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MECHANISMS OF RESISTANCE

Prevalence and Molecular Characterization of *mcr-1*-Positive *Salmonella* Strains Recovered from Clinical Specimens in China

Mingquan Cui,[a] Jinfei Zhang,[b] Zhen Gu,[c] Ruichao Li,[d] Edward Wai-chi Chan,[d] Meiyin Yan,[e] Congming Wu,[a] Xuebin Xu,[f] Sheng Chen[b,d]

Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agricultural University, Beijing, China[a]; Shenzhen Key Laboratory for Food Biological Safety Control, Food Safety and Technology Research Center, Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, People's Republic of China[b]; Emerging Infections Program China Office, People's Republic of China[c]; State Key Laboratory of Chirosciences, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong[d]; State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China[e]; Microbiology Laboratory, Shanghai Municipal Center for Disease Control and Prevention, Shanghai, People's Republic of China[f]

ABSTRACT The recently discovered colistin resistance element, *mcr-1*, adds to the list of antimicrobial resistance genes that rapidly erode the antimicrobial efficacy of not only the commonly used antibiotics but also the last-line agents of carbapenems and colistin. This study investigated the prevalence of the mobile colistin resistance determinant *mcr-1* in *Salmonella* strains recovered from clinical settings in China and the transmission potential of *mcr-1*-bearing mobile elements harbored by such isolates. The *mcr-1* gene was recoverable in 1.4% of clinical isolates tested, with the majority of them belonging to *Salmonella enterica* serotype Typhimurium. These isolates exhibited diverse pulsed-field gel electrophoresis (PFGE) profiles and high resistance to antibiotics other than colistin and particularly to cephalosporins. Plasmid analysis showed that *mcr-1* was carried on a variety of plasmids with sizes ranging from ~30 to ~250 kb, among which there were conjugative plasmids of ~30 kb, ~60 kb, and ~250 kb and nonconjugative plasmids of ~140 kb, ~180 kb, and ~240 kb. Sequencing of representative *mcr-1*-carrying plasmids revealed that all conjugative plasmids belonged to the IncX4, IncI2, and IncHI2 types and were highly similar to the corresponding types of plasmids reported previously. Nonconjugative plasmids all belonged to the IncHI2 type, and the nontransferability of these plasmids was attributed to the loss of a region carrying partial or complete *tra* genes. Our data revealed that, similar to the situation in *Escherichia coli*, *mcr-1* transmission in *Salmonella* was accelerated by various plasmids, suggesting that transmission of *mcr-1*-carrying plasmids between different species of *Enterobacteriaceae* may be a common event.

KEYWORDS *Salmonella*, *mcr-1*, transmission, IncX4, IncI2, IncHI2, plasmid

Polymyxins are considered the last-resort antibiotics used to treat carbapenem-resistant *Enterobacteriaceae* (CRE)-related infections due to their high efficacy and low resistance rate among clinical CRE strains. Polymyxins were discovered more than 50 years ago; however, due to their nephrotoxic and neurotoxic side effects, they are rarely used clinically (1). In recent years, due to the increasing prevalence of carbapenem-resistant *Enterobacteriaceae* and emergence of exten-

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Address correspondence to Congming Wu, wucm@cau.edu.cn, Xuebin Xu, xuxuebin@scdc.sh.cn, or Sheng Chen, sheng.chen@polyu.edu.hk.

M.C. and J.Z. contributed equally to this article.

sively drug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* strains, polymyxins have been reevaluated and are considered among the few remaining agents which exhibit bactericidal effects on multidrug-resistant (MDR) Gram-negative bacterial pathogens (2, 3).

The rate of bacterial resistance to polymyxins had previously been thought to be extremely low and mainly attributed to chromosomal mutations in specific two-component regulatory systems (e.g., *pmrAB* and *phoPQ* and their negative regulator *mgrB* in the case of *Klebsiella pneumoniae*), leading to structural modification of lipid A (4, 5). Recently, a new plasmid-mediated colistin resistance mechanism, mediated by the MCR-1 protein, a phosphoethanolamine transferase which can add the phosphoethanolamine moiety to lipid A, was discovered (6). The *mcr-1* gene was detectable in not only *Escherichia coli* but also other bacterial species such as *Klebsiella pneumonia* and *Salmonella* in isolates recovered from various countries (6, 7). The *mcr-1*-bearing plasmids were found to possess the ability to self-transmit between animal and human bacterial isolates and were also highly stable in bacteria even in the absence of polymyxin selection pressure (6). However, the *mcr-1* gene was found in only ~1% of clinical *E. coli* isolates, whereas the carriage rate could be as high as ~20% in bacteria isolated from farm animals and meat products (6). This discrepancy prompted us to make the hypothesis that only a small proportion of *mcr-1*-bearing strains can cause human infections.

