High incidence of multidrug-resistant *Escherichia coli* cohaboring *mcr-1* and *blaCTX-M-15* recovered from pigs

**Purpose:** The coexistence of mobile colistin (COL)-resistant gene *mcr-1* with extended-spectrum beta-lactamase (ESBL) gene in *Escherichia coli* has become a serious threat globally. The aim of this study was to investigate the increasing resistance to COL and in particular its coexistence with ESBL-producing *E. coli* recovered from pig farms in China.

**Materials and methods:** *E. coli* were isolated from 14 pig farms in Jiangsu China. Susceptibility testing was identified by micro-dilution method. PCR assay and nucleotide sequencing were used to detect COL-resistant genes, *mcr-1* to −5, as well as ESBL genes, *blaCTX-M*, *blaSHV* and *blaTEM*. Conjugation experiment, plasmid replicon typing of the multidrug resistance (MDR), S1-PFGE and DNA southern hybridization were performed to study the transferability of these genes.

**Results:** Overall, 275 *E. coli* isolates were recovered from a total of 432 cloacal and nasal swabs. More than 90% of the isolates were MDR, of which 70.18% were resistant to COL. Of these 275 isolates, *mcr-1* was identified as the most predominant gene carried by 71.63% (197/275) of isolates, 39.59% (78/197) of the isolates were harboring both *mcr-1* and ESBL genes (*blaCTX-M*, *blaSHV* and *blaTEM*). ESBL genotyping showed that *blaCTX-M* was the most predominant ESBL (68.49%) followed by *blaSHV* (16.4%) and *blaTEM* (15%). Sequencing revealed that the most common variants of *blaCTX-M* were found to be the most common Inc-types found both in donors and in transconjugants and were associated with the transfer of the *mcr-1* and ESBL encoding genes. Six strains carried a total of five different plasmids: approximately 97-, 130-, 160-, 227- and 242-kb plasmids.

**Conclusion:** The coexistence of the *mcr-1* - and *blaCTX-M-15*-carrying isolates displaying high MDR, recovered from *E. coli* of pig origin, is a major concern for both humans and veterinary medicine.

**Keywords:** *E. coli*, colistin, *mcr-1*, ESBL, coexistence

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**Introduction**

Antimicrobial resistance (AMR) has now been widely recognized as a crucial threat to human and animal health as the extensive use of antimicrobials in humans as well as in food-producing animals. Global consumption of antimicrobials in animal settings may rise up to 67% by 2030, determined predominantly by BRICS (Brazil, Russia, India, China and South Africa) countries, as large-scale and intensive farming operations are greatly in demand with the upsurge in revenue and animal protein consumption. This heavy antimicrobial practice creates a selective pressure that contributes to the emergence and spread of bacterial resistance. One of the major concerns is the rapid increase...
of the multidrug-resistant (MDR) Escherichia coli in animal settings and clinical medicine. This is not only because of the lessened number of useful antimicrobials for curing MDR E. coli infections, but also due to the potential transfer of MDR E. coli strains from animals to humans, especially which producing extended-spectrum β-lactamases (ESBLs) and carbapenemases, and display resistance to colistin (COL).

ESBLs are β-lactamases that confer resistance to oxyimino “second- and third-generation” cephalosporin’s (eg, cefotaxime (CTX), ceftriaxone and ceftazidime) and aztreonam. ESBL-producing bacteria were first reported in 1980, soon after the introduction of the third-generation cephalosporin’s (CTX and cefotiafur (CEF) into clinical settings. Currently, there are more than 350 ESBL genes that have been reported, and these genes are commonly developed through point mutations of the classical SHV-1 and TEM-1 β-lactamases and more increasingly prominent the CTX-M types. Among the CTX-M enzymes, blaCTX-M-55 has become the leading CTX-M type in ESBL-producing E. coli isolates of animal origin during the last decade. In contrast, blaCTX-M-15 seems to be the most extensive types in isolates of human origin. ESBL-producing E. coli are highly linked with multiple plasmids and studies have reported that ESBL genes are often carried on IncF, IncI1, IncN, IncH1 and IncH2 in food-producing animals worldwide. There is potential for ESBL genes/plasmid spreading between E. coli from animals, food and humans.

The co-occurrence of ESBL genes and mcr-1 in E. coli was reported from China in 2016. Rhouma and Letellier assumed that a historic relation existed between ESBL genes, carbapenemase genes and mcr-1. A recent study proposed that cephalosporin resistance is commonly spread in animals and humans through distinct plasmids. It is highly expected that food-producing animals have become the most significant reservoirs in disseminating these resistance genes in the community through horizontal gene transfer. To assess the co-occurrence and emergence of mcr and ESBL genes in E. coli of pig source, we examined 14 pig farms of Jiangsu province in China to evaluate the current scenario of these resistant genes in pigs and further clarified the predominant genotype and plasmids diversity of mcr and ESBL genes.

