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SHORT COMMUNICATION

Co-Transfer of *mcr-1* and *mcr-3* Variant in *Escherichia coli* ST1632 Isolate in China: Silence is not Always Golden

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Escherichia coli strain 22a1303, carrying both *mcr-1.1*/*IncX4* and *mcr-3.5*/*IncP1* plasmids, was isolated from an ongoing antimicrobial resistance monitoring program. We were concerned that the dual presence of the *mcr* genes could mediate more complex transmission of resistance, so we further analyzed 116 known *E. coli* strains carrying both genes in the public database. We found that *E. coli* 22a1303 carrying the *mcr-3*/*dgkA* combination and *mcr-1* gene, which should mediate high levels of resistance, was sensitive to polymyxin with a MIC value of only 1mg/L. Conjugation experiment demonstrated that the two plasmids can be transferred from the donor strain 22a1303 to the recipient strain *E. coli* J53 at the same time, and the colistin resistance of transconjugants was increased by 8-fold compared with *E. coli* 22a1303.

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INTRODUCTION

Antimicrobial resistance (AMR) is a global public health concern that poses a threat for the treatment of bacterial infections effectively (Antimicrobial Resistance Collaborators, 2022). Antimicrobial misuse and overuse are important drivers of the resistance phenomenon (Holmes *et al.*, 2016). Colistin is considered as one of the last choice antibiotics for the treatment of infections elicited by multidrug-resistant bacteria (Lim *et al.*, 2010; Tran *et al.*, 2016). Except *mcr-6*, all of the other *mcr* genes have been located on conjugative plasmids or ColE-type plasmids mobilizable with helper plasmids (Ling *et al.*, 2020; Hussein *et al.*, 2021). This means that *mcr* genes can easily be transmitted horizontally, resulting in a variety of bacterial species showing increased colistin tolerance. Adding to the tension, *mcr-1* and *mcr-3* evolved while spreading widely and have the largest number of variants, being identified in isolates from 61 and 22 countries across six continents, respectively (Ling *et al.*, 2020).

Surveillance of ARGs is necessary to mitigate the threat of AMR (Ovejero *et al.*, 2017). However, the effectiveness of monitoring is limited by the silencing of ARGs. The expression of ARGs reduces the competitive fitness of a bacterial strain in the absence of antibiotic selection. In addition to compensatory mutations, bacteria can sometimes "hide" their resistance genotype by silencing it in order to lower the potential fitness cost exerted by antibiotic resistance genes carried on mobile genetic elements (Humphrey *et al.*, 2012). Numerous studies have established the presence of silent ARGs in bacterial genomes (Stasiak *et al.*, 2021), but their distribution and prevalence are not well understood.

Escherichia coli (*E. coli*) is the most common conditional pathogenic bacterium and a poster child for One Health studies that attempt to understand plasmid flow (Nguyen *et al.*, 2021). In this study, we report the coexistence and silencing of the *mcr-1* and *mcr-3* genes in a single *E. coli.*

From 2016 to 2022, an ongoing surveillance program monitored mobile colistin resistance genes in *E. coli*. The program collected 1,366 *E. coli* without interruption from foodborne farms and associated abattoirs located in Hunan, China. The *E. coli* strain named 22a1303, which was found in swine feces, tested positive for *mcr-1/IncX4* and *mcr-3/IncP1*. Later, confirmation was achieved by

using Whole Genome Sequencing (WGS) as previously described (Lin *et al.*, 2022). The ResFinder-4.1, Virulence Finder-2.0.3, MLST-2.0.9 server, and SerotypeFinder-2.0.1 tools of the Center for Genomic Epidemiology (www.genomicepidemiology.org) were used to analyze. The strain belongs to the phylogenetic group B1 and was identified as serotype O182:H38 and CH-Type 11-0. ST1632 was determined as the strain type, with five replicons including IncFIB, IncP1, IncX1, IncX4, and p0111. The co-existence of both *mcr-1* and *mcr-3* in a single strain is concerning, as it could lead to a more complex spread of resistance. To determine the prevalence of *mcr-1* and *mcr-3* co-existence in *E. coli*, we compared all known *E. coli* strains carrying both genes. As of March 1, 2023, we compared the complete sequences of 116 publicly available *E. coli* strains carrying both genes were identified on NCBI to 22a1303. We found that East and Southeast Asia were the most heavily affected regions, with most of the strains being isolated from humans, companion animals, or foodborne animals. The *E. coli* 22a1303 in our experiment is closely related to the human *E. coli* from Laos, and belongs to the same cladistic branch as the environmental and porcine *E. coli* from China and Thailand (Fig. 1). The simultaneous presence of *mcr-1* and *mcr-3* genes has already been reported in food-producing animals in China, with a significantly higher prevalence in swabs taken from pigs and poultry (10.1%; 333/3290) across nine provinces (Zhang *et al.*, 2018). However, the prevalence of the co-occurrence of these genes was lower in our study (0.07%; 1/1366). The reason for this discrepancy could be attributed to differences in sampling time or the various conditions of antibiotic resistance in different farms. The significant difference in the incidence rates also leads us to speculate that the ban on colistin in breeding farms in China in 2016 may have had a significant control effect in Hunan Province, where our study was conducted, as in the other 23 provinces in China (Wang *et al.*, 2020).

