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Characteristics of colistin-resistant *Escherichia coli* from pig farms in Central China

Zhong Peng[1,2,3,4†], Xiaoxue Zhang[1,2,3,4†], Xiaosong Li[1,2,3,4], Zizhe Hu[1,2,3,4], Zugang Li[1,2,3,4], Chaoying Jia[1,2,3,4], Menghong Dai[2,3,4], Chen Tan[1,2,3,4], Huanchun Chen[1,2,3,4] and Xiangru Wang[1,2,3,4*]

Abstract

The emergence and dissemination of colistin resistance in *Enterobacteriaceae* mediated by plasmid-borne *mcr* genes in recent years now pose a threat to public health. In this study, we isolated and characterized colistin-resistant and/or *mcr*-positive *E. coli* from pig farms in Central China. Between 2018 and 2019, 594 samples were collected and recovered 445 *E. coli* isolates. Among them, 33 with colistin resistance phenotypes and 37 that were positive for *mcr* genes were identified, including 34 positive for *mcr-1*, one positive for *mcr-3*, and two positive for both *mcr-1* and *mcr-3*. An insertion of nine bases ("CTGGATACG") into *mcr-1* in four *mcr*-positive isolates led to gene dysfunction, and therefore did not confer the colistin resistance phenotype. Antimicrobial susceptibility testing revealed that 37 *mcr*-positive isolates showed severe drug resistance profiles, as 50% of them were resistant to 20 types of antibiotics. Multilocus sequence typing revealed a heterogeneous group of sequence types in *mcr*-positive isolates, among which ST10 (5/37), ST156 (5/37), and ST617 (4/37) were the predominant types. Plasmid conjugation assays showed that *mcr*-carrying plasmids of 25 *mcr*-positive isolates were conjugated with *E. coli* recipient, with conjugation frequencies ranging from 1.7×10^{-6} to 4.1×10^{-3} per recipient. Conjugation of these *mcr* genes conferred a colistin resistance phenotype upon the recipient bacterium. PCR typing of plasmids harbored in the 25 transconjugants determined six types of plasmid replicons, including IncX4 (14/25), FrepB (4/25), IncI2 (3/25), IncHI2 (2/25), FIB (1/25), and IncI1 (1/25). This study contributes to the current understanding of antibiotic resistance and molecular characteristics of colistin-resistant *E. coli* in pig farms.

Keywords: *Escherichia coli*, Colistin resistance, *mcr* positivity, Antimicrobial resistance, Sequence types, Plasmid types, Plasmid conjugation

* Correspondence: wangxr228@mail.hzau.edu.cn
†Zhong Peng and Xiaoxue Zhang contributed equally to this work.
[1]State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, No.1 Shizishan Street, Hongshan District, Wuhan, China
[2]Key Laboratory of Preventive Veterinary Medicine in Hubei Province, The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan, China
Full list of author information is available at the end of the article

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Background

Having first been discovered in the 1940s, polymyxins are an old family of chemically distinct lipopeptide antibiotics produced by the gram-positive bacterium *Paenibacillus polymyxa* (Li et al. 2019a). In recent years, with the rapid increase in multidrug-resistant gram-negative pathogens in the clinic, particularly the "superbugs" *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*, several polymyxin antibiotics, especially polymyxin B and polymyxin E (or colistin), have been used as the last therapeutic option for infections caused by these pathogens (Li et al. 2019b). Mechanistically, polymyxin antibiotics largely exert their primary antimicrobial mode of action by permeabilizing bacterial outer membrane through a direct interaction with lipopolysaccharide (Li et al. 2019c). However, bacteria have still successfully developed several resistance mechanisms to combat the effect of polymyxins (Wang et al. 2021). Of great concern is the mechanism conferred by plasmid-mediated *mcr* gene (Liu et al. 2016). *mcr* gene encodes a phosphoethanolamine (PEtN) transferase that helps to add a PEtN moiety to lipid A of lipopolysaccharide, increasing its cationic charges and consequently decreasing the binding of colistin to lipopolysaccharide (El-Sayed Ahmed et al. 2020; Sun et al. 2018). Since the report about a plasmid carrying *mcr-1* in *E. coli* from both humans and animals in China in 2016 (Liu et al. 2016), this colistin resistance mechanism has received extensive attention worldwide. In addition to *mcr-1*, nine plasmid-borne *mcr* genes (*mcr-2~mcr-10*) have been identified to date (Wang et al. 2020a). These 10 *mcr* genes have been detected in bacterial isolates from humans, animals, foods of animal origin, and environment, and they confer resistance to polymyxins (Andrade et al. 2020). The emergence of these genes may accelerate global movement towards a post-antibiotic era (Du et al. 2016; Paterson and Harris 2016). Therefore, it is of great importance to monitor the prevalence of *mcr*-bearing bacteria in clinical activities.