In *Salmonella*, the *mcr-1* gene was first described through analysis of whole-genome sequences of *Salmonella* available in GenBank, in which *mcr-1*-bearing plasmids were identified in 10 clinical *Salmonella enterica* isolates submitted between 2012 and 2015, including 8 *S. enterica* serotype Typhimurium, 1 *S. enterica* serotype Paratyphi B var. Java, and 1 *S. enterica* serotype Virchow strain (8). The *mcr-1* gene was subsequently reported to be recoverable from *Salmonella* strains isolated from food, animals, and clinical specimens in Europe, the United States, and China (9–14) and was found to be harbored mainly by IncX4 and IncI1 plasmids of various sizes (10, 11). However, these data remain scattered and do not provide a comprehensive view on either the prevalence of the *mcr-1* gene in *Salmonella* or the transmission kinetics of the different types of mobile elements that harbor such resistance determinants. To address these important issues, we conducted a nationwide surveillance on the prevalence of the *mcr-1* gene in *Salmonella* strains recovered from clinical specimens and investigated the transmission potential of the *mcr-1* gene recovered from these *Salmonella* isolates. Our data provide a comprehensive view of the extent of *mcr-1* contamination in *Salmonella* in China and essential insights into the features and routes of transmission of *mcr-1* among strains of this important foodborne pathogen.

RESULTS

Prevalence and characteristics of *mcr-1*-positive *Salmonella*. A total of 2,034 nonrepeated human clinical *Salmonella* isolates collected nationwide during the period of 2012 to 2015 by the Chinese Center for Disease Control and Prevention (CDC) were subjected to screening of the *mcr-1* gene, and 28 out of 2,034 (1.4%) isolates were found to contain this resistance gene. Among these 28 *mcr-1*-positive *Salmonella* isolates, 25 were *S.* Typhimurium and 3 were *S. enterica* serotype Enteritidis. All three *S.* Enteritidis isolates were collected in 2012, while *S.* Typhimurium was the only serotype found to be carrying the *mcr-1* gene afterward. All *mcr-1*-positive *Salmonella* isolates showed very diverse pulsed-field gel electrophoresis (PFGE) patterns, suggesting that transmission of *mcr-1*-carrying plasmids may be more common than clonal spread in *mcr-1*-positive *Salmonella* (Fig. 1). Antimicrobial susceptibility analysis revealed that these 28 *mcr-1*-positive *Salmonella* isolates generally displayed a high rate of resistance to other antibiotics in addition to colistin, in particular, to ceftriaxone (78%) (Table 1).

Mechanisms of transmission of *mcr-1* among *Salmonella* isolates. Conjugation experiments showed that 21 out of the 28 *mcr-1*-positive *Salmonella* isolates were able to transfer their colistin resistance phenotype to *E. coli* J53 (Fig. 1). PFGE using S1 nuclease (S1-PFGE) and Southern-hybridization analysis indicated that all 28 isolates,

