

Molecular Detection of the *mcr* Genes by Multiplex PCR

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Jiayang Liu^{1,*}
Zhiyuan Zhang^{1,*}
Yuyang Feng¹
Huimin Hu¹
Yu Yu¹
Lihao Qiu¹
Hongtao Liu¹
Zhimin Guo²
Jing Huang²
Chongtao Du¹
Jiazhang Qiu¹

¹Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun 130062, People's Republic of China;

²Department of Clinical Laboratory, The First Hospital of Jilin University, Changchun 130021, People's Republic of China

*These authors contributed equally to this work

Background: The emergence and prevalence of plasmid-mediated colistin-resistant bacterial strains in recent years have raised great concerns in clinical medicine. It is urgently needed to develop a cheaper, faster, simpler, sensitive, and specific molecular detection method to identify and monitor the dissemination of the transferable resistant determinants.

Methods and Results: Herein, eight pairs of primers were designed to set up a multiplex PCR method for the rapid and efficient determination of reported *mcr* genes. This assay can give results within 85 min (35 min for amplification and 50 min for electrophoresis). We validated the feasibility of this assay by testing the presence of *mcr* genes in 60 colistin-resistant isolates.

Conclusion: Our multiplex PCR technique exhibits remarkable advantages in the light of clear identification, efficiency of amplification, as well as the time consuming for detection, and thus could be useful for the surveillance and epidemiological research of plasmid-mediated colistin resistance, particularly for the under-resourced laboratories.

Keywords: multiplex PCR, colistin resistance, *mcr* genes

Introduction

Colistin is a polymyxin antibiotic originating from the gram-positive organism *Bacillus polymyxa*. Due to its significant nephrotoxicity and neurotoxicity,¹ the clinical application of colistin in the treatment of human bacterial infections has been restricted. The constantly increasing emergence and spread of multidrug-resistant bacteria is a serious threat to global health. The lack of effective antimicrobial agents in the treatment of diseases caused by resistant bacteria resulted in the reintroduction of colistin application in human infections. Unfortunately, colistin-resistant bacteria rapidly emerged owing to the improper use and abuse of colistin. Previously, resistance to colistin mostly involved chromosomal mutations.² However, since the first description of plasmid-borne resistance gene *mcr-1* by Liu et al in 2016, the acquired resistance mechanism of colistin has been placed on the center of focus.³ The prevalence of mobile colistin-resistant genes could be responsible for the rapid increase of colistin-resistant bacterial strains.³ In addition to *mcr-1*, nine other groups of *mcr* genes (*mcr-2* to *mcr-10*) were described within the past 4 years, adding to the complexities of colistin resistance.^{4,5} The novel genes showed distinct similarity with *mcr-1* in both nucleotide and amino acid sequences.⁶ Since *mcr-6* has 86% nucleotide identity with *mcr-2*, it has been considered as an *mcr-2* like gene.⁶ Most plasmid-mediated colistin-resistant strains carry a single *mcr* gene; however, co-presence of two *mcr* genes in a single isolate

Correspondence: Jiazhang Qiu;
Chongtao Du
Key Laboratory of Zoonosis, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun 130062, People's Republic of China
Email qiuiz@jlu.edu.cn;
duct@jlu.edu.cn

Table 1 Primers Used for Multiplex PCR Detection of *Mcr* Genes

Primer Name	Sequence (5'-3')	Target Gene	Size (bp)
<i>mcr-1_205F</i>	TCCAAAATGCCCTACAGACC	<i>mcr-1</i>	205
<i>mcr-1_205R</i>	GCCACCACAGGCAGTAAAT		
<i>mcr-2_279F</i>	CCTTTTGTGCTGATGGGTTT	<i>mcr-2</i>	279
<i>mcr-2_279R</i>	ATTTTGGAGCATGGTGGTGT		
<i>mcr-3_347F</i>	CTTGCTGAACCAATCCCATT	<i>mcr-3</i>	347
<i>mcr-3_347R</i>	CCATCGTTCTCCTTCCAAAA		
<i>mcr-4_426F</i>	GATCGAAGCTGTGTCTCG	<i>mcr-4</i>	426
<i>mcr-4_426R</i>	GCCAGCATTGGTACGCTAGT		
<i>mcr-5_522F</i>	GGTTGGCCGAGAAGATAACA	<i>mcr-5</i>	522
<i>mcr-5_522R</i>	ATGTTGCCAGAAGGTCCAAC		
<i>mcr-7_791F</i>	GTCAGTTACGCCATGCTCAA	<i>mcr-7</i>	791
<i>mcr-7_791R</i>	TTCTTGTCGCAGAACTGTGG		
<i>mcr-8_943F</i>	AAACTGAACCCGGTACAACG	<i>mcr-8</i>	943
<i>mcr-8_943R</i>	GCCATAGCACCTCAACACCT		
<i>mcr-9_635F</i>	GCGGTTGTAAAGGCGTATGT	<i>mcr-9</i>	635
<i>mcr-9_635R</i>	CAAATCGCGGTCAGGATTAT		