As the bacterium in which plasmid-carrying *mcr* gene was first determined (Liu et al. 2016), *E. coli* is an important zoonotic and foodborne pathogen that has a great capacity to accumulate resistance genes, mostly
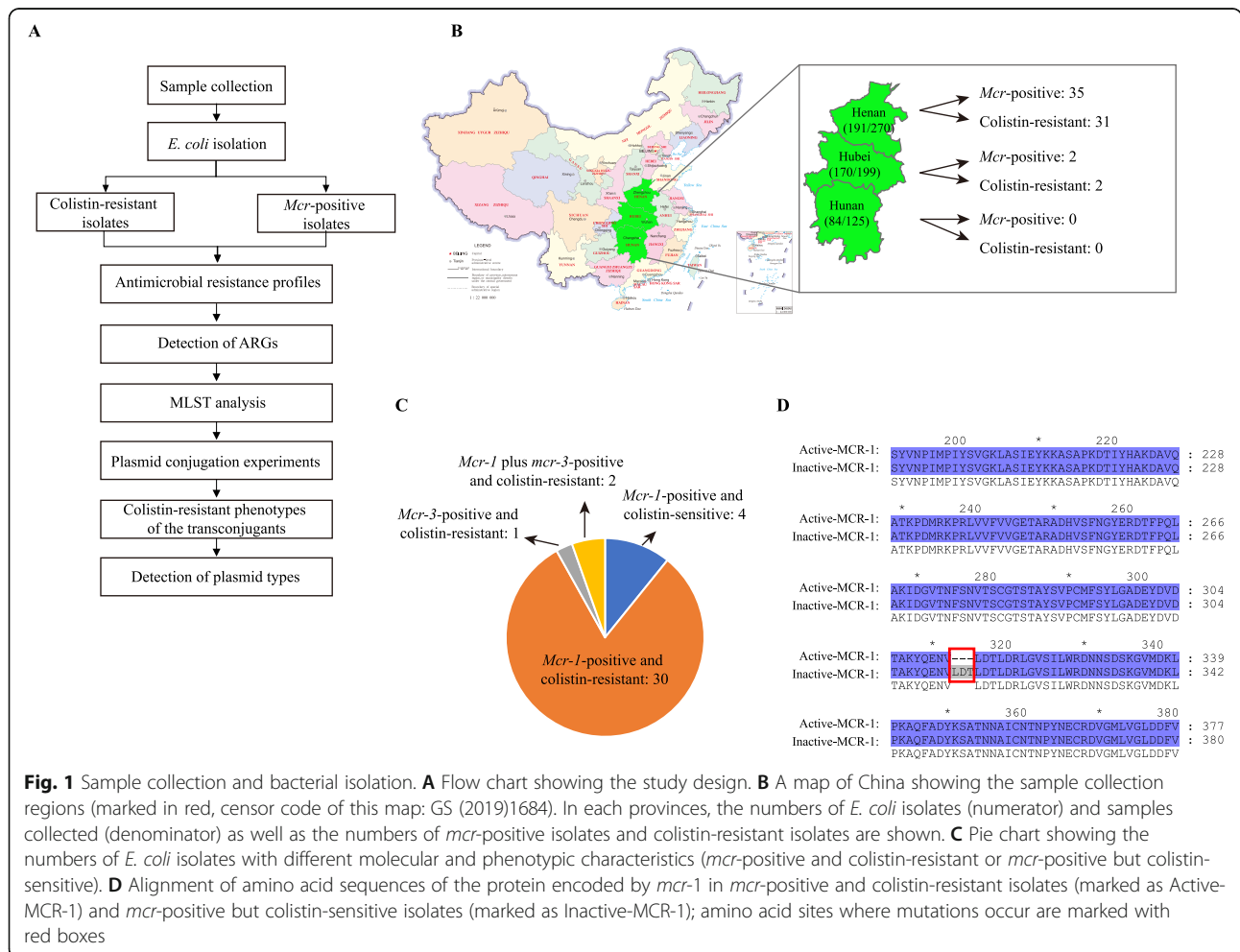
Fig. 1 Sample collection and bacterial isolation. **A** Flow chart showing the study design. **B** A map of China showing the sample collection regions (marked in red, censor code of this map: GS (2019)1684). In each provinces, the numbers of *E. coli* isolates (numerator) and samples collected (denominator) as well as the numbers of *mcr*-positive isolates and colistin-resistant isolates are shown. **C** Pie chart showing the numbers of *E. coli* isolates with different molecular and phenotypic characteristics (*mcr*-positive and colistin-resistant or *mcr*-positive but colistin-sensitive). **D** Alignment of amino acid sequences of the protein encoded by *mcr*-1 in *mcr*-positive and colistin-resistant isolates (marked as Active-MCR-1) and *mcr*-positive but colistin-sensitive isolates (marked as Inactive-MCR-1); amino acid sites where mutations occur are marked with red boxes

through horizontal gene transfer. This bacterial species is recognized as a "natural reservoir" of antimicrobial resistance genes (ARGs) (Poirel et al. 2018). It's also one of the most frequently recovered bacteria in livestock, in which the overuse and abuse of antimicrobials is proposed as a primary reason for the acceleration, development and spread of resistant bacteria (van Boeckel et al. 2017). From this point on, it will be necessary to monitor the epidemiological characteristics of drug-resistant *E. coli* in livestock. China is the largest pig-rearing country of the world. In this study, we report the isolation and antimicrobial resistance phenotypes as well as genetic characteristics of colistin-resistant *E. coli* and/or *mcr*-positive *E. coli* from pig farms in Central China, which is one of the primary pig-producing regions in China. The aim of this study is to reveal the current prevalence and diversion of colistin-resistant *E. coli* in Chinese pig farms.

Results

Isolation of colistin-resistant *E. coli* and *mcr*-positive *E. coli* from pig farms in Central China
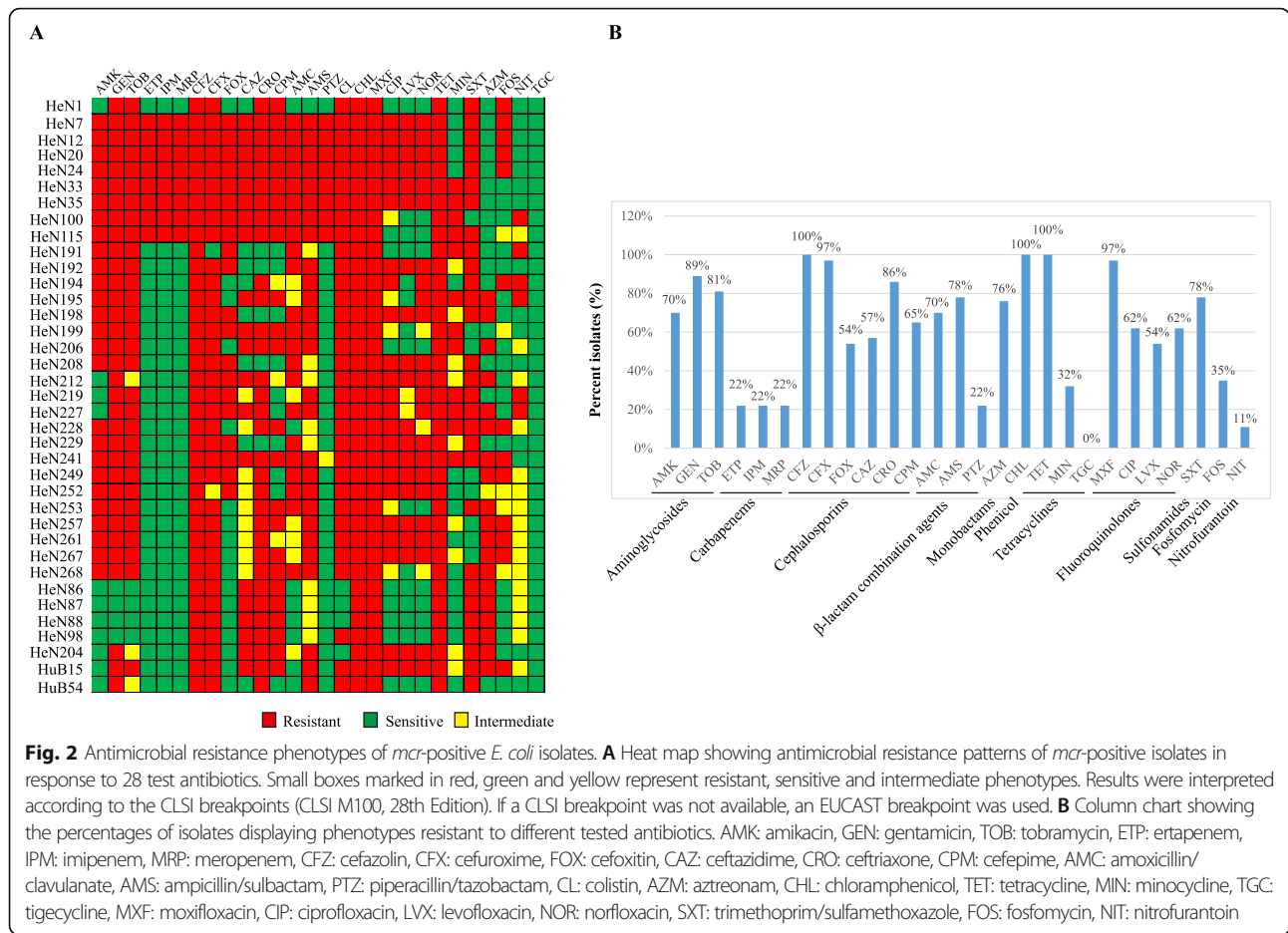
By performing bacterial isolation and identification (Fig. 1A), 445 *E. coli* strains were recovered from 594 farm-origin samples in Central China between 2018 and 2019 (Fig. 1B). The total isolation rate was 74.9%. Phenotype screening assays were performed in 33 *E. coli* isolates using agar containing 2 μg/mL colistin (7.42%; 33/

