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Prevalence and transmission of mobilized colistin resistance (*mcr-1*) gene positive *Escherichia coli* in healthy rural residents in Shandong province, China

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ABSTRACT

This study was conducted to explore the prevalence and transmission of *mcr-1 Escherichia coli* among healthy rural residents in Shandong, China, and to provide theoretical basis for the prevention and control of spread and treatment of multi-drug resistant *Escherichia coli*. A total of 218 healthy residents from 3 villages in Guan County, Shandong Province, China were included in this study, and their fecal samples were collected. Colistin-resistant *Escherichia coli* were selected, and their drug sensitivity and plasmids' transferability were measured. After analysis, some conclusions can be drawn. The *colistin-resistant Escherichia coli*, most strains of which are MDROs, is common and highly transmissible in healthy residents in rural areas in China. Interventions should be implemented to prevent the spread of *colistin-resistant Escherichia coli* through health education and tighter regulation of antibiotics.

1. Introduction

Currently, antibiotic resistance has become a major global public health challenge. Multidrug-resistant (MDR) pathogen infections are estimated to kill more than 700,000 people worldwide each year (Sun et al., 2017). Despite its high neurotoxicity and renal toxicity, colistin has replaced aminoglycoside drugs as one of the last approaches to treat MDR gram-negative bacterial infections (Rhouma et al., 2016). Colistin is a kind of cationic polypeptide belonging to the family of polymyxins, which is produced by Paenibacillus polymyxa. As the ultimate antibiotic in the treatment of Gram-negative bacterial infections, colistin works by disrupting the outer and inner membranes of bacterial. In June 2015, the National Medical Products Administration approved colistin as the oral medication of human beings, and in April 2018, colistin was treated as an injection drug to treat carbapenem-resistant *Escherichia*. But now strains that are resistant to colistin have appeared worldwide, and the well-known resistance mechanism is chromosome mutation. However, Jianzhong Shen et al., found a mechanism of plasmid-mediated colistin resistance (plasmids harboring *mcr-1*) in *Escherichia coli* from food-producing animals in China (Liu et al., 2016), which made the horizontal transmission of plasmids between bacteria possible. The mechanism of *mcr-1* mediated colistin-resistance is: *mcr-1* encodes proteases that mediate the addition of phosphoethanolamine to the lipid A of *Escherichia coli*, and the negative charge of lipid A is reduced, so that the cationic polymyxin peptide chain cannot be connected to the membrane by electrostatic action, thus losing its antibacterial effect. Gradually, many *mcr* variants have been reported worldwide. In 2016, another transferable colistin resistance gene, *mcr-2*, was found *in Escherichia coli* in pigs and cattle in Belgium (Basil et al., 2016). *mcr-3* was found in *Escherichia coli* from pigs in Shandong Province in 2017 (Wenjuan et al., 2017). *mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, mcr-9* have been discovered constantly (Wang et al., 2018) in *Escherichia coli* and other bacteria.

Regarding colistin resistance, most of the research subjects are

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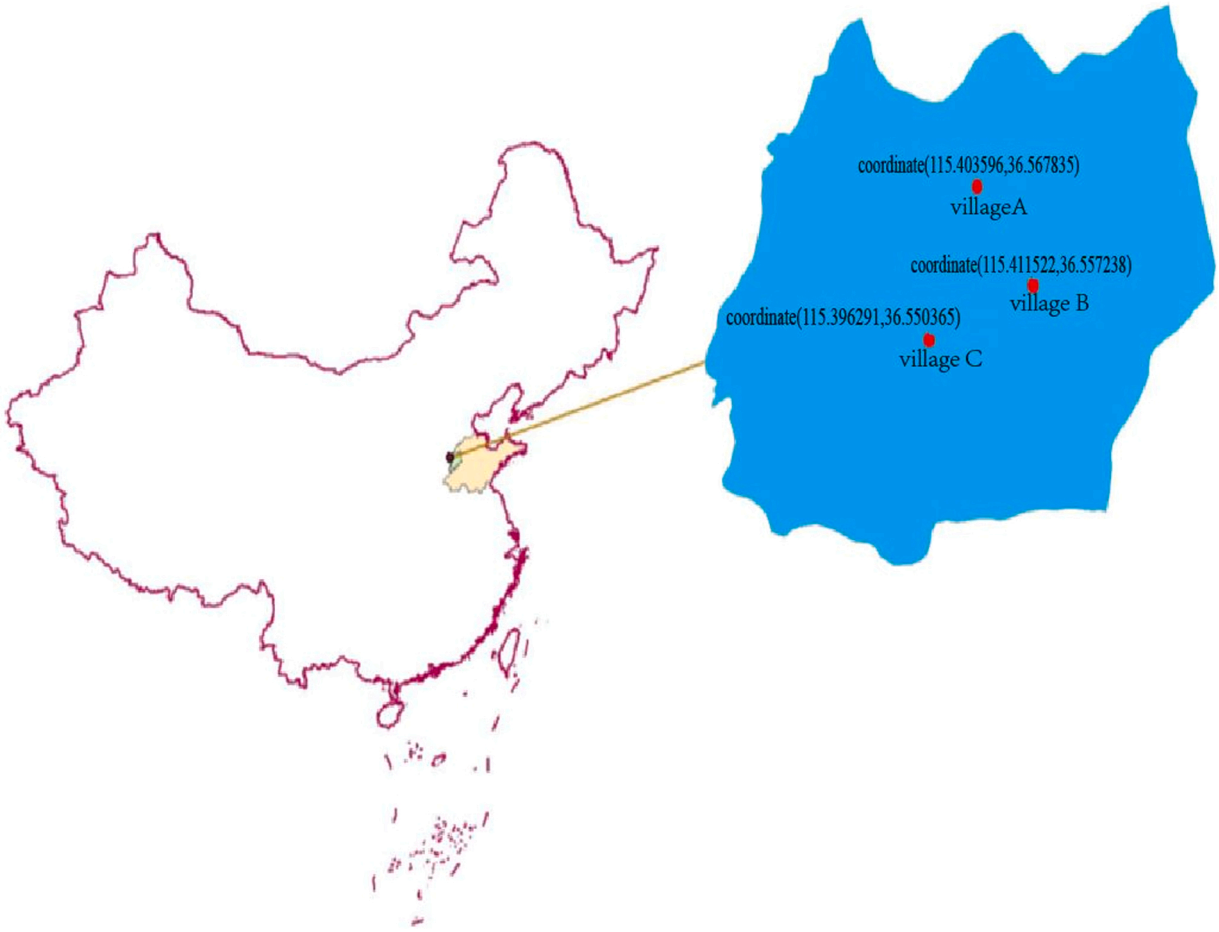

