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Molecular Characterization of *Escherichia coli* Isolates Carrying *mcr-1*, *fosA3*, and Extended-Spectrum-β-Lactamase Genes from Food Samples in China

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ABSTRACT This study surveyed the prevalence of *mcr-1* in extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* strains of food origin in China and identified strains that carried *mcr-1*, *fosA3*, and ESBL genes, which were carried in various plasmids. The *mcr-1* and ESBL genes could be cotransferred by one or more types of plasmids. The presence of these multidrug-resistant *E. coli* strains in food products might pose a huge threat to public health.

KEYWORDS *mcr-1*, *fosA3*, ESBL-producing *E. coli*, transmission, circular intermediate, Tn*6330*, plasmids

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Clinical and public health problems due to multidrug-resistant (MDR) bacterial infections have been further aggravated in recent years following the emergence of bla_{NDM-1}, a resistance gene that can mediate development of carbapenem resistance in the host strain and possesses the ability to disseminate rapidly among various species of bacterial pathogens worldwide (1, 2). Polymyxins have been regarded as the antibiotic of last resort to treat severe infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE). Recently, a new plasmid-mediated colistin resistance mechanism, mediated by the MCR-1 protein, a phosphoethanolamine transferase that modifies bacterial lipid A through modification of its phosphoethanolamine moiety, was discovered (3). Since the initial discovery of *mcr-1*-positive *Enterobacteriaceae* strains in China in 2015, this resistance mechanism has been reported in various parts of the world (3, 4). However, there is still a lack of comprehensive information regarding the prevalence of *mcr-1* in *Escherichia coli* of food origin. This study reports the isolation and characterization of foodborne *E. coli* strains that carried *mcr-1*, *fosA3*, and ESBL genes.

A total of 408 nonrepeated cefotaxime-resistant *E. coli* isolates were obtained from 828 retail food samples (484 pork, 76 beef, 143 chicken, and 125 shrimp) purchased from open-air markets and supermarkets in Shenzhen, China, during the period of 10 August 2015 to 22 February 2016. *E. coli* isolates were selected on MacConkey agar plates supplemented with 2 μg/ml cefotaxime and identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Bruker MicroFlex LT mass spectrometer (Bruker Daltonics). *E. coli* isolates were further confirmed by API20E test strips (bioMérieux, Inc.). All the *E. coli* strains were subjected to antimicrobial susceptibility testing for 11 antimicrobials using the agar dilution method according to the CLSI guidelines (5). All isolates were shown to be resistant to ampicillin, cefotaxime, ceftriaxone, and sulfamethoxazole-trimethoprim. They also exhibited resistance to other antibiotics, such as tetracycline (96%), nalidixic acid (84%), chloramphenicol (86%),

TABLE 1 MICs, antimicrobial resistance genes, and plasmid profiles of *E. coli* strains and their corresponding transconjugants[d]