Materials and methods

Collection of samples

A total of 432 samples (400 from healthy and 32 from dead pigs) were collected from 14 commercial pig farms in Jiangsu, China (Figure S1), during the period of August 2016 until December 2017. From each farm, samples were randomly collected. The anal swabs were collected by inserting the swab into the rectum and being rotated. To collect nasal swabs from swine, the nose was wiped with a piece of paper and a sterile swab was inserted into the nasal cavity and rotated for 3 s at 90°. From 32 dead pigs, all samples were aseptically obtained from different organs. All collected samples were immediately transported at 4°C to the laboratory for microbial examination and processed within 4 hrs.

Isolation and screening of ESBL-producing and COL-resistant E. coli

All samples were directly streaked onto MacConkey agar (Binhe Microorganism Reagent Co. Ltd., Hangzhou, China) supplemented with CTX (1 µg/mL) and COL (2 µg/mL) for the screening of possible ESBL-producing and COL nonsensitive E. coli as previously described. Plates were incubated at 37°C for 18–24 hrs. Presumptive E. coli colonies with dark pink to red colors were confirmed microscopically and further verified by species-specific PCR as described previously. Confirmed E. coli strains were stored in Luria–Bertani medium (Oxoid, United Kingdom) containing 40% (vol/vol) glycerol in aliquots at −80°C until further use.

ESBL-producing E. coli were further confirmed by double-disk synergy (DDS) testing as recommended by the Clinical and Laboratory Standards Institute (CLSI) guideline, using antibiotic discs of ceftazidime (30 µg), ceftazidime plus clavulanic acid (30/10 µg), CTX (30 µg) and CTX plus clavulanic acid (30/10 µg). DDS test was performed for phenotypic detection of ESBLs. The test result is considered as positive if the zone of inhibition is ≥5 mm larger with clavulanic acid than without.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed by minimum inhibitory concentration (MIC) determination using broth microdilution method against 11 antibiotics for all 275 isolates and 17 antibiotics for transconjugants. The MIC data was interpreted according to the CLSI recommendations. Antibiotics used in this study, comprised of 5 β-lactams – ampicillin (AMP), CTX, cefotaxin (CFX), CEF and meropenem (MEM) – and 12 non-β-lactams – COL, ciprofloxacin (CIP), chloramphenicol (CHL), enrofloxacin (ENR), gentamycin (GEN), kanamycin, nalidixic acid, polymyxin-B (POL-B), tetracycline (TET), trimethoprim, streptomycin and sulfamethoxazole. The MIC of COL was determined by broth
micro-dilution method recommended by the joint CLSI-EUCAST polymyxin breakpoints working group (www.EUCAST.org), CLSI VET01-A4 is used for CEF and ENR which are missing in the human CLSI M100-S27. *E. coli* ATCC 25922 was used as a quality control in antimicrobial susceptibility testing. Isolates that exhibited resistance to more than 3 antimicrobial agents were classified as MDR.

**PCR assays for detection of mcr and ESBL genes**

PCR assay was used to detect COL-resistant genes *mcr*-1 to *mcr*-5 as well as ESBL genes (*bla*CTX-M, *bla*SHV and *bla*TEM). Total DNA was isolated by conventional boiling method. All these resistant genes were screened via PCR-based diagnostics with specific primers, as previously described. All the primers and PCR conditions used in this study are listed in Table 1. All PCR positive amplicons of these targeted genes were sequenced by Sanger sequencing in TSINGKE Corporation (Nanjing, PR China).

**Conjugation experiment**

To determine the transferability of resistance genes, 15 COL-resistant *E. coli* isolates were selected as donors for conjugation. *E. coli* EC-600 (Nal<sup>R</sup>, Rif<sup>R</sup>) was used as recipient bacteria. Conjugation experiments were performed as previously described. These putative transconjugants were further confirmed using antibiotic susceptibility testing, PCR detection and plasmid incompatibility (Inc) groups typing carried by the transconjugants.

**Plasmid replicon typing**

Plasmid DNA was extracted from both donors and transconjugants using the Wizard Genomic DNA Purification kit (Promega) and was characterized by PCR-based replicon typing method (PBRT). Eighteen pairs of primers were designed to perform 5 multiplex and 3 simples PCRs targeting the FIA, FIB, FIC, HI1, HI2, IncI1, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIA replicons as previously mentioned. While primers for two other plasmids IncI2 and IncX4 were designed

