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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Establishment of PCR detection methods for drug-resistance gene *mcr-8***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group
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APPROVE
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Zhu Junjie

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Scientific supervisor Ph.D., Assoc. Prof. Iryna Voloshyna

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1	Introduction	until 11 April 2025	
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SUMMARY

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The emergence of bacterial resistance seriously threatens the sustainable development of public health and animal husbandry. Polymyxin is a key drug for clinical treatment of gram-negative bacteria infection and the last line of defense to prevent and control bacterial infection. The *mobile colistin resistance-8* (*mcr-8*) gene is one of the main genes that cause bacteria to develop polymyxin resistance. This study designed and established a specific PCR detection method for the *mcr-8* gene to be used to quickly screen and monitor the *mcr-8* gene. According to the *mcr-8* gene sequence, a pair of specific primers were designed using the primer design software Oligo 7 to amplify the conserved region of the target gene *mcr-8.1* (101-1798), and the theoretical amplification product length was 1268 bp. The physical and chemical properties of the designed primers meet the requirements, their GC content is moderate (47.6% and 52.6%), and the Tm value is stable at 59.4℃. Specific verification showed that samples containing only the *mcr-8* gene showed 1268 bp specific amplification products, and no target bands were observed in the negative control group; the positive control PCR product was sequenced, and the BLAST comparison showed that the sequence consistency with the *mcr-8* gene was 100%, which confirmed that the method was highly specific. Sensitivity analysis shows that the lower limit of detection of *mcr-8* plasmid DNA in this system can reach 10 pg. Through detection of 15 clinical isolates (including 10 *E. coli* and 5 *Salmonella* strains), the *mcr-8* gene was not detected in all the tested strains. The PCR detection method of *mcr-8* gene established in this study provides effective technical means for the rapid detection of bacterial resistance, and has important application value in the fields of clinical treatment and bacterial resistance monitoring.

Key words: mcr gene, mcr-8 gene, PCR, agarose gel electrophoresis, colistin resistance, Salmonella

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INTRODUCTION

This study is based on PCR technology and designed specific primers to target conserved regions of *mcr-8.1* gene (101-1798 bp). Primers (*mcr-8*-F/R) were designed using Oligo 7 software, a 20 μL reaction system was established, and the annealing temperature was optimized to 59.4 °C. The amplified product was verified by gel electrophoresis, specificity was confirmed by sequencing, and sensitivity was evaluated (detection limit 10 pg). This method was further used to detect 15 clinical isolates (Escherichia coli, *Salmonella*) and analyze the prevalence of *mcr-8*. The *mcr-8*-specific PCR detection method was successfully established, with amplified product 1268 bp, and the sequencing verification was 100% consistent with the target sequence. High sensitivity (lower detection limit of 10 pg), negative controls have no cross reaction. Clinical sample detection showed that *mcr-8* was not detected in none of the 15 strains of bacteria, suggesting that the gene has a low prevalence in the test bacterial population. The method is easy to operate and low cost, and is suitable for ordinary laboratories. This study provides efficient and low-cost technical means for rapid screening of *mcr-8* genes, fills the shortcomings of existing detection methods, and helps to identify polymyxin-resistant strains in the early stage of clinical practice, which has important practical value for ensuring public health safety and delaying the drug resistance crisis.

The relevance of the topic is establishment of PCR reaction system for *mcr-8* gene.

The purpose of this study is to establish an accurate and efficient detection method, which is applied to the clinical detection of *mcr-8* genes, and to establish a foundation for further research on the transmission pathways and drug resistance mechanisms of *mcr-8* genes, so as to develop new treatment methods to deal with the threat of sulfamicone-resistant bacteria.

The subject of the study was the drug resistance gene *mcr-8*.

The research method is based on PCR technology, designing specific primers to target conserved regions of *mcr-8* genes, optimizing annealing temperature, and establishing a detection system. The amplified product was verified by gel electrophoresis, specificity was confirmed by sequencing, sensitivity was evaluated, and it was used for detection of 15 clinical strains.

The research results are the successful development of a highly sensitive and specific *mcr-8* detection method to provide reliable tools for drug resistance monitoring. The clinical results of *mcr-8* were not detected to indicate its current low prevalence, but the method can quickly screen potential drug-resistant bacteria, guide the rational use of antibiotics, delay the spread of drug-resistant, and have important value for public health prevention and control.

The structure of the thesis is divided into four parts: introduction, materials and methods, results and analysis, summary and prospect. The introduction part systematically explains the research progress of bacterial drug resistance and *mcr* genes and the purpose and significance of the research. Materials and methods describe the experimental design in detail. The results partially demonstrate the reliability of verification methods such as electrophoresis and sequencing. The limitations of the proposed method and future research directions in the summary and prospects.

Chapter 1
LITERATURE REVIEW

1.1 Current status of bacterial drug resistance

Drug resistance refers to the tolerance of microorganisms, parasites and tumor cells to the effect of drugs, which in turn leads to the reduction or failure of drug efficacy. At the molecular level, drug resistance is often associated with gene changes, such as site mutations, deletions and gene amplification[1]. In the field of human medical and animal husbandry, excessive use of antibiotics has accelerated the screening and spread of *Salmonella* antibiotic genes. The spread of *Salmonella* resistance not only increases the difficulty of treating infectious diseases, but may also lead to increased medical costs, increased patient mortality, and even trigger a public health crisis of "no medicine available".

At present, it is reported that the *Salmonella* isolated from diarrhea patients are widely resistant to third-generation cephalosporin antibiotics, and also *Salmonella* resistant to aminoglycosides and carbapenem antibiotics[2].At the same time, *E. coli* accumulates a variety of drug-resistant genes through horizontal gene transfer, such as ESBLs, carbapenemase, PMQR gene and *mcr* gene, resulting in resistance to various antibiotics such as *β*-lactams, aminoglycosides, quinolones and polymyxins. Drug-resistant transmission is closely related to mobile genetic elements such as plasmids, transposons and integrons, and is widely present in livestock, poultry, companion animals and the environment. In particular, the transmission of the polymyxin-resistant gene *mcr-1* in animals poses a threat to public health. Therefore, it is necessary to conduct long-term and continuous drug resistance monitoring of *Salmonella* and *E. coli*. First, provide data support for clinical test drugs, and second, it can monitor the epidemic transmission status of drug-resistant bacteria and the emergence of new drug-resistant bacteria.