Fig. 1. The geographical location of three sample villages.

clinical patients (Rongsui et al., 2016; Kun-Jiao et al., 2016) or animals (Linxian et al., 2017), while few studies have selected healthy people as the research objects. In addition, compared with those of the urban population, the rural population may be more easily overlooked. Given that the coexistence of the *mcr-1* gene with other antibiotics is serious (Delgado-BlasJF et al., 2016), the infection status of colistin-resistant *Escherichia coli* carriage in the rural healthy population and the transmission mechanism of colistin-resistant *Escherichia coli* in healthy people are very important to study.

2. Materials and methods

In November 2018, 218 healthy rural residents from 3 villages in Guan County, Shandong Province, China, were included in the study. Relevant demographic information of rural population was gathered and the stool samples of population were collected. The samples were placed into Eswab tubes and then cultured on chromogenic medium with colistin, ECC color culture and PCR were used to screen *colistin-resistant Escherichia coli*. To assess the drug sensitivity of the isolated *colistin-resistant Escherichia coli*, antimicrobial susceptibility tests were performed by the broth microdilution method and agar dilution method following the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The *mcr-1* gene was detected by PCR and verified by whole-genome sequencing (WGS). The transferability of *mcr-1* and the size of the plasmid that carried *mcr-1* were determined by S1-PFGE and Southern blot. The transferability of the plasmid carrying *mcr-1* gene was verified by the conjugation experiment. In silico analysis, the software Easyfig 2.2.3 was used to compare the environment around the *mcr-1* gene of positive *Escherichia coli*, and the phylogenetic tree was constructed to understand the homology of *colistin-resistant Escherichia coli*. SPSS 24.0 was used to compare the basic demographic characteristics.

2.1. Sample collection and isolation of colistin-resistant Escherichia coli

Sampling points in villages were established and Eswab tubes (Copan, Brescia, Italy) were distributed to farmers by CDC staffs the previous day. Tubes with fecal samples were collected by staffs the next day according to the survey list of farmer households.

Fecal samples were enriched and cultured in brain heart infusion broth containing 1 μg/mL colistin and 30 μg/mL vancomycin overnight at 37 °C for 24 h. The turbid *mcr-1*-enriched cultures were screened by PCR and inoculated onto ECC chromogenic media (CHROMagar, France) containing 2 μg/mL colistin at 37 °C for 24 h. The blue colonies that grew on ECC medium were selected and inoculated onto brain heart infusion agar. PCR and matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) were used to confirm whether the presumptive colonies were *colistin-resistant Escherichia coli*. *Escherichia coli* ATCC 25922 was used as a negative control.

2.2. Antimicrobial susceptibility test

Polypeptides, tetracyclines, beta-lactams, quinolones, and aminoglycosides were selected as the five antibiotic classes for antimicrobial susceptibility tests of isolated colistin-resistant *Escherichia coli*. The minimum inhibitory concentrations (MICs) of the isolates were determined with using the microdilution broth method and agar dilution method. The results were interpreted according to the CLSI guidelines.

Table 1
Gender and age distribution of *colistin-resistant Escherichia coli* in three villages.

Variables	Village A			Village B			Village C			Total			χ^2	*P*
	N*	P**	%	N	P	%	N	P	%	N	P	%		
Gender														
Male	22	1	1.47	25	0	0.00	28	0	0.00	75	1	1.47	2.932	0.087
Female	46	3	4.41	47	2	2.78	50	3	3.85	143	8	11.04		
Subtotal	68	4	5.88	72	2	2.78	78	3	3.85	218	9	12.51		
Ages														
Youngly and wrinkly	15	0	0.00	11	1	1.39	11	0	0.00	75	1	1.39	0.668	0.414
Elderly	53	4	5.88	61	1	1.39	67	3	3.85	143	8	11.12		
Subtotal	68	4	5.88	72	2	2.78	78	3	3.85	218	9	12.51		

* Number of samples.
** Number of positive samples.

Escherichia coli ATCC 25922 was used as a quality control strain.

2.3. Statistical analysis

SPSS 24.0 was used as the statistical analysis tool. Baseline characteristics of the study population were compared by chi-square test. The inspection level (α) was set as 0.05.

2.4. Whole Genome Sequencing (WGS)

Genomic DNA was extracted with a TIANamp Bacteria DNA Kit (Beijing, China) and sequenced by Sangon Biotech (Shanghai, China) through the Illumina HiSeq sequencing platform. The quality of the high-throughput sequence data was assessed by FastQC. The reads were de novo assembled into contigs using SPAdes 3.11.1. Multilocus sequence typing (MLST) analysis was used to identify antimicrobial resistance genes. The genetic environment annotation and comparation were done with RAST and Easyfig version 2.2.3. A phylogenetic tree based on the genome of colistin-resistant *Escherichia coli* was constructed using MEGA. All isolates' accession numbers (WHLW00000000, WHLV00000000, WHLU00000000, WHLT00000000, WHLR00000000, WHLQ00000000, WHLP00000000, WHOK00000000, and WHLO00000000) can be found in GenBank.

2.5. Conjugation assay, S1-PFGE and Southern blotting

The transferability of the *mcr-1* colistin resistance gene was verified by using filter mating (Beiwen et al., 2017). The recipient strain (rifampicin-resistant *Escherichia coli* EC600) and donor strain (one of the 9 *colistin-resistant* isolates) were mixed at a ratio of 1:1 and cultured in LB liquid medium at 37 °C for 12 h. Transconjugants were screened on MH agar containing colistin and rifampicin (Aiqing et al., 2016). The bacteria were identified as *Escherichia coli* EC600 via MALDI-TOF MS, and the *colistin-resistant* transconjugants were tested by PCR.