| Primer name | PCR target | Sequence (5′- 3′) | Annealing temperature (°C) | Product size (bp) | Reference |
|-------------|------------|-------------------|-----------------------------|------------------|-----------|
| **E. coli-specific**<br>UAL UAR | *uidA* | TGGTAATTACGACGAAAACG GC ACG CGT GGT TAC AGT CTT GCG | 62 | 147 | 58 |
| **ESBL genes**<br>CTX-MA<br>CTX-MB | *bla*CTX-M | CGC TTT GCG ATG TGC AG ACC GCG ATA TCG TTG GT | 54 | 550 | 58 |
| SHV-F<br>SHV-R | *bla*SHV | GGG TTA TTC TTA TTT GTC GC TTA GCG TGG CCA GTG CTC | 58 | 930 | 58 |
| TEM-F<br>TEM-R | *bla*TEM | ATA AAA TTC TTG AAG ACG AAA GAC AGT TAC CAA TGC TTA ATC | 56 | 1086 | 58 |
| **MCR genes**<br>CLR5-F<br>CLR5-R | *mcr*-1 | CGG TCA GTC CGT TTG TTC CTT GGT CGG TCT GTA GGG | 58 | 309 | 41 |
| MCR-2-F<br>MCR-2-R | *mcr*-2 | TGT TGC TTG TCG CTA TTG GA AGA TGG TAT TGT TGG TTG CTG | 58 | 567 | 42 |
| MCR-3-F<br>MCR-3-R | *mcr*-3 | TCG CTA CGT TAT TTT GCA TTT TTA ACG AAA TTG GCT GGA ACA | 50 | 542 | 43 |
| MCR-4F<br>MCR-4R | *mcr*-4 | ATT GGG ATA GTC GCC TTT TT TTA CAG CCA GAA TCA TTA ATC | 58 | 487 | 44 |
| MCR-5-F<br>MCR-5-R | *mcr*-5 | ATG CGG TTG TCT GCA TTT ATC TCA TTG TTG TTC TCT G | 50 | 1644 | 45 |
separately which were missing in the previous replicon typing. All the PBRT primers and PCR conditions used in this study are listed in the Supplementary Table S1. PCR amplicons of plasmids were sequenced by Sanger sequencing (TSINGKE Corporation, Nanjing, PR China) and retrieved sequences were used to confirm replicon types by using BLAST tool available at NCBI web (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Pulsed field gel electrophoresis (PFGE) and Southern hybridization
To determine the genetic relatedness and location of transmissible mcr-1-positive elements, the six conjugative E. coli strains were characterized by S1-PFGE and Southern hybridization using a probe specific for mcr-1. Genomic DNA from each of the isolate was digested with S1 nuclease (Thermo scientific) and was examined by PFGE as previously described.31

Southern hybridizations of plasmid DNA were performed with a digoxin-labeled mcr-1-specific probe according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany) as previously described.32

Statistical analysis
Differences in the AMR profiles of E. coli isolates with or without mcr-1 were assessed by a two-tailed Chi-square test or Fisher’s exact test using the Statistical Packages of Social Sciences software for Windows, version 20.0 (IBM Corp., Armonk, NY), with P<0.05 set as the level of significant differences.

Results
Bacterial isolation and antimicrobial susceptibility
Overall, 275 E. coli isolates (243 from healthy and 32 from dead pigs) were recovered from 432 samples of 14 different pig farms. All isolates were observed to be ESBL-producing and COL nonsensitive as determined by phenotypic approaches, giving a carriage rate of 63.6% (275/432). Of these 275 E. coli isolates, 174 (63.27%) were from feces, 69 (25.09%) were from nasal and remaining 32 (11.63%) were from diseased and dead pigs.

The MICs were obtained from the antibiotic susceptibility testing for all isolates. To determine the resistance profiles of 275 representative E. coli strains, susceptibility of 11 antibiotics were used (Table 2). Of the 275 E. coli isolates, the resistant rate to TET was 97.81%, followed by AMP (96.72%), CHL (94.54%), CFX (86.18%), CTX (78.18%), CEF (77.81%), CIP (73.81%), POL-B (71.27%), GEN (70.54%), and COL (70.18%). In contrast, the most effective antibiotic against these isolates was MEM with 99.6% susceptibility. Majority of the E. coli strains showed considerable MDR to β-lactams and several non-β-lactams groups, including polypeptides group, fluoroquinolones, aminoglycosides, amphenicol, quinolone group, sulfonamides and TET.

mcr-1 and ESBL genes are prevalent among E. coli strains
Although 70.18% (193 of 275) swine E. coli isolates conferred resistance to COL, the mcr-1 carriage rate was 71.63% (197/275) (Table 3) and only mcr-1 gene was detected in these COL-resistant E. coli isolates. No other colistin-resistant gene (mcr-2 to mcr-5) could be detected in the study population of E. coli isolates. The mcr-1 gene was detected in all farms, and the prevalence rate was enormously high 71.6%, ranging from 47.8% to 100% in different farms, while 40.6% in diseased isolates (Table 3).

We recovered a total of 146 (53.09%) ESBL-producing E. coli strains from 275 samples collected from 14 different farms of pigs (Figure S2 and Table 5). Among the 146 ESBL-producing isolates, 68.49% (100/146) harbored blacTX-M genes, 22.60% (33/146) harbored blaSHV, while 18.49% (27/146) were carrying blaTEM genes. Among them, 6 isolates carried blaCTX-M and blaSHV, 3 isolates contained blaCTX-M and blaTEM, and one isolate had three genes of blaCTX-M, blaSHV, and blaTEM.