has been described recently.⁷⁻⁹ MCR family proteins are membrane-associated phosphoethanolamine (PEA) transferases which catalyze the addition of PEA moiety to the lipopolysaccharides (LPS)-lipid A, resulted in reduced affinity to colistin.¹⁰

To prevent the spread of colistin resistance, fast, cheap and efficient diagnostic approaches are desperately needed. Previously, several multiplex polymerase chain reaction (PCR) assays have been reported for the detection of *mcr* genes (*mcr-1* to *mcr-5*).^{6,11-13} Apparently, this method needs to be updated due to the discovery of novel *mcr* genes (eg *mcr-7*, *mcr-8*, and *mcr-9*). Here, we developed a multiplex PCR method which allowed us to quickly and accurately detect currently reported *mcr* genes in a single mixture.

Materials and Methods

Primers and Multiplex PCR Reaction

The nucleotide sequences of eight *mcr* genes were retrieved from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Specific primers (Table 1) for the amplification of the eight *mcr* genes were designed by Primer3Plus (www.bioinformatics.nl). The compatibility of these primers for the multiplex PCR was verified by the in-silico PCR modeling feature at the UCSC Genome Bioinformatics website (<http://genome.ucsc.edu>). The ability of each primer pairs to bind to its variants was verified by in-silico analysis using FastPCR software (<http://primer.digital.com/fastpcr.html>). The multiplex PCR was

performed in a 20 µL reaction mixture containing 10 µL of 2xFast Taq Master Mix (Novoprotein, Shanghai, China), 20 pmol of each primer for the amplification of the *mcr-1* to *mcr-5* genes, 10 pmol of each primer for the amplification of the *mcr-7* to *mcr-9* genes, and 20 ng of each template DNA. The multiplex PCR was performed with the following cycling conditions: denaturation at 94°C for 4 min, 20 cycles of amplification at 94°C for 5 seconds and 59°C for 15 seconds, and a final extension step at 72°C for 5 min. The resulting DNA products were analyzed by electrophoresis in a 2.0% agarose gel at 90 V for 50 min.

All of the *mcr* genes were synthesized by GenScript and cloned into pUC57 (Supplementary Table S1). To validate the specificity and efficiency of the primers and cycling conditions for the multiplex PCR assay, both isolated plasmid DNA of pUC57 harboring each *mcr* gene and the total DNA extracted from the liquid culture of *E. coli* Top10 containing the relevant plasmids by the boiling method were used as templates. In addition, different combinations of two or three *mcr* genes were applied to the multiplex PCR assay (Supplementary Table S2).

Detection of *mcr* Genes in Bacterial Isolates by the Multiplex PCR Assay

A total of 20 fecal samples of pigs were collected in 2019 from three different swine farms at Jilin province, China. Nineteen pet fecal samples (9 from cats and 10 from dogs)

were collected at the Veterinary Teaching Hospital of Jilin University. Colistin-resistant strains were selectively isolated on MacConkey agar (Hopebio, Qingdao, China) containing 2 mg/L colistin (Sigma-Aldrich, St Louis, MO, USA). The resulting colonies were purified three times and characterized by 16S rRNA sequencing. The sources and species of the isolates were listed in [Supplementary Table S3](#). Bacteria were cultured in 3 mL nutrient broth at 37°C for 16–24 hours. The total DNA was extracted by the boiling method. 0.5 µL of the total DNA was applied to the multiplex PCR assay described above to detect the presence of the currently reported *mcr* genes.