At the same time, studies have shown that the *mcr-8* gene has potential metastasis[3]. Given that *Salmonella* and *E. coli* are important zoonotic pathogens, PCR detection methods for drug-resistant gene *mcr-8* can be established to quickly

detect drug resistance of *Salmonella* and *E. coli*, effectively monitor and control the transmission dynamics of drug-resistant genes, and avoid ineffective treatment and further abuse of antibiotics.

1.2 mcr gene overview

1.2.1 mcr gene resistance mechanism

Polymyxin is a type of polypeptide antibiotic produced by *Bacillus polymyxin*. It has a lipophilic fatty acyl side chain and acts on lipid A in many Gram-negative bacteria lipopolysaccharides. Its mechanism of action is that positively charged α,γ-diaminobutyric acid and negatively charged phosphate groups of lipid A produce electrostatic effects, causing divalent cations such as Ca^{2+} and Mg^{2+}, increasing cell membrane permeability, cytoplasm leakage, and ultimately leading to cell death, thus exerting a bactericidal effect[4-6].

With the increasing prevalence of multidrug-resistant Gram-negative infections, the World Health Organization (WHO) listed polymyxin as the "Critical Antibiotic (HPCIA)" in 2024. However, polymyxins are overused as growth promoters in livestock industry, resulting in rapid spread of resistance.Studies have predicted that from 2025 to 2050, there will be more than 39 million deaths worldwide that can be directly attributed to antibiotic resistance or drug-resistant pathogens[7]. Infection of multidrug-resistant bacteria is the main cause of this phenomenon. At present, polymyxin is the last line of defense to prevent and control bacterial infections and one of the most important drugs.

The *mobile colistin resistance* (*mcr*) gene encodes phosphoethanolamine transferase, which can modify the lipid A domain in bacterial lipopolysaccharides. It transfers phosphoethanolamine (PEA) from the donor to the lipid A portion of the lipopolysaccharide (specifically acts on the glucosamine residue), resulting in a decrease in its net negative charge, thereby reducing the binding efficiency of polymyxin to bacterial cell membranes, resulting in bacterial resistance to polymyxin[6].There are more than 20 host bacteria of *mcr* reported, most of which belong to the *Enterobacteriaceae*, *Aeromonas* and Moraxaceae. In addition, most

host bacteria are widely distributed in nature and can achieve horizontal transfer between *mcr* species, resulting in the global epidemic of this drug-resistant gene.

Among them, the most popular *mcr-1* belongs to the YhjW/YjdB/YijP alkaline phosphatase family, mainly including the transmembrane domain located at the N-terminus, the catalytic domain located at the C-terminus, and the connecting region connecting the two regions. Through prediction of the protein structure of the *mcr-1* transmembrane domain, it was found that it contained 5 transmembrane helixes, which ensured that the *mcr-1* protein was anchored to the periplasmic side of the bacterial inner membrane[8]. The study found that deleting mutants in the transmembrane region can lead to a complete loss of drug resistance (MIC values drop from 16-32 mg/L to 2-4 mg/L), which fully demonstrates that transmembrane structure is crucial for the localization and function of the enzyme. Through extensive analysis of structural docking and sequence alignment, it was found that E246, T285, H395, D465 and H466 residues are crucial for substrate binding of *mcr-1*, which in turn determines the resistance of *mcr-1* encoded colistin. Point mutation experiments have shown that mutations in any of the above residues will lead to a complete loss of drug resistance, further confirming that they are involved in substrate binding or catalytic reactions. At the same time, *mcr-1* is highly similar to the EptA protein structure of Neisseria meningitidis, and both of them are dependent on zinc ions in their active centers. However, the domain of *mcr-1* and EptA lose their catalytic ability after interchangement, indicating that there is functional specificity between different *mcr* mutants. In addition, the catalytic domains of *mcr-1* and *mcr-2* still have enzymatic activity after being interchanged, suggesting that their functional domains have certain compatibility[9].

The phosphoethanolamine transferase encoded by the *mcr-8* gene is only 31.08% homology with *mcr-1*, and its homology with *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-6*, and *mcr-7* was 30.26%, 39.96%, 37.85%, 33.51%, 30.43% and 37.46% respectively, which implies that it has a unique evolutionary path. Sequence alignment showed that its active sites (E246, T285, K333, H395, D465, H466,

E468, H478) and 6 cysteine residues were highly conserved in protein ORF. These results suggest that the corresponding gene may encode a functional ethanolamine transferase mediates the resistance of *Klebsiella pneumoniae* and its *E. coli* to colistin. The resistance function of *mcr-8* gene was verified and found that it can be expressed in a single gene, which increases the minimum inhibitory concentration (MIC) value of *E. coli* DH5*α* by 4 times (MIC value increased from 0.25 mg/L to 1 mg/L), or increases the minimum inhibitory concentration (MIC) value of *E. coli* J53 by 16 times (MIC value increased from 0.25 mg/L to 4 mg/L) through ligation transfer[10].

For 122 strains of *Klebsiella pneumoniae* isolated from 300 chicken cloaca, the minimum inhibitory concentration (MIC) value of colistin ranged from 4-128 μg/ml, and the minimum inhibitory concentration (MIC) value of 4 strains (KP4, KP49, KP700, KP744) was >32 μg/ml, suggesting that there is an additional resistance mechanism for the *mcr-8* gene. Through S1 nuclease pulse field gel electrophoresis, Southern blotting and coupling assays, the results showed that all *mcr-8*-containing plasmids were transferred from *Klebsiella pneumoniae* isolates to *E. coli* J53 receptor strains at a frequency of 10^{-6}-10^{-8}, and resulted in a 2-32 fold increase in resistance to colistin by all transconjugates[11].

Although there have been many studies on the resistance mechanism of *mcr* family genes, no literature has been reported on the resistance mechanism mediated by *mcr-8* gene, and its specific targets and regulatory pathways still need to be further explored.