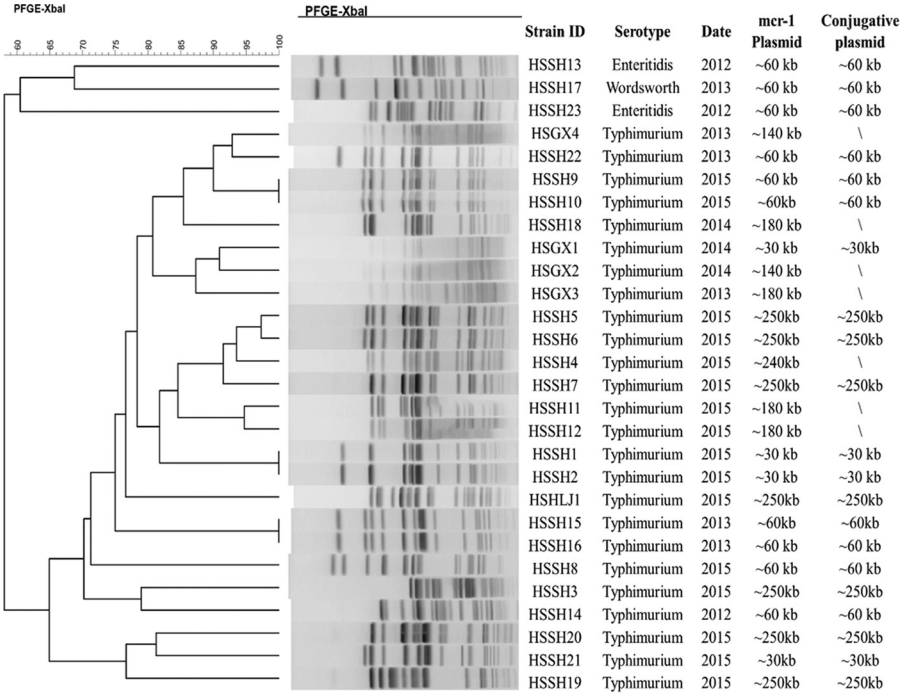

FIG 1 Summary of genetic characteristics of *mcr-1*-bearing *Salmonella* strains isolated from different sources.

regardless of their conjugative status, carried only one *mcr-1*-carrying plasmid. Most of the conjugative plasmids carrying *mcr-1* belonged to three major groups, ~30 kb (n = 3), ~60 kb (n = 11), and ~250 kb (n = 7). All nonconjugative plasmids had sizes of ~140 kb (n = 2), ~180 kb (n = 4), and ~240 kb (n = 1) (Fig. 1).

Genetic features of *mcr-1*-carrying plasmids recovered from *Salmonella* strains. Representative plasmids were selected from different strains, including plasmids of ~30 kb, ~60 kb, ~140 kb, ~180 kb, ~240 kb, and ~250 kb, for sequencing using the Illumina platforms. Two of them, ~60-kb and ~250-kb plasmids, were further sequenced by the PacBio platform to obtain the complete maps (Table 2). Illumina contigs of the ~30-kb plasmid harbored by *Salmonella* strain HSGX1 were obtained and aligned to several previously reported plasmids, including pOW3E1 (GenBank accession number KX129783.1) and pECJP-B65-33 (GenBank accession number KX084392.1). This plasmid from HSGX1 was shown to belong to the IncX4 type and could be aligned very well to pOW3E1 (KX129783.1) (>99% in both identity and coverage) (data not shown).

TABLE 1 Antimicrobial susceptibility of *mcr-1*-positive *Salmonella* strains isolated from different sources

Antibiotic	Breakpoint (μg/ml)	% of resistance[a]	
		WT (n = 28)	TC (n = 21)
Ampicillin	≥32	89	52
Amoxicillin-clavulanic acid	≥32/16	50	14
Ceftriaxone	≥4	64	62
Chloramphenicol	≥32	64	19
Gentamicin	≥16	54	24
Nalidixic acid	32	68	24
Ciprofloxacin	≥1	25	0
Trimethoprim-sulfamethoxazole	≥4/76	39	14
Tetracycline	≥16	79	0
Colistin	>2	100	100
Azithromycin	≥16	14	0

[a]"% of resistance" refers to the no. of resistant strains/no. of total strains (n). WT, wild type; TC, transconjugant.

TABLE 2 Origin and genetic features of *mcr-1*-bearing plasmids in *Salmonella* strains subjected to sequence analysis in this study

Strain name	Serotype	Yr of isolation	Approx size by S1-PFGE (kb)	Conjugative nature	Plasmid type	Sequence(s) analyzed
HSSH21	Typhimurium	2014	30	Conjugative	IncX4	Contigs
HSSH23	Enteritidis	2012	60	Conjugative	IncI2	pHSSH23-MCR1
HXGX4	Typhimurium	2013	140	Nonconjugative	IncHI2	Contigs
HXGX3	Typhimurium	2015	180	Nonconjugative	IncHI2	Contigs
HSSH4	Typhimurium	2015	240	Nonconjugative	IncHI2	Contigs
HSHLJ1	Typhimurium	2015	250	Conjugative	IncHI2	pHSHLJ1-MCR1