The resistance patterns of E. coli are different in mcr-1-positive and mcr-1-negative isolates
The mcr-1-positive E. coli isolates displayed more resistance to other antimicrobials than those of mcr-1-negative isolates (Table 4). For mcr-1-positive E. coli, all isolates, 100% (197/197), possessed not less than 3 antibiotics resistance pattern, while about 96.15% (75/78) of COL-negative isolates did (P=0.006), with 3 COL-sensitive isolates which displayed resistance to not more than 2 antibiotics. In addition, about two-thirds of mcr-1-positive E. coli isolates, 68.52% (135/197), showed resistance to at least 9 drugs, but only 3.84% (3/78) of mcr-1-negative isolates did (P=0.000). Interestingly, 68.02% (134/197) of mcr-1-positive strains presented resistance profiles to 10 drugs, but no COL-negative E. coli did (P=0.000).
Table 2: Distribution of MICs of 11 antibiotics for 275 MDR *Escherichia coli* isolates

| Antibiotics    | MIC (mg/L) | 0.008 | 0.016 | 0.032 | 0.063 | 0.125 | 0.25  | 0.5   | 1    | 2    | 4    | 8    | 16   | 32   | 64   | 128  | 256  | >256 | MIC50 | MIC90 | Resistance |
|----------------|------------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|------|------|------|------|------|------|-------|-------|-----------|
| Ampicillin     | -          | -     | -     | -     | -     | -     | -     | -     | 0    | 1    | 1    | 1    | 6    | 4    | 12   | 23   | 38   | 189  | >256 | >256 | 96.72% |
| Cefotaxime     | -          | -     | -     | -     | 0     | 8     | 9     | 11    | 14   | 18   | 15   | 14   | 19   | 31   | 45   | 44   | 33   | 14   | 32   | 256  | 78.18% |
| Cefoxitin      | -          | -     | -     | -     | 0     | 3     | 2     | 11    | 3    | 1    | 18   | 72   | 51   | 56   | 30   | 9    | 19   | 32   | 128  | 86.18% |
| Ceftiofur      | -          | -     | -     | -     | 0     | 3     | 21    | 23    | 14   | 12   | 26   | 19   | 28   | 33   | 56   | 31   | 9    | 32   | 256  | 77.81% |
| Chloramphenicol| -          | -     | -     | -     | -     | -     | -     | 0     | 0    | 2    | 1    | 7    | 5    | 17   | 34   | 35   | 61   | 113  | 256  | >256 | 94.54% |
| Ciprofloxacin  | -          | 0     | 15    | 19    | 5     | 6     | 3     | 14    | 10   | 25   | 26   | 42   | 47   | 38   | 23   | 2    | -    | 16   | 64   | 73.81% |
| Colistin       | -          | -     | 0     | 9     | 29    | 8     | 14    | 22    | 29   | 110  | 41   | 7    | 4    | 2    | 0    | -    | -    | 4    | 8    | 70.18% |
| Gentamycin     | -          | -     | -     | 0     | 12    | 14    | 9     | 17    | 13   | 4    | 12   | 25   | 54   | 56   | 27   | 16   | 16   | 32   | 256  | 70.54% |
| Meropenem      | 0          | 16    | 158   | 75    | 10    | 4     | 3     | 2     | 3    | 1    | 0    | 0    | -    | -    | -    | 0.032| 0.0625| -    | 0.36% |
| Polymyxin-B    | -          | -     | -     | 3     | 12    | 12    | 15    | 37    | 41   | 87   | 49   | 16   | 2    | 1    | 0    | -    | -    | 4    | 8    | 71.27% |
| Tetracycline   | -          | -     | -     | -     | -     | 0     | 3     | 0     | 3    | 0    | 1    | 16   | 41   | 47   | 69   | 96   | 256  | >256 | >256 | 97.81% |

Notes: Red vertical lines indicate the breakpoints between intermediate and resistant values. White areas indicate range of tested dilutions for each antibiotic; the MIC50 and MIC90 values are concentrations at which ≥50% and ≥90% of isolates are inhibited.

Abbreviations: MIC, minimum inhibitory concentration.
Table 3  Prevalence of mcr-1 & or ESBL-producing E. coli in swine samples collected from different farms of Jiangsu China

| Farm numbers | No. of positive E. coli samples | No. of mcr-1 positive E. coli isolates (%) | No. of mcr-1 & ESBL producing E. coli isolates (%) |
|---------------|---------------------------------|------------------------------------------|-----------------------------------------------|
| Farm 1        | 32                              | 22 (68.7)                               | 10 (31.2)                                     |
| Farm 2        | 11                              | 7 (63.6)                                | 3 (27.2)                                      |
| Farm 3        | 10                              | 8 (80.0)                                | 4 (40.0)                                      |
| Farm 4        | 26                              | 18 (69.2)                               | 4 (15.3)                                      |
| Farm 5        | 14                              | 14 (100)                                | 2 (14.2)                                      |
| Farm 6        | 14                              | 13 (92.8)                               | 2 (14.2)                                      |
| Farm 7        | 7                               | 5 (71.4)                                | N.D.                                          |
| Farm 8        | 14                              | 12 (85.7)                               | 6 (42.8)                                      |
| Farm 9        | 16                              | 14 (87.5)                               | 9 (56.2)                                      |
| Farm 10       | 43                              | 37 (86.0)                               | 11 (25.5)                                     |
| Farm 11       | 23                              | 11 (47.8)                               | 5 (21.7)                                      |
| Farm 12       | 12                              | 9 (75.0)                                | 6 (50)                                        |
| Farm 13       | 14                              | 7 (50.0)                                | 4 (28.5)                                      |
| Farm 14       | 7                               | 7 (100)                                 | 5 (71.4)                                      |
| Diseased      | 32                              | 13 (40.6)                               | 7 (21.8)                                      |
| Total         | 275                             | 197                                     | 78                                            |

Abbreviations: ESBL, extended-spectrum β-lactamase; N.D., not determined.

