



Transmission and stable inheritance of carbapenemase gene (*bla*_{KPC-2} or *bla*_{NDM-1})-encoding and *mcr-1*-encoding plasmids in clinical Enterobacteriaceae strains

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ABSTRACT

Objectives: The aim of this study was to investigate the potential for transmission and heritability of carbapenemase gene (*bla*_{KPC-2} or *bla*_{NDM-1})-encoding or *mcr-1*-encoding plasmids in clinical Enterobacteriaceae strains.

Methods: Potential for transmission of carbapenemase gene (*bla*_{KPC-2} or *bla*_{NDM-1})-encoding or *mcr-1*-encoding plasmids in clinical Enterobacteriaceae strains was tested in three conjugation models, namely filter-mating conjugation in laboratory conditions, a meat product model and the gastrointestinal (GI) tract of rats. Plasmid stability in Enterobacteriaceae strains was also determined.

Results: We demonstrated that plasmids carrying a carbapenemase gene (*bla*_{KPC-2} or *bla*_{NDM-1}) could be efficiently conjugated to strains carrying the *mcr-1* gene and vice versa, and that these plasmids could stably co-exist in clinical Enterobacteriaceae strains. These findings suggest that Enterobacteriaceae can readily acquire phenotypic resistance to both carbapenems and colistin in natural environments such as food products and the GI tract of human and animals.

Conclusion: Gene transfer events are common among members of the Enterobacteriaceae and serve as a key mechanism facilitating adaptation to new environments. Development of innovative strategies and surveillance measures to curtail the dissemination of multidrug resistance plasmids is necessary. Transmission and stable inheritance of these two types of plasmids would lead to the emergence of multidrug-resistant pathogens that are resistant to all currently available last-line antibiotics for Gram-negative bacterial infections.

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1. Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) strains, an important group of bacterial pathogens, have become an increasingly common causative agent of hospital infections. Production of *Klebsiella pneumoniae* carbapenemase (KPC) or New Delhi metallo- β -lactamases (NDM) is a major mechanism of carbapenem resistance in CRE [1]. The *bla*_{KPC} gene is known to be harboured by plasmids of various types, such as IncI2, IncX, IncR and ColE1 plasmids,

whereas *bla*_{NDM-1} is mainly located on plasmids of IncX3 type [2–4]. Global dissemination of KPC-producing and NDM-producing Enterobacteriaceae and the emergence of mobile genetic elements (MGEs) that possess the ability to disseminate rapidly among a broad range of species have rendered KPC- and NDM-producing organisms a worldwide public-health threat in recent years.

Colistin has been regarded as an antibiotic of last resort for the treatment of serious infections caused by CRE [5,6]. However, discovery of the conjugative plasmid-mediated *mcr-1* gene implied the possibility of horizontal transfer of colistin resistance [5,7]. Until now, several reports have documented carriage of the *mcr-1* gene on plasmids of IncX4, IncI2, IncHI2, IncFII and IncP replicon

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types in different species of Enterobacteriaceae. Importantly, such plasmids were found to exhibit high transmission rates among various pathogenic strains [8,9]. Emergence of the plasmid-borne *mcr-1* gene has seriously limited future therapeutic options against CRE and prompted researchers to investigate the underlying mechanisms of transmission of such colistin resistance determinants.

Horizontal gene transfer (HGT) is a key mechanism by which bacteria evolve to become resistant to antibiotics through acquisition of novel resistance genes [10]. In the present study, we studied the transferability of plasmids carrying a carbapenemase gene (*bla_{KPC-2}* or *bla_{NDM-1}*) or the *mcr-1* gene among clinical Enterobacteriaceae strains in various models in order to investigate the potential for transmission and heritability of these plasmids to converge in one bacterial host to produce a multidrug-resistant organism.

2. Materials and methods

2.1. Bacterial isolates

A total of seven different types of *bla_{KPC-2}*-bearing plasmids of various sizes and four different types of *bla_{NDM-1}*-bearing plasmids, which were recovered from clinical CRE strains from our previous national surveillance study [11], were selected for this study after confirmation by PCR [12] and NextSeq 500 (Illumina Inc., San Diego, CA, USA) and MinION (Oxford Nanopore Technologies, Oxford, UK) sequencing (Supplementary Table S1). Three major types of conjugative plasmids carrying the *mcr-1* gene, namely an ~33-kb IncX4 plasmid, an ~66-kb IncI2 plasmid and an ~250-kb IncHI2 plasmid, all recovered from *Escherichia coli* strains isolated from food samples, were also tested (Supplementary Table S1) [7,13,14].