Strain	MIC (μg/ml) of[a]:						AMR[b] gene(s)	Plasmid(s) (kb)	*mcr-1* plasmids (kb)	bla_{CTX-M} plasmid (kb)	CI[c] form
	CLS	AMK	CTX	CIP	CRO	FOS					
14	8	4	8	0.12	>16	>512	$bla_{CTX-M-14}$, *fosA3*	~104, ~230	~230, IncHI2	~230	+
TC14	4	0.5	8	0.06	>16	<4	$bla_{CTX-M-14}$	~230	~230, IncHI2	~230	+
25	8	2	>16	>16	>16	>512	$bla_{CTX-M-55}$, *fosA3*	~33, ~60, ~104	~33, IncX4	~60	−
TC25	4	1	4	0.015	>16	<4	$bla_{CTX-M-55}$	~33, ~60	~33, IncX4	~60	−
149	8	>128	>16	>16	>16	>512	$bla_{CTX-M-55}$, *fosA3*	~33, ~60, ~90	~33, IncX4	~60	−
TC149	8	0.5	>16	0.015	>16	<4	$bla_{CTX-M-55}$	~33, ~60	~33, IncX4	~60	−
163	8	4	>16	>16	>16	>512	$bla_{CTX-M-55}$, $bla_{CTX-M-65}$, *fosA3*	~60, ~78, ~104, ~138	~60, IncI2	~60	−
TC163	8	0.5	>16	0.015	>16	<4	$bla_{CTX-M-55}$	~60	~60, IncI2	~60	−
199	8	1	>16	0.5	>16	<4	$bla_{CTX-M-14}$	~90, ~120, ~280	~280, IncHI2	~280	+
TC199	8	0.5	>16	0.015	>16	<4	$bla_{CTX-M-14}$	~280	~280, IncHI2	~280	+
328	4	2	>16	2	>16	>512	$bla_{CTX-M-55}$, *fosA3*	~33, ~90	~33, IncX4	~90	−
TC328	4	0.5	>16	0.0075	>16	<4	$bla_{CTX-M-55}$	~33, ~90	~33, IncX4	~90	−
383	8	4	>16	>16	>16	>512	$bla_{CTX-M-55}$, *fosA3*	~33, ~60, ~78, ~138, ~200	~33, IncX4	~60	−
TC383	4	0.5	>16	0.0075	>16	<4	$bla_{CTX-M-55}$	~33, ~60	~33, IncX4	~60	−
384	4	>128	>16	>16	>16	>512	$bla_{CTX-M-55}$, *fosA3*	~60, ~120	~60, IncI2	~60	−
TC384	2	0.5	>16	0.0075	>16	<4	$bla_{CTX-M-55}$	~60	~60, IncI2	~60	−
391	4	2	>16	0.5	>16	<4	$bla_{CTX-M-14}$	~33, ~104, ~250	~250, IncHI2	~250	+
TC391	4	0.5	4	0.015	>16	<4	$bla_{CTX-M-14}$	~250	~250, IncHI2	~250	+
J53	1	0.03	0.015	0.5	1	<4	−	−	−	−	−

[a]CLS, colistin; AMK, amikacin; CTX, cefotaxime; CIP, ciprofloxacin; CRO, ceftriaxone; FOS, fosfomycin.
[b]AMR, antimicrobial resistance.
[c]CI, circular intermediate.
[d]−, negative; +, positive.

kanamycin (61%), and ciprofloxacin (59%). However, all isolates were susceptible to meropenem, and only 5% of these strains were resistant to amikacin.

These *E. coli* isolates were subjected to screening for the presence of the *mcr-1* gene by PCR assay as previously described (3). A total of 109 out of 408 (27%) cefotaxime-resistant *E. coli* strains were found to harbor the *mcr-1* gene. Out of the 109 *mcr-1*-positive *E. coli* strains, 106 were resistant to colistin (MIC $\geq$ 4 μg/ml). Conjugation experiments were performed for all *mcr-1*-positive *E. coli* isolates as previously described (10) and showed that 35 out of the 109 isolates could successfully transfer their colistin resistance phenotypes to the recipient strain, *E. coli* J53. The MICs of colistin for these 35 transconjugants were mainly either 4 or 8 μg/ml, with the majority exhibiting resistance to multiple antimicrobial agents except for meropenem (Table 1). DNA linearization with S1 nuclease followed by pulsed-field gel electrophoresis (S1-PFGE) and Southern hybridization analyses was performed on these 35 *E. coli* strains and their transconjugants using the *mcr-1* probe as previously described (6), with results confirming that the *mcr-1* gene of 34 isolates was located on three major types of conjugative plasmids (~33, ~60, and ~216 to 280 kb); interestingly, some isolates were found to harbor more than one *mcr-1*-bearing plasmid.