Coexistence of mcr-1 and ESBL genes screened among the strains

Based on the results of this study, among 197 mcr-1 positive isolates, 39.59% (78/197) were identified as carrying both mcr-1 and ESBL genes. Distribution of all mcr-1 positive E. coli isolates (n=78) harboring ESBL genes are analyzed and presented in Figure 1 and Table 5. Our findings indicate that the combination of mcr-1 with blaCTX-M was the most prevalent with the rate of 70.51% (55/78) followed by the combination of mcr-1 and blaSHV (14.10%, 11/78). Finally, 7.69% (6/78) isolates were identified carrying both mcr-1 and blaTEM. Furthermore, combination of mcr-1 with two or more than two ESBL genes was also identified. Results showed that a total of 5.12% (4/78) of mcr-1-positive isolates also carried blaCTX-M and blaSHV, while 1.28% (1/78) of mcr-1-positive isolates carried blaCTX-M and blaTEM. Interestingly, a single isolate was carrying (mcr-1+ blaCTX-M + blaSHV + blaTEM). In this study, mcr-1 and blaCTX-M were identified as the dominant genes (Table 5).

As mcr-1 in combination with blaCTX-M were identified as the most prevalent (70.51%, 55/78), we further sequenced the blaCTX-M genes to explore the subtypes. Sequencing analysis of these 55 blaCTX-M isolates showed that all these mcr-1-positive isolates were harboring blaCTX-M-1 group. The most prevalent variants identified in these 55 isolates belonged to this group were blaCTX-M-15 in 38/55 (69%) isolates, followed by blaCTX-M-55 in 16/55 (29%) isolates and blaCTX-M-1 in one isolate (1.8%).

mcr-1 and ESBL genes could be conjugative transfer by plasmids with different replicon type

Conjugation experiments were performed on random 15 mcr-1 positive isolates. Of the 15 mcr-1 resistant isolates, 10 isolates were carrying additional blaCTX-M, while a single isolate was harboring blaTEM. Of these 15 isolates, 12 were successfully transferred to E. coli EC-600. The resistance profiles of the 12 transconjugants were identical to those of the mcr-1 and blaCTX-M carrying E. coli donor isolates, indicating the transfer of antibiotic resistance. In addition, resistant to several non-β-lactam antibiotics, such as aminoglycosides, fluoroquinolones, TET, macrolides and sulfonamides, were also co-transferred along with COL and β-lactam resistance. MICs of COL of these transconjugants revealed 4- to 8-fold increase as compared with the recipient EC-600 (0.125 μg/mL).

PCR-based replicon typing (PBRT) showed that in the E. coli isolates carrying mcr-1 and blaCTX-M, the plasmids with different replicons, including IncHI2 (n=7), IncFIB (n=7), IncFIC (n=4), IncP (n=4), IncFepB (n=4), IncN (n=3), IncX4 (n=2) IncY (n=2) and IncI1 (n=1), were detected in the donor strains (Figure 2 and Table 6).

However, PBRT of the transconjugants confirmed only five replicons, IncHI2, IncFIB, IncFIC, IncN and IncX4, which were present in both donors and transconjugants and were associated with the transfer of the mcr-1 and ESBL genes.

S1-PFGE analysis demonstrated that these six strains carried multiple plasmids varying in sizes ranging from...
Southern hybridization assay confirmed that the \textit{mcr-1} gene recovered from these six strains was positioned on the following five different types of plasmids: with the size of approximately 97, 130, 160, 227 and 242 kb, respectively (Figure 3B).

Table 5 Distribution of various resistance genes among 275 MDR \textit{Escherichia coli} isolates from pigs

| Colistin resistant gene | Extended-spectrum beta-lactamase genes | No. of isolates |
|-------------------------|----------------------------------------|-----------------|
| \textit{mcr-1}          | \textit{bla}_{CTX}          | \textit{bla}_{SHV} | \textit{bla}_{TEM} |
| +                      | +                        | +                | 119   |
| +                      | +                        | +                | 1     |
| +                      | +                        | +                | 34    |
| +                      | +                        | +                | 13    |
| +                      | +                        | +                | 15    |
| +                      | +                        | +                | 55    |
| +                      | +                        | +                | 11    |
| +                      | +                        | +                | 6     |
| +                      | +                        | +                | 1     |
| +                      | +                        | +                | 4     |
| +                      | +                        | +                | 1     |
| +                      | +                        | +                | 2     |
| +                      | +                        | +                | 2     |
| +                      | +                        | +                | 1     |
| +                      | +                        | +                | 1     |
| +                      | +                        | +                | 9     |

Abbreviations: +, positive; N.D., not determined.