2.2. In vitro filter-mating experiment

An in vitro filter-mating assay to assess the HGT potential of plasmids was performed at 37°C for 16 h as previously described [14] with modifications. Eleven different types of plasmids harbouring carbapenemase genes (*bla_{NDM-1}* or *bla_{KPC-2}*) and three types of plasmids carrying the *mcr-1* gene were involved in the pairwise conjugation experiment (Supplementary Table S1). The pairwise combinations of donor and recipient strain used in the filter-mating experiment are listed in Table 1. Strains (1×10^9 CFU) were mixed in a donor:recipient ratio of 1:1 and transconjugants were selected using China Blue agar containing colistin (2 µg/mL) and meropenem (1 µg/mL). Controls were also performed by plating recipient and donor cells on China Blue agar containing single and double antibiotics. All transconjugants were verified for the presence of the *mcr-1* and carbapenemase (*bla_{NDM-1}* or *bla_{KPC-2}*) genes by PCR using primers as previously described [5,12]. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method based on Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. All conjugation experiments were repeated at least twice, and the conjugation frequencies of strains in three conjugation models were calculated by the following equation [5]:

$$\text{Conjugation frequency} = \frac{T}{D + R} \times 100\%$$

where *T*, *D* and *R* denote the densities of transconjugants, donors and recipients, respectively.

2.3. Pulsed-field gel electrophoresis (PFGE) and Southern hybridisation

Transconjugants co-harboring the *mcr-1* and carbapenemase (*bla_{KPC-2}* or *bla_{NDM-1}*) genes were subjected to further characteri-

sation by *Xba*I-PFGE as previously described to identify the donor and receptor strains in all three models in this study [7]. *S*1-PFGE and Southern hybridisation were also performed as previously described to confirm the transmission of plasmids [7]. Examples are shown in Supplementary Figs S1 and S2.

2.4. In vitro conjugation on meat product at different temperatures

Based on the results of the filter-mating conjugation experiment, *bla_{NDM-1}*-bearing NBJE55 and *bla_{KPC-2}*-bearing K84 CRE strains and three *mcr-1*-positive *E. coli* strains (M25, M163 and M14) were selected and conjugated with each other on food samples in vitro. Pork samples were purchased from a supermarket in Shenzhen and were sterily cut into cubes with a size of approximately 6 cm × 6 cm × 0.8 cm. Bacterial suspensions ($100 \mu\text{L}$ with 1×10^9 CFU) were pipetted onto the surface of pork samples at a ratio of 1:1 and were mixed well. Petri dishes were then gently rotated to make sure that the bacterial suspensions covered the whole surface of the pork samples. To see whether the plasmids carrying a carbapenemase gene (*bla_{KPC-2}* or *bla_{NDM-1}*) and those carrying the *mcr-1* gene could be conjugated between bacteria at a storage temperature of 4°C, ambient temperature of 25°C or optimum growth temperature of 37°C, each piece of pork placed in a culture dish was incubated at the respective temperatures. Following overnight incubation, pork samples were cut into small pieces under sterile conditions and were re-suspended in 50 mL of sterile saline. Transconjugants were selected as described above. Single colonies were picked and identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF), and carriage of plasmids was confirmed by PCR [5,12], *S*1-PFGE and Southern hybridisation [14]. Control experiments were conducted and the conjugation frequencies of strains were measured as mentioned above. Three replicates were used for meat product experiments.

2.5. In vivo transconjugation in the rat gastrointestinal (GI) tract

Animals were purchased from Guangdong Medical Laboratory Animal Center and were allowed free access to food and water. They were examined twice daily for any clinical signs such as behaviour, GI function, respiratory distress, food and water intake, etc. The experimental protocol was approved by the Research Animal Care and Use Committee of the Hong Kong Polytechnic University. Briefly, bacteria that grew to a dose of 10^9 CFU were centrifuged at 4°C and were then resuspended with sterile saline. Sterile saline solution of donor and recipient strains for each pairwise was mixed and immediately inoculated into the GI tract of a rat. Thirty specific pathogen-free (SPF) male Sprague–Dawley (SD) rats (11–15 weeks old) were randomly divided into five groups after a background check: one group was infected intragastrically with strains K84 and M14, one group was infected with strains K84 and M163, one group was infected with strains NBJE55 and M14, one group was infected with strains NBJE55 and M163, and the remaining group was treated with sterile saline as a control. Each of one group was infected intragastrically at 0, 24 and 72 h with 1×10^9 CFU of a mixture of CRE and *mcr-1*-bearing strain pairs and one with saline as control. Fresh faeces (500–1000 mg) was collected after 24 h of inoculation at three time points (24, 48 and 96 h). Faecal samples were diluted 10-fold in sterile saline and re-suspended. Then, $100 \mu\text{L}$ of the suspension was plated on China Blue agar without antibiotics and China Blue agar containing colistin (2 µg/mL) and meropenem (1 µg/mL), respectively. The conjugation frequency of strains was measured as described above. Three replicates were used for rat GI tract experiments.

