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# Prevalence and Characteristics of *Escherichia coli* MCR-1 - Like in Rabbits in Shandong, China

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## Abstract

The antibiotic resistance gene mcr-1 is widespread in domestic and wild animals. Therefore, continuous monitoring of its prevalence and characteristics is required. In this study, we developed a Polymerase Chain Reaction (PCR)-based method to detect mcr-1 of *Escherichia coli* in rabbits of Tai'an, China, and determined the characteristics of mcr-1-bearing plasmids. A total of 55 non-duplicated *E. coli* was recovered from the swabs of rabbit feces. Plasmid profiling, plasmid and chromosome PCR, a conjugation experiment, lactose fermentation experiment, multilocus sequence typing, and polymyxin resistance tests were performed to determine the characteristics of mcr-1-bearing plasmids. Bacterial plasmids and chromosome DNA were separately extracted and amplified by PCR with mcr-1-specific primers. Eight of the 55 specimens were mcr-1-positive, for a positive rate of 14.6%. Although mcr-1 was successfully amplified with PCR from bacterial plasmids, it could not be amplified from bacterial chromosome DNA. The mcr-1-positive *E. coli* harbored more drug-resistant genes compared with the mcr-1-negative specimens, and results showed diverse sequence types. Overall, these findings suggest the possible threat of the transmission of mcr-1 from rabbits to humans, especially since the gene is located on transferable plasmids making horizontal transfer relatively easy. Since food-producing animals are necessary for our daily diet, worldwide cooperation is needed in fighting the spread of this drug resistance gene to avoid human infections with multidrug-resistant pathogenic bacteria.

**Keywords: Rabbits; MCR-1; Prevalence; Plasmids; *Escherichia coli***

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## Introduction

With the widespread use of antibiotics in farming, drug-resistant genes are now widely distributed in the intestines of farm animals, which are continuously being identified [1-3]. Following this pattern, it is likely that drug-resistant bacteria are present in rabbit feces [4,5]. Polymyxin is a promising antimicrobial peptide, and very few bacteria show polymyxin resistance at present [6]. However, Chinese researchers recently identified mcr-1 as a gene conferring resistance to colistin and polymyxin [7,8]. Although mcr-1 has been reported and detected worldwide, its global prevalence remains largely unknown. Liu et al. [8] screened for mcr-1 in *Escherichia coli* in raw pork and found that the gene was located on a plasmid. The prevalence of *Escherichia coli* mcr-1 in rabbits in China has not been reported. In these studies, the key methods to detect the location of genes were based on Southern blotting or whole genome sequencing. However, their detection methods were not based on Polymerase Chain Reaction (PCR) amplification, which can help in estimating the prevalence of mcr-1. Therefore, in the present study, we employed a simpler and economical method to determine the location and characteristics of *E. coli* mcr-1 among rabbits in China. We also developed a method of combination of conjugation, PCR and fermentation test to further prove that plasmid harboring mcr-1. For the final determination, we applied plasmid whole genome sequencing to the mcr-1 positive strains.

## Methods

### Sample collection and identification of *E. coli*

Rabbit feces were collected in aseptic tubes [9,10], and plated on MacConkey agar as well as placed in microchemical tubes to select and identify *E. coli*. The suspicious colonies were identified by bacterial biochemical tests (Table 1). After biochemical identification, Gram stain and microscopic examination were performed to observe the morphology of the bacteria for confirmation. Positive colonies were then chosen for further biochemical identification using the automated API 20E system (Sysmex bioMérieux, Tokyo, Japan).

A total of 55 non-duplicated *E. coli* strains were recovered from rabbit farms. *E. coli* was cultivated in Luria-Bertani liquid medium containing 2 μg/mL polymyxin B antibiotics, and positive specimens were selected as the PCR detection templates.

The rabbits had been raised on large rabbit farms free from thirst or starvation, and without signs of anxiety, fear, or depression. The formula for rabbit feed is 17% corn, 24% bran, 21% soybean meal, 5% imported fish meal, 3% active yeast and 30% grass powder. Fecal samples were randomly collected from the diarrhea of rabbits on three farms. The three farms were separately selected in three administrative counties. The samples were independently collected from individual animals. Because the sampling process did not harm the rabbits, ethical approval was not required for the study.