Antimicrobial resistance phenotypes were determined by broth-microdilution assays, the results are shown in Table 1. We discovered multiple AMR genes, beyond *mcr-1.1* and *mcr-3.5* (Table 1). These genes provide resistance against important antibiotic classes used in clinical settings, such as aminoglycosides, tetracyclines, polymyxins, and sulfonamide. This suggests a potential limitation of treatment options for human patients. Especially, antibiotic resistance in bacteria, is an urgent threat to the lives of immunocompromised individuals. One real concern, different virulence genes were detected from the *E. coli* 22a1303 and we observed were along with the presence of 540 proteins assigned to pathogenic families and the probability of being a human pathogen up to 93.6% (https://cge.food.dtu.dk/services/PathogenFinder/). The coexistence of AMR genes and high pathogenic potential underscores the strong threat of *E. coli 22a1303* to public health, especially the lives of cancer patients.

The *mcr-1* gene and *mcr-3* gene were located on a plasmid with an IncX4 (33130 bp) and IncP1 (50637 bp) replicon signature respectively. Our study found that *mcr-1* regions which lacked IS*Apl1* were unable to form the composite transposon Tn*6330*, which reduces the likelihood of plasmid destruction. Additionally, this plasmid contained members of the IS6 family and type IV secretion systems, which are important for the evolution and mobilization of ARGs globally (Fig. 2) (Christie, 2016; Massella *et al.*, 2020). The *mcr-3* gene can be traced back, along with its linked sequences to the *dgkA* gene, other organisms such as *Aeromonas* (Zhang *et al.*, 2019). The *E. coli* DAGK protein is integral to the plasma membrane, and when the *dgkA* sequence is closely linked to the *mcr-3* gene and co-expressed, colistin resistance can be improved compared to the expression of single coding sequences from the *mcr-3* gene (Gallardo *et al.*, 2020). This emphasizes the risk of transmission of the plasmid carrying the *mcr-3*/*dgkA* combination, which increases the likelihood of developing higher levels of colistin resistance (Fig. 3). In our experiments, however, *E. coli* 22a1303 carrying the *mcr-3*/*dgkA* combination and *mcr-1* did not mediate polymyxin resistance.

Table 1: Antimicrobial susceptibility profiles, resistance genes and virulence genes of *E. coli* 22a1303 from swine feces at a foodborne farm in Hunan, China

Classes	Antimicrobial	MIC (mg/L)	Resistance gene	Virulence gene
Penicillins	*Amoxicillin*	8	*mcr-1.1, mcr-3.5, OqxA, OqxB, qnrS1, tet(B), floR*	*aslA, csgA, fdeC, hlyE, nlpI, terC, tia*
	Cefotaxime	0.0625		
Cephalosporins	*Ceftriaxone*	0.0625		
	Ceftiofur Sodium	1		
Carbapenems	*Meropenem*	0.015625		
Monobactams	*Aztreonam*	0.125		
Quinolones	*Ciprofloxacin*	0.125		
	Levofloxacin	0.5		
Aminoglycosides	*Amikacin*	2		
	Gentamicin	1		
Tetracyclines	*Tigecycline*	0.125		
	Tetracycline	0.5		
Polypeptide	*Colistin*	1		
Amphenicol	*Florfenicol*	128		

