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Research Article

Prevalence of *Escherichia coli* Plasmid *mcr-1* 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 applied a PCR-based method to detect *mcr-1* of *Escherichia coli* in rabbits of Tai'an, China. A total of 55 non-duplicated *E. coli* samples were recovered from the swabs of rabbit feces. Plasmid and chromosome PCR, a conjugation experiment, lactose fermentation experiment, multilocus sequence typing, and antibiotic 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%. The *mcr-1*-positive *E. coli* harbored more drug-resistant genes compared with the *mcr-1*-negative specimens and results showed diverse sequence types. All the *E. coli* isolates were sensitive to ceftazidime, ceftriaxone, imipenem and amikacin while 14.6% of the isolates showed resistance against polymycin and 65.5% were resistant against ampicillin. Although *mcr-1* was successfully amplified with PCR from bacterial plasmids, it could not be amplified from bacterial chromosome DNA. Overall, *mcr-1* has been first isolated from rabbits, and these findings suggest the possible threat of the transmission of *mcr-1* from rabbits to humans, primarily since the gene is located on transferable plasmids making horizontal transfer relatively easy. Since food-producing animals are necessary for our daily diet, global 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*; Plasmids; *Escherichia coli*; Shandong Province

Novelty Statement: *mCR-1* gene is a polymycin-resistant gene discovered in recent years, which is an important discovery in the world. After that, *mcr-1* were found in *Escherichia coli* and *Klebsiella pneumoniae* in chickens, ducks, geese, pigs, and other omnivorous animals. Rabbits belong to herbivores, and this is the first time that *mcr-1* has been found in rabbits in China.

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]. Furthermore, antimicrobial resistant bacteria can be transferred to the humans through the food chain, thus affecting human health. Therefore, in the present study, we employed a simpler and more 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 PCR and lactose fermination test to prove further that the plasmid is harboring *mcr-1*. For the final determination, we applied plasmid whole genome sequencing to the *mcr-1* positive strains.

Polymyxin is a promising antimicrobial peptide, and very few bacteria show polymyxin resistance at present. However, Chinese researchers recently identified *mcr-1* as a gene conferring resistance to colistin and polymyxin [6,7]. Although *mcr-1* has been reported and detected worldwide, its global prevalence remains mostly unknown. Liu et al. 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*.

Materials and Methods

Sample collection and identification of *E. coli*

The rabbits had been raised on large rabbit farms free from thirst or starvation. 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. Because the sampling process did not harm the rabbits, ethical approval was not required for the study.

Rabbit feces were collected in aseptic tubes [8] and plated on MacConkey agar as well as placed in micro chemical tubes to select and identify *E. coli*. The suspicious colonies were identified by

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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		

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

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

bacterial biochemical tests (Table 1).

PCR detection of *mcr-1*

The DNA from 55 *E. coli* strains was amplified by PCR with *mcr-1* whole sequence specific primers. The PCR products of *mcr-1* were then subjected to electrophoresis. The positive specimens were sent to Sangon for direct sequencing for confirmation [9], 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. PCR was then carried out with the extracted plasmid as a template using primers specific to *mcr-1* and other resistance genes under the reference 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

Those plasmids 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. A whole-genome shotgun strategy was used to construct libraries of different inserted fragments. Paired-end sequencing was performed on the Illumina MiSeq platform. Finally, a complete plasmid sequence was obtained by assembling overlapping groups and filling vacancy sequences by a combinatorial PCR or step-by-step method.

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 [10]. The resulting transconjugants were selected on brain heart infusion agar plates supplemented with polymyxin B (2mg/L) [11]. 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.

Conjugated strains of *E. coli* were also subjected to a lactose fermentation experiment, with the *mcr-1*-positive strain J53 as the negative control.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *E. coli* isolates was tested according to the determination of the Minimal Inhibitory Concentration (MIC) of several antibiotics. The susceptibility of the isolates was then tested with 15 kinds of commonly used antimicrobial agents. *E. coli* isolates resistant to more than three classes of antimicrobials were defined as Multidrug-Resistant (MDR) isolates [12,13].

Multilocus sequence typing (MLST)

According to Zhao et al. eight pairs of primers for housekeeping genes 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 DNA Star software and then submitted to the Pasteur online database for processing Zhao et al. The allele number of each housekeeping gene was obtained and the Sequence Type (ST) of each strain was acquired [14].

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 transferability *in vitro*, and that the mobile plasmid plays an essential role in the process of drug resistance transmission in *E. coli*.