Table 1

Genetic characteristics of transconjugants obtained by the in vitro filter-mating method

Conjugation between bacterial strains*		Transconjugants**	MIC ($\mu\text{g/mL}$)		Plasmid frequency†	Conjugation frequency†
			COL	MEM		
M14/M25/M163	K26	K26-M25	8	≥ 4	$2.3 \times 10^{-2} \pm 1.7 \times 10^{-3}$ (fg)	$2.2 \times 10^{-3} \pm 1.5 \times 10^{-4}$ (de)
		M14-K26	8	2	$8.0 \times 10^{-2} \pm 3.1 \times 10^{-3}$ (ef)	$7.7 \times 10^{-4} \pm 5.0 \times 10^{-5}$ (e)
	K2	M25-K2	8	≥ 4	$5.0 \times 10^{-2} \pm 7.2 \times 10^{-3}$ (fg)	$5.1 \times 10^{-3} \pm 3.1 \times 10^{-4}$ (bc)
		M163-K2	16	≥ 4	$1.1 \times 10^{-2} \pm 1.0 \times 10^{-3}$ (g)	$1.2 \times 10^{-3} \pm 1.5 \times 10^{-4}$ (e)
	K4	M25-K4	8	≥ 4	$8.7 \times 10^{-3} \pm 3.5 \times 10^{-5}$ (g)	$8.6 \times 10^{-4} \pm 6.6 \times 10^{-5}$ (e)
		M163-K4	8	2	$9.6 \times 10^{-2} \pm 5.3 \times 10^{-3}$ (e)	$1.2 \times 10^{-3} \pm 2.1 \times 10^{-4}$ (e)
	K5	K5-M25	8	2	$3.0 \times 10^{-2} \pm 3.1 \times 10^{-3}$ (fg)	$2.7 \times 10^{-3} \pm 2.0 \times 10^{-4}$ (d)
		K5-M163	16	≥ 4	$2.0 \times 10^{-2} \pm 2.8 \times 10^{-3}$ (g)	$1.7 \times 10^{-3} \pm 4.0 \times 10^{-4}$ (de)
	K5-M14		16	≥ 4	$2.1 \times 10^{-2} \pm 3.9 \times 10^{-3}$ (g)	$1.7 \times 10^{-3} \pm 4.0 \times 10^{-4}$ (de)
	K39	M25-K39	16	2	$1.6 \times 10^{-1} \pm 6.9 \times 10^{-3}$ (d)	$1.6 \times 10^{-3} \pm 5.0 \times 10^{-4}$ (de)
		M163-K39	4	≥ 4	$4.2 \times 10^{-3} \pm 3.7 \times 10^{-3}$ (g)	$2.2 \times 10^{-3} \pm 3.5 \times 10^{-4}$ (de)
	M14-K39		4	≥ 4	$2.9 \times 10^{-1} \pm 2.0 \times 10^{-2}$ (c)	$3.0 \times 10^{-3} \pm 1.5 \times 10^{-4}$ (cd)
	K84	K84-M25	4	≥ 4	$3.8 \times 10^{-2} \pm 4.5 \times 10^{-2}$ (fg)	$4.0 \times 10^{-3} \pm 2.7 \times 10^{-3}$ (c)
		K84-M163	4	≥ 4	$3.7 \times 10^{-2} \pm 3.6 \times 10^{-3}$ (fg)	$4.3 \times 10^{-3} \pm 7.0 \times 10^{-4}$ (bc)
	K84-M14		8	≥ 4	$4.7 \times 10^{-2} \pm 3.7 \times 10^{-2}$ (fg)	$4.7 \times 10^{-3} \pm 5.0 \times 10^{-4}$ (bc)
	K7	K7-M25	4	≥ 4	$5.5 \times 10^{-3} \pm 9.9 \times 10^{-4}$ (g)	$4.2 \times 10^{-3} \pm 3.1 \times 10^{-4}$ (bc)
		K7-M163	4	≥ 4	$9.7 \times 10^{-3} \pm 2.4 \times 10^{-3}$ (g)	$6.7 \times 10^{-3} \pm 5.7 \times 10^{-4}$ (a)
	K7-M14		4	≥ 4	$1.3 \times 10^{-1} \pm 1.8 \times 10^{-2}$ (d)	$1.5 \times 10^{-3} \pm 5.1 \times 10^{-4}$ (e)
	NBJE55	M25-NBJE55	8	≥ 4	$4.8 \times 10^{-2} \pm 2.6 \times 10^{-2}$ (fg)	$1.8 \times 10^{-3} \pm 9.4 \times 10^{-4}$ (de)
		M163-NBJE55	4	≥ 4	$4.7 \times 10^{-2} \pm 7.6 \times 10^{-3}$ (fg)	$1.9 \times 10^{-3} \pm 1.7 \times 10^{-3}$ (de)
	NBJE55-M14		4	≥ 4	$5.4 \times 10^{-2} \pm 4.1 \times 10^{-2}$ (f)	$1.7 \times 10^{-3} \pm 2.0 \times 10^{-4}$ (de)
	NJXR7	NJXR7-M25	8	≥ 4	$1.0 \times 10^{-2} \pm 9.7 \times 10^{-4}$ (g)	$3.1 \times 10^{-3} \pm 2.0 \times 10^{-4}$ (cd)
		NJXR7-M163	8	≥ 4	$5.1 \times 10^{-3} \pm 5.6 \times 10^{-4}$ (g)	$4.9 \times 10^{-3} \pm 2.0 \times 10^{-4}$ (bc)
	NJXR7-M14		8	≥ 4	$3.3 \times 10^{-1} \pm 2.0 \times 10^{-2}$ (b)	$2.7 \times 10^{-3} \pm 5.6 \times 10^{-4}$ (d)
	NCQ17	M25-NCQ17	4	≥ 4	$2.7 \times 10^{-2} \pm 2.8 \times 10^{-3}$ (fg)	$4.6 \times 10^{-3} \pm 3.1 \times 10^{-4}$ (bc)
		M163-NCQ17	4	≥ 4	$3.5 \times 10^{-1} \pm 5.5 \times 10^{-2}$ (b)	$2.9 \times 10^{-3} \pm 6.4 \times 10^{-4}$ (cd)
	NCQ17-M14		4	≥ 4	$4.9 \times 10^{-1} \pm 2.8 \times 10^{-2}$ (a)	$3.9 \times 10^{-3} \pm 2.1 \times 10^{-4}$ (c)
	NCQ10	NCQ10-M25	4	≥ 4	$4.8 \times 10^{-1} \pm 3.6 \times 10^{-2}$ (a)	$5.3 \times 10^{-3} \pm 3.6 \times 10^{-4}$ (b)
		NCQ10-M163	4	≥ 4	$7.5 \times 10^{-2} \pm 1.7 \times 10^{-3}$ (ef)	$7.8 \times 10^{-3} \pm 1.2 \times 10^{-4}$ (a)
	NCQ10-M14		8	≥ 4	$2.6 \times 10^{-2} \pm 5.3 \times 10^{-4}$ (fg)	$2.6 \times 10^{-3} \pm 5.0 \times 10^{-4}$ (de)