### PCR detection of MCR-1

The DNA from 55 *E. coli* strains was amplified by PCR with mcr-1 whole sequence specific primers, F: 5'AGTAGGCGTTTATTTGATAAATACGGCA3'; R: 5'TTATATCAGATAAATTGTACTGGATTTC3' designed by Prime Premier 5. PCR systems included 25 μL PCR mix, 21 μL deionized water, 1 μL forward primer, 1 μL reverse primer, and 2 μL template, for a total of 50 μL. The reaction program was as follows: 94°C pre-denaturation for 5 min, followed by 28 cycles of 94°C denaturation for 30 sec, annealing at 55°C for 30 sec, and 72°C extension for 30 sec. A final extension step was conducted at 72°C for 7 min. The PCR products of mcr-1 were then subjected to electrophoresis at 140V for 30 min. The positive specimens were sent to Sangon for direct sequencing for confirmation [11], and the sequences of mcr-1-positive strains were compared by the Blastn tool of the National Center for Biotechnology Information website.

We further attempted to amplify the mcr-1 gene from extracted plasmids and bacterial chromosomes, respectively. The samples of mcr-1-positive strains were separated and the plasmids were extracted with the OMEGA plasmid kit and subjected to electrophoresis. The extracted plasmid was used as the template for PCR, and one single plasmid electrophoresis strip of one sample was recycled. PCR was then carried out with the extracted plasmid as a template using primers specific to mcr-1 and other resistance genes under the same PCR conditions described above (Supplementary Table S1). Similarly, bacterial genomic chromosomes were extracted and purified from the samples of mcr-1-positive strains, and PCR analysis was performed with mcr-1-specific primers as described above.

### Plasmid characterization and sequencing

The concentrations of the extracted plasmids were determined. Those meeting the requirements of sequencing were sent to Shanghai Pinoson Biological Co. Ltd. for whole-genome sequencing, and the coding genes and structure were analyzed by bioinformatics. Through comparative analysis of the extracted plasmids, the *E. coli* R45 strain carrying the mcr-1, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$, and *qnrS* genes was ultimately selected; the extracted plasmid from this strain was named pR45. A whole-genome shotgun strategy was used to construct libraries of different inserted fragments. Paired-end sequencing was performed on the Illumina MiSeq platform. SPAdes genome assembler (v 3.7.1) was used to construct contigs and the scaffold by the ab initio assembly of sequencing data, which were then removed and corrected. Finally, a complete plasmid sequence was obtained by assembling overlapping groups and filling vacancy sequences by a combinatorial PCR or step-by-step method. After sequencing, Bacterial Annotation System (BASys; http://wishart.biology.ualberta.ca/basys/cgi/submit.pl) (Van Domselaar et al. 2005) was used to predict and annotate the open reading frame of the plasmid sequence, which was confirmed with DNA MAN 5.2.10 software. BlastP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare each predicted protein-coding gene with protein databases. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to further align the gene sequences with reference sequences in the GenBank database; the target and reference plasmid sequences were aligned with the Blastnt-Blast2 algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *E. coli* strain PGR46 plasmid pPGRT46 (GenBank accession no. KM023153.1) served as the reference plasmid for annotation. The plasmid map was drawn with SnapGene Viewer 3.2.1.

**Table 1:** Biochemical test results of *E. coli* strains.

| Test item | Test result | Test item | Test result |
|---|---|---|---|
| Sucrose | Positive | M-R Test | Positive |
| Lactose | Positive | V-P Test | Negative |
| Glucose | Positive | $H_2S$ Test | Negative |
| Maltose | Positive | Indole Test | Positive |
| Mannitol | Positive | | |