Discussion

China alone produces and consumes roughly half the planet's pigs, about 500 million annually, and has been the leading consumer of antibiotics in the world. The increased usage of antibiotics may trigger the emergence of AMR. Reports on emergence of AMR particularly resistance of \(\beta\)-lactam and COL are increasing all over the world. The prevalence of ESBL in animal origin has been rising since 2003, with slight variances amongst terrestrial regions and different animal species. We report on the high incidence of \textit{mcr-1}-carrying ESBL-producing \textit{E. coli} recovered from pigs in Jiangsu, China. Our results indicated that all the \textit{mcr-1} and ESBL-producing \textit{E. coli} isolates showed MDR. The majority of these isolates (77–86%) showed resistance to cephalosporin (Table 2). In addition, high resistance was also observed to common \(\beta\)-lactam and non-\(\beta\)-lactam antimicrobials such as AMP, fluoroquinolones, aminoglycosides, amphenicol, quinolones, sulfonamides and TET which are commonly used in human as well in veterinary practice. Many recent studies have reported MDR ESBL-producing \textit{E. coli} isolated from poultry, pigs, cattle and humans.

Recently, plasmid-mediated COL-resistant genes \textit{mcr-1} to \textit{mcr-8} have been widely discovered around the world. Herein, we screened 275 MDR \textit{E. coli} isolated from 14 pig farms from Jiangsu province for the presence of \textit{mcr-1} to \textit{mcr-5} genes. Only \textit{mcr-1} gene was detected in isolates from every farm, and the carriage rate was extremely high in 71.6% (197/275). The high prevalence rate of the \textit{mcr-1} found in this study.

Figure 1 Distribution of various resistance genes in combination.
from pigs in Jiangsu is consistent with very recent reports from China.48,49 These recent studies in pigs reported similarly high mcr-1-positive carriage (79.2% and 76.2%), ranging from 45% to 100% in different provinces, while the mcr-1 rate in Jiangsu province reported by 48 was 71.9% which is very similar to our findings 71.6%. The present study and, together with the previous studies, confirmed a surprisingly high rate of mcr-1 in swine farms and is likely associated with the prolonged and extensive practice of COL as a growth promoter in pigs.

The coexistence of mcr-1 with other resistance genes in an E. coli was reported in China.50 One recent study also assumed that a historic bridge existed between mcr-1 and ESBL.23 However, there is scarcity in the incidence of the coexistence of mcr-1 and ESBL in the pig origin. Herein, we screened 275 MDR E. coli-resistant strains from pig farms in Jiangsu during 2016–2017 and found a high occurrence of mcr-1-positive strains with ESBL in the swine 39.5% (78/197), which was very high from the previous report.51 A very recent longitudinal study from China investigated the co-rising of mcr-1 and ESBL in chicken isolates.14 Among ESBL-positive strains, we found that blactx-m is the most predominant.

In this study, 39.5% mcr-1-positive E. coli strains were detected which coexist in different ESBL genes. Among them, blactx-m was the predominant one which was found in 55 mcr-1-positive E. coli 70.5% (Figure 1). On sequence-based analysis of these 55 blactx-m isolates, interestingly the blactx-m15 gene was found to be the most prevalent blactx-m gene (69%) followed by blactx-m55 (29%). The spread of the blactx-m15 gene is a common blactx-m enzyme and detected widely in Enterobacteriaceae of human origin.19,52 Very few studies have reported the co-occurrence of mcr-1 and blactx-m15 of human origin.22,53 From China, one recent study in dairy cows also found blactx-m15 as the second prevalent ESBL gene 21.4% (62/275), but there was no mcr-1 gene detected in those blactx-m15 isolates.54 Another study also reported the coexistence of mcr-1 and blactx-m15 in Turkey hen meat.55 Herein, this is the first investigation that reveals the coexistence of mcr-1 and blactx-m15 in E. coli strains of pig origin in a very high proportion.