Results

Validation of the Multiplex PCR Method

Eight pairs of primers were designed to amplify the internal fragments of *mcr* genes. The sizes of the amplicons were 205 bp (*mcr-1*), 279 bp (*mcr-2*), 347 bp (*mcr-3*), 426 bp (*mcr-4*), 522 bp (*mcr-5*), 791 bp (*mcr-7*), 943 bp (*mcr-8*), and 635 bp (*mcr-9*). As shown in [Figure 1](#), when plasmid DNA of pUC57 carrying each *mcr* gene was used as template in the multiplex PCR mixture, unique and specific bands were visualized by agarose gel electrophoresis, suggesting the specificity of the primer sets. Additionally, we prepared the total DNA through boiling the broth cultures of *E. coli* strains harboring plasmid containing *mcr* genes. Similarly, amplification of the expected gene fragment was also observed when the total DNA was utilized as a template ([Figure S1](#)). The PCR products were recovered from the

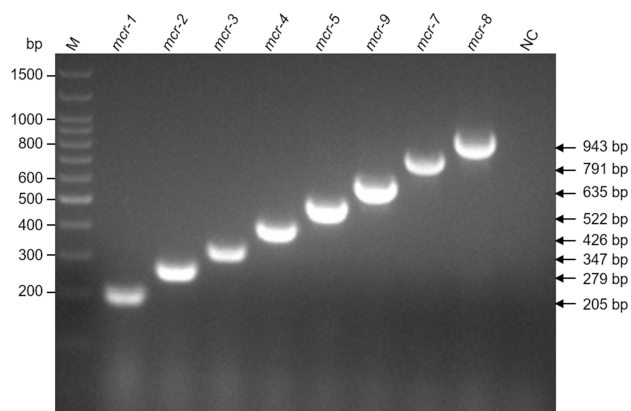


Figure 1 Multiplex PCR detection of *mcr* genes. Purified plasmid pUC57 carrying each *mcr* gene was used as templates. Agarose gel electrophoresis (2.0%) was used to separate multiplex PCR products. M indicates the molecular size marker (Trans100bp plus DNA ladder, TransGen Biotech, China). The size of each amplicon is indicated on the right side. NC, empty vector pUC57 was included as negative control.

gel and the specific amplification was confirmed by sequencing. In order to test the detection limit of the method, different amounts of plasmid templates were applied to the reaction mixture. The detection limits for *mcr-2*, *mcr-4*, *mcr-5*, *mcr-7*, *mcr-9* were less than 10 pg ([Figure S2](#)). Although at a lower efficiency, amplification of *mcr-1*, *mcr-3* and *mcr-8* could also be detected in the presence of 10 pg of plasmid templates ([Figure S2](#)). In addition, we tested the specificity of the method against other antibiotic resistance genes. No non-specific product or interference was detected when plasmid DNA harboring *dim-1*, *spm-1*, *imp-1* or *aim-1* was added to the multiplex PCR mixture ([Figure S3](#)). The PCR fragments were designed to be at least 60 bp difference in size, allowing us to easily differentiate the products. In addition, we utilized a 2% agarose gel and run for a longer time (50 min) to assure each band could be clearly distinguished. To accelerate the multiplex PCR detection process, we optimized the cycling conditions by reducing the denaturation time during cycling, annealing time and number of cycles. Since all the amplicons were less than 1000 bp in size, a final extension step was sufficient to allow extension of the PCR products. Apparently, omission of the extension step during cycling did not affect the amplification. Taken together, our method could allow the rapid identification of *mcr* genes with total detection time less than 85 min (amplification and electrophoresis).

Since the co-occurrence of two *mcr* genes in a single isolate has been reported,^{7–9} we tested the ability of our multiplex PCR method to simultaneously detect mixed DNA templates in a single tube. As shown in [Figure 2](#), the multiplex PCR system can effectively detect 28 combinations of two genes and 56 combinations of three genes. The band amplified for each gene was as expected.

Each of the *mcr* genes has multiple variants. We performed in-silico analysis to predict the ability of each primer pairs to bind to their variants. The results suggested that except for *mcr-2.2* and *mcr-3.25*, all the *mcr* variants could specifically bind to the primers ([Supplementary Table S4](#)).