445). However, PCR detection identified 37 *E. coli* isolates were positive for *mcr* genes (8.31%; 37/445), including 34 isolates positive only for *mcr-1*, one isolate positive only for *mcr-3*, and two isolates positive for both *mcr-1* and *mcr-3* (Fig. 1C). These 37 *mcr*-positive isolates included the 33 isolates determined by phenotype screening assay. Tests on MIC (minimum inhibitory concentration) of colistin among these 37 *mcr*-positive isolates revealed that MIC of 33 isolates ranging from 4 μg/mL to 8 μg/mL, while the remaining four isolates lower than 0.5 μg/mL (Table 1).

To understand why the four *mcr-1*-carrying *E. coli* isolates were sensitive to colistin, *mcr-1* genes were cloned from both colistin-resistant and colistin-sensitive isolates. Nucleotide sequencing and sequence alignments revealed that *mcr-1* harbored by the four colistin-sensitive *E. coli* had an insertion of nine bases ("CTGGATACG") at sites 946~954 bp compared to *mcr-1* in colistin-positive isolates, which led to an insertion of three amino acid residues ("LDT") at sites 314~316 in mobile colistin resistance (MCR) protein as encoded by four colistin-sensitive *E. coli* (Fig. 1D). Next, *mcr-1* genes harbored by colistin-resistant *E. coli* (*mcr-1*[R]) and colistin-sensitive *E. coli* (*mcr-1*[S]) were cloned into the commercially available plasmid pMD™19-T (TAKARA, Japan). pMD19-*mcr-1*[R], pMD19-*mcr-1*[S], and pMD19-T were then transformed into TOP10 chemically competent *E. coli* (Thermo-Fisher, US).

Table 1 Minimum inhibitory concentration (MIC) values for colistin of 37 *mcr*-positive *E. coli* isolates

Strain	*Mcr* profile	MIC (μg/mL)	Strain	*Mcr* profile	MIC (μg/mL)
HeN1	*mcr-1*	8	HeN212	*mcr-1*	4
HeN7	*mcr-1*	8	HeN219	*mcr-1+ mcr-3*	4
HeN12	*mcr-1*	8	HeN227	*mcr-3*	4
HeN20	*mcr-1*	4	HeN228	*mcr-1+ mcr-3*	4
HeN24	*mcr-1*	4	HeN229	*mcr-1*	4
HeN33	*mcr-1*	4	HeN241	*mcr-1*	4
HeN35	*mcr-1*	4	HeN249	*mcr-1*	4
HeN98	*mcr-1*	4	HeN252	*mcr-1*	4
HeN100	*mcr-1*	4	HeN253	*mcr-1*	4
HeN115	*mcr-1*	4	HeN257	*mcr-1*	4
HeN191	*mcr-1*	4	HeN261	*mcr-1*	4
HeN192	*mcr-1*	4	HeN267	*mcr-1*	4
HeN194	*mcr-1*	4	HeN268	*mcr-1*	4
HeN195	*mcr-1*	4	HuB15	*mcr-1*	4
HeN198	*mcr-1*	4	HuB54	*mcr-1*	4
HeN199	*mcr-1*	4	HeN86	*mcr-1*	<0.25
HeN206	*mcr-1*	4	HeN87	*mcr-1*	<0.5
HeN208	*mcr-1*	4	HeN88	*mcr-1*	<0.25
			HeN204	*mcr-1*	<0.5

Fig. 2 Antimicrobial resistance phenotypes of *mcr*-positive *E. coli* isolates. **A** Heat map showing antimicrobial resistance patterns of *mcr*-positive isolates in response to 28 test antibiotics. Small boxes marked in red, green and yellow represent resistant, sensitive and intermediate phenotypes. Results were interpreted according to the CLSI breakpoints (CLSI M100, 28th Edition). If a CLSI breakpoint was not available, an EUCAST breakpoint was used. **B** Column chart showing the percentages of isolates displaying phenotypes resistant to different tested antibiotics. AMK: amikacin, GEN: gentamicin, TOB: tobramycin, ETP: ertapenem, IPM: imipenem, MRP: meropenem, CFZ: cefazolin, CFX: cefuroxime, FOX: cefoxitin, CAZ: ceftazidime, CRO: ceftriaxone, CPM: cefepime, AMC: amoxicillin/ clavulanate, AMS: ampicillin/sulbactam, PTZ: piperacillin/tazobactam, CL: colistin, AZM: aztreonam, CHL: chloramphenicol, TET: tetracycline, MIN: minocycline, TGC: tigecycline, MXF: moxifloxacin, CIP: ciprofloxacin, LVX: levofloxacin, NOR: norfloxacin, SXT: trimethoprim/sulfamethoxazole, FOS: fosfomycin, NIT: nitrofurantoin