Among these 35 strains carrying conjugative *mcr-1*-encoding plasmids, 26 strains transferred only *mcr-1*-positive plasmids, while 9 were able to transfer both *mcr-1*-positive plasmids and ESBL-gene-encoding plasmids. These 9 *E. coli* strains were further characterized. Multilocus sequence typing (MLST) was performed for all these strains, and the results showed that strains 199 and 383 belonged to sequence type 10 (ST10), strain 25 belonged to ST641, and strain 328 belonged to ST4015, whereas the remaining 5 strains belonged to new different STs, suggesting the genetic diversity of these *E. coli* strains. β-Lactamase genes were screened in these 9 isolates and their transconjugants as previously described (7). The results showed that 6 out of 9 transconjugants were positive for the $bla_{CTX-M-1}$ group gene and that the other three were positive for the $bla_{CTX-M-9}$ group gene. Further sequencing of the full length of these β-lactamase genes confirmed that all $bla_{CTX-M-1}$ group genes were $bla_{CTX-M-55}$, with strain 149 also carrying a $bla_{CTX-M-65}$, whereas the $bla_{CTX-M-9}$ group genes were $bla_{CTX-M-14}$. For

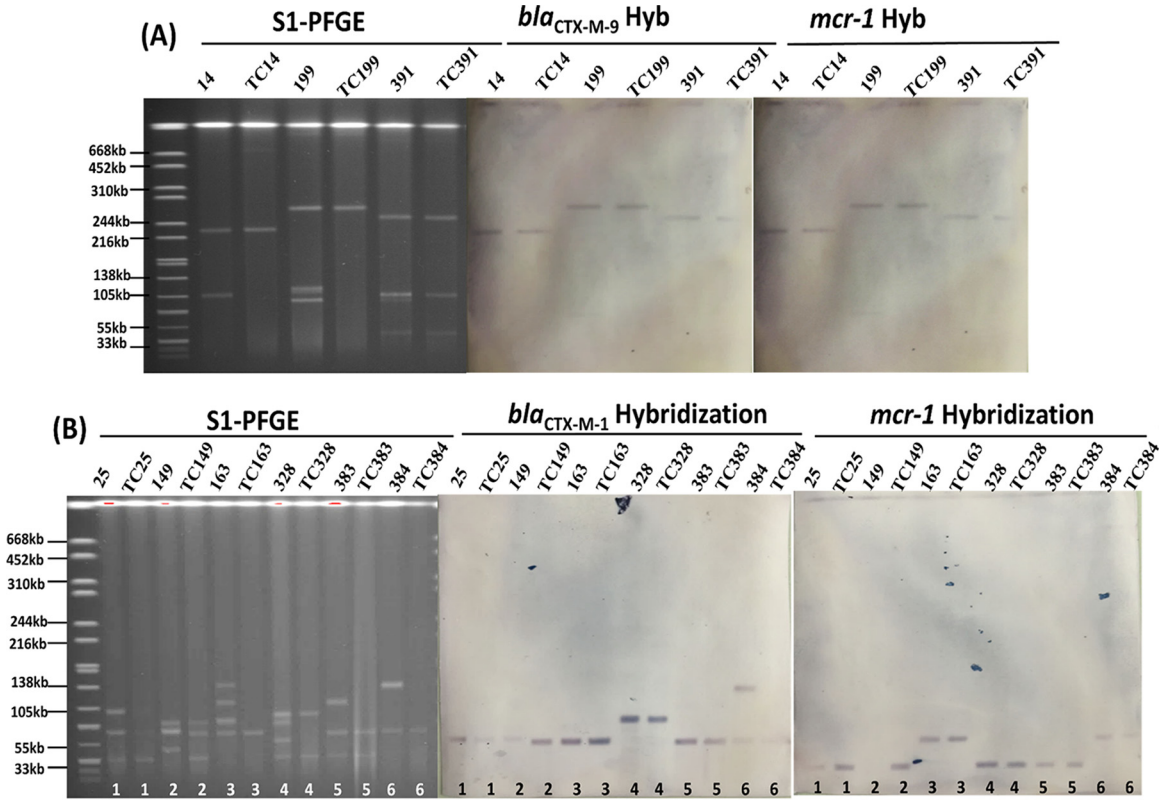

FIG 1 S1-PFGE and Southern hybridization analyses of *E. coli* strains and their corresponding transconjugants carrying the *mcr-1* and various bla_{CTX-M} genes.