The complete sequence of a ~60-kb conjugative plasmid, recovered from the *S.* Enteritidis HSSH23 strain, pHSSH23-MCR1, was obtained. It was found to belong to an IncI2-type plasmid with a backbone structure similar to that of pHNSHP45 (GenBank accession number KP347127) and other plasmids reported previously (8, 9). Compared to plasmid pHNSHP45, pHSSH23-MCR1 with a size of 60,035 bp was found to lack an IS*683*-mediated fragment (Table 2 and Fig. 2). The two types of IncI2 plasmids differentiated by the presence or absence of IS*Apl1* and IS*683* are known to have been

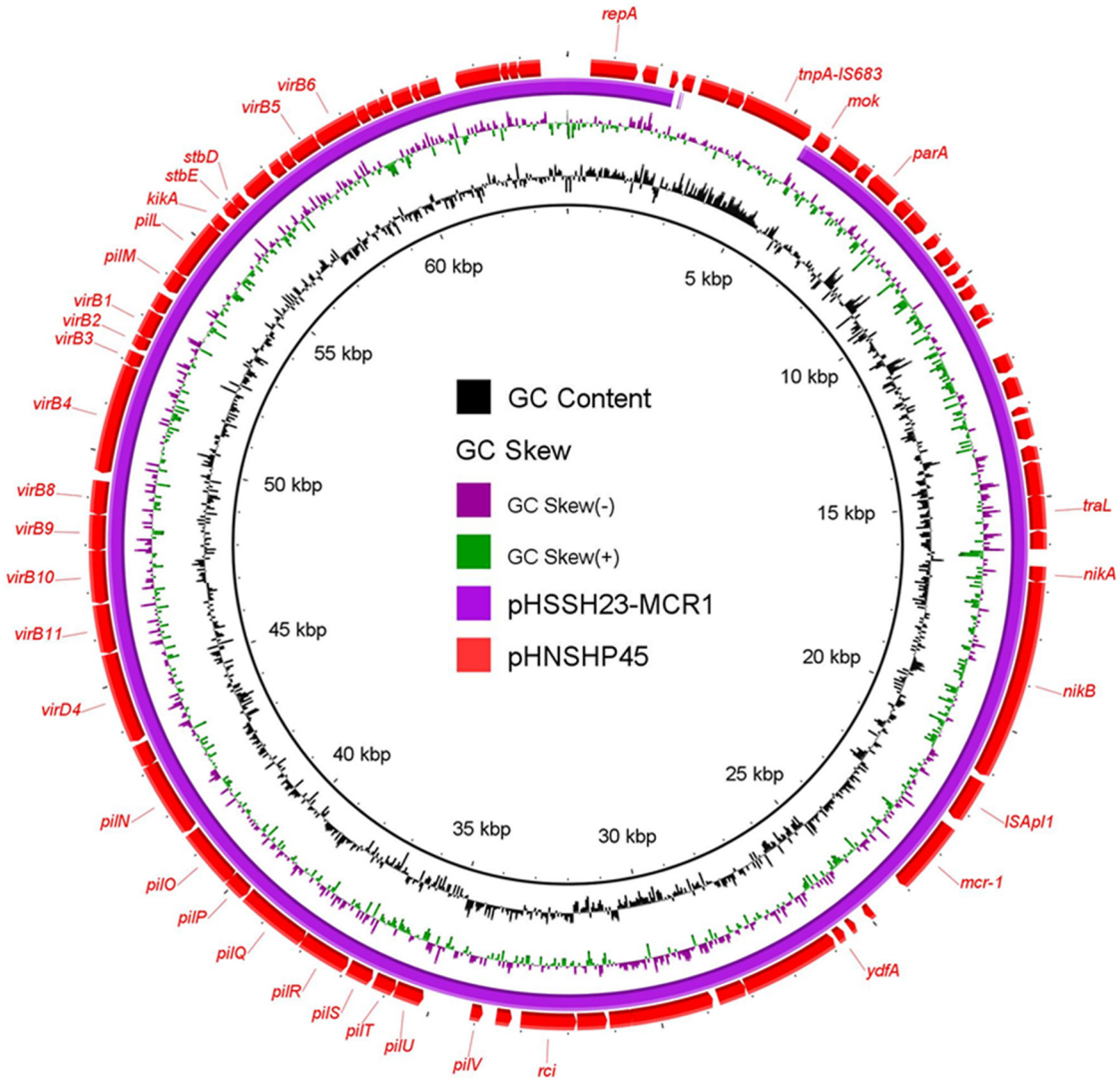

FIG 2 Alignment of one *mcr-1*-bearing IncI2 plasmid against pHNSHP45. The reference plasmid, the previously reported pHNSHP45, is indicated by red arrows. pHSSH23-MCR1, labeled in blue, was aligned to the reference plasmid using BLAST Ring Image Generator (BRIG) software. The gaps in pHSSH23-MCR1 represent missing sequences compared to the sequence of the reference plasmid.