MIC, minimum inhibitory concentration; COL, colistin; MEM, meropenem.

* Strains carrying the *bla*_{KPC-2}, *bla*_{NDM-1} and *mcr-1* genes are denoted by the initials 'K', 'N' and 'M', respectively. Detailed genetic information of the test strains is provided in Supplementary Table S1.** First strain name represents the donor strain and second strain name represents the recipient strain, which was confirmed by *Xba*I-PFGE.† Frequencies are the mean $\pm$ standard deviation of triplicate analyses. Values followed by different letters in parentheses in the same column are significantly different ($P < 0.05$).

2.6. Confirmation of plasmid stability

A single colony of each transconjugant obtained in different models was cultured overnight in Luria–Bertani (LB) broth without antibiotics. The overnight culture was then diluted at 1:100 in 10 mL of fresh LB broth. Dilution and serial passage was conducted every 24 h for 20 consecutive days. Culture collected on the 1st, 5th, 10th, 15th and 20th passage was diluted to 10^{-2} , 10^{-4} and 10^{-6} after a 10-fold serial dilution, respectively, and then spread onto three China Blue agar plates with and without colistin (2 µg/mL) and meropenem (1 µg/mL). CFU from plates containing antibiotics were considered as plasmid-positive colonies, while CFU from antibiotic-free plates were considered as the total population in the culture. The plasmid frequency was determined by dividing plasmid-positive CFU by the total population CFU and was representative of plasmid stability of passaged transconjugants of each type. A higher plasmid frequency suggested higher stability of the plasmid in the host.

2.7. Statistical analysis

All analyses were performed in triplicate. Experimental data are expressed as the mean $\pm$ standard deviation. Statistical analysis of all data was performed using IBM SPSS Statistics v.25.0 (IBM Corp, Armonk, NY, USA). Analysis of variance (ANOVA) and Duncan's multiple range test were used to determine significant differences ($P < 0.05$).