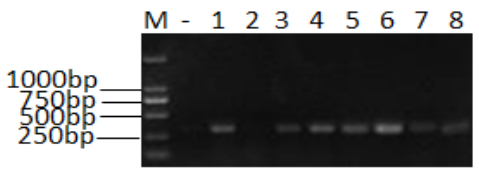

Figure 1: *mcr-1*-positive *E. coli*.

Table 3: Characteristics of *mcr-1* of *E. coli* in rabbits.

Strain	Genbank	ST	Resistance phenotype	Resistance
R45	MH602237	ST88	AML-AMP-C-CIP-GEN- NA-SXT-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *cmlA, flor, sul2, sul3, tetB,* ***mcr-1***
R48	MH602238	ST88	AMP-C-CIP-GEN-NA-SXT-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *cmlA, flor, sul2, sul3, tetB,* ***mcr-1***
R49	MH602239	ST2	AMP-C-CIP-NA-SXT-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *cmlA, flor, sul3,* ***mcr-1***
R50	MH602240	ST88	AMP-C-CIP-GEN-NA-SXT-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *cmlA, flor, sul2, sul3, tetB,* ***mcr-1***
R51	MH602241	ST353	C-TET-**PB**	bla_{TEM}, *flor, qnrS, sul2,* ***mcr-1***
R54	MH602242	ST88	C-CIP-NA-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *flor, sul2, sul3, tetB,* ***mcr-1***
R54	MH602243	ST24	AML-AMP-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *flor, sul1,* ***mcr-1***
R55	MH395740	ST88	AMP-C-CIP-GEN-NA-SXT-TB-TET-**PB**	bla_{CTX-M}, bla_{TEM}, *cmlA, flor, sul2, sul3, tetB,* ***mcr-1***

Table 4: Lactose fermentation results.

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

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, Figure 3, Table 4).

In addition, the plasmid DNA of *mcr-1*-positive *E. coli* strongly amplified *mcr-1*. The target band was purified and subjected to PCR detection using primers for both *mcr-1* and bla_{TEM}, which showed positive results indicating the two resistant genes coexist on the same plasmid. Bla_{TEM} 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 (Table 3). 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 5 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%) Fabrizio et al. and is markedly higher than that reported for humans (1~2%) [15]. 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

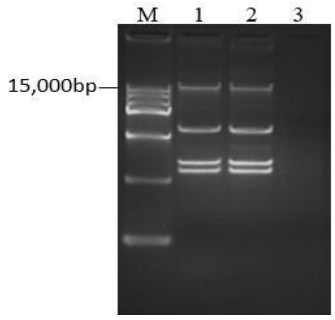

Figure 2: Identical plasmid profile of the donor and conjugant.

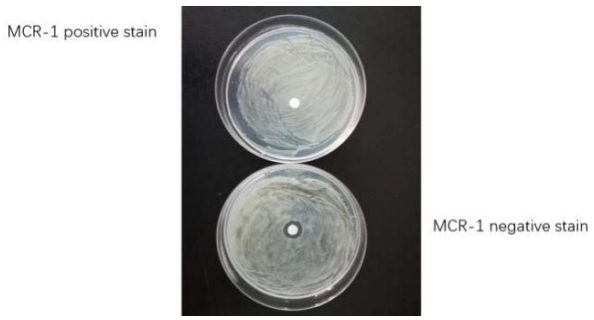

Figure 3: Drug sensitivity tests.

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*. Of note, *mcr-1* has been found in lots of animals such as livestock and poultry, birds, and wild animals. As far as we know, this is the first time that *mcr-1* was isolated from rabbits.

This study showed that *E. coli* isolated from diarrheic farmed rabbits in the Tai'an area exhibit sometimes very frequent resistance to antimicrobials important to human medicine, which further highlights the need for reasonable use of antibiotics. In this study, the highest isolation rate of 40% (22/55) was found for ST302 which had not yet been reported relating to infections, while the highest isolation rate of 37.5% (3/8) was found for ST88 among *mcr-1* positive strains.

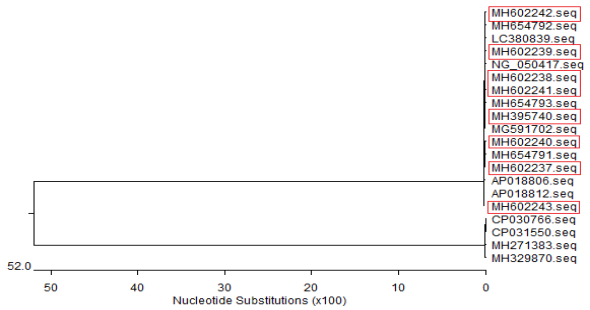

Figure 4: Phylogenetic tree of *mcr-1* detected in *E. coli* isolated from rabbits.