Antimicrobial susceptibility testing (AST) revealed that MIC of colistin in *E. coli* strains harboring pMD$_{19}$-*mcr*-1^R, pMD$_{19}$-*mcr*-1^S and pMD$_{19}$-T were 2 μg/mL, 0.5 μg/ mL and 0.25 μg/mL, respectively.
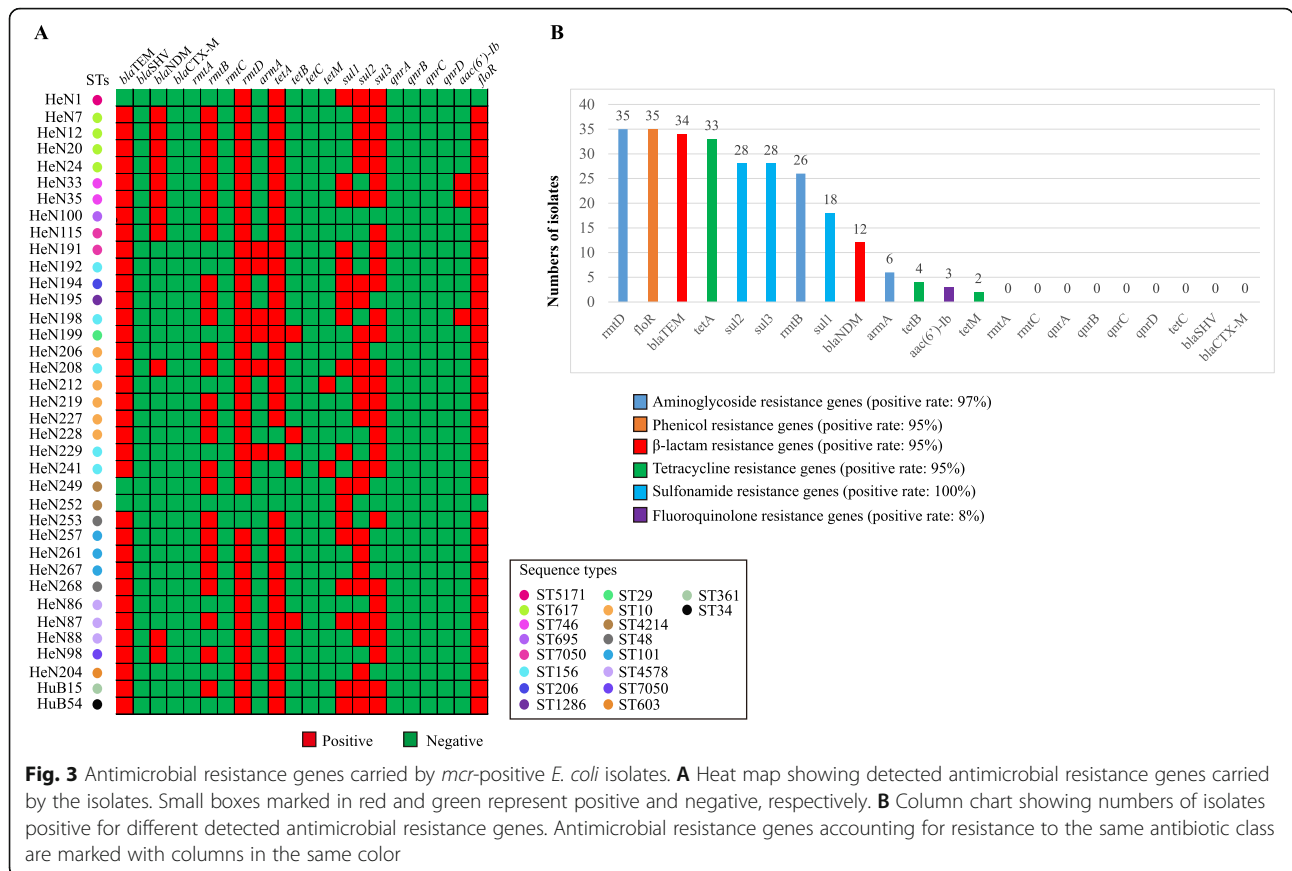
Antimicrobial resistance phenotypes of colistin-resistant *E. coli* and *mcr*-positive *E. coli*

AST revealed that the 37 *mcr*-positive isolates showed severe resistance profiles. All of them were resistant to more than nine types of tested antibiotics; over 80% (81.08%, 30/37) of the isolates were resistant to more than 15 types of tested antibiotics; and approximately 24% (24.32%, 9/37) isolates were resistant to 20 types of antibiotics (Fig. 2A). Among antibiotics tested here, resistance to cefazolin (CFZ), cefuroxime (CFX), chloramphenicol (CHL), moxifloxacin (MXF) and tetracycline (TET) were the common phenotypes, and all *mcr*-positive isolates were resistant to these five types (Fig. 2B). In particular, approximately 22% *mcr*-positive isolates were resistant to three carbapenem antibiotics tested here: imipenem (IPM), meropenem (MRP) and ertapenem (ETP) (Fig. 2B).

Detection of antimicrobial resistance genes

detection of ARGs showed that over 70% *mcr*-positive isolates were positive for *rmtD* (94.59%, 35/37), *floR* (94.59%, 35/37), *bla*$_{TEM}$ (91.89%, 34/37), *tetA* (89.19%, 33/37), *sul2* (75.68%, 28/37), *sul3* (75.68%, 28/37) and *rmtB* (70.27%, 26/37). However, fewer than 50% isolates were positive for *sul1* (48.65%, 18/37), *bla*$_{NDM}$ (29.73%, 11/37), *armA* (16.22%, 6/37), *tetB* (10.81%, 4/37), *aac (6')-Ib* (8.11%, 3/37), and *tetM* (5.41%, 2/37). None of the isolates were positive for *rmtA*, *rmtC*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *tetC*, *bla*$_{SHV}$ or *bla*$_{CTX-M}$ (Fig. 3A).