3. Results

3.1. Carbapenemase gene (*bla*_{KPC-2} or *bla*_{NDM-1})-bearing plasmids and *mcr-1*-bearing plasmids are compatible with each other in Enterobacteriaceae

Klebsiella pneumoniae and *E. coli* strains carrying seven and four *bla*_{KPC-2}- and *bla*_{NDM-1}-bearing plasmids, respectively, were subjected to two-way in vitro conjugation analysis with *E. coli* strains carrying three different types of *mcr-1*-bearing plasmids. These plasmids were all of the different types of plasmids that we have in our laboratory after the Illumina NextSeq 500 and MinION sequencing confirmation and they represented the most common MGEs that harbour carbapenemase genes and the *mcr-1* gene. Except for three pairs of strains (K26/M163, K2/M14 and K4/M14) for which conjugation was not successful, all plasmids carrying a carbapenemase gene in these Enterobacteriaceae strains could undergo conjugation with strains harbouring three types of *mcr-1*-bearing plasmids to form transconjugants that co-harboured *bla*_{KPC-2}/*mcr-1* or *bla*_{NDM-1}/*mcr-1* plasmids (Table 1). To determine the genetic identity of donor and recipient strains of these transconjugants, one transconjugant collected from each pair of strains in the conjugation experiment was selected for cluster analysis by *Xba*I-PFGE. Our data revealed that plasmids carrying a carbapenemase gene could be transferred to strains carrying an *mcr-1*-bearing plasmid, and vice versa (Table 1; Supplementary Fig. S1). The conjugation frequency of plasmids harboured by these strains was determined to be ~ 8 to 80×10^{-4} , and significant differences ($P < 0.05$) were observed in different transconjugants. Co-carriage of these plasmids in each transconjugant was confirmed by *S*1-PFGE and Southern hybridisation analysis (Supplementary Fig. S2). The stability study revealed that plasmids acquired by the test transconjugants collected from the in vitro filter-mating model remained stable after serial passage, confirming that plasmids carrying a carbapenemase gene could indeed be stably transferred between clinical CRE strains and foodborne *mcr-1*-bearing *E. coli* strains (Table 1). Overall, the plasmid frequency, which reflects the stability of plasmids in transconjugants after

serial passage of transconjugants without antibiotic pressure, was found to be significantly different ($P < 0.05$). However, there was no significant difference ($P < 0.05$) in plasmid and conjugation frequency of K5-M25/M163/M14, K84-M25/M163/M14, M25/M163-NBJE55 and NBJE55-M14, indicating a relatively stable transmission between *bla*_{NDM}/*bla*_{KPC}-harbouring plasmids of K5, K84 and NBJE55 and *mcr-1*-bearing plasmids (Table 1).

3.2. Plasmid transmission in food model

To investigate the transmission of plasmids carrying the carbapenemase gene and plasmids bearing the *mcr-1* gene in the natural environment, the efficiency of plasmid transmission among bacterial strains located in meat samples was tested. The *bla*_{NDM}-bearing strain NBJE55 and *bla*_{KPC-2}-bearing strain K84 and the *mcr-1*-bearing strains M25, M14 and M163 were selected to investigate plasmid transmission in a food model for showing good growth, high conjugation frequency and plasmid stability under in vitro conjugation experiments. After the confirmation of PCR and *Xba*I-PFGE, it was shown that the *bla*_{KPC-2}-bearing plasmid from K84 could be conjugated to M25 and M14 at 37°C and to M163 at 25°C and that the *mcr-1*-bearing plasmid from M14 could be conjugated to K84 at 25°C. No conjugation between K84 and *mcr-1*-bearing plasmid *E. coli* strains was observed at 4°C. On the other hand, the *mcr-1*-bearing plasmid from M25 could be conjugated to NBJE55 at 25°C and the *mcr-1*-bearing plasmid from M163 could be conjugated to NBJE55 at all three temperatures tested. In addition, the *mcr-1*-bearing plasmid from M14 and the *bla*_{NDM-1}-bearing plasmid from NBJE55 could be conjugated to each other at 37°C and 25°C, respectively (Table 2). These findings indicated that most of the plasmids harbouring carbapenemase genes in this study could be transmitted to strains carrying an *mcr-1*-bearing plasmid, and vice versa, and that plasmids carrying carbapenemase genes can co-exist with plasmids carrying the *mcr-1* gene in a single bacterial host at 25°C. Conjugation efficiencies were in the range of $\sim 10^{-1}$ to 10^{-5} . Interestingly, the conjugation frequency of 25-M14-K84 and 25-M14-NBJE55 was significantly higher than other transconjugants, suggesting that temperature played an important role in the efficiency of plasmid transmission. Moreover, transmission of transconjugants remained stable after serial passage, confirming that plasmids harbouring carbapenemase genes (*bla*_{KPC-2} or *bla*_{NDM-1}) and those carrying the *mcr-1* gene could stably co-exist in Enterobacteriaceae strains (Table 2).