Silencing of resistant genes in bacteria is rarely reported (Deekshit *et al.*, 2012; Ovejero *et al.*, 2017). We concluded that this is mainly due to that in most genotypic investigations, only resistant isolates are screened for the presence of specific genes that confer antibiotic resistance. Because we did not use colistin to select the *mcr*-positive isolates, we were able to detect *mcr-1* and *mcr-3*-positive *E. coli* 22a1303 that was susceptible to colistin and had MIC of only 1mg/L. Rare instances of gene silencing in prokaryotes mediated by the *H-NS* protein have been reported (Göransson *et al.*, 1990; Hommais *et al.*, 2001). We suspect that this may be one of the reasons for the silencing of the *mcr-1* gene in *E. coli* 22a1303. It is worth noting that the *H-NS* protein was not found in the genetic environment surrounding many AMR genes, except for *mcr-1.1* in *E. coli* 22a1303. The conjugation experiment was performed using the colistin-resistant 22a1303 as donor and the sodium azide-resistant *E. coli* J53 as recipient (Lin *et al.*, 2022). Conjugation transfer experiments showed that *mcr-1*/IncX4 and *mcr-3*/IncP1 plasmids successfully transfered colistin resistance to *E. coli* J53, increasing MIC value from 1mg/L to 8mg/L. This suggesting that gene silencing is a property of the host rather than the plasmid itself. This emphasizes that silent genes are dormant in non-resistant strains like a "time bomb." We speculated that *mcr-1* gene silencing was related to the cell wall structure, but whether this change is mediated by bacterial initiative or its precise mechanism is yet to be elucidated. The occurrence of gene silencing can

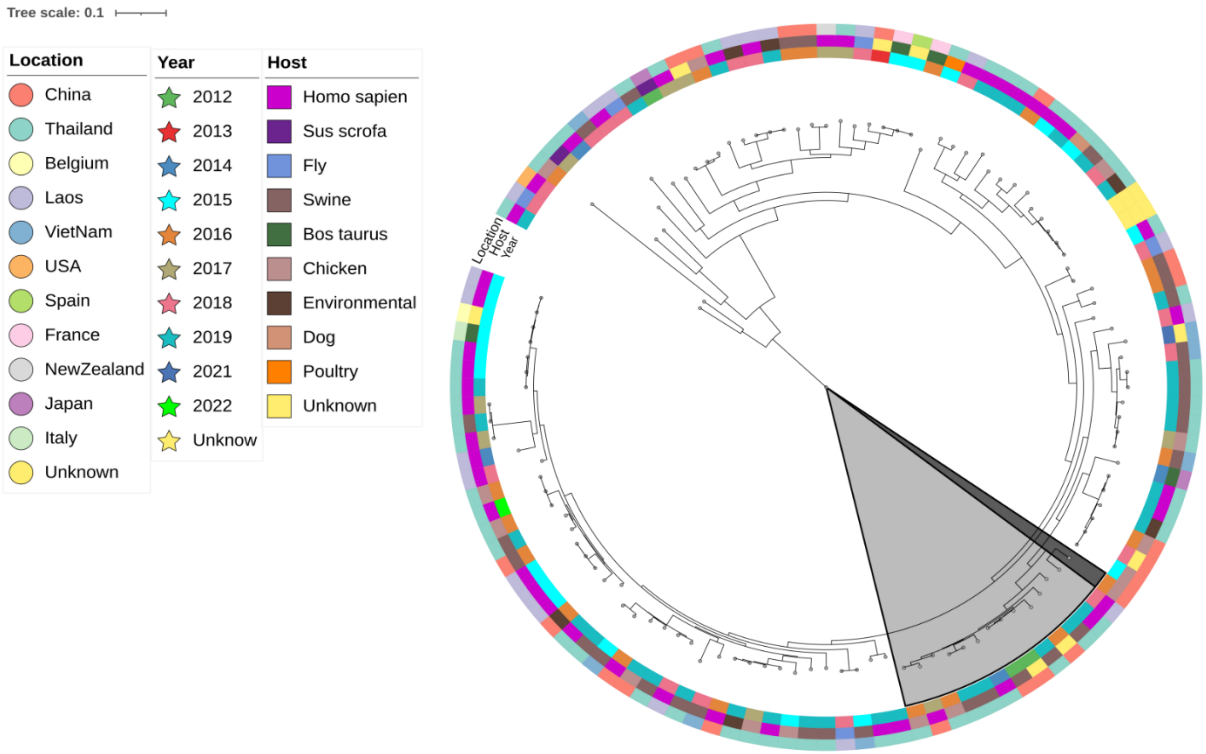

Fig. 1: Comparison of *E. coli* 22a1303 with publicly available *E. coli* strains carrying both the *mcr-1* and *mcr-3* genes was used to show the origin of samples, sampling time, and genetic relationships among 117 *E. coli* strains. The dark gray area represents *E. coli* 22a1303, and the light gray area represents the same or similar branched *E. coli* strains.

