACCCGCCGTAAAGG	*gyrB*	309	(34)
gyrB-R	GCTGTGATAACGCAGTTTGTCCGGG			
parC-F	GTACGTGATCATGGACCGTG	*parC*	531	(34)
parC-R	TTCGGCTGGTCGATTAATGC			
parE-F	ATGCGTGCGGCTAAAAAAGTG	*parE*	290	(34)
parE-R	TCGTCGCTGTCAGGATCGATAC			
Plasmid-mediated quinolone resistance				
qnrA-F3	ATTTCTCACGCCAGGATTTG	*qnrA*	516	(35)
qnrA-R3	GATCGGCAAAGGTYAGGTCA			
qnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	469	(35)
qnrB-R	ACGAYGCCTGGTAGTTGTCC			
qnrD-F	CGAGATCAATTTACGGGGAATA	*qnrD*	656	(31)
qnrD-R	AACAAGCTGAAGCGCCTG			
qnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	417	(35)
qnrS-R	TAAATTGGCACCCTGTAGGC			

aac(6')-Ib-cr-F	GCAACGCAAAAACAAAGTTAGG	*aac(6')-Ib-cr*	560	(36)
aac(6')-Ib-cr-R	GTGTTTGAACCATGTACA			

425

426

427 Table 3. Primers used in PCR amplification to confirm the *>ISApl1-mcr-1-ISApl1<*

428 arrangement

Primers	**Nucleotide sequence (5' to 3')**	**Target**	**Length (bp)**	**Reference**
IS-1:	TACTTCCTACCGCCATCTTACA	The whole length of Tn6390	4537	This study
IS-4	TACTTCCTACCGACATCTTAC			
MCR1-RC-F	CTTGGTCGGTCTGTAGGG	To test the ability of Tn*6390* to generate circular intermediate	1598	(23)
MCR1-R	TGTCCACGGTTGATGCG			
IS-5	TCTGTTTGGGGTTGATT	IS*Apl1* and HP1 in the upstream *of mcr-1*	1904	This study
IS-7	AAAGTCAAAGACCGCACC			
IS-2	GAGCCATACGGTGGTGT	The intact IS*Apl1* and a part of *nik*B	1293	This study
IS-6	CGAATCCGATTTGCTTA			

IS-8	CACAAGAACAAACGGACTGAC	IS*Apl1* in the downstream *of* *mcr-1* and a part of *mcr-1*	This study
IS-a	AACGCCTACTGGCTGAGATGAG	To sequence Tn*6390*	This study
IS-b	GGTCGCAACCAGCAAG	To sequence Tn*6390*	This study
IS-c	GTGGCGTTCAGCAGTCATT	To sequence Tn*6390*	This study
IS-d	GCTTACCCACCGAGTAGATT	To sequence Tn*6390*	This study
IS-e	TGGTCGCTGATTGGTTTT	To sequence Tn*6390*	This study
IS-f	GACACCACCGTATGGCTCA	To sequence Tn*6390*	This study

429