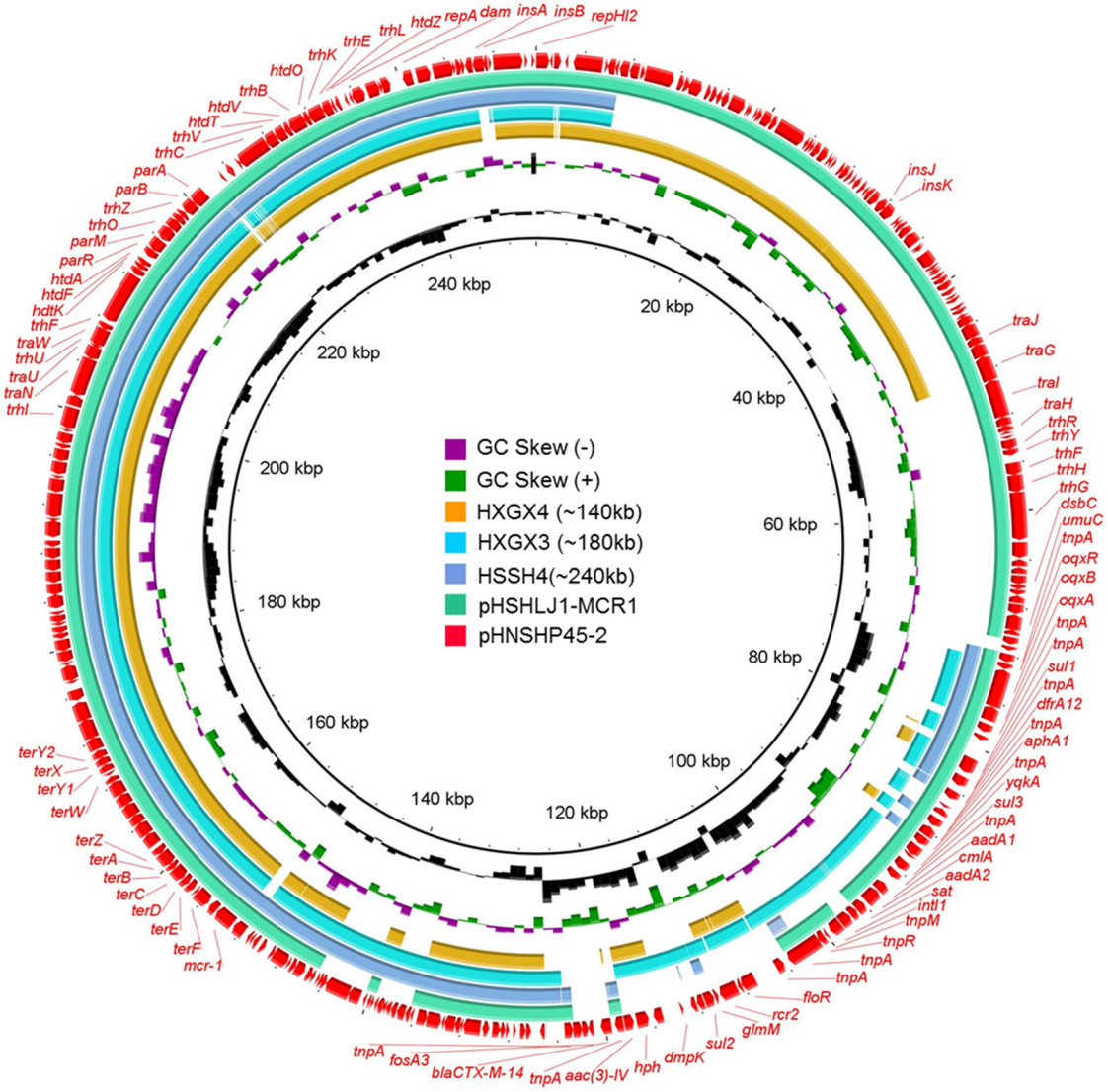

FIG 3 Alignment of conjugative and nonconjugative IncHI2 plasmids/contigs against pHNSHP45-2. The circular map was created using BLAST Ring Image Generator (BRIG) tools; the linear map was generated by EasyFig. Genes in the reference plasmid, pHNSHP45-2, which was reported previously, are labeled by red arrows. Plasmid pHSHLJ1-MCR1 and contigs of other plasmids labeled with different colors were aligned to sequence of the reference plasmid. The gaps in the plasmid sequences represent the missing sequences compared to the sequence of the reference plasmid.

disseminated worldwide and have also been recovered from *E. coli* and *Salmonella* spp. strains in the United Kingdom (8).

The complete sequence of a conjugative ~250-kb plasmid recovered from *S.* Typhimurium HSHLJ1, plasmid pHSHLJ1-MCR1 (238,539 bp), was obtained and compared to that of pHNSHP45-2; results showed that this conjugative plasmid displayed a high degree of sequence homology to pHNSHP45-2 (Fig. 3). Antimicrobial resistance gene analysis showed that pHSHLJ1-MCR1 contained some additional insertion sequences (ISs) and one *mph*(A) gene (Fig. 4). Interestingly, alignment of Illumina contigs of the nonconjugative ~240-kb plasmid recovered from the *S.* Typhimurium strain HSSH4 to pHNSHP45-2 revealed that this plasmid aligned very well with pHNSHP45-2 except that a region containing the *tra* genes, which were responsible for plasmid conjugation, was absent in the plasmid of strain HSSH4 (Fig. 3). Antimicrobial resistance gene analysis showed that plasmids from *S.* Typhimurium strain HSSH4 also lacked the mobile elements carrying $bla_{CTX-M14}$, *oqxAB*, *fosA3*, and other antimicrobial resistance genes but gained other mobile