Application of the Multiplex PCR Method to Bacterial Isolates

To test the feasibility of this method for clinical use, we isolated 60 colistin-resistant strains (43 isolates of *E. coli*

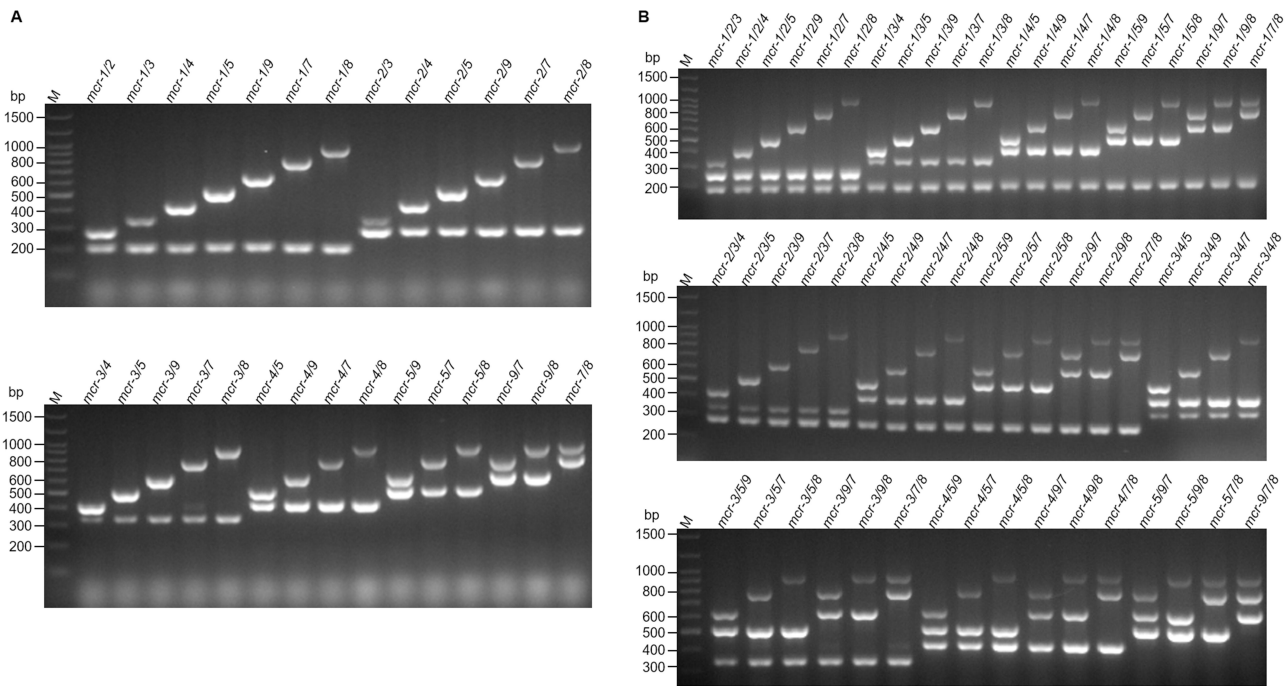


Figure 2 Multiplex PCR detection of two (A) or three (B) *mcr* genes. Different mixtures of double or triple plasmid DNA carrying a single *mcr* gene were used as templates. M indicates the molecular size marker.

and 17 isolates of *Klebsiella pneumoniae*) from fecal samples of pigs and pets by selective medium containing 2 mg/L of colistin (Supplementary Table S3). The total DNA of these strains was made by the boiling method from liquid cultures and added to the multiplex PCR mixture. Twenty out of the

60 isolates were determined to contain *mcr* genes (Figure 3). These included 19 strains positive for *mcr-1* and 1 isolate positive for *mcr-3* (Figure 3). Collectively, the results indicated the reliability of our method for the determination of *mcr* genes in bacterial isolates.