To determine whether *mcr-1* was on a plasmid or the chromosome, S1-PFGE and Southern blot hybridization were conducted, *Escherichia coli* H9812 was used as a reference marker. (1) To make gels for electrophoresis, the membrane proteins were dissolved, and the DNA was released by placing the bacterial suspension and protease K at 37 °C for 15 min; the mixture was then mixed with boiled agarose to make a gel block for electrophoresis. (2) For pulsed-field gel electrophoresis, the reference marker (*Escherichia coli* H9812) was digested by *Xba*I, and the sample strains were digested by S1 RNase; the DNA bands of the reference marker and sample strains were then analyzed by electrophoresis. (3) For Southern blot hybridization, the electrophoretic DNA was transferred to a nylon membrane and fixed; a digoxin probe was hybridized with the DNA and detected (DIG High Prime DNA Labeling and Detection Starter Kit II).

3. Results

3.1. General characteristics of the study population

Three villages of Guan County, Shandong Province, China (village A, village B, village C) were selected in this study (Fig. 1). A total of 240 fecal samples from healthy people (80 samples per village) were planned to collected. However, 22 (9.17 %) people were lost to follow-up because the stool sample or questionnaire was not up to standard of sufficient quality, so the final total sample size was 218, with 68, 72 and 78 residents from villages A, B and C respectively. The age of the study population ranged from 29 to 93, with an average age of 61.82 ± 12.83. The average ages of village A, B, C were 59.13 ± 13.35 (29–88), 62.4 ± 13.33 (33–85), 63.63 ± 11.60 (29–93) respectively ($\chi^2 = 1.851$, $P = 0.396$). There were more female than male, 143 women and 75 men, with a F:M ratio of 1.91. The F:M ratios of village A, B and C were 2.09, 1.88 and 1.79 respectively ($\chi^2 = 0.207$, $P = 0.902$).

Table 2
MICs of nine antibiotics.

Strains	MIC (mg/l)[a]								
	CT	TGC	CTX	AMC	MEM	FOX	CIP	TE	AMK
A065	**4**	0.25	0.016	8	0.032	4	0.25	**128**	1
A009	**4**	0.125	**128**	8	0.064	4	0.5	**32**	1
A049	**4**	0.125	0.016	4	0.032	4	0.25	**128**	1
A067	**>32**	0.125	**64**	16	0.064	16	**64**	**32**	**>128**
B042	**8**	0.125	0.016	8	0.064	4	**128**	**32**	1
C034	**4**	0.064	**64**	8	0.032	4	**64**	**32**	1
B020	**4**	0.125	**>128**	16	0.064	**32**	**128**	**16**	**>128**
C063	**8**	0.064	0.5	4	0.125	2	0.25	**32**	1
C073	**4**	0.125	0.032	4	0.032	4	**1**	**32**	1

[a] CT, colistin; TGC, tigecycline; CTX, cefotaxime; AMC, amoxicillin-clavulanic acid; MEM, meropenem; FOX, cefoxitin; CIP, ciprofloxacin; TE, tetracycline; AMK, amikacin. Resistance is highlighted in bold.
[b] The bold text in the table shows drug resistance, and the nonbold text shows drug sensitivity.

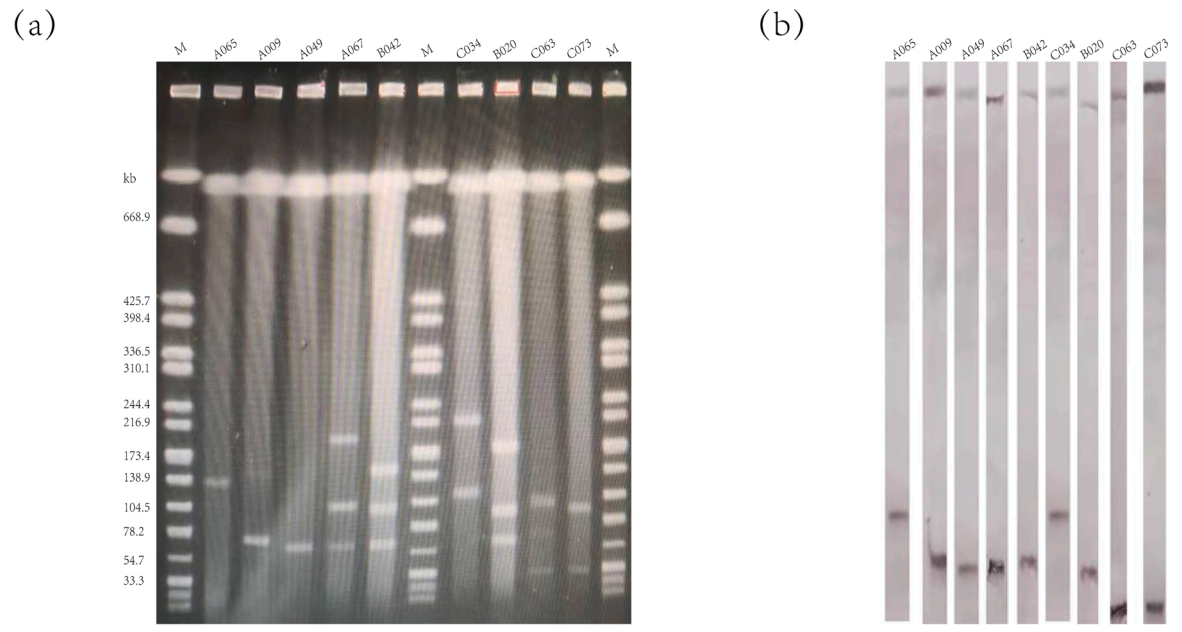

Fig. 2. S1-PFGE and Southern blotting of a DNA marker (*Escherichia coli* H9812) and 9 sample strains. (a) The size of the plasmid in each strain. (b) Transferable plasmid containing *mcr-1* in 9 sample strains.

3.2. Screening for the colistin-resistant Escherichia coli

According to the size of *mcr-1* of 328bp, 9 strains of *mcr-1* harboring *Escherichia coli* were obtained by PCR, capillary gel electrophoresis and MALDI-TOF MS, with the carriage rate of *mcr-1* harboring *Escherichia coli* being 4.13 % (9/218) in rural healthy residents (Table 1).