1.2.2 mcr gene detection method

The methods for detecting *mcr* genes are divided into two categories: phenotypic detection and molecular detection[12]. Phenotype detection determines drug resistance by observing bacterial growth or metabolic characteristics, which can detect whether bacteria are resistant, but it is difficult to determine its drug resistance mechanism.

Nordmann et al. established a method (RPNP) for rapid detection of polymyxin resistance in *Enterobacteriaceae* bacteria in 2016. The principle is to judge drug resistance by detecting the glucose metabolism of bacteria in the presence of colistin or polymyxin B at a specific concentration. The test was 99.3% sensitive to 135 drug-resistant bacteria and 65 sensitive bacteria, with a specificity of 95.4%, and took less than 2 hours, which was faster, economical and easy to operate than traditional micro broth dilution method (BMD). Its limitations are also obvious. They are not suitable for BACs such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, and rely on naked eyes to interpret them. More experiments may be required for low-level drug resistance[13].

Coppi et al. developed a colistin MAC test in 2017 to screen for *mcr-1*-mediated colistin-resistant *Enterobacteriaceae* bacteria by detecting minimal inhibitory concentration (MIC) changes in colistin in the presence of 2,6-pyridindicarboxylic acid (DPA). The test of 74 drug-resistant bacteria (61 strains carrying the *mcr-1-like* gene and 13 strains were *mcr*-negative) showed that the minimum inhibitory concentration (MIC) value of colistin was reduced by 8 times in 59 strains of *mcr-1*-positive strains, while there was no significant change in the minimum inhibitory concentration (MIC) value of *mcr*-negative strains and 2 strains of *mcr-1*-positive *Klebsiella pneumoniae*. This method is suitable for laboratories that lack molecular detection conditions, but the sample size is only 74 strains, and no tests are conducted for other *mcr* genotypes (such as *mcr-2* to *mcr-10*), so further verification is required[14]. See Table 1 for specific phenotypic detection methods.

Table 1 Phenotype detection methods, principles and characteristics

Detection method	Principle	Features
Culture medium	Contains specific ingredients to inhibit the growth of sensitive	Low cost, simple operation, but long detection time, low specificity and sensitivity to *mcr* gene

	bacteria, and adds color developer in part of the culture medium to assist in identifying bacteria	
MIC Determination Tool	For example, micro broth dilution method (BMD), E test method (E-test), etc., drug resistance is evaluated by determining the minimum inhibitory concentration (MIC)	The micro broth dilution method (BMD) is a standard method for in vitro drug sensitivity tests recommended by the laboratory standardization association, but the operation is cumbersome; the E-test method (E-test) is relatively simple to operate, but the accuracy is limited due to the diffusion of antibiotics in agar.
New detection method	For example, RPNP, colistin MAC test, etc., to detect drug-resistant bacteria based on other principles	Some methods have high detection efficiency, but different methods have different detection effects on different bacterial species.

Molecular diagnosis is characterized by detecting the *mcr* gene, which has high sensitivity and specificity, but it cannot confirm the phenotype resistance of bacteria, and it is difficult to detect unknown drug resistance mechanisms based on primers and probes.

Bernasconi et al. used the CT103XL microarray platform to detect *β*-lactamase and *mcr-1*/*mcr-2* resistance genes in *Enterobacteriaceae* bacteria, which can complete the detection within 6.5 hours. Tests on 106 isolates (including 80 *E. coli*, 14 *Klebsiella pneumoniae*, etc.) showed that 32 strains carrying the *mcr-1*/*mcr-2* gene were correctly identified with an accuracy rate of 100%. The advantage of this method is that it is rapid and high throughput, suitable for epidemiological monitoring of multidrug-resistant bacteria, but cannot detect emerging drug-resistant genes such as *mcr-3*[15].

Imirzalioglu et al. used a loop-mediated isothermal amplification (LAMP) instrument and the eazyplex SuperBug *mcr-1* kit to detect 67 *mcr-1* positive strains

and 37 negative strains (including 9 inherently resistant bacteria). The results showed that the sensitivity and specificity were 100%. The core advantage of this method is that the total inspection time is only 20 minutes, the instrument is easy to carry, and is suitable for on-site inspection and epidemiological monitoring of breeding farms, food processing plants and medical institutions. Its locality includes the limited sample size of a single test, only targeting *mcr-1* (not covering other *mcr* genes), etc.[16]. Specific molecular detection methods are shown in Table 2.

Table 2 Molecular detection methods, principles and characteristics

Detection method	Principle	Features
PCR and WGS	Conventional PCR is used to detect known *mcr* genes; whole genome sequencing (WGS) can identify all drug resistance mechanisms; real-time and multiplex PCR can detect multiple *mcr* genes	Conventional PCR is easy to operate and inexpensive, but can only detect one specific gene at a time; WGS is costly and time-consuming; real-time and multiple PCR can detect multiple genes at the same time, but some methods still need further optimization.
Microarray	For example, CT103XL, multiple ligation detection reactions are used to detect *β*-lactamase and *mcr-1*/*2* genes simultaneously	Can detect multiple genes, but it is costly and cannot detect emerging drug-resistant genes such as *mcr-3* gene
Loop-mediated isothermal amplification (LAMP)	Eazyplex SuperBug detects *mcr-1*, sample preparation is simple, and the relevant instruments are movable	The detection time is short, but the detection genome type is limited, so it is impossible to directly detect the original sample.

1.2.3 Research progress of mcr gene

In 2015, the first study reported that the plasmid-mediated *mcr-1* gene can make bacteria resistant to polymyxins. Liu et al. used whole plasmid sequencing and subcloning techniques to identify the *mcr-1* gene of *E. coli SHP45* strain. The *mcr-1* mechanism was studied through sequence comparison, homology modeling