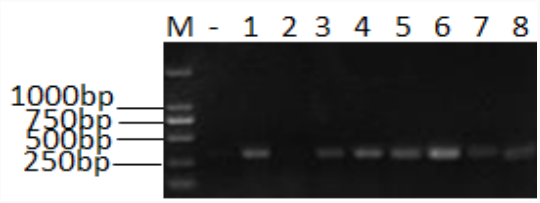


**Figure 1:** *MCR-1*-positive *E. coli*.

### Conjugation experiments

The transferability of mcr-1-bearing plasmids from isolates was determined using filter mating with *E. coli* J53 as the recipient strain, mixing at a ratio of 1:1 in broth culture, as previously described [12]. The resulting transconjugants were selected on brain heart infusion agar plates supplemented with polymyxin B (2 mg/L) [13]. Subsequently, the positive bacteria were cultivated together with the mcr-1-negative receptor J53, which contained no plasmid. The conjugated bacteria were observed using plasmid extraction and electrophoresis analysis. Transfer of the resistance gene was considered to have taken place when the workable plasmids were transferred from the wild type mcr-1-positive bacterium to the recipient bacterium.

Conjugated strains of *E. coli* were also subjected to a lactose fermentation experiment, with the mcr-1-positive strain J53 as the negative control, and to confirm transfer of the functional gene of conjugated strains. The strains were inoculated in lactose fermentation

tubes overnight; the results were recorded as positive if the color of the purple microchemical tubes turned yellow.

### Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *E. coli* isolates was tested according to determination of the Minimal Inhibitory Concentration (MIC) of several antibiotics. The bacteria were cultured at 37°C in Luria-Bertani broth medium for 6 h. The concentration of *E. coli* was adjusted to $1.5 \times 10^8$ colony forming units/mL in sterile saline. The susceptibility of the isolates was then tested with 15 kinds of commonly used antimicrobial agents, including Ciprofloxacin (CIP), Chloramphenicol (C), Nalidixic Acid (NA), Amoxicillin/clavulanic Acid (AML), Tobramycin (TB), Ceftazidime (CAZ), Ceftriaxone (CRO), Gentamicin (GEN), Sulfamethoxazole/trimethoprim (SXT), Imipenem (IMP), Tetracycline (TET), Ampicillin (AMP), Cefoxitin (FOX), Polymycin-B (PB), and Amikacin (AMK), using the disc diffusion test recommended by the Clinical and Laboratory Standards Institute [14]. For this assessment, the *E. coli* strain ATCC25922 was used as the quality control strain. *E. coli* isolates resistant to more than three classes of antimicrobials were defined as Multidrug Resistant (MDR) isolates [15,16].

### Multilocus sequence typing (MLST)

According to Zhao et al. [5], eight pairs of primers for housekeeping genes (*dinB*, *icdA*, *pabB*, *polB*, *putP*, *trpA*, *trpB*, and *uidA*) were designed and used for PCR. The products of PCR amplification were then sequenced by Shanghai Sangon Biotech Co., Ltd. The results were amended using Chromas and DNAstar software and then submitted to the Pasteur online database for processing [5]. The allele number of each housekeeping gene was obtained and the Sequence Type (ST) of each strain was acquired [17].

### Phylogenetic analysis

The phylogenetic tree of the complete mcr-1 sequences from mcr-1 positive *E. coli* was constructed by a maximum likelihood method using DNAstar Megalign software to determine the relationships among strains.

## Results

### Prevalence of MCR-1

Eight of the 55 specimens were found to be mcr-1-positive, representing a positivity rate of 14.6% (Figure 1). Although mcr-1 was successfully PCR-amplified from bacterial plasmids, it could not be amplified from bacterial chromosome DNA, suggesting that the mcr-1 resistant gene may locate on the plasmid and not on genomic chromosomes.

The mcr-1-positive strains harbored significantly more drug-resistant genes other than mcr-1 compared to the mcr-1-negative strains (chi square test, $P<0.05$; Table 2). Accordingly, the mcr-1-positive *E. coli* had a greater probability of being MDR than mcr-1 negative *E. coli* ($P<0.05$).