3.3. Plasmid transmission in the rat gastrointestinal (GI) tract

To gain further understanding of the efficiency of plasmid transmission in the GI tract, a preeminent incubator for development of antimicrobial-resistant organisms and a perfect place for plasmid transmission under various selection pressures, a SD rat GI tract transmission model was established. Strains K84 and NBJE55 were subjected to two-way conjugation analysis in the rat GI tract with M163 and M14 in this study. All rats used in this experiment were checked for the presence of bacteria carrying *bla*_{KPC-2}-, *bla*_{NDM-1}- or *mcr-1*-bearing plasmids, with the results showing that all animals were clear of these antimicrobial resistance genes. After 2 days of background check, SD rats were co-infected with four different sets of bacterial strains, namely K84-M14, K84-M163, NBJE55-M14 and NBJE55-M163, by oral gavage with culture of a bacterial concentration of $\sim 10^9$ CFU/mL at 24, 48 and 96 h, respectively (Supplementary Table S2). Following confirmation by PCR, *Xba*I-PFGE, *S*1-PFGE and Southern hybridisation analysis, our results showed that the *bla*_{KPC-2}-bearing plasmid from strain K84 could be conjugated to strain M14 at 24, 48 and 96 h post-inoculation; it could also be conjugated to strain M163 at 24 h and 48 h post-inoculation, with the *bla*_{KPC-2}-bearing strain serving as the donor strain. Likewise,

Table 2

Genetic characteristics of transconjugants obtained from the meat-based plasmid transmission model

Conjugation between bacterial strains*		Conjugation T_m and status**			Transconjugants†	Plasmid frequency††	Conjugation frequency††
		37°C	25°C	4°C			
K84	M25	✓			37-K84-M25	$2.3 \times 10^{-3} \pm 7.4 \times 10^{-4}$ (h)	$1.4 \times 10^{-5} \pm 5.6 \times 10^{-7}$ (c)
	M163		✓		25-K84-M163	$6.3 \times 10^{-3} \pm 1.4 \times 10^{-4}$ (g)	$6.1 \times 10^{-5} \pm 2.5 \times 10^{-6}$ (c)
	M14	✓			37-K84-M14	$6.2 \times 10^{-4} \pm 5.7 \times 10^{-5}$ (i)	$3.2 \times 10^{-5} \pm 3.0 \times 10^{-6}$ (c)
	M14		✓		25-M14-K84	$6.1 \times 10^{-2} \pm 1.4 \times 10^{-3}$ (c)	$6.0 \times 10^{-1} \pm 2.5 \times 10^{-2}$ (a)
NBJE55	M25	✓			37-M25-NBJE55	$7.9 \times 10^{-2} \pm 8.2 \times 10^{-4}$ (a)	$2.1 \times 10^{-5} \pm 2.5 \times 10^{-6}$ (c)
	M163	✓			37-M163-NBJE55	$3.3 \times 10^{-2} \pm 8.8 \times 10^{-4}$ (d)	$1.6 \times 10^{-5} \pm 2.5 \times 10^{-6}$ (c)
	M163		✓		25-M163-NBJE55	$7.3 \times 10^{-2} \pm 1.6 \times 10^{-3}$ (b)	$7.1 \times 10^{-5} \pm 2.0 \times 10^{-6}$ (c)
	M163			✓	4-NBJE55-M163	$8.4 \times 10^{-3} \pm 3.6 \times 10^{-4}$ (f)	$1.3 \times 10^{-5} \pm 2.6 \times 10^{-6}$ (c)
	M14	✓			37-NBJE55-M14	$2.8 \times 10^{-3} \pm 1.8 \times 10^{-4}$ (h)	$8.1 \times 10^{-5} \pm 2.5 \times 10^{-6}$ (c)
	M14		✓		25-M14-NBJE55	$1.8 \times 10^{-2} \pm 3.4 \times 10^{-4}$ (e)	$1.7 \times 10^{-1} \pm 2.0 \times 10^{-2}$ (b)

 T_m , temperature.* Strains carrying the *bla*_{KPC-2}, *bla*_{NDM-1} and *mcr-1* genes are denoted by the initials 'K', 'N' and 'M', respectively.

** ✓ Indicates the condition where transconjugants were successfully obtained.

† First strain name represents the donor strain and second strain name represents the recipient strain; all transconjugants were resistant to colistin and meropenem.