$bla_{CTX-M-55}$-positive transconjugants, the sizes of plasmids observed in these strains were ~60 and ~90 kb, with five plasmids being ~60 kb (Table 1, Fig. 1). Plasmids harboring the *mcr-1* gene were two sizes, ~33 and ~60 kb. Isolates 163 and 384 were found to harbor plasmids of ~60 kb, which carried the *mcr-1* and $bla_{CTX-M-55}$ genes. For *E. coli* strains carrying the $bla_{CTX-M-14}$ gene, the $bla_{CTX-M-14}$ and *mcr-1* genes were found to be located in plasmids of ~230, ~250, and ~280 kb (Table 1, Fig. 1).

Recently, renewed attention has been paid to fosfomycin for the treatment of both urinary and systemic infections due to rapid dissemination of multidrug-resistant Gram-negative bacteria, especially strains of *Enterobacteriaceae* species that are resistant to traditionally used agents (8). Compared to other agents, fosfomycin seems to have retained antimicrobial activity against a substantial percentage of clinical isolates, particularly *E. coli* isolates. Therefore, the presence of a fosfomycin-resistant determinant commonly detected in China, the *fosA3* gene, was screened for in these 9 *E. coli* isolates and their transconjugants as previously described. Surprisingly, 7 out of the 9 *E. coli* strains harbored the *fosA3* gene, which was not cotransferred with the *mcr-1* and ESBL genes. Plasmid profile analysis of both parental and transconjugants of these 9 *E. coli* strains suggests that the *fosA3* gene might be located on additional plasmids of various sizes, with plasmids of ~104 kb being the most dominant. Further research will be needed to understand more about the genetic features of the *fosA3* gene in these *E. coli strains*.

The total genomic DNA from the 9 transconjugants carrying the *mcr-1* and bla_{CTX-Ms} genes was extracted and sequenced using the Illumina platform as previously described (6). For the 6 *E. coli* transconjugants carrying $bla_{CTX-M-55}$, two types of *mcr-1*-bearing plasmids were detected, the ~30-kb IncX4 type and the ~60-kb IncI2 type. Alignment of Illumina contigs of the four ~30-kb plasmids harbored by *E. coli* strains TC25, TC149, TC383, and TC328 to several previously reported plasmids showed that they aligned very well to pOW3E1 (KX129783.1) (>99% in both identity and coverage;