A significant increase in the blactx-m55 was found in E. coli by over a period of ten years.4 Many previous studies reported that blactx-m55 in human strains in China has become the second dominant blactx-m type and even the occurrence of blactx-m55 was higher than blactx-m15. A high rate of mcr-1 and blactx-m55 was recently detected from the chicken origin in China,14 which was consistent with our results. Thus, the concurrent dissemination of the mcr-1 harboring blactx-m15 and blactx-m55 mediated by a single bacterial clone is existing which suggests that mcr-1 is found in the diverse reservoirs.
| Strain   | CTX MIC (mg/L) | COL MIC (mg/L) | Plasmid (Inc) types | Resistance genes | Resistance profile for non-beta-lactam antibiotics |
|----------|----------------|----------------|---------------------|------------------|--------------------------------------------------|
| EC-9     | 128            | 16             | HI2,II,Y,FIC        | MCR-1,CTX-M-15   | GEN, ENR, NAL, SUL, CIP, KEN, CHL, TET, TRM, STR, POL-B |
| EC-9-T   | 128            | 8              | HI2, FIC            | MCR-1,CTX-M-15   | GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, POL-B     |
| EC-37    | 256            | 8              | N, P                | MCR-1,CTX-M-55   | NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, STR, POL-B |
| EC-37-T  | 32             | 4              | N                   | MCR-1,CTX-M-55   | NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, STR, POL-B |
| EC-48    | 256            | 4              | FIB, P, FrepB, N    | MCR-1,CTX-M-55   | GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR, POL-B |
| EC-48-T  | 32             | 2              | FIB, N              | MCR-1,CTX-M-55   | GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR, POL-B |
| EC-S2    | 128            | 4              | FIB,N, P            | MCR-1,CTX-M-55   | GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR, POL-B |
| EC-S2-T  | 256            | 2              | FIB,N               | MCR-1,CTX-M-55   | GEN, ENR, NAL, SUL, KEN, CHL, TET, TRM, STR, POL-B |
| EC-29    | 256            | 8              | HI2, FIB            | MCR-1,CTX-M-15   | GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B         |
| EC-29-T  | 0.5            | 2              | HI2, FIB            | MCR-1,CTX-M-15   | GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B         |
| EC-34    | 256            | 8              | FIB, HI2, FrepB     | MCR-1,CTX-M-15   | ENR, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-34-T  | >256           | 8              | HI2,FIB             | MCR-1,CTX-M-15   | ENR, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-40    | 128            | 4              | HI2, FIB, FrepB     | MCR-1,CTX-M-15   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-40-T  | 64             | 2              | HI2, FIB            | MCR-1,CTX-M-15   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-1     | 256            | 4              | X4,FIC              | MCR-1,TEM        | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-1-T   | 256            | 2              | X4,FIC              | MCR-1,TEM        | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-25F   | >256           | 2              | HI2,FIB, P          | MCR-1,CTX-M-15   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-25F-T | >256           | 4              | HI2, FIB            | MCR-1,CTX-M-15   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-55    | 64             | 8              | HI2,FIB, FIC,Y      | MCR-1,CTX-M-55   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-55-T  | 64             | 4              | HI2,FIC             | MCR-1,CTX-M-55   | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-20    | 2              | 4              | X4,FIC, FrepB       | MCR-1            | GEN, NAL, SUL, CFX, CEF, KEN, CHL, TET, TRM, POL-B |
| EC-20-T  | 0.25           | 4              | X4,FIC              | MCR-1            | GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B         |
| EC-19    | 128            | 32             | HI2                 | MCR-1            | GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B         |
| EC-19-T  | 256            | 4              | HI2                 | MCR-1            | GEN, NAL, SUL, KEN, CHL, TET, TRM, POL-B         |
| EC-600   | 0.25           | 0.125          | ND                  | ND               | ND, not determined.                              |

Abbreviations: COL, colistin; CTX, cefotaxime; GEN, gentamycin; ENR, enrofloxacin; NAL, nalidix acid; SUL, sulfamethoxazole; CIP, ciprofloxacin; KEN, kenamycin; CHL, chloramphenicol; TET, tetracycline; TRM, trimethoprim; STR, streptomycin; POL-B, polymyxin-B; ND, not determined.
In addition, \textit{bla}_{\text{CTX-M-15}} \text{ and } \textit{bla}_{\text{CTX-M-55}} \text{ were previously reported on conjugative plasmids, ie, FIB, IncI1, IncHI2, IncK, IncP and IncN.}^{35,56} \text{ Therefore, we also detected these incompatibility types by PCR typing. While } \textit{mcr-1} \text{ gene was often found on conjugative plasmids like IncF, IncI2, IncHI2, IncN, IncP and X4, which exhibit an unexpected diversity.}^{57} \text{ In conjugation experiment, IncHI2, IncFIB, IncFIC, IncN and IncX4, were found in both donors and transconjugants and were associated with the transfer of the } \textit{mcr-1} \text{ and ESBL encoding genes.}

Genetic representation of } \textit{mcr-1}-\text{carrying plasmids demonstrated that this gene is located on different conjugative elements of } \sim 97 \text{ kb and } 242 \text{ kb in size. The fact that } \textit{mcr-1}\text{-carrying } \textit{E. coli} \text{ isolates display divergent PFGE profiles suggests that these elements may play a vital role in } \textit{mcr-1} \text{ transmission. The incidence of closely related plasmids that carry } \textit{mcr-1} \text{ and ESBL resistance genes among genotypically varied } \textit{E. coli} \text{ strains from various origins is a threat for alarm as it indicates that plasmids can easily disseminate from animals to humans and the spread of these plasmids may be remarkably challenging to control.}

Considering that } \textit{bla}_{\text{CTX-M}} \text{ has become the most prevalent ESBL type of animal origin in the last few years, this situation may suggest that } \textit{mcr-1} \text{ and } \textit{bla}_{\text{CTX-M}} \text{ emerged and arose due to the extensive use of antimicrobial practice in animal farming in the last decade. Our results also suggested that } \textit{bla}_{\text{CTX-M-15}} \text{ and } \textit{bla}_{\text{CTX-M-55}} \text{ and other } \beta\text{-lactamase genes coharboring with } \textit{mcr-1} \text{ positive isolates is a potential threat to public health as the pig carrying these genes may enter the food chain. It is recommended that we should pay high consideration in monitoring the incidence of ESBL-producing & COL-resistant } \textit{E. coli} \text{ in both clinical and food-producing animals.}

\textbf{Figure 3 (A)} \textit{S1-nuclease pulsed-field gel electrophoresis profiles of six } \textit{E. coli} \text{ and (B) Southern Blot hybridization of six } \textit{E. coli} \text{ carrying the } \textit{mcr-1} \text{ plasmid.}

\textbf{Notes:} M – Salmonella H9812 (15.0–242.5 kb); 1: E-55; 2: E-01; 3: E-09; 4: E-02; 5: E-44; and 6: E-37. The arrows in the figure represent the position of the } \textit{mcr-1} \text{ plasmid.
Conclusion
Our study reported a high incidence of the mcr-1-carrying ESBL-producing E. coli recovered from pigs in Jiangsu, China. The coexistence of the mcr-1- and blaCTX-M-15-carrying isolates displaying MDR, recovered from pig origin is a major concern for both humans and veterinary medicine. The presence of these genes on the conjugative plasmids with the ability to transfer between similar strains which contain other drug resistance genes emphasizes on urgent intervention.