and electrospray ionization mass spectrometry[17]. Subsequent studies found that the drug resistance mechanism had spread to Southeast Asia and was widely spread around the world[18]. In 2016, the *mcr-2* gene was first identified in *E. coli* from Belgian porcine and bovine origin. This is a 1617 bp phosphoethanolamine transferase located on the IncX4 plasmid, with a homology of 76.7% with *mcr-1* nucleotide and a high transfer frequency. It often coexists with multiple drug-resistant genes (such as CTX-M type ultra-wide-spectrum *β*-lactamase, CMY-2), which may further aggravate drug-resistant transmission [19]. The plasmid-mediated colistin resistance gene *mcr-3* was reported in 2017. This gene is present in porcine-derived plasmid pWJ1, 261-kb Inch12 plasmid porcine, and has been detected in *Enterobacteriaceae* and *Aeromonas* in several countries[20]. In the same year, the *mcr-4* gene was detected in *Salmonella typhimurium* isolated from the cecum of pigs in Italian slaughterhouses. Through whole genome sequencing (WGS) and comparative genome analysis, the *mcr-4* gene was found to be on the ColE10 type plasmid[21]. Also in 2017, the *mcr-5* gene was found in *Salmonella* para*typhimurium* fermented by tartaric acid in Germany. *mcr-5* has low amino acid sequence similarity to other known *mcr* genes, suggesting that *mcr-5* may have an independent evolutionary source. In 2017, *mcr-6.1* was detected on the multi-animal *Moraxella* chromosome, which had 87.9% homology to *mcr-2* of *E. coli*. Unlike other *mcr* genes, *mcr-6.1* is located on the chromosome and no complete insertion element is found around it[22]. In 2018, the *mcr-7.1* gene was identified in *Klebsiella pneumoniae* from chick origin in Chinese, which was located on the Incl2 type plasmid, and no insertion sequence (IS) or transposon was found around it, which was speculated that it may be integrated into the plasmid through recombinant events[23]. In the same year, the *mcr-8* gene was first discovered on the transferable IncF type II plasmid of *Klebsiella pneumoniae* (*KP91*). Functional cloning showed that the acquisition of a single *mcr-8* gene significantly increased the resistance of *E. coli* and *Klebsiella pneumoniae* to colistin. The minimum inhibitory concentration (MIC) value of colistin for strains carrying this gene is 16 μg/mL, and it also carries a multidrug-resistant gene[10]. In

2019, the *mcr-9* gene carried by a novel plasmid was found in *Salmonella typhimurium*, which is multidrug-resistant but colistin-sensitive. Through bioinformatics search, it was found that *mcr-9* is widely distributed in the InclH2 plasmid of *Enterobacteriaceae*[24]. In 2020, the *mcr-10* gene was found in *Enterobacter roggenkampii*, which is located on the InFIA plasmid. *mcr-10* can increase the minimum inhibitory concentration (MIC) value of colistin-sensitive strains by 4 times, demonstrating its ability to mediate colistin resistance[25].

At present, *mcr-1* and *mcr-9* are the most widely transmitted in the *mcr* gene, and are found in isolates in 61 countries on six continents, respectively; *mcr-3* and *mcr-5* are followed by, respectively, and other genes have a small transmission range or are distributed in dispersed[26]. The emergence of *mcr* genes has had a profound impact on human health and public health worldwide. These genes are usually located on plasmids and can be transmitted between different strains through horizontal gene transfer, further aggravating the spread of drug resistance.

1.2.4 Current status of *mcr-8* gene research

Compared with *mcr-1*, the drug resistance and transmission mechanism of *mcr-8* gene have not been fully understood, but its role in polymyxin resistance cannot be ignored. Studies have found that 9.83% of the isolates of *Klebsiella pneumoniae* are present in *mcr-8*. According to S1 nuclease pulse field gel electrophoresis and Southern blot analysis, the *mcr-8* gene of all isolates is located on the plasmid. This gene is located on plasmids of different sizes. These plasmids can be divided into three types: IncFIA, IncA/C and IncFIIK, and their genetic environment is complex and diverse. Moreover, these plasmids also contain genes that are resistant to p-lactams, tetracyclines, aminoglycosides, sulfonamides, macrolides, chloramphenicol and frefenicol. In some strains, mutations in the two-component system may work in concert with *mcr-8*. Even if the plasmid carrying *mcr-8* is eliminated, other resistance mechanisms still exist, which keeps the strains resistant to colistin[11]. *mcr-8* positive strains are usually resistant to a variety of antibiotics. Studies have shown that the *Klebsiella pneumoniae* 17R293

isolate carrying the *mcr-8.1* gene is widely resistant to colistin, *β*-lactam, quinolones, fosfomycin and other antibiotics[27]. At the same time, the same genetic environment of *mcr-8.1* exists between animals and humans[28], which reminds us to pay close attention to and monitor the spread of *mcr-8*. Hu Jun et al.'s research found that movable genetic elements such as insertion sequences and transposons play a crucial role in *mcr* transmission, and *mcr-8* transmission is related to IS elements IS903B and ISEcl1[29]. In addition, the pKP91-00067 gene carried by *Klebsiella pneumoniae* IncF type II plasmid can regulate the sensitivity of strains to colistin and cefoxitin, and can also regulate the ligation and transfer of strain plasmids[30]. In 2022, studies have found that the *mcr-8* gene can form a multidrug-resistant hybrid plasmid with bla_{NDM-1} (carbapenem resistance gene) or tmexCD1-toprJ1 (tigecycline resistance gene) through IS26 and ltrA-mediated plasmid hybridization. This plasmid can be efficiently transferred in *E. coli* and *Klebsiella pneumoniae* and has increased stability under colistin pressure, resulting in a carbapenem or tigecycline resistance phenotype, posing a major threat to clinical treatment[31].

Nabti et al. designed a real-time fluorescence quantitative PCR detection method for TaqMan probes targeting the *mcr-8* gene in 2020. The specificity of the designed primers and probes was first detected by BlastN analysis and PCR. Sensitivity and specificity assays were performed in vitro on 290 Gram-negative bacteria genomic DNA and 250 personal fecal samples of metagenomic DNA. 120 clinical Gram-negative bacteria were screened by real-time PCR, and the minimum inhibitory concentration of colistin (MIC) was determined on the positive strain. It was found that one *Klebsiella pneumoniae* isolate (KP95) was positive, and its minimum inhibitory concentration (MIC) value was 8 mg/mL. This real-time fluorescence quantitative PCR detection method has high specificity and sensitivity, with a detection limit of 55 CFU/mL, and can detect the *mcr-8* gene from DNA samples within 2 hours, providing an effective tool for clinical detection[32].