Among the detected ARGs, all *mcr*-positive isolates were positive for ARGs accounting for resistance to sulfonamides (*sul1*, *sul2* and/or *sul3*), while 97% isolates were positive for ARGs accounting for resistance to aminoglycosides (*rmtD*, *rmtB* and/or *armA*). Approximately 95% isolates were positive for ARGs for resistance to tetracyclines (*tetA*, *tetB* and/or *tetM*) and for resistance to phenicol (*floR*). Totally 92% isolates were positive for ARGs accounting for resistance to β-lactams in addition to carbapenems (*bla*$_{TEM}$), and 32% isolates were positive for ARGs for resistance to carbapenems (*bla*$_{NDM}$). Only

Fig. 3 Antimicrobial resistance genes carried by *mcr*-positive *E. coli* isolates. **A** Heat map showing detected antimicrobial resistance genes carried by the isolates. Small boxes marked in red and green represent positive and negative, respectively. **B** Column chart showing numbers of isolates positive for different detected antimicrobial resistance genes. Antimicrobial resistance genes accounting for resistance to the same antibiotic class are marked with columns in the same color

8% isolates were positive for fluoroquinolone-resistant ARGs (*aac(6')-Ib*) (Fig. 3B).

Sequence types of colistin-resistant *E. coli* and *mcr*-positive *E. coli*

MLST (multilocus sequence typing) analysis identified 17 types of sequence for 37 *mcr*-positive isolates, including ST10 (5/17), ST156 (5/17), ST617 (4/17), ST101 (3/17), ST7050 (3/17), ST4578 (3/17), ST48 (2/17), ST746 (2/17), ST4214 (2/17), ST34 (1/17), ST603 (1/17), ST29 (1/17), ST1286 (1/17), ST206 (1/17), ST695 (1/17), ST5171 (1/17), and ST361 (1/17) (Fig 4). Phylogenetic trees constructed based on MLST data revealed that several sequence types showed close relationships (Fig. 4).

Conjugation of *mcr*-carrying plasmids

To assess the transferability of *mcr*-bearing plasmids, bacterial conjugation experiments were performed between *mcr*-carrying *E. coli* and recipient *E. coli* C600. Results showed that *mcr*-bearing plasmids from 25 isolates in this study were conjugated, and the conjugation frequencies ranged from 1.7×10^{-6} to 4.1×10^{-3} per recipient (Table 2). AST results revealed that conjugating *mcr*-bearing plasmids conferred a colistin resistance phenotype to the recipient *E. coli*. MIC of colistin for
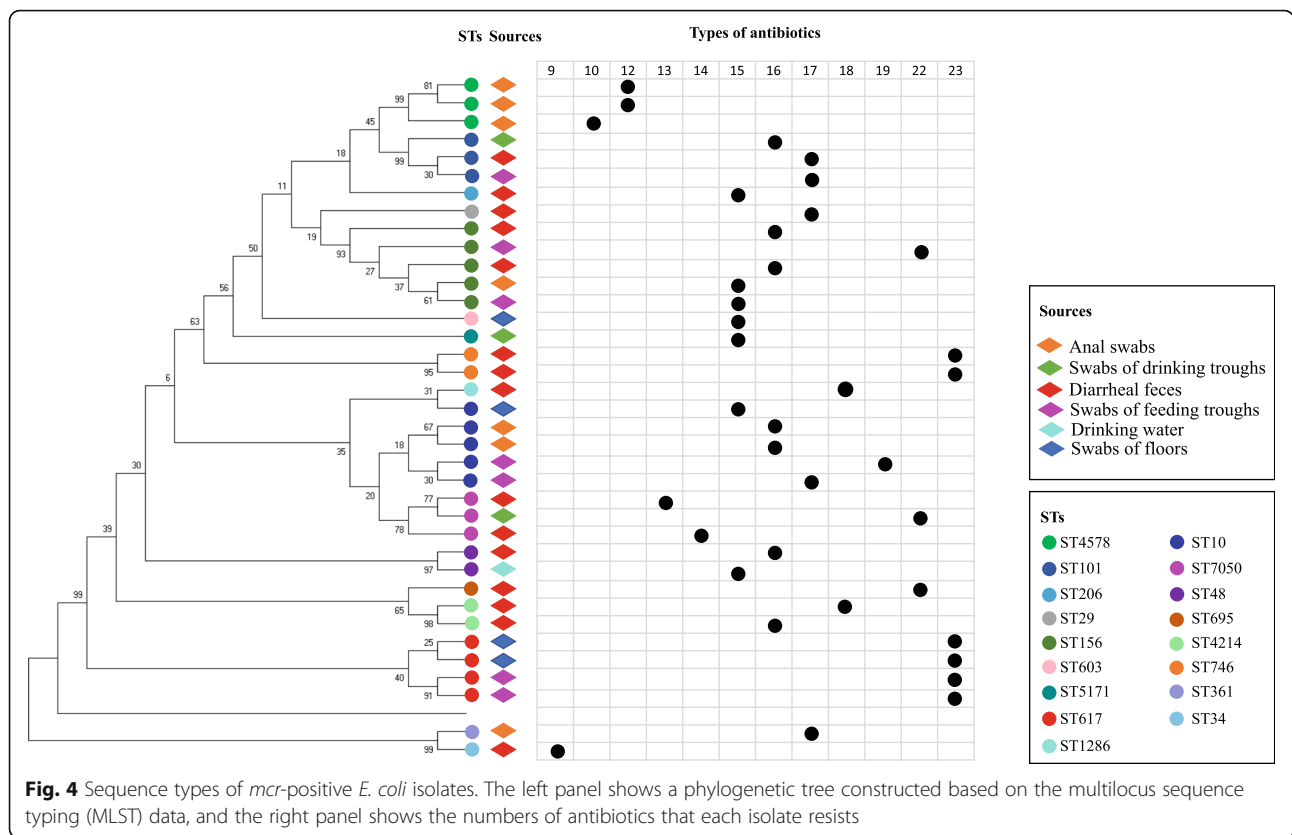
strain C600 was lower than 1 μg/mL while it increased to 4 μg/mL for transconjugants (Table 3).

Types of *mcr*-carrying plasmids

PCR detection of above 25 transconjugants revealed there were six plasmid types, namely IncX4 (14/25), FrepB (4/25), IncI2 (3/25), IncHI2 (2/25), FIB (1/25) and IncI1 (1/25). Among these plasmid types, IncX4 was the most frequently detected, being found in 14 of the 25 transconjugants.

