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

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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 Incl1 (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
**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
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).

| 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        |
Antimicrobial susceptibility testing (AST) revealed that MIC of colistin in \textit{E. coli} strains harboring pMD19-\textit{mcr}-1\textsubscript{R}, pMD19-\textit{mcr}-1\textsubscript{S} and pMD19-T were 2 μg/mL, 0.5 μg/mL and 0.25 μg/mL, respectively.

**Antimicrobial resistance phenotypes of colistin-resistant \textit{E. coli}**

AST revealed that the 37 \textit{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 \textit{mcr-positive} isolates were resistant to these five types (Fig. 2B). In particular, approximately 22% \textit{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% \textit{mcr-positive} isolates were positive for \textit{rmtD} (94.59%, 35/37), \textit{floR} (94.59%, 35/37), \textit{bla}\textsubscript{TEM} (91.89%, 34/37), \textit{tetA} (89.19%, 33/37), \textit{sul2} (75.68%, 28/37), \textit{sul3} (75.68%, 28/37) and \textit{rmtB} (70.27%, 26/37). However, fewer than 50% isolates were positive for \textit{sul1} (48.65%, 18/37), \textit{bla}\textsubscript{NDM} (29.73%, 11/37), \textit{armA} (16.22%, 6/37), \textit{tetB} (10.81%, 4/37), \textit{aac (6')-Ib} (8.11%, 3/37), and \textit{tetM} (5.41%, 2/37). None of the isolates were positive for \textit{rmtA}, \textit{rmtC}, \textit{qnrA}, \textit{qnrB}, \textit{qnrC}, \textit{qnrD}, \textit{tetC}, \textit{bla}\textsubscript{SHV} or \textit{bla}\textsubscript{CTX-M} (Fig. 3A).

Among the detected ARGs, all \textit{mcr-positive} isolates were positive for ARGs accounting for resistance to sulfonamides (\textit{sul1}, \textit{sul2} and/or \textit{sul3}), while 97% isolates were positive for ARGs accounting for resistance to aminoglycosides (\textit{rmtD}, \textit{rmtB} and/or \textit{armA}). Approximately 95% isolates were positive for ARGs for resistance to tetracyclines (\textit{tetA}, \textit{tetB} and/or \textit{tetM}) and for resistance to phenicol (\textit{floR}). Totally 92% isolates were positive for ARGs accounting for resistance to β-lactams in addition to carbapenems (\textit{bla}\textsubscript{TEM}), and 32% isolates were positive for ARGs for resistance to carbapenems (\textit{bla}\textsubscript{NDM}). Only...
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 $1.7 \times 10^{-6}$ to $4.1 \times 10^{-3}$ 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
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                   | HeN199C    | 2.0×10⁻⁵                   |
| 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                   |            |                            |
resistant \textit{mcr-1}-harboring \textit{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 \textit{mcr-1}-harboring \textit{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 \textit{E. coli} were positive for \textit{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 \textit{mcr-1}-harboring \textit{E. coli} isolates determined in this study were low (4 $\mu$g/ml or 8 $\mu$g/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 \textit{mcr-1} generally confers low-level resistance (Poirel et al. 2017; Zhu et al. 2021). However, four \textit{mcr-1}-positive isolates that did not display colistin resistance phenotypes were also identified (Fig. 1C, Table 1). Notably, this type of \textit{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, \textit{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 \textit{E. coli} transformants containing various plasmids (pMD19-\textit{mcr-1}R, pMD19-\textit{mcr-1}S and pMD19-T), which revealed that MIC of transformants containing pMD19-\textit{mcr-1}R was 0.5 $\mu$g/ml, while that of transformants containing pMD19-\textit{mcr-1}R was 2 $\mu$g/mL (interpreted as colistin-resistant according to the EUCAST breakpoint).

This work also revealed that all \textit{mcr-1}-positive \textit{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 $\beta$-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; Zajac et al. 2019). The presence of these multidrug-resistant \textit{E. coli} strains at farm level poses a high probability of spreading them to humans along pork supply chain since \textit{E. coli} is a common food contaminating bacterium as well as a foodborne pathogen (Batz et al. 2011). In particular, eight \textit{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 (\textit{mcr-1} and \textit{bla}_{\text{TEM}}) 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 \textit{mcr-positive} isolates. More than 90% isolates were positive for ARGs accounting for resistance to sulfonamides ($\text{sul1}$, $\text{sul2}$ and/or $\text{sul3}$), aminoglycosides ($\text{rmtD}$, $\text{rmtB}$ and/or $\text{armA}$), tetracyclines ($\text{tetA}$, $\text{tetB}$ and/or $\text{tetM}$), phenicol ($\text{floR}$) and \textit{$\beta$-lactams} ($\text{bla}_{\text{TEM}}$) (Fig. 3). Since these ARGs are frequently disseminated through

| Table 3 Minimum inhibitory concentration (MIC) analysis of colistin for transconjugants and the recipient \textit{E. coli} C600 |
| Conjugants | MIC ($\mu$g/mL) | Conjugants | MIC ($\mu$g/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              |
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 (Poirol 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 blaTEM (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 (Gededjerg 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 blaNDM 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 $1.7 \times 10^{-6}$ to $4.1 \times 10^{-3}$ 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, funC, gyrA, icd, mdh, purA and recA) in E. coli, as described previously (Wirth et al. 2006). On each agar plate, 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 ddH2O. 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), tigecycline (TGC), amoxicillin (AMC), ampicillin (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/](https://enterobase.warwick.ac.uk/species/ecoli/)). Sequence types were assigned based on the alleles of seven housing-keeping genes in E. coli (adk, funC, 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 2x Phanta Master Mix (Vazyme, Nanjing, China), and 12 μL ddH2O. 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/](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 (OD600 = 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. Cefazidime; CRO: Ceftriaxone; CPM: Cefepime; AMC: Amoxicillin/clavulanate; AMS: Ampicillin/sulbactam; PTZ: Piperacillin/
Not applicable.

Availability of data and materials

The funder had no role in the study design, edges the financial support from the China Postdoctoral Science Foundation and the Walmart Foundation (Project # 61626817). Zhong Peng acknowl-

This work was supported in part by the National Key R&D Program of China (grant number: 2018 M640719). The funder had no role in the study design, experiments and data analysis; Z.P., and X.W. drafted the manuscript; and Z.P., H.C., and X.W. revised the manuscript. All the authors read and approved the final manuscript.

Funding

This work was supported in part by the National Key R&D Program of China (grant numbers: 2017YFC1600103 and 2017YFC1600101), the Natural Science Foundation of Hubei Province (grant number: 2020CFF525), the China Foundation of Hubei Province (grant number: 2020CFB525), the China Postdoctoral Science Foundation and the Walmart Foundation (Project # 61626817). Zhong Peng acknowledg-

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

Authors’ contributions

Z.P., H.C., and X.W. contributed to the conception and design of this work; Z.P., Z.X., X.L., Z.H., Z.L., and C.J. participated in sample collection, laboratory experiments and data analysis; Z.P., and X.W. drafted the manuscript; and Z.P., M.D., C.T., H.C., and X.W. revised the manuscript. All the authors read and approved the final manuscript.

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.

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Received: 15 April 2021 Accepted: 25 May 2021

Published online: 12 July 2021

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