Li Yiming and others also constructed a real-time fluorescence quantitative PCR detection method for the polymyxin resistance gene *mcr-4/5/8* in 2021. Based

on the gene reference sequences of the polymyxin-resistant genes *mcr-4*, *mcr-5* and *mcr-8* downloaded by GenBank, specific primers were designed and their specificity was verified, and a real-time fluorescence quantitative detection method was established using the SYBR Green I dye method. The *mcr-4*, *mcr-5* and *mcr-8* genes were cloned onto the pMD19-T vector, and the standard plasmid was constructed, and the amplification curve, standard curve and melting curve of the fluorescence quantitative PCR reaction were established using the standard plasmid as a template. The results show that the method has good specificity, the amplification curve, standard curve and melting curve perform excellently. The blind selection results for known samples are 100% consistent with the sequencing results, but the target gene is not detected in the environmental samples, making it difficult to evaluate its prevalence in livestock and poultry farms[33].

At present, China has detected 8 *mcr* genes except *mcr-6*[26]. Selecting *mcr-8* genes as research objects will not only help to in-depth exploration of their transmission mechanisms and drug resistance characteristics, fill the gap in current detection technology, but also provide a scientific basis for formulating targeted prevention and control strategies and reserve technical strength for monitoring their potential trends.

1.3 Purpose and significance of research

The long-term abuse of antibiotics has led to the increasing resistance of bacteria, significantly reducing the therapeutic effect. Currently, polymyxin is one of the most important drugs to treat bacterial infections, and the emergence of the *mcr* gene has made bacteria resistant to colistin. Among them, the *mcr-8* gene may have a unique evolutionary pathway and can form a multidrug-resistant hybrid plasmid with bla_{NDM-1} (carbapenem resistance gene) or tmexCD1-toprJ1 (tigecycline resistance gene), resulting in a carbapenem or tigecycline resistance phenotype, which seriously threatens clinical treatment. Therefore, a PCR detection method for the *mcr-8* gene can be constructed to monitor the spread of *mcr-8* and provide a practical solution to ensure public health safety.

This study is committed to developing a PCR detection method for the *mcr-8* gene. Compared with other methods, PCR detection methods have the advantages of high sensitivity and strong specificity, and the detection time is faster and the cost is lower than that of traditional culture methods. The detection of *mcr-8* gene by this PCR method can quickly and accurately identify strains carrying the gene, providing an important reference for clinical treatment and infection control. In addition, detection of *mcr-8* genes can help monitor its distribution in different regions and strains, evaluate the prevalence of polymyxin resistance, and provide scientific basis for formulating antibiotic use policies and antibiotic prevention and control strategies. To sum up, establishing an efficient and reliable PCR detection method for *mcr-8* gene is of great significance to respond to the challenges of polymyxin resistance and ensure public health safety.

Summary of chapter 1

1. Antibiotic abuse has led to an increase in bacterial resistance, especially *Salmonella* is widely resistant to antibiotics such as ampicillin, tetracycline, carbapenems and other antibiotics. It is urgent to establish drug-resistant gene monitoring technology to guide clinical drug use and prevention and control strategies.

2. The *mcr* gene encodes phosphoethanolamine transferase, which reduces the binding efficiency of polymyxin by modifying the lipopolysaccharide lipid A structure, leading to drug resistance. Currently, the methods for detecting *mcr* genes are divided into: phenotypic detection (fast but low specificity, and the drug resistance mechanism cannot be clarified), and molecular detection (high sensitivity, but relying on known gene sequences, it is difficult to cover new genes). After the discovery of *mcr-1* in 2015, *mcr* family genes (*mcr-2* to *mcr-10*) have been discovered around the world and have a wide range of transmission.

3. *mcr-8* and *mcr-1* have low homology (31.08%), have unique active sites and conserved structures, and can enhance colistin resistance through plasmid transfer (MIC value is increased by 4-16 times). Although existing detection

methods (such as real-time fluorescence quantitative PCR) are sensitive, they cover limited gene types and *mcr-8* standardized detection schemes are still lacking.

4. The purpose and significance of the research is to develop a specific PCR detection method for *mcr-8* to quickly screen drug-resistant strains and monitor their spread dynamics. Make up for the gap in existing technology and provide scientific basis for clinical treatment, antibiotic policy formulation and drug resistance prevention and control.

Chapter 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental materials

2.1.1 Source of materials

The *mcr-8* gene was synthesized by Shanghai Shenggong Biotechnology Company, and the corresponding gene fragment was integrated into the plasmid pUC57 as a positive control. The negative control used *E. coli* ATCC 25922 without the *mcr* gene as a template, and the strain was provided by the Institute of Microbiology and Immunity, Qilu University of Technology. The blank control uses an equal amount of ddH2O as a template.

2.1.2 Main reagents

2×Taq Master Mix (Norvezan, Suzhou, China); Mini Kit (Vazyme, China); EasyPure® Bacteria Genomic DNAKit (TransGen Biotech, China); FastPure Plasmid Mini Kit (Vazyme, China); trypsin, yeast extract, sodium chloride, nucleic acid dye; agarose and 50×TAE buffer; DL1500 DNAMarker; FastPure Gel DNAExtraction, etc.

2.1.3 Main instruments

Table 2-1 Main instruments of experiment

Instrument	Model	Company
Constant temperature culture machine	DHP-9082	Shanghai Yiheng Technology Co., Ltd.
Ultra-clean workbench	SW-CJ-2F	China Suzhou Purification Group
Electronic analysis balance	SL601	Shanghai Minqiao Electronic Instrument Factory
Electrophoresis instrument	Agaro-PowerTMe	Beijing Liuyi Biotechnology

		Co., Ltd.
Sterilization pot	LDZX-50KBS	Shanghai Shen'an Medical Technology Co., Ltd.
PCR amplification instrument	Mycycler	Bio-Rad, USA
Gel imaging system	2020D	Beijing New Technology Company
Nucleic acid protein analyzer	NanoDrop2000	ThermoFisher Company in the United States

2.2 Experimental methods

2.2.1 Preparation of bacterial DNA

Bacterial genomic DNA extraction kit was used to standardize nucleic acid extraction of bacterial cultures. According to the operating specifications of the instructions, the bacterial lysis, protein denaturation, nucleic acid adsorption and elution steps are completed in turn to obtain high-quality genomic DNA. Nucleic acid quality analysis was performed using an ultraviolet spectrophotometer to measure the ratio of A260/A280 to A260/A230. The ideal value of A260/A280 for high-purity DNA is about 1.8, and the ideal value of A260/A280 is about 2.0. Based on the measured values, determine whether significant protein, carbohydrate, salt or organic solvent contamination is detected in the sample to ensure that the PCR experimental requirements are met.