Discussion

The rapid increase and dissemination of colistin-resistant *Enterobacteriaceae* as well as other gram-negative bacteria carrying plasmid-borne *mcr* genes in both humans and animals pose a major threat to global public health (Paterson and Harris 2016). Therefore, an extensive number of studies have monitored the prevalence and isolation of bacteria carrying plasmid-borne *mcr* genes in both medical and veterinary environments in recent years (Ilbeigi et al. 2021; Snyman et al. 2021; Tufic-Garutti et al. 2021). In this study, we performed an isolation and microbiological characterization of colistin-resistant and *mcr*-positive *E. coli* isolates from three provinces in Central China. Our study revealed

Fig. 4 Sequence types of *mcr*-positive *E. coli* isolates. The left panel shows a phylogenetic tree constructed based on the multilocus sequence typing (MLST) data, and the right panel shows the numbers of antibiotics that each isolate resists

total isolation rates of 5.56% (33/594) for colistin-resistant *E. coli* and 6.23% (37/594) for *mcr*-positive *E. coli*. Both rates were much lower than those reported previously in China. In a recent study, frequency of colistin resistance in *E. coli* from pigs was 24.1% in 12 provinces of China from 2013-2014 (Huang et al. 2017), while in another study, prevalence of colistin-resistant *mcr-1*-harboring *E. coli* isolates in pigs from 14 Chinese provinces was as high as 45% in 2016 (Shen et al. 2020). The relatively low prevalence rate determined in this study may be due to China's policy of banning the use of colistin as a growth promoter in livestock in China (Wang et al. 2020b). Following its implementation in 2017, it showed significant effects on reducing colistin resistance in both animals and humans in China. For example, a recent study showed that prevalence of colistin-

Table 2 Conjugation frequencies of *mcr*-carrying plasmids

Conjugants	Frequencies (per recipient)	Conjugants	Frequencies (per recipient)
HeN1C	2.1×10^{-5}	HeN208C	1.6×10^{-5}
HeN7C	1.1×10^{-5}	HeN219C	1.3×10^{-4}
HeN20C	4.8×10^{-5}	HeN227C	6.9×10^{-4}
HeN24C	2.2×10^{-5}	HeN228C	7.6×10^{-4}
HeN33C	3.9×10^{-3}	HeN241C	3.8×10^{-5}
HeN35C	1.6×10^{-3}	HeN249C	2.3×10^{-3}
HeN115C	2.6×10^{-3}	HeN252C	4.1×10^{-3}
HeN191C	1.9×10^{-4}	HeN253C	8.1×10^{-4}
HeN194C	1.2×10^{-5}	HeN12C	2.0×10^{-5}
HeN195C	1.0×10^{-5}	HeN268C	1.6×10^{-4}
HeN198C	1.7×10^{-6}	HeN98C	3.4×10^{-4}
HeN199C	3.9×10^{-5}	HuB15C	4.2×10^{-4}
HeN206C	3.6×10^{-4}		

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Table 3 Minimum inhibitory concentration (MIC) analysis of colistin for transconjugants and the recipient *E. coli* C600

Conjugants	MIC (µg/mL)	Conjugants	MIC (µg/mL)
HeN1C	4	HeN208C	4
HeN7C	4	HeN219C	4
HeN20C	4	HeN227C	4
HeN24C	4	HeN228C	4
HeN33C	4	HeN241C	4
HeN35C	4	HeN249C	4
HeN115C	4	HeN252C	4
HeN191C	4	HeN253C	4
HeN194C	4	HeN12C	4
HeN195C	4	HeN268C	4
HeN198C	4	HeN98C	4
HeN199C	4	HuB15C	4
HeN206C	4	C600	≤1

resistant *mcr-1*-harboring *E. coli* isolates in pigs from 14 Chinese provinces decreased from 45% (in 2016) to 19% after one year banning, with a remarkable reduction occurring in 10 of 14 surveyed provinces ($P < 0.0001$) (Shen et al. 2020). In the same study, a significant decrease in prevalence of *mcr-1*-harboring *E. coli* among farm pigs, from 76% in 2016 to 24% in 2018 ($P < 0.0001$), was also observed in Guangzhou, the capital of Guangdong Province in South China.

PCR detection results of this research revealed that all colistin-resistant *E. coli* were positive for *mcr-1* (Fig. 1C, Table 1), suggesting that colistin resistance phenotype in these isolates was conferred by this gene (Liu et al. 2016). It's worth noting that MIC of colistin on colistin-resistant *mcr-1*-harboring *E. coli* isolates determined in this study were low (4 µg/ml or 8 µg/ml; Table 1), which is consistent with those of other studies (Liu et al. 2016; Quan et al. 2017). This low value might have occurred because plasmid-mediated colistin resistance gene *mcr-1* generally confers low-level resistance (Poirel et al. 2017; Zhu et al. 2021). However, four *mcr-1*-positive isolates that did not display colistin resistance phenotypes were also identified (Fig. 1C, Table 1). Notably, this type of *E. coli* has been reported recently, but the underlying mechanism remains to be elucidated (Li et al. 2018). By nucleotide sequencing and sequence alignment analyses, *mcr-1* gene carried by colistin-sensitive isolates showed an insertion of several bases, which caused an insertion of three amino acid residues ("LDT") at sites 314~316 in the protein (Fig. 1D). These insertions were located in the catalytic domain of MCR-1 (residues 215-541) (Stojanoski et al. 2016), which may thereby lead to the disruption of catalytic activity of this protein. Next, we intend to analyze structure of these inactive MCR

proteins to reveal the non-working mechanisms. However, this hypothesis might be partly supported by our AST assays in different TOP10 *E. coli* transformants containing various plasmids (pMD19-*mcr-1*^{R}, pMD19-*mcr-1*^{S} and pMD19-T), which revealed that MIC of transformants containing pMD19-*mcr-1*^{S} was 0.5 µg/mL, while that of transformants containing pMD19-*mcr-1*^{R} was 2 µg/mL (interpreted as colistin-resistant according to the EUCAST breakpoint).

This work also revealed that all *mcr-1*-positive *E. coli* isolates from pig farms had severe antimicrobial resistance profiles. They were also resistant to antibiotics commonly used in clinic, including World Health Organization (WHO)-listed important antibiotics such as aminoglycosides, broad-spectrum cephalosporins and other β-lactams, and fluoroquinolones (Fig. 2). All of these isolates could be defined as multidrug-resistant bacteria and even extensively resistant bacteria, according to the international expert proposal for interim standard definitions for acquired resistance (Magiorakos et al. 2012). Similar findings have also been reported in other parts of China (Cheng et al. 2020; Tong et al. 2018; Yuan et al. 2021), as well as other countries around the world (Clemente et al. 2019; Oh et al. 2020; Zając et al. 2019). The presence of these multidrug-resistant *E. coli* strains at farm level poses a high probability of spreading them to humans along pork supply chain since *E. coli* is a common food contaminating bacterium as well as a foodborne pathogen (Batz et al. 2011). In particular, eight *mcr*-positive isolates were also resistant to three tested carbapenems (IPM, MRP, ETP) (Fig. 2).