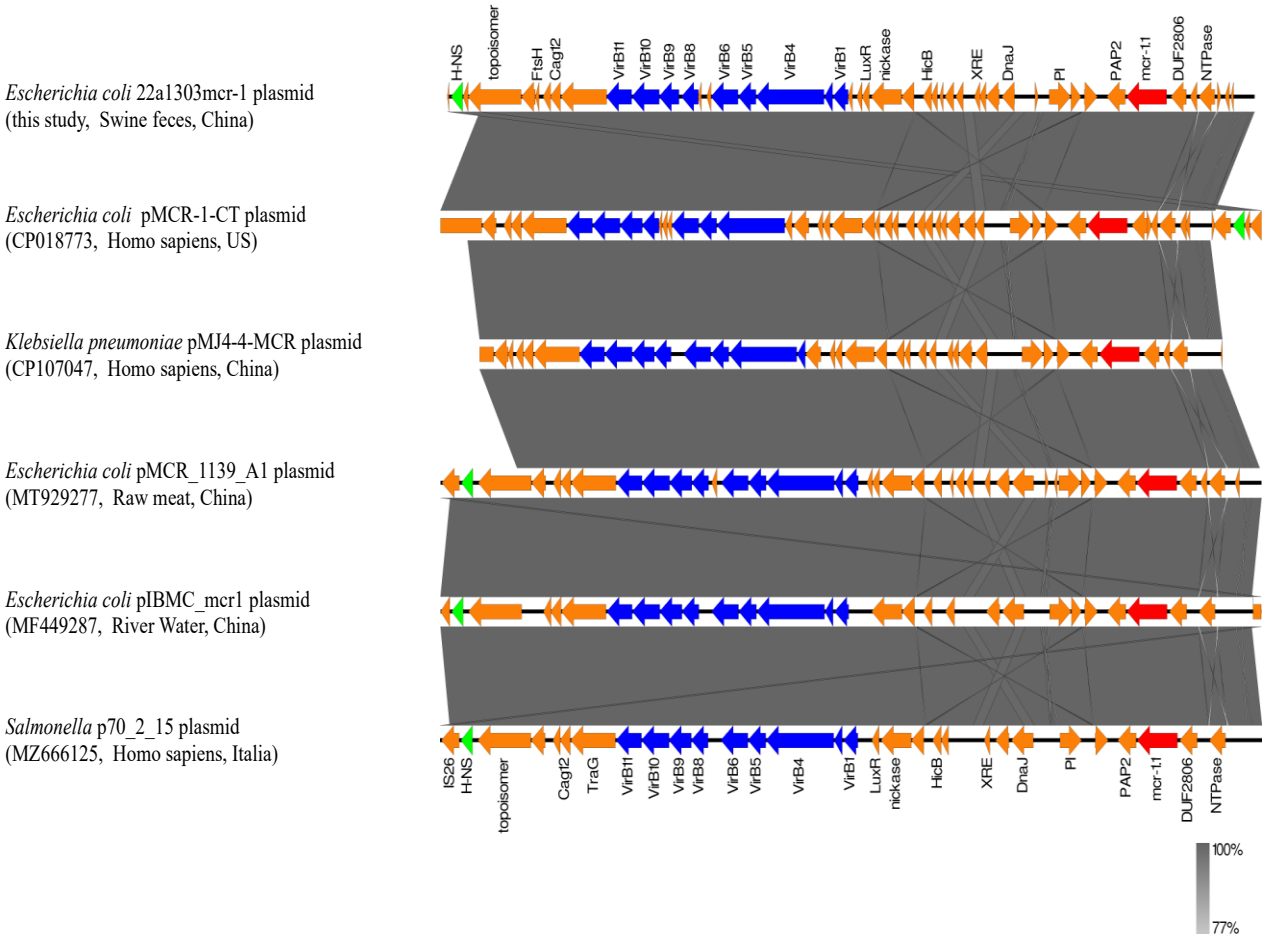

Fig. 2: Genetic environment analysis of plasmid containing *mcr-1* gene in *E. coli* 22a1303 and reference plasmids. Arrows indicate the transcription direction. Shared regions with a high degree of sequence similarity are indicated in gray. Red arrows denote *mcr-1.1*, green arrows indicate the H-NS protein, blue arrows indicate type IV secretion system genes, and orange arrows indicate other genes.