2.2.2 Primer design and synthesis

Search and download *mcr-8.1* from NCBI (https://www.ncbi.nlm.nih.gov/genbank/), and find the conserved regions of the *mcr-8.1* gene using CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Based on the conserved region sequence, the primer design software Oligo 7 was designed according to the principles of primer

parameters of 18-30 bp in length and 40-60% GC content, and the primers were obtained in Table 2.2.

The conserved region of the target gene is amplified (101-1798) of *mcr-8.1* (NCBI reference sequence: NG_061399.1), the forward primer binding site is 501-521, the reverse primer binding site is 1750-1768, and the expected product length is 1268 bp, covering the core region of the phosphoethanolamine transferase domain.

Primers are verified by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and can fully match the *mcr-8.1* reference sequence, with good specificity and practical application effects. The primers used in this experiment were synthesized by Beijing Bomaide Co., Ltd.

Table 2-2 *mcr-8* gene design primers

Primer name	Sequence (5'-3')	Primer binding site	Size（bp）
*mcr-8*_F	TACCAGCAATTATCCTGGCGT	501-521	1268
*mcr-8*_R	AACTGCGGAAGACAGTGGT	1750-1768	

2.2.3 Establishment of PCR detection method

（1）Construction of PCR reaction system

Table 3 20 μL PCR reaction system

Reagents	Volume
ddH_2O	8.6 μL
2×Taq Master Mix (Dye Plus)	10 μL
Upstream primers	0.2 μL
Downstream primers	0.2 μL
Template DNA	1.0 μL

（2）PCR reaction program parameter setting

Table 4 PCR reaction program parameters

Circulation steps	Temperature	Time	Number of loops
Pre-transformation	95℃	5 min	1
transsexual	95℃	15 sec	35
annealing	59.4℃	15 sec	
extend	72℃	50 sec	
Finally extended	72℃	10 min	

（3）Optimize primer annealing temperature

A 20 μL system was constructed, and a temperature gradient of 54-66℃ was set, with a total of 10 gradients (55.0℃, 55.3℃, 55.9℃, 56.8℃, 58.1℃, 59.4℃, 60.6℃, 61.9℃, 63.2℃, 64.1℃, 64.7℃, 65.0℃), PCR amplification was performed according to the parameters of Table 2-4 and detected by agarose gel electrophoresis.

2.2.4 Electrophoretic identification of PCR amplification products

Weigh 2 g of agarose and add it to a 250 ml conical flask; take 2 ml 50×TAE buffer and 98 ml of distilled water, and add it to the conical flask for initial mixing; put the mixture into the microwave and heat it, and wait until the glue is completely dissolved, let cool to about 45-55℃; add nucleic acid dye and shake it quickly.

Insert comb teeth into the glue tank and pour in the shaken gel. After the gel is completely solidified, carefully remove the comb, pour TAE buffer into the electrophoresis tank, put the gel into the electrophoresis tank, and do the sampling. First, spot the DNAMarker, then absorb the PCR product and mix it with the loading buffer, and slowly add it to the spot well with the sample loading gun. Determine the direction of the sample hole, cover the electrophoresis tank, set the voltage to 130 V, then turn on the power, and electrophoresis for 20 minutes.

After the electrophoresis is completed, the gel electrophoresis image is observed using a gel imaging system. Compared with DNAMarker, the bar band size is consistent with the expectations, and the quality of the PCR product is judged. If the band meets the expected size, it is initially judged that the expansion product meets the expected target.

2.2.5 Gene sequencing verification of PCR amplification products

Using a DNA recovery kit, the DNA in the gel was recovered according to the standard operating procedures and handed over to Shanghai Shenggong for sequencing. After obtaining the sequencing results, nucleotide sequence alignment and result interpretation were performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to evaluate the specificity of the PCR method.

2.2.6 Verification of PCR method sensitivity

The plasmid solution carrying the *mcr-8* gene was diluted to 10^{-11} at a 10-fold ratio, and the plasmid solution with 3 dilutions was taken, 10^{-9}, 10^{-10}, and 10^{-11}. The solution was tested by PCR, and each dilution was repeated more than 3 times to determine the sensitivity of the detection method.

2.2.7 Application of PCR method in detection of isolates

In order to detect the presence of *mcr-8* gene in the strain, this study conducted PCR detection of *E. coli* strains (including 10 chicken-derived E100, E106, E118, E49, E63, E64, E65, E72, E85, E95) and *Salmonella* strains (including 5 human clinical samples isolated *Salmonella* QLUF110, QLUF111, QLUF112, QLUF113, QLUF114). Bacterial cultures were standardized by EasyPure® Bacteria Genomic DNAKit (TransGen Biotech, China), and a 20 μL PCR reaction system was constructed. PCR amplification was performed according to the PCR reaction procedure parameters in Table 2-4. The product was

obtained for agarose gel electrophoresis, and then the gel electrophoresis image was observed using the gel imaging system.

Summary of chapter 2

The positive control was plasmid pUC57 containing the *mcr-8* gene. Negative controls used *E. coli* ATCC 25922 without the *mcr* gene as a template. The blank control uses an equal amount of ddHO. The prepared reagents and instruments were tested according to the following methods.

1. DNA extraction: Bacterial genomic DNA extraction kit was used to standardize nucleic acid extraction of bacterial cultures. Nucleic acid quality analysis was performed using an ultraviolet spectrophotometer to ensure that the extract meets the PCR experimental requirements.

2. Primer design: The target region is *mcr-8.1* conserved region (101-1798). Primer sequence F is TACCAGCAATTATCCTGGCGT; R is AACTGCGGAAGACAGTGGT. Specificity was confirmed by BLAST verification.

3. PCR system and program: Configure 20 μL PCR system, set the program to 95℃ prededenatment for 5min → 35 cycle (95℃ 15sec → 59.4℃ annealing → 72℃ extension) → final extension for 10 min.