3.3. The susceptibility profiles of colistin-resistant Escherichia coli

9 *colistin-resistant Escherichia coli* strains were tested for drug sensitivity (Table 2) to 9 antibiotics (colistin; tigecycline; cefotaxime; amoxicillin-clavulanic acid; meropenem; cefoxitin; ciprofloxacin; tetracycline; amikacin), the results showed that 6 strains (A009, A067, B042, B020, C034 and C073) were multidrug-resistant (MDR). These MDR isolates were most frequently resistance to tetracycline (n = 9, 100 %), ciprofloxacin (n = 5, 55.56 %), and cefotaxime (n = 4, 44.44 %). It was worth noting that *colistin-resistant Escherichia coli* strains were all resistant to tetracycline. On the contrary, all isolates were sensitive to tigecycline, amoxicillin-clavulanic acid and meropenem. Notably, cefotaxime resistance occurred. As a third-generation cephalosporin, cefotaxime is often used as the last line of defense in severe infections, and the emergence of resistance to it challenges biosecurity.

3.4. Characterization of plasmids harboring the mcr-1 gene

For conjugation assays, a single colony collected from an antibiotic-resistant plate containing rifampicin and colistin was applied for PCR, an amplification product sized 320 bp was obtained, which means that the transconjugants containing *mcr-1* and the gene was transferable. S1-PFGE and Southern blotting were also conducted. In graph (a) of Fig. 2, all strains contained plasmids and 6 out of 9 colistin-resistant isolates contained more than one plasmid. In graph (b) of Fig. 2, all strains contained transferable plasmids carrying *mcr-1*, which meant that the Southern hybridization was successful. The size of the plasmid carrying *mcr-1* was from 33.3 kb to 138.9 kb. However, the size of the plasmids carrying *mcr-1* in most samples was around 70 kb.

3.5. Genetic structures analysis of plasmids carrying mcr-1

The surrounding genetic environment of *mcr-1* in the 9 strains was analyzed and found to be polymorphic, consisting of three types (Fig. 3). The first type was shown in graph (a): C063 and C073. In graph (b), the *mcr-1* surrounding genetic environments in the A049, A067 and B042 strains were highly similar. BLASTn analysis showed that A067 had a query coverage of 88 % to A049, and A049 had a query coverage of 87 % to B042. Notably, all three isolates had genes that mediate conjugation transfer, namely, *virB, traL* and *traG*. Moreover, A049 and A067 had insertion elements (IS family), but B042 did not. Unlike those in graph (b) strains, the *mcr-1*-surrounding genetic environments in the A065, A009, C034 and B020 were quite different (graph (c)). A009 had a typical structure: IS*Apl1*-*mcr-1*-IS*Apl1*.

To analyze single nucleotide polymorphism (SNP)-based phylogeny, 15 strains of colistin-resistant *Escherichia coli* from different sources in MLST were downloaded. The origin and distribution of 9 strains of *colistin-resistant Escherichia coli* in this research and 15 strains of *colistin-resistant Escherichia coli* in MLST were listed in Table 3.

The phylogenetic tree could be classified into three clades (Fig. 4). The first clade had only one strain (C063), which was the most distantly related to the other strains. Most of our samples from three villages (B042, A065, C073, C034, and B020) were in the second clade, which meant that the samples from the three villages may related and *mcr-1* harboring plasmids may circulate among the villages. The third clade had three sources (healthy human feces, clinical sources, and the environment), which might indicate *mcr-1* spread between humans and the environment.

4. Discussion

Most *Escherichia coli* are harmless and even can benefit their hosts by preventing the colonization of harmful bacteria. But with the overuse of antimicrobial agents, the emergence of multi-drug resistant *Escherichia coli* becomes more and more frequently recent years. Colistin gradually returning to the public's view as a last defense line to treat multi-drug resistant *Escherichia coli* especially carbapenem-resistant *Escherichia coli*.

Following the application of colistin, the problem of colistin resistance appears in public view. In previous studies, colistin resistance was