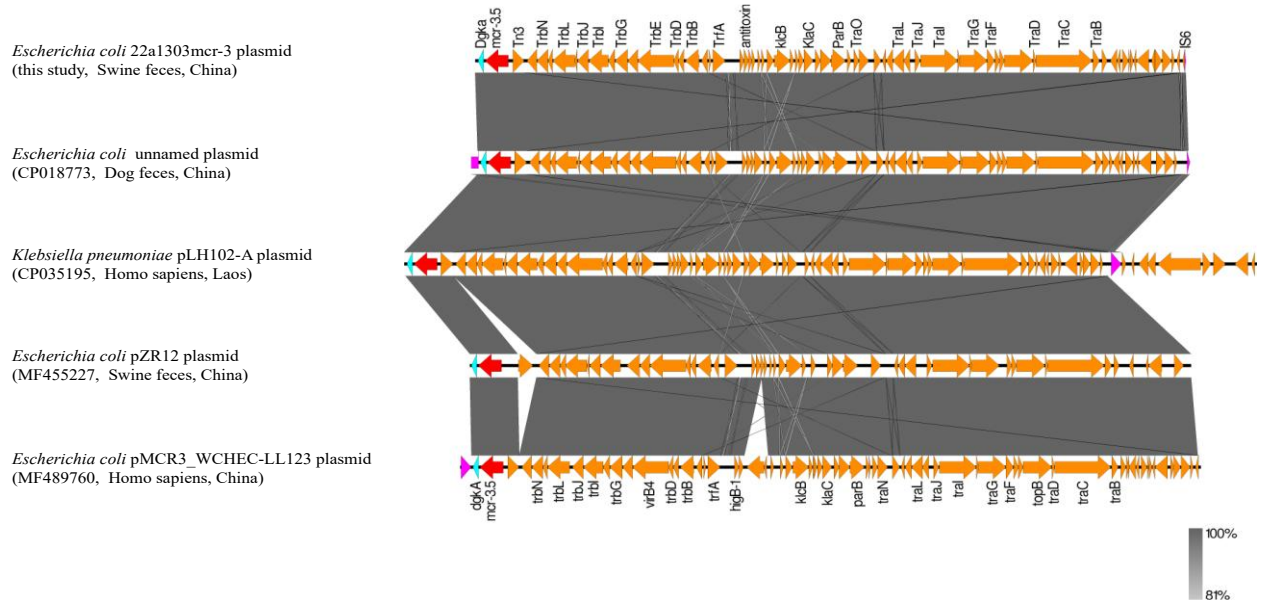

Fig. 3: Genetic environment analysis of plasmid containing *mcr-3* gene in *E. coli* 22a1303, and reference plasmids. Arrows indicate the transcription direction. Shared regions with a high degree of sequence similarity are indicated in gray. Red arrows denote *mcr-3.5*, Tiffany green arrows indicate the sequences dgkA, pruple arrows indicate IS6 genes, and orange arrows indicate other genes.

not only help ARGs evade detection of their phenotype, but also create a fitness advantage for bacteria, which increases the risk of ARG transmission. Due to the reversibility of silencing, strains carrying silencing genes are more dangerous to public health than common multidrug-resistant bacteria. We strongly recommend that special attention be given to "gene silencing" in monitoring ARGs, and that more rigorous surveillance plans be developed based on molecular approaches to track genes, not just bacteria. To better monitor ARGs, the exact mechanism of active or passive silencing of ARGs, and cross-sectional epidemic investigation of silencing genes should be investigated in a timely manner.

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Authors contribution: Z L Sun and J Y Li conceived and designed the study. H Y Li, H G Lin, Y Fu, Q F Fu, J Tan,Y N Wang and R S Zhou executed the experiment. H Y Li and H G Lin analyzed the data. and wrote the article.

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