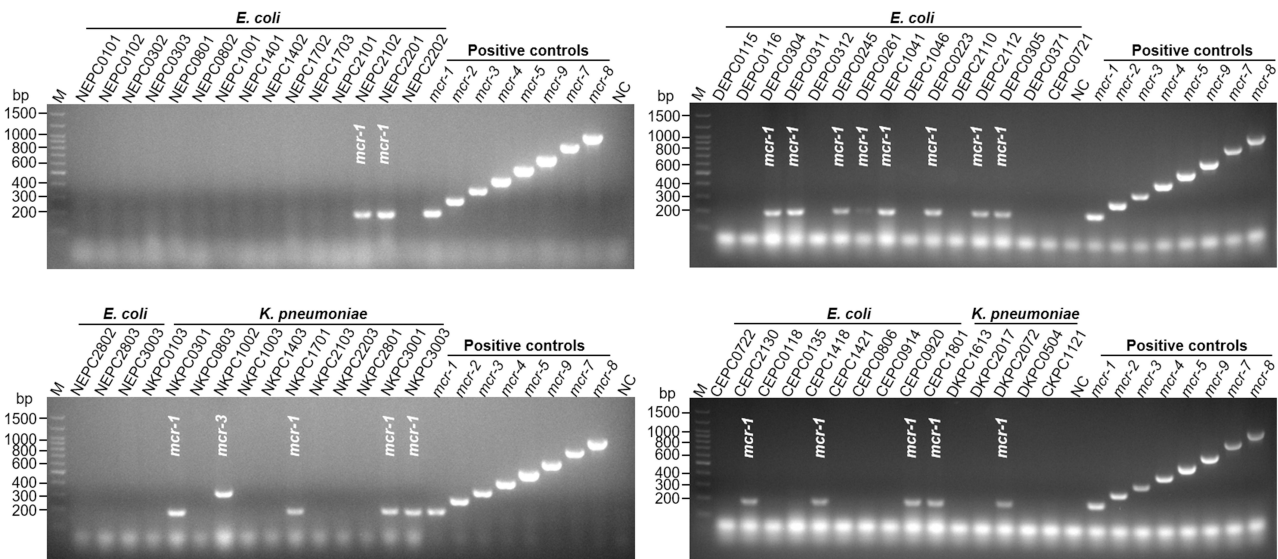


Figure 3 Molecular detection of *mcr* genes in bacterial isolates by the multiplex PCR method. Colistin-resistant bacterial strains (43 isolates of *E. coli* and 17 isolates of *K. pneumoniae*) were tested for the presence of *mcr* genes. Total bacterial DNA was obtained by the boiling method and applied to the multiplex PCR assay. *E. coli* strain Top10 harboring the *mcr*-encoding plasmid was included as a positive control. NC, *E. coli* TOP10 with empty vector pUC57. M shows the molecular size marker.

Discussion

Colistin is one of the last-resort antimicrobial agents for the treatment of human infections caused by fatal and multidrug-resistant gram-negative bacteria. However, colistin resistance has increased significantly worldwide in the recent years, majorly attributed to the emergence and dissemination of plasmid-mediated resistance mechanisms.³ Since the first report of the plasmid-borne polymyxin resistance determinant *mcr-1*, nine groups of *mcr* genes with diverse similarities in nucleotide and amino acid sequences have been described.⁴

The development of efficient molecular diagnostics is urgently necessary to identify and constrain this fast-spreading menace. Recently, a series of molecular tests have been designed to detect MCR-producing strains, including microarray,¹⁴ loop-mediated isothermal amplification (LAMP),¹⁵ conventional PCR,³ multiplex PCR^{6,11–13} and real-time PCR.^{16,17} Considering the sensitivity, specificity, skill and equipment requirements, cost, as well as turnaround time, each detection approach has its own advantages and disadvantages. Apparently, most of the techniques only detect a single *mcr* gene. The microarray detection method and the real-time PCR method are sensitive, and capable of detecting multiple *mcr* genes, but their application is restricted due to the requirement of specific and expensive equipment. Previously, four multiplex PCR assays capable of screening for *mcr-1* to *mcr-5* with different sets of primers and cycling conditions were reported.^{6,11–13} This method represents an efficient and inexpensive way for the rapid determination of *mcr*-encoding bacterial isolates. However, novel groups of *mcr* genes were not included in these assays.^{6,11–13} Another multiplex PCR method aimed to amplify *mcr-6* to *mcr-9* was reported recently, which is a valuable supplement to the available *mcr-1* to *mcr-5* multiplex PCR assays.¹⁸ In this study, we developed a multiplex PCR technique that could detect the *mcr* genes in a single mixture. Apparently, our method shows a striking advantage in the time consuming for detection. Recently, *mcr-10* was described as a novel group of *mcr* genes that has the highest similarity with *mcr-9*.⁵ In the future, it is necessary to include this gene for the development of a multiplex PCR method to detect all reported *mcr* genes.

Conclusion

Here we developed a multiplex PCR method that allowed efficient and rapid (less than 35 min for the amplification)

detection of all described families of *mcr* gene (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-7*, *mcr-8* and *mcr-9*) within a single tube. We believe that this technique can be applied to periodic surveillance and epidemiological research in hospitals, farms, foods, and the environment.

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Disclosure

The authors declare no conflicts of interest for this work.

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