Dissemination characteristics of *mcr-1*

Because of the limitation of the total number 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 the *mcr-1*-negative plasmid (Table 2). 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 from different sources indicated that the *mcr-1* gene was most likely derived from one ancestor, 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 a minimal flow of people. Additionally, the rabbit feed contains the 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 [16,17]. Resistance genes not only transfer from one bacterium to another or from one bacterium specy to other species but also move geographically consequencely. 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 challenging 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 the ability for horizontal transfer *in vitro*, indicating that the mobile plasmid plays a vital 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. Therefore, the 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.

References

1. Briñas L, Zarazaga M, Saenz Y, Ruiz-Larrea F, Torres C. Beta-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob. Agents Chemother. 2002; 46: 3156-3163.
2. Naseer U, Sundsfjord A. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. Microb. Drug Resist. 2011; 17: 83-97.
3. Bryan A, Shapir N, Sadowsky MJ. Frequency and distribution of tetracycline resistance genes in genetically diverse, non-selected, and nonclinical *Escherichia coli* strains isolated from diverse human and animal sources. Appl. Environ. Microbiol. 2004; 70: 2503-2507.
4. Zhao X, Ye C, Chang W, Sun S. Serotype distribution, antimicrobial resistance, and class I integrons profiles of *Salmonella* from animals in slaughterhouses in Shandong Province, China. Front. Microbiol. 2017; 8: 1-9.
5. Gao LL, Hu, JQ, Zhang, XD, Wei L, Li S, Miao Z, et al. Application of swine manure on agricultural fields contributes to extended-spectrum β-lactamase-producing *Escherichia coli* spread in Tai'an, China. Front. Microbiol. 2015; 6: 313.
6. Agnoletti F, Brunetta R, Bano L, Drigo I, Mazzolini E. Longitudinal study on antimicrobial consumption and resistance in rabbit farming. Int. J. Antimicrob. Agents. 2018; 51: 197-205.
7. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism *MCR-1* in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect. Dis. 2016; 16: 161-168.
8. Agnoletti F. Update on rabbit enteric diseases: despite improved diagnostic capacity, where does disease control and prevention stand? Proc. "10th World Rabbit Congr", Sharm El-Sheikh, Egypt. 2012; 113-1127.
9. White P, Mclver CJ, Deng YM, Rawlinson WD. Characterisation of two new gene cassettes, aadA5 and dfrA17. FEMS Microbiol. Lett. 2006; 182: 265-269.
10. Zhang H, Zhou Y, Guo S, Chang W. Multidrug resistance found in extended-spectrum beta-lactamase-producing Enterobacteriaceae from rural water reservoirs in Guantao, China. Front. Microbiol. 2015; 6: 1-4.
11. Guardabassi L, Schwarz SD, Lloyd H. Pet animals as reservoirs of antimicrobial-resistant bacteria. J. Antimicrob. Chemother. 2004; 54: 321-332.
12. Valat C, Forest K, Billet M, Polizzi C, Saras E, Madec JY, et al. Absence of co-localization between pathovar-associated virulence factors and extended-spectrum β-lactamase (blaCTX-M) genes on a single plasmid. Vet. Microbiol. 2016; 192: 163-166.
13. Moawad AA, Hotzel H, Awad O, Tomaso H, Neubauer H, Hafez HM, et al. Occurrence of *Salmonella enterica* and *Escherichia coli* in raw chicken and beef meat in northern Egypt and dissemination of their antibiotic resistance markers. Gut Pathog. 2017; 9: 57.

14. Dotto G, Giacomell M, Grilli G, Ferrazzi V, Carattoli A, Fortini D, et al. High prevalence of *oqxAB* in *Escherichia coli* isolates from domestic and wild lagomorphs in Italy. Microb. Drug Resist. 2014; 20: 118-123.

15. Yi LX, Liu YY, Wu RJ, Liang ZS, Liu JH. Research progress on the plasmid-mediated colistin resistance gene *mcr-1*. Yi Chuan. 2017; 39: 110-126.

16. Kun QF, Geng Y, Yu ZH, et al. Detection of quinolone resistance and plasmid mediated quinolone gene in *Escherichia coli* isolated from rabbit. Chinese J. Prevent. Vet. Med. 2006; 38: 944-948.

17. Silva N, Igrejas G, Figueredo N, Gonçalves A, Radhouani H, Rodrigues J, et al. Molecular characterization of antimicrobial resistance in *enterococci* and *Escherichia coli* isolates from European wild rabbit (*Oryctolagus cuniculus*). Sci. Total Environ. 2010; 408: 4871-4876.