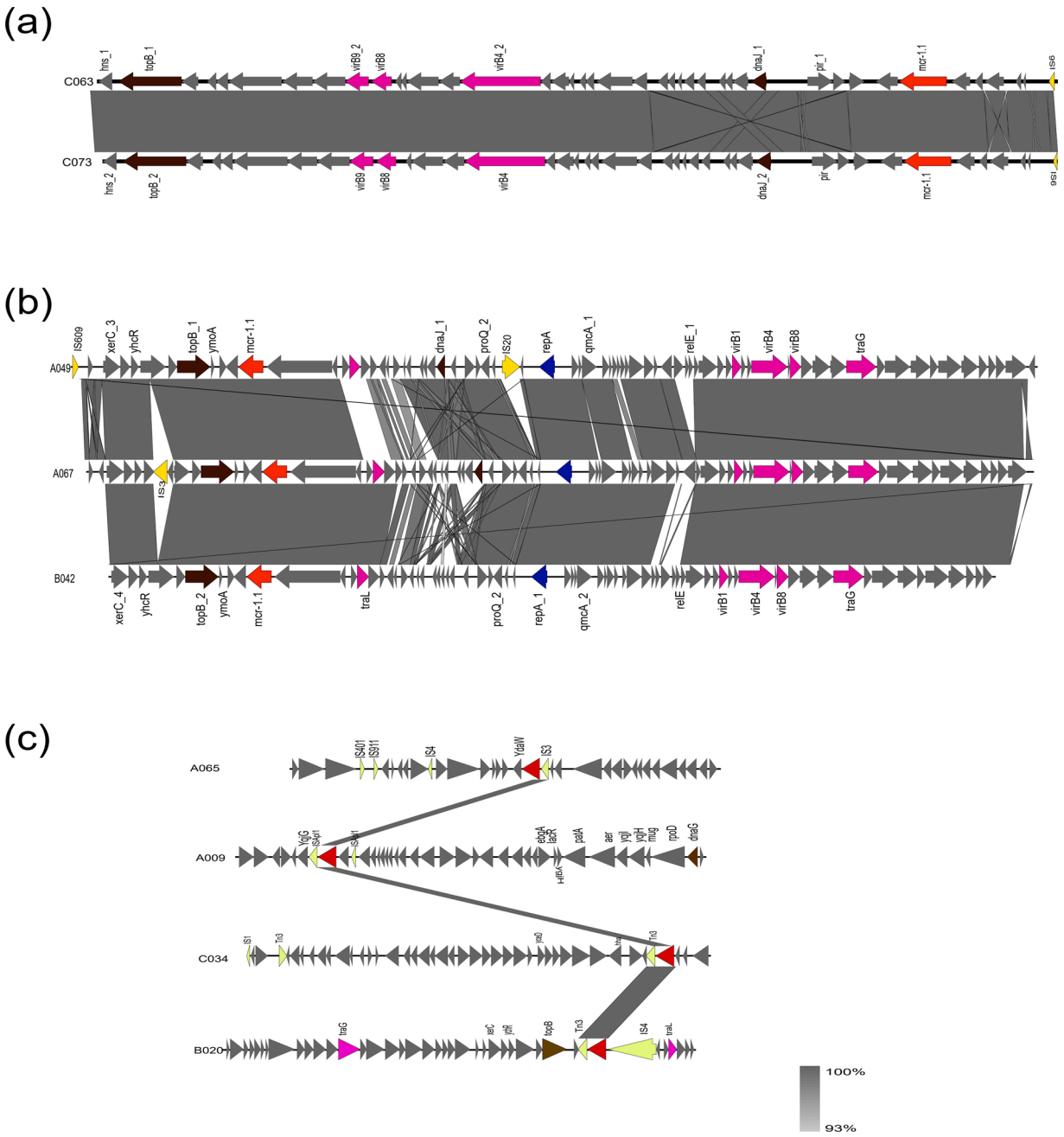

Fig. 3. Genetic structures of three representative *mcr-1*-carrying plasmid types (a) The first type: The genetic structure of two essentially identical strains. (b) The second type: The genetic structures of the three strains with high similarity. (c) The third type: The genetic structure of the three strains is completely different except for the presence of *mcr-1*. Open reading frames (ORFs) are indicated by colored arrows, with different colors representing different functions: red arrows represent *mcr-1*, yellow arrows represent insertion elements, blue arrows represent gene-related replicons, fuchsia arrows represent mobile elements, brown arrows indicate genes associated with plasmid stability and gray arrows represent hypothetical proteins.

mainly caused by chromosome mutation, but the cccchromosome is relatively stable and not easy to spread between bacteria, so it has not attracted widespread attention. In 2015, however, studies have shown that plasmid can mediate the spread of colistin resistance gene, which caused a great sensation in the society. Unlike the brief clinical application, colistin has been used as a growth promoter in animals for a long time. And many colistin-resistant *Escherichia coli* have also been found in animals. Following the discovery of the plasmid-mediated *mcr-1*, the Chinese Ministry of Agriculture and Rural Affairs formally banned colistin as an animal growth promoter on April 30, 2017. According to announcement number 2428 issued by the Chinese Ministry of Agriculture and Rural Affairs, colistin sulfate premix was prohibited as a growth promoter in mainland China. Compared with chromosomes, plasmids are more unstable and easier to transfer (Toleman et al., 2015). If plasmids harboring *mcr-1* keep increasing in *Escherichia coli* (Otter et al., 2019), super bacteria will be more common in the future (Jung et al., 2013). All these findings make it urgent to study the transmission mechanism of *Escherichia coli* with plasmid harboring *mcr-1* in humans. So far, most studies have focused on clinical patients and ignored healthy people that carry colistin-resistant *Escherichia coli*. If we are not paying attention to these healthy carriers, colistin-resistant *Escherichia coli* would flood by ignoring them. Therefore, it is necessary to study the transmission and mechanism of colistin-resistant *Escherichia coli* in healthy people.

Table 3
The origin and distribution of *mcr-1* gene positive *Escherichia coli*.

	organism group	strain	distribution	origin
1	*Escherichia coli* and *Shigella*	CO63	China: Shandong	healthy human feces
2	*Escherichia coli* and *Shigella*	B042	China: Shandong	healthy human feces
3	*Escherichia coli* and *Shigella*	A065	China: Shandong	healthy human feces
4	*Escherichia coli* and *Shigella*	C073	China: Shandong	healthy human feces
5	*Escherichia coli* and *Shigella*	295B	Bolivia	healthy human feces
6	*Escherichia coli* and *Shigella*	CO34	China: Shandong	healthy human feces
7	*Escherichia coli* and *Shigella*	Ec.47460	China: Hangzhou	healthy human feces
8	*Escherichia coli* and *Shigella*	BO20	China: Shandong	healthy human feces
9	*Escherichia coli* and *Shigella*	A067	China: Shandong	healthy human feces
10	*Escherichia coli* and *Shigella*	224A	Bolivia	healthy human feces
11	*Escherichia coli* and *Shigella*	SZH29-1	China: Shenzhen	clinical patients
12	*Escherichia coli* and *Shigella*	PN47	Thailand: Phitsanulok	healthy human feces
13	*Escherichia coli* and *Shigella*	PN71	Thailand: Phitsanulok	healthy human feces
14	*Escherichia coli* and *Shigella*	PN46	Thailand: Phitsanulok	healthy human feces
15	*Escherichia coli* and *Shigella*	PN49	Thailand: Phitsanulok	healthy human feces
16	*Escherichia coli* and *Shigella*	PN51	Thailand: Phitsanulok	healthy human feces
17	*Escherichia coli* and *Shigella*	SZM334-1	China: Shenzhen	environment
18	*Escherichia coli* and *Shigella*	A009	China: Shandong	healthy human feces
19	*Escherichia coli* and *Shigella*	L935	Thailand: Phitsanulok	healthy human feces
20	*Escherichia coli* and *Shigella*	PN60	Thailand: Phitsanulok	healthy human feces
21	*Escherichia coli* and *Shigella*	A049	China: Shandong	healthy human feces
22	*Escherichia coli* and *Shigella*	SZH3951	China: Shenzhen	clinical patients
23	*Escherichia coli* and *Shigella*	PN41	Thailand: Phitsanulok	healthy human feces
24	*Escherichia coli* and *Shigella*	PN45	Thailand: Phitsanulok	healthy human feces

Among the 218 residents included in the study, 68 people belonged to village A, 72 people belonged to village B and 78 people belonged to village C, 34.40 % were male and 65.60 % were female. Their ages ranged from 29 to 93 years old, the average age was 61.82 ± 12.83 years old. A total of 9 strains of *colistin-resistant Escherichia coli* were isolated from samples of 218 healthy residents, with a prevalence rate of 4.13 %. The prevalence rates of *colistin-resistant Escherichia coli* in animals and people are different. In terms of animals, the isolation rate of *colistin-resistant Escherichia coli* is relatively high. Among the 17 strains of *Escherichia coli* isolated from Venezuelan pigs (Roer et al., 2018), the positive rate of *mcr-1* is 5.88 %. Of the 100 strains of *Escherichia coli* isolated from chickens in Pakistan, the positive rate of *mcr-1* is 8.00 %. Among 804 strains of *Escherichia coli* isolated from pigs in Guangdong (Morten et al., 2010), China, the positive rate of *mcr-1* is as high as 21.00 %. In terms of people, the carrying rate of *colistin-resistant Escherichia coli* is lower than animal carrying rate. Among the 315 strains of *Escherichia coli* clinically isolated from Zhejiang province (Abigail et al., 2004), China, the positive rate of *mcr-1* is 1.60 %. Among the 1988 strains of *Escherichia coli* isolated from Shangqiu, Henan province, China, the positive rate of *mcr-1* is 0.30 %.