4. Electrophoresis identification: 2% agarose gel, 130 V electrophoresis for 20 min. After the electrophoresis is completed, the gel electrophoresis image is observed using a gel imaging system, compared with the DNA Marker, to observe whether the band size is consistent with the expectations, and to judge the quality of the PCR product.

5. Sequencing verification: positive product sequencing.

6. Sensitivity: The plasmid solution with three dilution gradients of 10^{-9}, 10^{-10} and 10^{-11} was selected for PCR detection. Repeat detection of each dilution for more than 3 times

7. Actual detection: PCR was performed on 10 *E. coli* strains and 5 *Salmonella* strains.

Chapter 3
EXPERIMENTAL PART

3.1 PCR amplification and gel electrophoresis

PCR identification was performed on plasmid solution carrying the *mcr-8* gene (positive control) and *E. coli* ATCC 25922 (negative control) as templates. A 20 μL PCR reaction system was configured according to Table 3, and an equal amount of ddH2O was set as a blank control. PCR amplification was performed according to Table 4 PCR reaction program parameters. The amplified product was electrophoresised at 130V in 2% agarose gel for 20 minutes, and the band size was analyzed by a gel imaging system. The results are shown in Figure 3.1.

Under the reference of DNAMarker, a single band that was consistent with the expected size was observed in the positive control group, indicating that PCR successfully amplified the target product. There were no bands in the blank control group and the negative control group, indicating that the experimental specificity was high and the system was not contaminated.

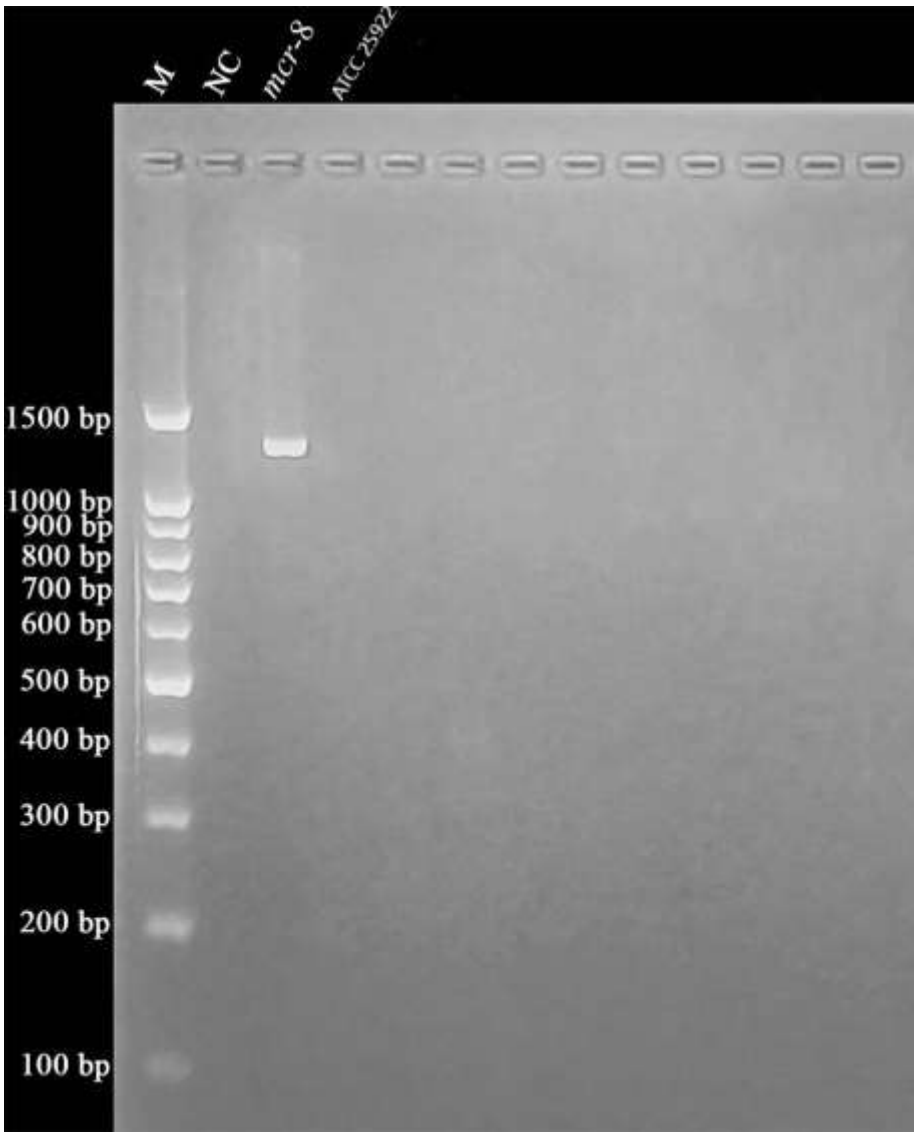

Figure 3.1 Gel electrophoresis diagram of PCR detection results for *mcr-8* primer specific verification

The primer annealing temperature was optimized, and a temperature gradient of 54-66℃ was set, with a total of 10 gradients (55.0℃, 55.3℃, 55.9℃, 56.8℃, 58.1℃, 59.4℃, 60.6℃, 61.9℃, 63.2℃, 64.1℃, 64.7℃, 65.0℃). PCR amplification was performed according to the parameters of Table 4 and detected by agarose gel electrophoresis. The results are shown in Figure 3.2. The band is clearest when the annealing temperature is 59.4°C, so the optimal annealing temperature is determined to be 59.4°C.