### Plasmid sequencing results

Plasmid whole-genome sequencing was conducted on the mcr-1 positive strains. Blastn showed that mcr-1 was located on the plasmid. The extracted plasmid, designated pR45, was found to be a closed-loop DNA molecule with 83,157 bp and a 52.74% GC content, encoding 45 predicted genes, including four known resistance genes: mcr-1, $bla_{CTX-M}$, $bla_{TEM-1}$, and *qnrS1*. To prove the transferability of mobile plasmids *in vitro*, *E. coli* strain R45, carrying the mcr-1, $bla_{CTX-M}$, $bla_{TEM-1}$, and *qnrS1* genes, was selected for comparing and analyzing the extracted plasmids. The results of drug resistance phenotyping and resistance gene detection of conjugated bacteria *in vitro* were consistent with the results of plasmid sequencing, demonstrating that the *E. coli* resistance gene has transfer ability *in vitro*, and that the mobile plasmid plays an important role in the process of drug resistance transmission in *E. coli*.

**Table 2:** Comparison of multiple drug-resistant isolates detected in *MCR-1*-positive and -negative strains.

| MDR | Yes | | No | |
|---|---|---|---|---|
| | No. | Rate | No. | Rate |
| *MCR-1* Positive | 7 | 87.50% | 1 | 12.50% |
| *MCR-1* Negative | 23 | 48.94% | 24 | 51.06% |

**Table 3:** Lactose fermentation results.

| | Lactose fermentation | Plasmid | *MCR-1* (Plasmid) |
|---|---|---|---|
| donor | + (yellow) | + | + |
| recipient | - (purple) | - | - |
| zygote | + (yellow) | + | + |


**Figure 2:** Drug sensitivity tests.

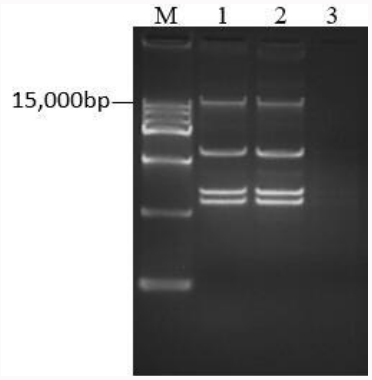


**Figure 3:** Identical plasmid profile of the donor and conjugant.

### Conjugation tests

The conjugation tests confirmed the horizontal transfer of mcr-1 in *E. coli* strains obtained from rabbit feces, therefore proving that mcr-1 was located on plasmids. The mcr-1-positive bacteria were then cultivated together with the mcr-1-negative strain J53, which contained no plasmid. The transfer of the resistance gene was found to take place when the workable plasmid was transferred from the wild type mcr-1 positive bacterium to the recipient. Moreover, the conjugated bacteria acquired lactose fermentation ability and showed an increase in polymyxin resistance ability (Figure 2 and Table 3).

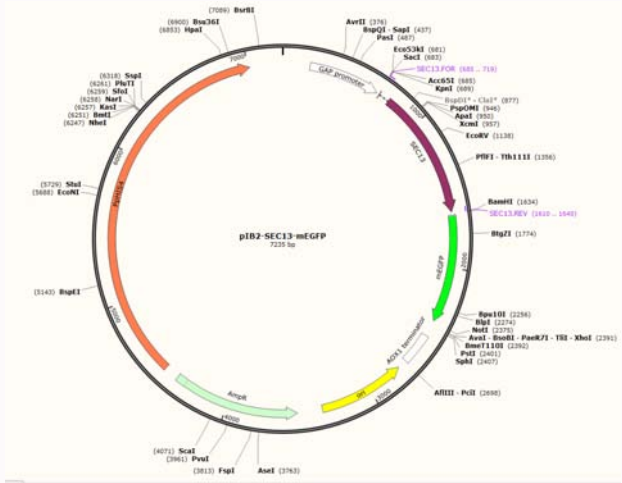


**Figure 4:** Plasmid map of *MCR-1 positive E. Coli.*

In addition, the plasmid DNA of mcr-1-positive *E. coli* strongly amplified mcr-1. The positive band was purified and subject to PCR detection using primers for both mcr-1 and blaTEM, which showed positive results indicating the two resistant genes, coexist on the same plasmid (Figure 3). BlaTEM was included in this analysis as it is the most common AMR genes in the samples, with a positive rate of 98.2%.

### Characteristics of MCR-1

Thirteen different STs were identified among the 55 strains, with the most prevalent being ST302 (22/55, 40.0%), ST370 (12/55, 21.8%), and ST468 (Supplementary Table S2). Of note, the mcr-1-positive *E. coli* strains also showed a wide diversity of STs, although the dominant type was ST88 (62.5%).