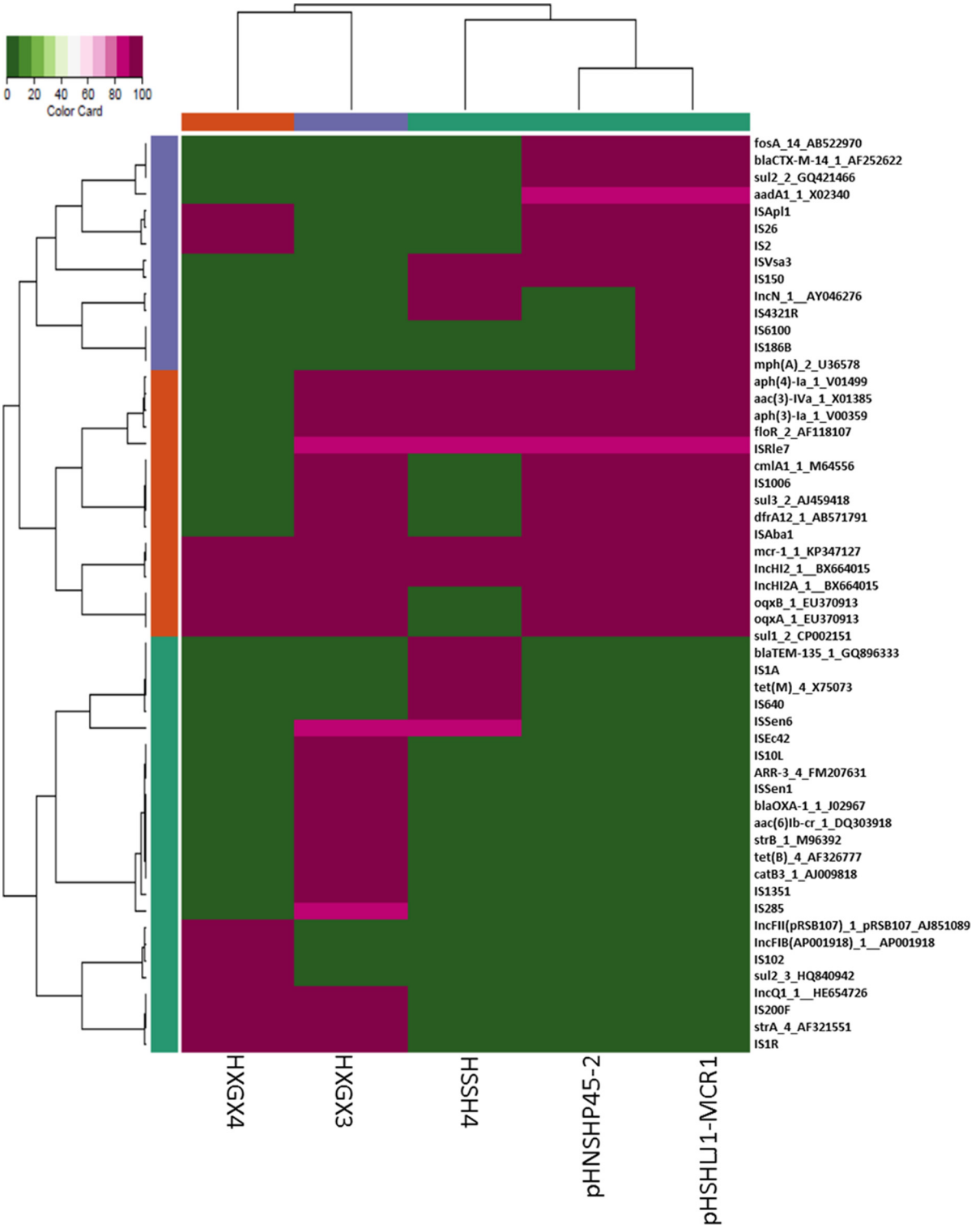

FIG 4 Antimicrobial resistance-related gene analysis for all IncHI2 plasmids of various sizes. Plasmid pHNSHP45-2 was reported previously; plasmid pHSHLJ1-MCR1, with a size of 238,539 bp, was isolated from *S.* Typhimurium strain HSHLJ1. Illumina sequencing was performed for contigs of nonconjugative plasmids of ~240 kb, ~180 kb, and ~140 kb recovered from *S.* Typhimurium strains HSSH4, HXGX3, and HXGX4, respectively.

elements carrying other antimicrobial resistance genes and transposase genes (Fig. 4). The genetic differences resulting from the absence of the *tra* region may explain why the ~240-kb plasmid of *S.* Typhimurium strain HSSH4 was nonconjugative.

Alignment of Illumina contigs of the nonconjugative ~180-kb plasmid recovered from *S.* Typhimurium strain HXGX3 to pHNSHP45-2 revealed that this plasmid aligned very well

with pHNSHP45-2 except that a large region of ca. 70 kb harboring the *tra* region was missing compared to the sequence of pHNSHP45-2. In addition, this ~180-kb plasmid also lacked the mobile element carrying $bla_{CTX-M14}$ and *fosA3* but gained a mobile element carrying bla_{OXA-1}, *aac(6′)-Ib-cr*, and *catB3*, according to antimicrobial resistance gene analysis (Fig. 3). These different genetic features, detectable among the two plasmids, depicted the evolution