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Disclosure
The authors report no conflicts of interest in this work.

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**Supplementary materials**

**Figure S1** Map of sampling sites in Jiangsu province.

**Figure S2** Screening of mcr-1 and ESBL encoding genes in *E. coli*.  
**Notes:** PCR product was separated on 1% agarose gel. Lane 1 and 6 shows 2000 bp molecular marker (Vazyme, Beijing, China); Lane 2, (mcr-1); Lane 3 (blaCTX-M) isolate; Lane 4, (blaTEM); and Lane 5 shows (blaSHV).
| Primers | Sequence (5’ to 3’) | Target sites/genes | Annealing temperature | Amplicons size | References |
|---------|----------------------|---------------------|-----------------------|----------------|------------|
| **PBRT** primers | | | | | |
| HI1-F | HI1-R | GGAGCGATGGATTACCTTCAGTAC TGCCGTTTCACTCCTGAGTA | parA-parB | 58 °C | 471-bp |
| HI2 -F | HI2- R | GGCTCCTACCGTCTGATTCTT TGAAACCGCAGCGGCGAGA | RNAI | 58 °C | 644-bp |
| I1-F | I1-F | CGAAAGCCGGACCGCAGAA TCGTGGTTCCCGCGAGTTG | iterons | 58 °C | 139-bp |
| X-F | X-R | AACCTTTAGGCTTTAAGGTTGCTGAT TGAGATCTTTTTATCTCAGTTTACTG | ori | 58 °C | 376-bp |
| L/M-F | L/M-F | GGATGAAAACTACACGCTGAG CTGGCGGAGGCATTCTTTAGG | repA, B, C | 58 °C | 785-bp |
| N-F | N-R | GTCTAAGCAGCTTACGGAAG GTTTCACTCTGGCAAGTTT | repA | 58 °C | 559-bp |
| FIA- F | FIA- R | CCATGCTGTTCATGAGGATGAG GTATATCCTCTTACGCGAGG | iterons | 58 °C | 462-bp |
| FIB -F | FIB -R | GGAGTTCTGACACACGCTGAG CTCCGCGCTTCCGCGGAT | repA | 58 °C | 702-bp |
| W-F | W-R | CCTAAGAAACAAAAAGCGCGCAG | repA | 58 °C | 242-bp |
| Y-F | Y-R | AAATTCAACACACTTGCGCTGCA GCGAGATGAGCGATACGTAATACGTAAT | repA | 58 °C | 765-bp |
| P-F | P-R | CTATGGGCCGCGACCGCGCAGAAA TCACCGCGCGCCGCGCCG | iterons | 58 °C | 534-bp |
| FIC -F | FIC -R | GTGAACCTGGCAGGAGGAGG TTTCCTCTGCGCCAAAACTAGAT | repA2 | 58 °C | 262-bp |
| A/C -F | A/C -R | GAGAACCAAAAAAGACCGCTGGA ACGAGAAACCTGGAATGTGCACTC | repA | 58 °C | 465-bp |
| T-F | T-R | TTGGCTGTGTTGTGCTAAACCAT CGTGATTACCTCTGTTGAC | repA | 58 °C | 750-bp |
| FIIIS--F | FIIIS -R | CTGTCGGTAAGCTGTAGGCC CTTCGCCCCAACCTTCAGC | repA | 58 °C | 270-bp |
| FrepB-F | FrepB-R | TGATGGTTAAGGAAATTGG GAAGATCAGTCACCCACCATCC | RNAI/repA | 58 °C | 270-bp |
| K/B -F | K/B-R | GCCGGTCCCGGAAGCCCGAGAAAC TCTTGCACGCGCGCGCAAA | RNAI | 58 °C | 160 bp |

(Continued)
Table S1 (Continued).

| Primers | Sequence (5’ to 3’) | Target sites/genes | Annealing temperature | Amplicons size | References |
|---------|----------------------|---------------------|-----------------------|----------------|------------|
| B/O-F   | GCGGTCCGGAAGCCAGAAAAC| RNAI                | 58 °C                 | 159 bp         | 1          |
| B/O-R   | TCTGCCTCCGGAAGCCAGAAAAC|                      |                       |                |            |
| X4- F   | AGCAAAACAGGAAAGGAAGAGACT| -                   | 62 °C                 | 569 bp         | 2          |
| X4-R    | TACCCAAAATCGTAACCTG |                      |                       |                |            |
| IncI2-F | ATTGTGCGTGCTTCA       | RNAI                | 60 °C                 | 353 bp         | This study |
| IncI2-R | TGGAGAGATTAAGGAGAA    |                      |                       |                |            |

References

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