Nowadays, *colistin-resistant Escherichia coli* research mainly focus on the animals and clinical populations, and few researchers study the prevalence of *colistin-resistant Escherichia coli* in healthy people. In this study, fecal samples from 218 healthy residents in rural China are collected and isolated for *colistin-resistant Escherichia coli*. The positive rate of *mcr-1 Escherichia coli* is 4.13 %, even higher than the *colistin-resistant* rate of some clinical patients, which indicates that its prevalence in healthy people is not optimistic and it has the potential to cause a certain scale of epidemic in the population.

The antimicrobial susceptibility results showed that the *mcr-1* positive strains were all resistant to colistin and tetracycline. However, the resistance to tetracycline was higher than that to polymyxin as seen from the MIC. That may be because *colistin-resistant Escherichia coli* were more resistant to other antibiotics than colistin (Corbella et al., 2017; Nordmann et al., 2016). 6 isolates displayed a wide profile of antimicrobial resistance, which indicated that the bacterial resistance was very serious in healthy residents in this region. All our colistin-resistant strains were susceptible to meropenem, a carbapenem antibiotic. Consistent with our drug sensitivity test, colistin-resistant *Escherichia coli* were all sensitive to carbapenems (Li et al., 2018) in the study of diarrhea patients. A study of clinical patients in Vietnam found that *c* NCGM-EC88 and NCGM-EC89 were resistant to ciprofloxacin and colistin, whereas both isolates were susceptible to carbapenems (Tada et al., 2017). In a study of patients with bloodstream infections, all colistin-resistant *Escherichia coli* were susceptive to carbapenems (Zhong et al., 2019). The Carbapenems are forbidden to use in livestock production and banning carbapenems from the livestock production has curbed the spread of antibiotic-resistant *Escherichia coli* in animals and humans. These suggest that IMP could be used to treat infection caused by colistin-resistant *Escherichia coli*.

Our strain B020 had β-lactamase gene $bla_{CTX\text{-}M}$, which means that B020 is colistin-resistant ESBL. Study has shown that colistin-resistant ESBL are more resistant to antibiotics (Amin et al., 2020), and this finding is consistent with the high MIC of cefotaxime and amikacin obtained in our tests.

Some multidrug-resistant *Escherichia coli* did not have corresponding resistance genes. Our 9 strains are all resistant to tetracycline, but the tetracycline-resistant gene *tet* is only exist in 8 strains (there is no *tet* in the B042). This phenomenon can be explained by hetero-resistance. Since heterogeneous drug resistance cannot be detected by drug sensitivity tests, the diagnosis and treatment of patients with bacterial infections presents great challenges. A study finds that the combination of other antibiotics like ceftazidime improves the antibacterial efficacy of colistin against hetero-resistance and reduces the emergence of colistin-resistant subpopulation. This discovery provides new ideas for the treatment of multidrug resistance and hetero-resistance in bacteria. Therefore, in the next stage, several strains of multidrug-resistant *Escherichia coli* from this study would be selected for a combination antibiotic susceptibility test to determine whether the bacteria can be effectively inhibited. There are some resistance phenotypes that are inconsistent with the presence of resistance genes, but there are also some that are consistent. For example, our strains presented no carbapenem resistance genes and were susceptible to meropenem.

MLST analysis assigned these 9 colistin-resistant isolates to nine distinct sequence types (STs): every isolate had a different sequence type (ST). The strain C073 belongs to ST101 *Escherichia coli*, which is the most common genotype of *NDM-1*-producing *Escherichia coli* and is also regarded as a reservoir for antibiotic resistance genes (*tet, sul, aadA* et al). In addition, ST101 is considered to have an environmental (Wang et al., 2017; Toleman et al., 2015; Shen et al., 2018) lineage (water, sewage and poultry). And ST101 *Escherichia coli* always appears in clinical patients but is rarely found in healthy people. The isolation of ST101 *Escherichia coli* from a healthy person may indicate that it can be transmitted between the environment, poultry and healthy people. The multidrug-resistant strain B042 pertains to *Escherichia coli* ST410. Some data suggest that *Escherichia coli* ST410 is another pandemic extra-intestinal pathogenic *Escherichia coli*. Our clinical comparative strain