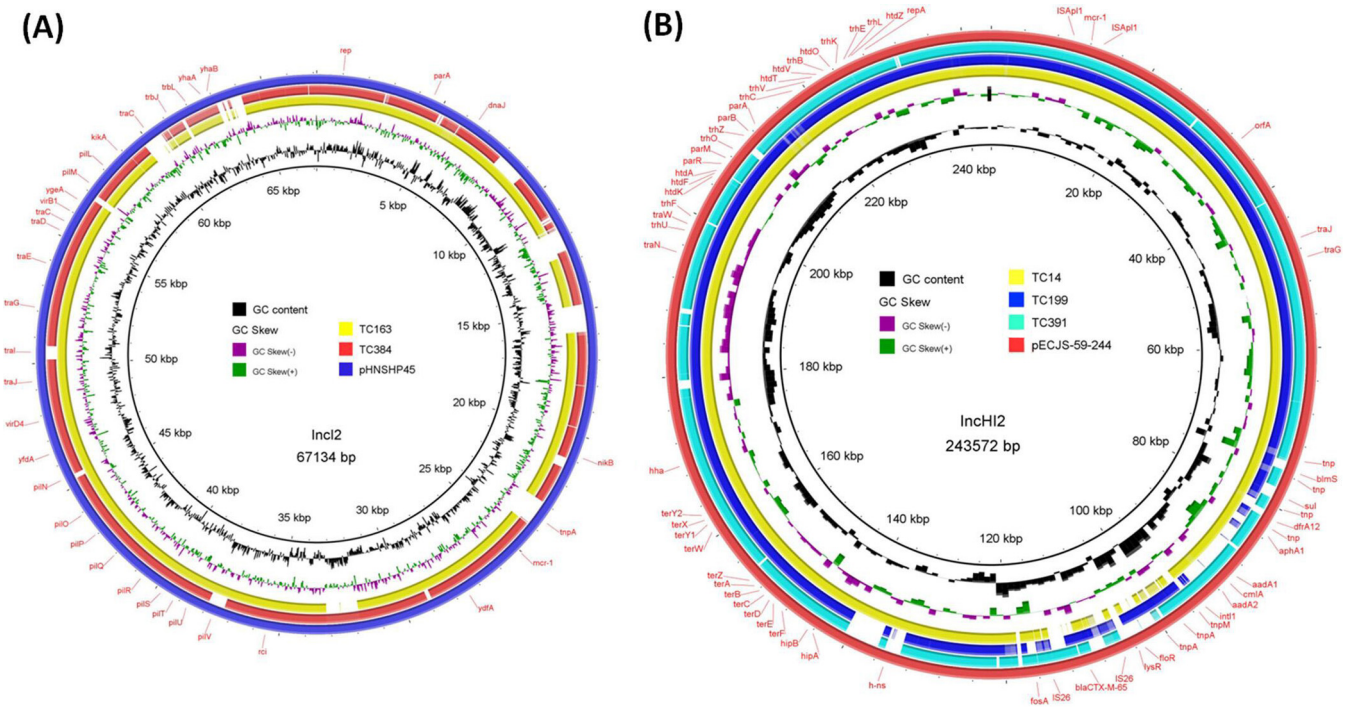

FIG 2 Sequence alignment of IncI2- and IncHI2-type *mcr-1*-bearing plasmids. (A) The plasmid pA31-12 (KX034083) was used as a reference to compare with two *mcr-1*- and $bla_{CTX\text{-}M\text{-}55}$-bearing plasmids which possess the IncI2 replicon. The outer circle with red arrows signifies annotation of the reference sequence. Gaps in the circle refer to plasmid regions which are missing compared to the reference. (B) The plasmid pECJS-59-244 (KX084394.1) was used as reference to compare with three *mcr-1*- and $bla_{CTX\text{-}M\text{-}14}$-positive strains. The outer circle with red arrows denotes annotation of the reference plasmid. The three IncHI2 strains (TC14, TC199, and TC391) exhibited sequence similarity with the reference, but alignment was not successful because of the low overall sequence homology. The number of IS*Apl1* repeats is not depicted. One MDR region was observable in the backbone of all IncHI2 plasmids.

data not shown). Two transconjugants, TC163 and TC384, carried an ~60-kb IncI2-type plasmid that carried the *mcr-1* and $bla_{CTX\text{-}M\text{-}55}$ genes. Illumina contigs of these two plasmids aligned well to IncI2 plasmid pA31-12 (KX034083), suggesting that these two plasmids were highly homologous to pA31-12 (9). Illumina contigs of plasmids carrying both *mcr-1* and $bla_{CTX\text{-}M\text{-}14}$ from 3 *E. coli* transconjugants, TC14, TC199, and TC391, exhibited a high degree of sequence homology with pECJS-59-244 (KX084394.1) and harbored a large MDR region which rendered IncHI2 the most genetically diverse plasmid type (Fig. 2). All three IncHI2 plasmids were found to harbor IS*Apl1*–*mcr-1*–*orf*–IS*Apl1*, the intact composite transposon Tn*6330* (6). Tn*6330* is known to form a circular intermediate comprising two direct repeats of IS*Apl1* and the *mcr-1* gene, which may facilitate the transmission of *mcr-1* in different plasmid backbones (10). All 9 transconjugants were then screened for the presence of the circular intermediate as previously described (10). Consistent with previous reports, circular intermediates could be detected only in *E. coli* strains that carried IncHI2 plasmids carrying intact Tn*6330*, not in strains carrying IncX4 or IncI2 plasmids. This confirmed that the circular intermediate can be generated only when the *mcr-1-orf* gene cassette is surrounded by two copies of IS*Apl1* to form intact Tn*6330*.

This study reports the comprehensive surveillance of *mcr-1* in ESBL-producing *E. coli* strains isolated from food products in Shenzhen, China. Our data show that *mcr-1* was very common in ESBL-producing *E. coli* strains from various food products, with some strains carrying *mcr-1*, *fosA3*, and ESBL genes. These *E. coli* isolates may pose a huge threat to public health and thus warrant further investigation.

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We declare no conflicts of interest.

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