routes of the IncHI2 class of *mcr-1*-carrying plasmids and indicated that the loss of the ca. 70-kb *tra* region in plasmid from strain HXGX3 could contribute to the loss of transferability of this plasmid. Illumina reads of the ~140-kb plasmid recovered from *S.* Typhimurium strain HXGX4 were aligned to pHNSHP45-2. This alignment of this plasmid was very similar as the alignment of the ~180-kb plasmid from HXGX3 to pHNSHP45-2 except for the further deletions in the MDR region, but the plasmid also gained some more antimicrobial resistance genes that were not detected in other IncHI1 plasmids reported in this study (Fig. 3).

DISCUSSION

Similar to the situation in other clinical *Enterobacteriaceae* isolates, the prevalence rate of *mcr-1* in clinical *Salmonella* isolates was found to remain very low (1.4%). Interestingly, our data showed that most of the *Salmonella* strains that harbored *mcr-1*-bearing plasmids were *S.* Typhimurium, suggesting that a specific genetic background is required for acquisition and maintenance of *mcr-1*-bearing mobile elements. The close association between *S.* Typhimurium and *mcr-1*-bearing plasmids requires further investigation (8). These human clinical isolates exhibited more diverse genetic backgrounds, suggesting that clonal spread is not the major mechanism of *mcr-1* transmission in *Salmonella*.