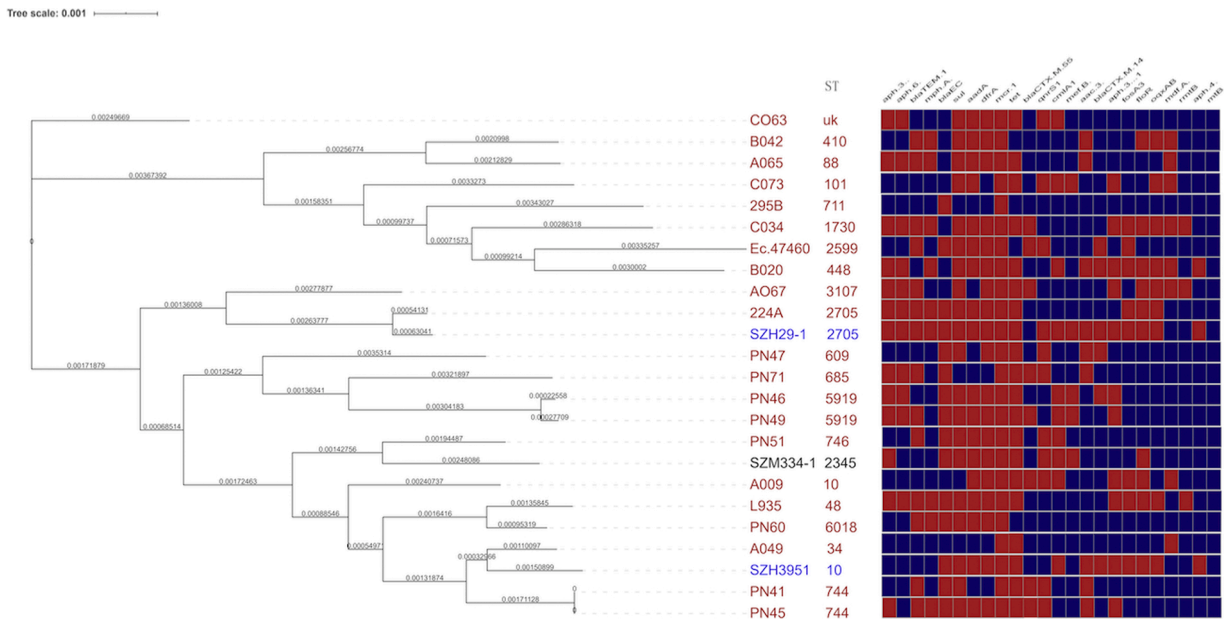

Fig. 4. Phylogenetic tree. The tree scale bar corresponds to a 0.1 % difference. The strains in red came from healthy human feces, the strains in blue were from clinical sources, and the strain in black was from the environment.

SZH3951 belongs to ST10 which is widely distributed in human diarrheagenic *Escherichia coli* infection in Hangzhou of China. And strain A009 isolated from healthy person also pertains to ST10. This proves that ST10 *Escherichia coli* exists not only in clinical patients but also in healthy people.

The hypothesis that *mcr-1* was located on plasmids and could be passed on to other bacteria was verified by conjugation assay, S1-PFGE and Southern blotting. Results of S1-PFGE and Southern blot showed that most of the plasmids carrying *mcr-1* were small plasmids with a size of 70 kb. Furthermore, the analysis in the clinical strains in China revealed that *mcr-1* is the only drug resistance gene found on *mcr-1* carrying plasmids. It means that other resistance genes existed in other plasmids rather than *mcr-1* harboring plasmids. Whether the same problem exists in our strains remains to be further analyzed by plasmid sequencing. In addition, although most of our colistin-resistant isolates contained more than one plasmid, the *mcr-1* containing plasmid was only one per strain. Our strains A067, B042 and C063 all carried three plasmids, although there was only one plasmid carrying *mcr-1*, the MIC of colistin for 3 strains, especially the A067 (MIC>32 μg/mL), was very high, which might be due to the co-resistance of other resistance genes in other plasmids. *mcr-1* on chromosomes rather than plasmids also increase colistin-resistance (Delannoy et al., 2017) in Gram-negative bacteria, thus the increase of colistin-resistance may also be caused by *mcr-1* on the chromosomes. In addition to increasing drug resistance, the antimicrobial resistance mediated by mobile plasmids was reported to be an important reason for the generation of extensively drug-resistant bacteria (Magiorakos and Srinivasan, 2012), that might explain why most multi-resistant bacteria contain plasmids.

The surrounding environments of *mcr-1* give us some discoveries and revelations. *mcr-1* often forms part of a composite transposon, Tn6330 (ISA*pl1*-*mcr-1*-*pap2*-ISA*pl1*), which is responsible for its mobilization. Only one of our colistin-resistant strains(A009) contains similar structure (ISA*pl1*-*mcr-1*- hypothetical protein-ISA*pl1*). In another study, they found that 205 of 300 (68·3%) *mcr-1* positive *Escherichia coli* had entirely lost ISA*pl1*, particularly isolates with some plasmids. This suggests that ISA*pl1* is not an essential mobile element in *mcr-1* transfer.

When the *colistin-resistant Escherichia coli* isolates were analyzed by phylogenetic tree, strains from three village in this study were closely related to each other and were closely related to foreign strains from different sources (clinical patients, the environment, and healthy people) that found in NCBI. This result may be explained by the following reasons: First, some bacteria have natural antibiotic resistance mechanisms. When they infect or colonize humans, they interact with human *Escherichia coli* and undergo gene exchange. Second, colistin is used as a veterinary drug for animals. Humans consume food animal that carries antibiotic-resistant bacteria, and these exotic bacteria in meat give the intestinal flora (such as *Escherichia coli*) resistance genes by horizontal transfer. Clinical isolates of colistin-resistant *Escherichia coli*, NGMM-EC88 and NGMM-EC89, were found to be identical with food-producing animals' *Escherichia coli*, which could prove that colistin-resistant *Escherichia coli* could be transmitted from animals to humans (Tada et al., 2017). In 2015, a single case of colistin-resistant *Escherichia coli* isolated from urinary tract infection patient might be the evidence that *mcr-1* from animals has spread to humans (Izdebski et al., 2016). Third, colistin-resistant *Escherichia coli* in the environment may be transmitted from the environment to animals through their ingestion of food. Colistin-resistant *Escherichia coli* was found in free-range chicken that were rarely given antibiotics. Free-range chickens might be exposed to antibiotic residues and antibiotic-resistant bacteria from the environment where they were out forage for foods (Mohammed and Ajrin, 2020).

Although the ministry of agriculture decided to stop the colistin sulfate used for animal growth, it can be used as a veterinary prescription drug. The use of subtherapeutic doses of antibiotics in farm animals to therapy may accelerated the spread of colistin-resistant *Escherichia coli,* and reducing colistin production may help control the generation and spread of drug resistant bacteria. With the decrease of colistin sulfate premix production, the prevalence of colistin-resistant *Escherichia coli* in pigs and chickens also decreased in most of the 23 selected provinces and municipalities across China. In recent years, the problem of antibiotic resistance is increasing severely, and the rate of it still shows no signs of slowing down. Bacterial drug resistance is often accompanied by the emergence of fitness cost, including growth rate mitigation and virulence decline. However, bacteria can also make up for the fitness cost produced by drug resistance or genetic choice. Therefore, in the current circumstance, it is relatively unable to eliminate drug resistant bacteria. By studying the fitness cost of bacterial, we can select antibacterial agents that have high fitness cost and less likely to cause bacterial compensatory mutations, and thereby prevent or delay the production of bacterial drug resistance. A multicentre case

Table 4
Previous study and present study results.