†† Frequencies are the mean $\pm$ standard deviation of triplicate analyses. Values followed by different letters in parentheses in the same column are significantly different ($P < 0.05$).**Table 3**

Genetic characteristics of transconjugants obtained from the rat gastrointestinal tract-based transmission model

Conjugation between bacterial strains*		Administration time**			Transconjugants†	Plasmid frequency††	Conjugation frequency††
		1 st	2 nd	3 rd			
K84	M14	✓			1-K84-M14	$6.9 \times 10^{-4} \pm 9.1 \times 10^{-5}$ (b)	$2.5 \times 10^{-5} \pm 4.0 \times 10^{-6}$ (de)
	M14		✓		2-K84-M14	$7.4 \times 10^{-3} \pm 7.5 \times 10^{-3}$ (b)	$7.3 \times 10^{-4} \pm 5.5 \times 10^{-5}$ (a)
	M14			✓	3-K84-M14	$1.2 \times 10^{-2} \pm 1.5 \times 10^{-2}$ (b)	$8.8 \times 10^{-6} \pm 4.0 \times 10^{-7}$ (e)
	M163	✓			1-K84-M163	$9.5 \times 10^{-3} \pm 5.9 \times 10^{-3}$ (b)	$3.1 \times 10^{-5} \pm 2.6 \times 10^{-6}$ (de)
	M163		✓		2-K84-M163	$5.4 \times 10^{-3} \pm 7.2 \times 10^{-3}$ (b)	$5.5 \times 10^{-5} \pm 4.0 \times 10^{-6}$ (d)
NBJE55	M163	✓			1-M163-NBJE55	$1.5 \times 10^{-1} \pm 7.3 \times 10^{-2}$ (a)	$3.1 \times 10^{-4} \pm 2.5 \times 10^{-5}$ (b)
	M163			✓	3-M163-NBJE55	$5.0 \times 10^{-2} \pm 2.1 \times 10^{-3}$ (b)	$1.7 \times 10^{-4} \pm 2.1 \times 10^{-5}$ (c)

* Strains carrying the *bla*_{KPC-2}, *bla*_{NDM-1} and *mcr-1* genes are denoted by the initials 'K', 'N' and 'M' respectively.

** ✓ Indicates the condition where transconjugants were successfully obtained.

† First strain name represents the donor strain and second strain name represents the recipient strain; all transconjugants were resistant to colistin and meropenem.

†† Frequencies are the mean $\pm$ standard deviation of triplicate analyses. Values followed by different letters in parentheses in the same column are significantly different ($P < 0.05$).

the *bla*_{NDM-1}-bearing plasmid harboured by strain NBJE55 could be conjugated to strain M163 at 24 h and 96 h post-inoculation, with the *bla*_{NDM-1}-bearing strain acting as the receipt strain (Table 3; Supplementary Table S2). The conjugation frequency ranged from 8.8×10^{-6} to 7.3×10^{-4} , with significant differences observed in transconjugants obtained from the GI tract of rats. These findings suggested that plasmids harbouring carbapenemase genes (*bla*_{KPC-2} or *bla*_{NDM-1}) in clinical CRE strains could be effectively transmitted to *mcr-1*-bearing *E. coli* strains in the animal GI tract to produce organisms resistant to both colistin and carbapenems, suggesting that the animal GI tract can serve as an incubator for development of antimicrobial resistance. The plasmid frequency of transconjugants obtained from the GI tract of rats was shown to be significantly different ($P < 0.05$) and the plasmid frequency of 1-

M163-NBJE55 was significantly higher than other transconjugants. However, transconjugants collected from the in vivo rat GI tract model remained genetically stable after serial passage, confirming that plasmids carrying carbapenemase genes (*bla*_{KPC-2} or *bla*_{NDM-1}) can stably co-exist with those carrying *mcr-1* in Enterobacteriaceae strains (Table 3).

4. Discussion

Since the emergence of *K. pneumoniae* strains producing the carbapenemase KPC [1], the New Delhi metallo- β -lactamase NDM [16] and the MGE-encoded colistin resistance determinant MCR-1 [5], several reports have documented that transmission of these genes can occur via clonal spread and HGT [2,7,17], posing a se-

rious public-health threat worldwide. Moreover, simultaneous acquisition of *mcr-1* and carbapenemase genes by *K. pneumoniae* and *E. coli* strains has recently been reported in several studies [18–20]; however, little is known about the molecular mechanisms underlying the transition from a monodrug-resistant strain into a multidrug-resistant status via HGT. Currently, dissemination of resistance genes among bacterial pathogens by HGT is considered a key mechanism that not only gives rise to an alarming increase in the incidence of antibiotic resistance both in hospital settings and animal husbandry, but also drives genetic diversification and evolution of pathogens [21,22]. In the present study, we demonstrated, using both in vitro and in vivo models, that plasmids harbouring carbapenemase genes (*bla*_{KPC-2} and *bla*_{NDM-1}) and *mcr-1*-bearing plasmids obtained both from environmental and clinical settings could be acquired by a single bacterial strain and stably inherited by its offspring.