Figure 4 shows the phylogenetic tree to display the evolutionary relationships among the eight mcr-1 sequences, demonstrating that although the eight positive strains were non-duplicated *E. coli*; their mcr-1 sequences were very similar.

## Discussion

### Prevalence of MCR-1 in *E. coli*

The prevalence of mcr-1 (8/55, 14.6%) detected in *E. coli* strains obtained from rabbits in Tai'an, China is similar to that reported in a study conducted in Italy (50/320, 15.6%) [7], and is markedly higher than that reported for humans (1% ~ 2%) [18]. This high rate may be due to the greater use of polymyxin in farms than in clinical practice. More importantly, all of the mcr-1-positive strains obtained in the present study were isolated from a single farm among the three sampled farms. This may be related to several factors. First, the sample size might not have been large enough to reflect the actual situation at all farms. Second, the horizontal transfer of mcr-1 was confined within each relatively closed farm, thereby preventing gene transfer among farms, especially farms from different regions. Finally, but potentially most important, the amount of polymyxin use varied across the different farms, which would impose different selection pressures on mcr-1.

### Dissemination characteristics of MCR-1

Because of the limitation of the total amount of specimens, it is difficult to generalize the results overall. Nevertheless, the antibiotic resistance tests demonstrated that the mcr-1-positive plasmids were more likely to harbor other resistant genes than mcr-1-negative plasmid. Bacteria without plasmids readily gained donor bacterium plasmids and the mcr-1 gene along with the ability for lactose fermentation and polymyxin resistance at the same time. Therefore, these results strongly suggest the high horizontal dissemination potential of mcr-1.

Moreover, the low diversity of mcr-1 sequences among the *E. coli* strains indicated that the mcr-1 gene was most likely derived from same source, further suggesting clonal transmission of *E. coli* and horizontal transmission of mcr-1-bearing plasmids in this area. This may be related to the fact that this region is relatively isolated, far from the city, with minimal flow of people. Additionally, the rabbit feed contains same fish meal, which may contain mcr-1 positive bacteria and thus infect the rabbit when eaten.

The resistance gene mcr-1 was found in eight strains of bacteria, which shows that the presence of plasmids for bacteria makes it possible to produce drug resistance and survive in adversity [19,20]. Resistance genes not only transfer from one bacterium to another or from one bacterium species to other species but also move geographically consequently. Therefore, the threat of drug resistance is not localized to a given animal farm or region, but represents a worldwide concern requiring global cooperation. Indeed, the fact that the bacterial resistant gene is located on the plasmid makes it potentially more difficult to control than a chromosomal gene. Plasmid transmission makes the spread of drug resistance genes easier and faster, and since the same plasmid can carry a variety of resistance genes, the recipient can immediately become resistant to multiple drugs. This finding suggests that it would be very difficult to cure humans infected with multiple drug-resistant pathogenic bacteria.

## Conclusion

The conjugation test and whole-genome sequence analysis of the ligated plasmid demonstrated that the *E. coli* resistance gene mcr-1 is circulating in rabbits of China, with ability for horizontal transfer *in vitro*, indicating that the mobile plasmid plays an important role in the process of antibiotic resistance of *E. coli*. As the AMR positive bacterial strains can survive in the presence of antibiotics, they may acquire additional drug resistance genes, resulting in a new MDR phenotype for the donor bacteria. The bacteria can readily acquire additional drug resistance genes, resulting in a new MDR phenotype for the donor bacteria. Therefore, continuous selective pressure of antibiotics in farms will result in the production of new drug resistance genes that can readily circulate among domestic and wild animals, and even humans.

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## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contribution

Weishan Chang and Jing Zhai designed the study. Xinxing Wang,

Xiaonan Zhao, Hanming Jiang, Hongna Zhang, Shuying Yi, JW, and DH performed the experiments and analyzed the data. Xinxing Wang, Xiaonan Zhao, Weishan Chang, and Jing Zhai wrote and revised the manuscript. All authors contributed to the revision of the manuscript and read and approved the submitted version.

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