Study titles	study results
Antimicrobial Resistance Profile of *mcr-1* Positive Clinical Isolates of *Escherichia coli* in China From 2013 to 2016	This study showed a high occurrence of *mcr- 1* positive plasmids in patients with diarrheal diseases of Guangzhou in China and the abolishment of the *mcr- 1* mediated colistin resistance in one *E. coli* isolate.
Changes in colistin resistance and *mcr-1* abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study	The colistin withdrawal policy and the decreasing use of colistin in agriculture have had a significant effect on reducing colistin resistance in both animals and humans in China. However, continuous colistin monitoring is essential, in particular to act as an early warning system for colistin stewardship in Chinese hospitals.
Clinical relevance and plasmid dynamics of *mcr-1*-positive *Escherichia coli* in China: a multicentre case-control and molecular epidemiological study	Health-care contact was the most probable risk factor. Plasmids are likely to have played a critical role in *mcr-1* transmission, rather than clone dissemination and lateral transfer of ISA*pl1*. Our findings underscore the importance of continued surveillance of E coli strains positive for *mcr-1* and potentially other resistance associated genes, particularly in hospital settings.
Epidemiology and molecular characterization of *mcr-1* in *Escherichia coli* recovered from patients with bloodstream infections in Changsha, central China	The prevalence of the *mcr-1* gene in patients with E. coli bloodstream infection was 2.1% in Changsha, China. The *mcr-1*-positive E. coli isolates had varied susceptibility profiles, although all three were susceptible to carbapenems. This therapeutic window is crucial given the risk of rapid deterioration in high-incidence areas worldwide.
Extensive antimicrobial resistance and plasmid-carrying resistance genes in *mcr-1*- positive *E. coli* sampled in swine, in Guangxi, South China	Thirty-three *mcr-1*-positive E. coli isolates in Guangxi displayed a wide profile of antimicrobial resistance. Plasmid-carrying resistance genes might be the main cause of MCRPEC multidrug resistance. This study highlighted the necessity for long-term surveillance of *mcr-1*-positive E. coli in pigs.
Occurrence and Characterization of *mcr-1*-Positive *Escherichia coli* Isolated From Food-Producing Animals in Poland, 2011–2016	Our findings show that *mcr-1.1* has spread widely among production animals in Poland, particularly in turkeys and appears to be transferable mainly by IncX4 and IncHI2 plasmids spread across diverse *E. coli* lineages.
Occurrence and genetic characteristics of *mcr-1*-positive colistin-resistant *E. coli* from poultry environments in Bangladesh	We report a 13.5 % prevalence of mcr-1-positive MDR *E. coli* in poultry fecal samples predominantly from LBMs in Bangladesh accentuating the need for safe disposal of poultry feces and hygiene practices among people exposed to poultry.
The global distribution and spread of the mobilized colistin resistance gene *mcr-1*	Our results provide the first systematic phylogenetic analysis of the origin and spread of *mcr-1*, and emphasize the importance of understanding the movement of antibiotic resistance genes across multiple levels of genomic organization.
Prevalence, risk factors, outcomes, and molecular epidemiology of *mcr-1*-positive Enterobacteriaceae in patients and healthy adults from China: an epidemiological and clinical study	In 2017, colistin will be formally banned from animal feeds in China and switched to human therapy. Infection with MRCPEC is associated with sex, immunosuppression, and previous antibiotic exposure, while colonization is also associated with antibiotic exposure. MLST and plasmid analysis shows that MCRPEC are diversely spread throughout China and pervasive in Chinese communities.
mcr-1 and *mcr-2* variant genes identified in Moraxella species isolated from pigs in Great Britain from 2014 to 2015	Our results add further evidence for the mobilization of the mcr-pap2 unit from Moraxella via composite transposons leading to its global dissemination. The presence of mcr-pap2 from recent Moraxella isolates indicates they may comprise a reservoir for *mcr*.
Transmission of *mcr-1*-Producing Multidrug-resistant Enterobacteriaceae in Public Transportation in Guangzhou, China	This study was the first to demonstrate that public transportation is a source of *mcr-1*-producing E. coli and K. pneumoniae isolates.
Colistin resistance prevalence in *Escherichia coli* from domestic animals in intensive breeding farms of Jiangsu Province	Co-occurrence of *mcr-1* and *mcr-2* was identified in 20% (88/440) in pigs, 7.22% (32/443) in chickens, and 9.52% (4/42) in cattle. Interventions and alternative options are necessary to minimise further dissemination of mcr between food-producing animals and human.
Prevalence of *mcr-1* in *Escherichia coli* and Klebsiella pneumoniae recovered from bloodstream infections in China: a multicentre longitudinal study	Prevalence of *mcr-1* in *Escherichia coli* and Klebsiella pneumoniae recovered from bloodstream infections in China: a multicentre longitudinal study

control and molecular epidemiological study shows that health-contact probably the underlying risk factor for colistin-resistant *Escherichia coli* infections, and adjustments to health care may help curb this infection. In patients with colistin-resistant *Escherichia coli* infection, they did not exposure to colistin or farm animals but had chronic disease, thus chronic disease may be related to colistin-resistant *Escherichia coli* infection for their reduced immunity. Therefore, focusing attention on people with chronic diseases may be effective in preventing colistin-resistant *Escherichia coli* infections. In China, the majority of antibacterial agents are prescription drugs, and patients can only get antibiotics with a doctor's prescription. This policy is conducive to the current problem of antibiotic abuse.

In conclusion, the prevalence and transmission of *colistin-resistant Escherichia coli* are explored in a rural healthy population through experiment and data analysis. The prevalence of *colistin-resistant Escherichia coli* in healthy rural people is relatively high and more than half of the *colistin-resistant Escherichia coli* are multidrug-resistant. All the *colistin-resistant Escherichia coli* in this study contain the *mcr-1* plasmid and the plasmid could transmit between *Escherichia coli* from different sources, which presents a new challenge for interrupting the transmission of the *colistin-resistant Escherichia coli* in the future. Selecting antibacterial agents that have high fitness cost, adjustments to health care, focusing attention on chronic patients, antibiotics as prescription drugs may be effective measures to prevent colistin-resistant *Escherichia coli* infections (Table 4).

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