Previously, we reported the genomic characteristics of these colistin and carbapenem co-resistant isolates, and showed that these worrisome phenotypes were conferred by plasmid-borne ARGs (*mcr-1* and *bla*$_{NDM-1}$) with conjugation capacity (Peng et al. 2019a, b). It should be remembered that both colistin and carbapenems are recognized as last-resort antibiotics for treating infections caused by multidrug-resistant gram-negative pathogens (Du et al. 2016). The existence of these isolates in food animals may lead to having no antibiotics available in clinical settings if they are transmitted to humans. The continuous monitoring of these colistin and carbapenem co-resistant isolates in both medical and veterinary environments is necessary and important. Corresponding to the serious resistance phenotypes determined here, ARG detection also revealed a serious condition in which resistance genes were found among these *mcr*-positive isolates. More than 90% isolates were positive for ARGs accounting for resistance to sulfonamides (*sul1*, *sul2* and/or *sul3*), aminoglycosides (*rmtD*, *rmtB* and/or *armA*), tetracyclines (*tetA*, *tetB* and/or *tetM*), phenicol (*floR*) and β-lactams (*bla*$_{TEM}$) (Fig. 3). Since these ARGs are frequently disseminated through

horizontal transfer, with the help of plasmids and other mobile genetic elements, these *mcr*-positive isolates harboring multiple ARGs may represent a major reservoir of resistance genes that may be responsible for treatment failures in both human and veterinary medicine (Poirel et al. 2018).

By performing MLST analysis, a heterogeneous group of sequence types were determined for 37 *mcr*-positive isolates. ST10 (5/37), ST156 (5/37) and ST617 (4/37) were the most predominant (Fig. 4). It has been reported that *E. coli* ST10 and its related types were frequently recovered from livestock, food and human intestinal samples. A higher prevalence of plasmid-borne ARGs was found in these types compared to others (Manges et al. 2017; Matamoros et al. 2017; Oteo et al. 2009). Similar to these reports, *E. coli* ST10 and its related types (ST1286, ST7050) showed severe AMR profiles and possessed many ARGs in addition to *mcr-1*, including ESBL-encoding gene bla_{TEM} (Figs. 2 and 3), suggesting that these sequence types pose a threat to public health. In addition, 11 NDM and MCR co-producing *E. coli* isolates were determined in this study (Fig. 3), and they were assigned to ST617 (4/11), ST746 (2/11), ST7050 (2/11), ST156 (1/11), ST695 (1/11) and ST4578 (1/11) (Fig. 4). Although most of these sequence types represent novel *E. coli* co-producing MCRs and NDMs, *E. coli* strains such as ST617 and ST746, have been recovered from both patients and diseased pigs (Gedebjerg et al. 2015; Hayer et al. 2020; Tian et al. 2020; Wu et al. 2018). Considering that *mcr* commonly confers resistance to the last-resort antibiotic colistin and bla_{NDM} commonly confers resistance to another last-resort antibiotic class, carbapenems, for treating infections caused by gram-negative pathogens (Du et al. 2016), the recovery of MCR and NDM co-produced in *E. coli* with potentially pathogenic sequence types should receive more attention. These bacteria might cause infections for which there are no effective antibiotics available in clinic. Continuous monitoring of these bacteria in both humans and animals is necessary.

mcr genes are generally disseminated by plasmids (Liu et al. 2016; Yi et al. 2017). To understand whether *mcr* gene harbored in these *mcr*-positive isolates is carried by plasmids and their plasmid types, plasmid conjugation experiments were first performed. Results revealed that *mcr* gene in 25 *mcr*-positive isolates was conjugated to recipient *E. coli*, with conjugation frequencies ranging from 1.7×10^{-6} to 4.1×10^{-3} per recipient (Table 2). More importantly, the conjugation of these *mcr* genes conferred a colistin resistance phenotype to the recipient bacterium (Table 3). These findings indicate that *mcr* genes carried by the isolates from pig farms in this study possess transferability and could mediate the transmission of colistin resistance. PCR typing analysis of

plasmids harbored in the 25 transconjugants revealed six types of plasmid replicons, specifically, IncX4 (14/25), FrepB (4/25), IncI2 (3/25), IncHI2 (2/25), FIB (1/25) and IncI1 (1/25). These findings are in part consistent with those from other reports, in which researchers also found that *mcr* genes were mostly detected in *E. coli* harboring plasmids IncX4, IncHI2, IncI2 and/or IncI1 (Al-Mir et al. 2021; Höfle et al. 2020; Olowo-Okere and Yacouba 2020; Song et al. 2020; Zelendova et al. 2020). While less common, *mcr* gene in *E. coli* carried by FIB and FrepB plasmids have also been documented (Shafiq et al. 2021).

Conclusion

In summary, we characterized drug resistance phenotypes, ARG profiles, sequence types, and putative plasmid types of colistin-resistant and/or *mcr*-positive *E. coli* isolates from pig farms in Central China in this study. Results revealed that several *mcr*-positive *E. coli* did not display a colistin resistance phenotype, which might be because base mutations were present, thereby leading to gene dysfunction. Notably, *mcr*-positive *E. coli* isolates determined in this study displayed severe AMR profiles, carried multiple ARGs, including those associated with great public health concerns, indicating that these *E. coli* isolates pose a threat to human health. This research also revealed a heterogeneous group of sequence types for *mcr*-positive *E. coli* isolates and several sequence types, such as ST617 and ST746, which reportedly correlated with diseases in both humans and pigs. These clones should receive more attention. In addition, we found that *mcr* genes in *E. coli* isolates from pig farms in Central China were most likely to carry several types of plasmids, and most of these plasmids possessed transferability and could help disseminate colistin resistance. Prevalence of colistin-resistant and/or *mcr*-positive *E. coli* in pig farms of China will be continuously monitored in the future.

Materials and methods
Sample collection and bacterial isolation
The study design is shown in Fig. 1A. Between 2018 and 2019, a total of 594 samples were collected, including fecal samples from diarrheal pigs, anal swabs from healthy pigs, and swabs of feeding and drinking troughs and floors from nine farms in Henan, Hubei and Hunan provinces in Central China for *E. coli* isolation (Fig. 1B). Samples were maintained in sterilized buffered peptone water (BPW) and shipped on ice to laboratory for immediate treatment. To improve isolation efficacy, each collected samples was pre-incubated in Luria-Bertani (LB) broth (Sigma-Aldrich, MO, USA) at 37°C for 12 h. Afterwards, sample culture was streaked on MacConkey agars and incubated at 37°C for 12 h. Presumptive

colonies with similar morphological characteristics to those of *E. coli* were selected for further confirmation by 16S rRNA gene sequencing and PCR amplification of seven housekeeping genes (*adk*, *fumC*, *gyrA*, *icd*, *mdh*, *purA* and *recA*) in *E. coli*, as described previously (Wirth et al. 2006). On each agar plates, five colonies were selected, but only one confirmed *E. coli* colony was included for further study.