Clinical *Salmonella* isolates also carried various conjugative plasmids reported in other *Enterobacteriaceae* so far, including a ~30-kb IncX4, ~60-kb IncI1, and ~250-kb IncHI2. Surprisingly, we detected several nonconjugative plasmids with sizes of ~140 kb, ~180 kb, and ~240 kb in these clinical *Salmonella* isolates. These plasmids all belonged to IncHI2 and were very similar to IncHI2 plasmids reported earlier. Sequence analysis showed that the inability of ~240-kb plasmids to undergo conjugation was attributed to deletion of part of the *tra* region, which is present in the conjugative ~250-kb plasmids. The ~140-kb and ~180-kb plasmids showed further deletions of the *tra* region and MDR regions while they gained some other MDR regions which were different from those of pHNSHP45-2. In fact, the IncHI2 type of plasmids without *mcr-1* have not been reported in other bacterial species so far. The backbone of the IncHI2 plasmid lacking the *mcr-1*-carrying mobile element, namely, pHXY0908 (GenBank accession number KM877269.1), has been reported only in *Salmonella*. The presence of nonconjugative plasmids of ~140 kb, ~180 kb, and ~240 kb in these *Salmonella* isolates together with the presence of a prototype of these IncHI2 plasmids in *Salmonella* may suggest that insertion of an *mcr-1*-carrying mobile element into the backbone of this type of plasmid might be responsible for one of the modes of *mcr-1* transmission in *Salmonella*.

In conclusion, results of this nationwide surveillance of the *mcr-1* carriage rate and vector structure in clinical *Salmonella* isolates provide a comprehensive understanding of the features and mechanisms of transmission of *mcr-1* in *Salmonella* and, hence, lay the foundation for future development of strategies to control the transmission of this colistin resistance determinant among Gram-negative bacterial pathogens.

MATERIALS AND METHODS

***Salmonella* strains.** *Salmonella* strains were collected from human clinical samples nationwide in China by Shanghai Municipal Center for Disease Control and Prevention, Shanghai and National Institute for Communicable Disease Control and Prevention (ICDC), and Chinese Center for Disease Control and Prevention, Beijing. All test strains were isolated in CHROMagar *Salmonella* agar (CHROMagar Company, Paris, France) and XLT4 agar (Oxoid). Suspected *Salmonella* colonies were selected for biochemical confirmation using an API 20E system (bioMérieux, Marcy l' Etoile, France), as well as via molecular identification by PCR assay targeting the *invA* gene, followed by sequencing. *Salmonella* serotyping was conducted by performing a slide agglutination test, using *Salmonella* antiserum (S&A Reagents Lab, Ltd., Bangkok, Thailand) according to the Kaufman-White scheme.

Screening of the *mcr-1* gene in *Salmonella*. *Salmonella* genomic DNA was prepared using the boiling method. PCR was performed using primers targeting *mcr-1* as reported previously (6). The genetic identity of all amplification products was confirmed by nucleotide sequencing.

Antimicrobial susceptibility tests. All *Salmonella* isolates were subjected to antimicrobial susceptibility tests by the standard agar dilution method as described by the Clinical and Laboratory Standards Institute (15, 16). Sixteen antimicrobials, as shown in Table 1, were tested. *Escherichia coli* strain ATCC 25922 was used as the quality control.

Conjugation, PFGE, S1-PFGE, and Southern hybridization. Conjugation experiments were carried out using the mixed broth method as previously described (17). PFGE, S1-PFGE, and Southern hybridization were performed as previously described (18).

Plasmid sequencing. Representative plasmids with sizes of ~30 kb, ~60 kb, ~140 kb ~180 kb, ~240 kb, and ~250 kb recovered from *Salmonella* parental strains and transconjugants were subjected to plasmid sequencing using Illumina and PacBio platforms and analyzed as previously described (19).

Accession number(s). The complete nucleotide sequences of the ~60-kb (pHSSH23-MCR1) and ~250-kb (pHSHLJ1-MCR1) plasmids were submitted to GenBank under accession numbers KX856068 and KX856066, respectively.

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We have no conflicts of interest to declare.

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