Among the three known canonical gene transfer mechanisms (conjugation, transduction and transformation), conjugation has been demonstrated to be the most efficient in mediating transfer of antimicrobial resistance genes between various bacterial species [23], during which plasmids that harbour resistance genes can be acquired by a recipient cells through direct contact with a donor organism [21]. The conjugation process therefore enables bacteria to acquire the ability to express new phenotypes, including resistance and virulence [10]. In the present study, the ability of bacteria to undergo conjugation was tested both in in vitro and in vivo assays. Overall, seven types of *bla*_{KPC-2}-bearing plasmids, four types of *bla*_{NDM-1}-bearing plasmids with various sizes, and three major types of *mcr-1*-bearing plasmids were tested in this study. Our data confirm that these plasmids were compatible with each other, forming strains resistant to both carbapenems and colistin. To assess the efficiency of transmission between plasmids harbouring carbapenemase genes (*bla*_{KPC-2} and *bla*_{NDM-1}) and *mcr-1* genes in the natural environment, we developed a meat-based model and a rat GI tract transmission model. Different from filter-mating conjugation, bacteria are naturally present on the surface of meat products and inside the GI tract of humans and animals [24]. Our data showed that plasmids harbouring carbapenemase genes (*bla*_{KPC-2} and *bla*_{NDM-1}) could be transferred to strains that contained *mcr-1*-bearing plasmids in the in vitro food model. In particular, temperature was found to be a determining factor of conjugation efficiency since some plasmids could only be conjugated at a certain temperature, while not others. It is noted that filter-mating conjugation exhibited the highest conjugation efficiency, which is probably due to the tight interaction on filter paper compared with natural conditions such as on a meat surface or in the GI tract. The data might suggest that meat and GI tract conjugation models might be better models to study the transmission of multidrug resistance (MDR) plasmids in foodborne pathogens, since natural plasmid transmission would normally occur in food and the animal/human GI tract. Conjugation frequencies in these two models are much lower than that of the filter-mating method, while plasmid frequency remains at a similar level. In addition, some conjugation could be observed in the meat model at ambient temperature and even 4°C, which are normally not tested by the filter-mating method. It is also very interesting to see that 25-M14-K84 and 25-M14-NBJE55 could be conjugated at a high conjugation frequency on meat, which is not found in the other two conjugation models. This is consistent with a previous report that HI-type plasmids exhibit higher conjugation frequencies at ambient temperature [25], however, some transconjugants appear to have acquired the HI2 plasmid from strain M14 at 37°C in our study. The conditions tested in this model allow us to closely mimic the real-life situation in the natural environment compared with the filter-mating conjugation assays performed in a laboratory. The data also imply for the first time that the storage temperature of food prod-

ucts, if not low enough, can actually promote the transmission of MDR plasmids among organisms residing in the products. In addition to natural transmission among organisms in food samples, our data also showed that the animal GI tract is a perfect niche in which bacteria undergo conjugative plasmid transfer. Conjugative transfer of resistance genes has been reported in different animal models, e.g. transfer of plasmids in the gut of chickens [26] and interspecies transfer of resistance determinants in the human GI tract [27–29]. None the less, our data show that the efficiency of transmission of resistance and virulence genes among members of the human and animal microbiota is underestimated. Our data regarding plasmid transmission in the animal GI tract confirm that both plasmids carrying carbapenemase (*bla*_{KPC-2} and *bla*_{NDM-1}) and *mcr-1* genes could be transmitted effectively in the animal GI tract, allowing Enterobacteriaceae strains to acquire MGEs that encode phenotypic resistance to both carbapenems and colistin. Transmission and stable inheritance of these two types of plasmids would lead to the emergence of multidrug-resistant pathogens that are resistant to all currently available last-line antibiotics for Gram-negative bacterial infections.

In summary, carbapenemase-producing plasmids could be transmitted into strains harbouring *mcr-1* genes via HGT, and vice versa, contributing to the emergence of strains with new and inheritable phenotypes and genotypes. Gene transfer events are likely to be common among members of the Enterobacteriaceae and serve as a key mechanism facilitating adaptation to new environments and evolution. Development of innovative strategies and surveillance measures to curtail the dissemination of MDR plasmids is necessary.

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Competing interests

None declared.

Ethical approval

All experimental rats were obtained from Guangdong Medical Laboratory Animal Center and had free access to food and water. All experiments were conducted strictly in accordance with the guidelines of the Chinese Association for the Accreditation of Laboratory Animals Care (CAALAC) issued by mainland China, and the Care and Use of Animals for Experimental Purpose issued by the Agriculture, Fisheries and Conservation Department of Hong Kong SAR, as well as the relevant local animal welfare bodies in China. The permit number of the animal work was 44007200040682. The experimental protocol was approved by the Research Animal Care and Use Committee of the Hong Kong Polytechnic University.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.05.022.

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