Screening colistin-resistant isolates and *mcr*-positive isolates

To screen isolates with a colistin resistance phenotype, recovered *E. coli* isolates were streaked onto Müller-Hinton (MH) agar containing 2 μg/mL colistin (concentration chosen based on the European Committee on Antimicrobial Susceptibility Testing [EUCAST] clinical breakpoint 2018) and cultured at 37°C for 12 h. *E. coli* ATCC 25922 was used as a quality control. In parallel, all *E. coli* isolates were also selected as *mcr*-positive strains by using PCR with primers listed in Table S1. PCR assays were performed in a 20 μL mixture containing 1 μL bacterial DNA template, 1 μL forward or reverse primers, 10 μL 2× Master Mix (Vazyme, Nanjing, China), and 7 μL ddH$_2$O. Thermocycling conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, annealing at 51~60°C for 30 s (Table S1), and 72°C for 2 min 30 s, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1% agarose gel.

Antimicrobial susceptibility testing

The resistance phenotypes of colistin-resistant isolates and *mcr*-positive isolates were determined using broth microdilution method in accordance with the protocol published by the United States Clinical & Laboratory Standard Institute (CLSI document M100, 28th Edition). A total of 28 types of antibiotics, namely amikacin (AMK), gentamicin (GEN), tobramycin (TOB), imipenem (IPM), meropenem (MRP), ertapenem (ETP), colistin (CL), cefazolin (CFZ), cefuroxime (CFX), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (CPM), chloramphenicol (CHL), fosfomycin (FOS), nitrofurantoin (NIT), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), norfloxacin (NOR), minocycline (MIN), tetracycline (TET), aztreonam (AZM), tigecycline (TGC), amoxicillin/clavulanate (AMC), ampicillin/sulbactam (AMS), piperacillin/tazobactam (PTZ) and trimethoprim/sulfamethoxazole (SXT) were assessed. Results were interpreted using the CLSI breakpoints (CLSI M100, 28th Edition). If a CLSI breakpoint was not available, a EUCAST breakpoint was used. *E. coli* ATCC 25922 was used for quality control.

Detection of antimicrobial resistance genes

Antimicrobial resistance genes (ARGs) harbored in screened colistin-resistant *E. coli* were detected by PCR with primers listed in Table S1. A total of 22 types of ARGs that conferred resistance to six antibiotic classes were detected. PCR assays were performed the same as isolate screening described above.

Multilocus sequence typing

Multilocus sequence typing (MLST) was performed by following protocol published in the *E. coli* MLST database (https://enterobase.warwick.ac.uk/species/ecoli/). Sequence types were assigned based on the alleles of seven housing-keeping genes in *E. coli* (*adk*, *fumC*, *gyrA*, *icd*, *mdh*, *purA* and *recA*). Primers used for amplifying these genes were listed in Table S1. PCR assays were performed in a 30 μL mixture containing 1 μL bacterial DNA template, 1 μL forward or reverse primers, 15 μL 2× Phanta Master Mix (Vazyme, Nanjing, China), and 12 μL ddH$_2$O. PCR assays were performed the same as isolate screening described above. The confirmed products were sent for nucleotide sequencing and DNA sequences were submitted to the *E. coli* MLST database (https://enterobase.warwick.ac.uk/species/ecoli/) for sequence type determination.

Plasmid conjugation

Plasmid conjugation assays between *mcr*-positive *E. coli* (donor) and rifampin-resistant *E. coli* C600 (recipient) were performed on a nitrocellulose membrane, as described previously (Peng et al. 2019a). In brief, a mid-log phase donor and the recipient strains (OD$_{600}$ = 0.5~0.6) were mixed at a ratio of 1:3 (*v/v*). The bacterial mixture was then spotted onto a nitrocellulose membrane that was pre-plated on LB agar. After a 12 h incubation at 37°C, bacteria on the membrane were washed off using LB broth and were shaken at 37°C for 4 h. Lastly, the transconjugants were selected on LB agar plates laced with rifampin (1000 μg/mL; our pretests showed that all the *E. coli* donor strains could be inhibited by this concentration) plus colistin (2 μg/mL). MIC for colistin of transconjugants were determined using broth microdilution method as mentioned above. *E. coli* ATCC 25922 was used as quality control.

Plasmid typing

Putative types of *mcr*-carrying plasmids were determined by PCR assays with primers listed in Table S1. PCR assays were performed the same as isolate screening described above.

Abbreviations
AMK: Amikacin; GEN: Gentamicin; TOB: Tobramycin; ETP: Ertapenem; IPM: Imipenem; MRP: Meropenem; CFZ: Cefazolin; CFX: Cefuroxime; FOX: Cefoxitin; CAZ: Ceftazidime; CRO: Ceftriaxone; CPM: Cefepime; AMC: Amoxicillin/clavulanate; AMS: Ampicillin/sulbactam; PTZ: Piperacillin/

tazobactam; CL: Colistin; AZM: Aztreonam; CHL: Chloramphenicol; TET: Tetracycline; MIN: Minocycline; TGC: Tigecycline; MXF: Moxifloxacin; CIP: Ciprofloxacin; LVX: Levofloxacin; NOR: Norfloxacin; SXT: Trimethoprim/sulfamethoxazole; FOS: Fosfomycin; NIT: Nitrofurantoin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-021-00009-5.

> **Additional file 1: Table S1.** Primers used in the present study.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There is no conflict of interest to declare.

Author details

[1]State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, No.1 Shizishan Street, Hongshan District, Wuhan, China. [2]Key Laboratory of Preventive Veterinary Medicine in Hubei Province, The Cooperative Innovation Center for Sustainable Pig Production, Huazhong Agricultural University, Wuhan, China. [3]Key Laboratory of Development of Veterinary Diagnostic Products, Ministry of Agriculture and Rural Affairs, Huazhong Agricultural University, Wuhan, China. [4]International Research Center for Animal Disease, Ministry of Science and Technology, Huazhong Agricultural University, Wuhan, China.

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