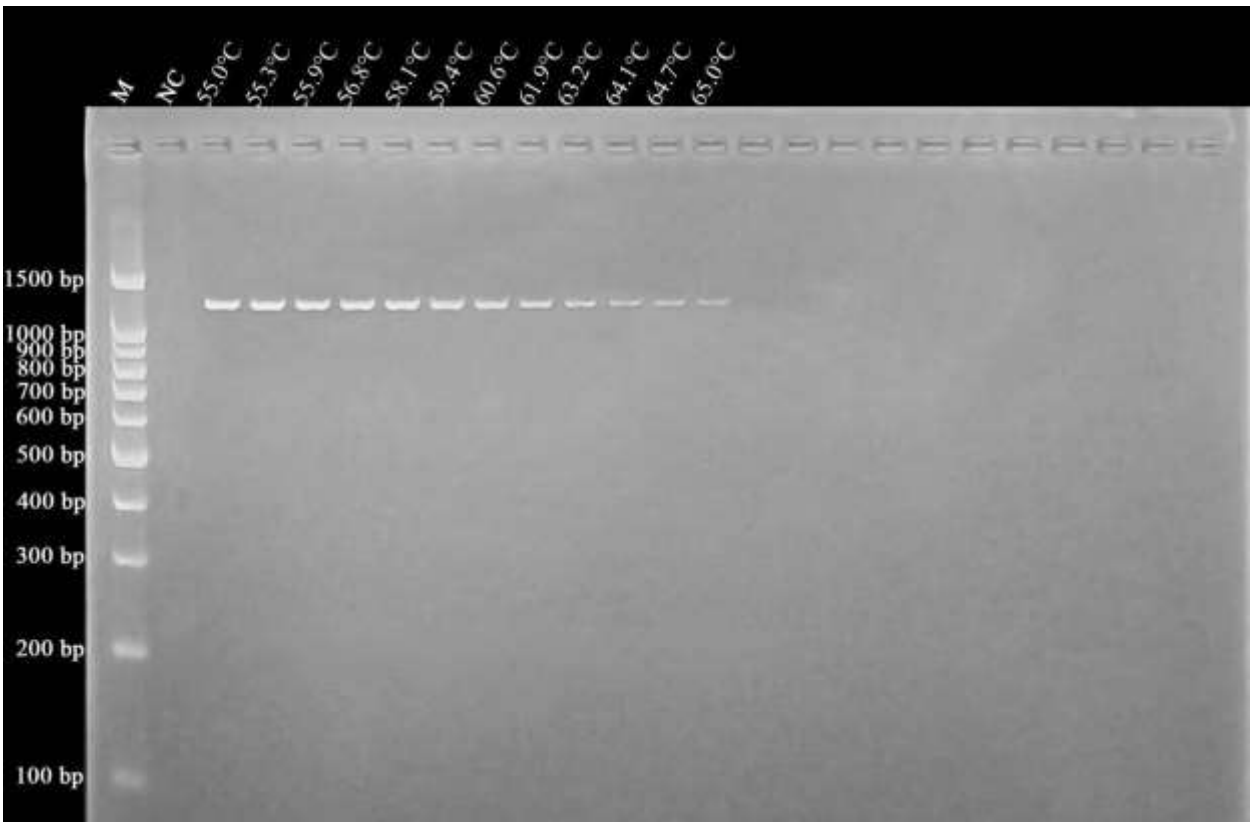

Figure 3.2 Optimized gel electrophoresis diagram of *mcr-8* primer annealing temperature

3.2 Gene sequencing verification of PCR amplification products

The sequencing of positive control PCR products was completed by Shanghai Shenggong Bioengineering Company, and the sequencing results were presented in FASTA format (see Figure 3). Nucleotide sequence alignment was performed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the sequencing sequence was 100% consistent with the *mcr-8* gene sequence, indicating that the PCR method has high specificity.

>refNG_061399.1|:500-1768 Klebsiella pneumoniae KP91 pKP91 mcr-8 gene for phosphoethanolamine--lipid A transferase MCR-8.1, complete CDS

TACCAGCAATTATCCTGGCGTTGCCTTCAACAGACAATAAGCGGGGAGCTTTTAGAATT
GAATTGTGGTGGTTGGCGCATATTTGCATAGCTGTAGTCTTATTAGCCATGGTTACCATG
GTGTTTTATAAGGATTACGCATCTCTCATACGAAACAATATGCAGATTAAAGACCAGGC
TTTACCTTTTAACTTTGTGCGTAATACGAATGGTTACCTTAAAAGAAAATACCAGGCATC
TTCAACAATTCTACAAAGCGTGGGGGAGGATGCTGTACGTCCAATATATTCAAATGCTC
CACCGAAACTGGTGGTTGTCGTCGTGGGCGAAACCGCCAGAGCACAGAATTTCCAGC
TGAATGGCTATTCGCGGGTAACCAACCCCTATCTTTCCAGACGACATGATGTTATCAGTT
TCAAAAATGTGTCGTCATGCGGAACGGCTACCGCAATATCACTACCCTGCATGTTCTCG
CGAATGTCACGTAACGAATACAATGAAGTCCGTGCCGCATCAGAAGAAAACTTGCTGG
ATATCCTTAAACGTACAGGTGTTGAGGTGCTATGGCGCAACAATAACAATGGTGGTTGT
AAGGGAATCTGCAAGCGAGTACCCACAGATGATATGCCGGCAATGAAAGTAATTGGGG
AATGTGTTAACAAAGATGGTACATGCTTTGATGAGGTGTTATTAAATCAACTCTCATCCC
GAATTAATGCAATGCAGGGTGATGCGCTTATTGTTTTACATCAAATGGGCAGTCATGGA
CCAACATATTTTGAACGTTATCCGTCTACAAGTAAAGTCTTTAGCCCAACTTGCGACAG
CAACCTGATCGAAAAATGCTCAAATAAAGAACTGGTCAATACATACGACAATACGCTA
GTTTATACTGATCGTATGCTGAGCAAAACTATTGAACTGTTGCAACGTTATTCCGGGATG
CGTGACGTTGCTATGATATATCTTTCTGATCATGGAGAATCGCTGGGGGAAAGCGGAAT
ATATCTTCATGGCACACCATATATTATTGCCCCCAATGAACAAACACACATCCCGATGTT
TATGTGGTTTTCGTCTTCATTCGCGCAGCATTCCAAATTAAATCTAGAATGCCTGACCGG
TAATGCCGACAAACAATACAGTCATGATAATTTTTATCATTCAATACTTGGTCTCTTCAA
CGTAAAAACCAGTGTATATAAACCGGAGTTAGATATGTTTACTCTATGTCGACAATCTGA
CCACACACCACTGTCTTCCGCAGTT

Figure 3 Sequencing results of positive control PCR products

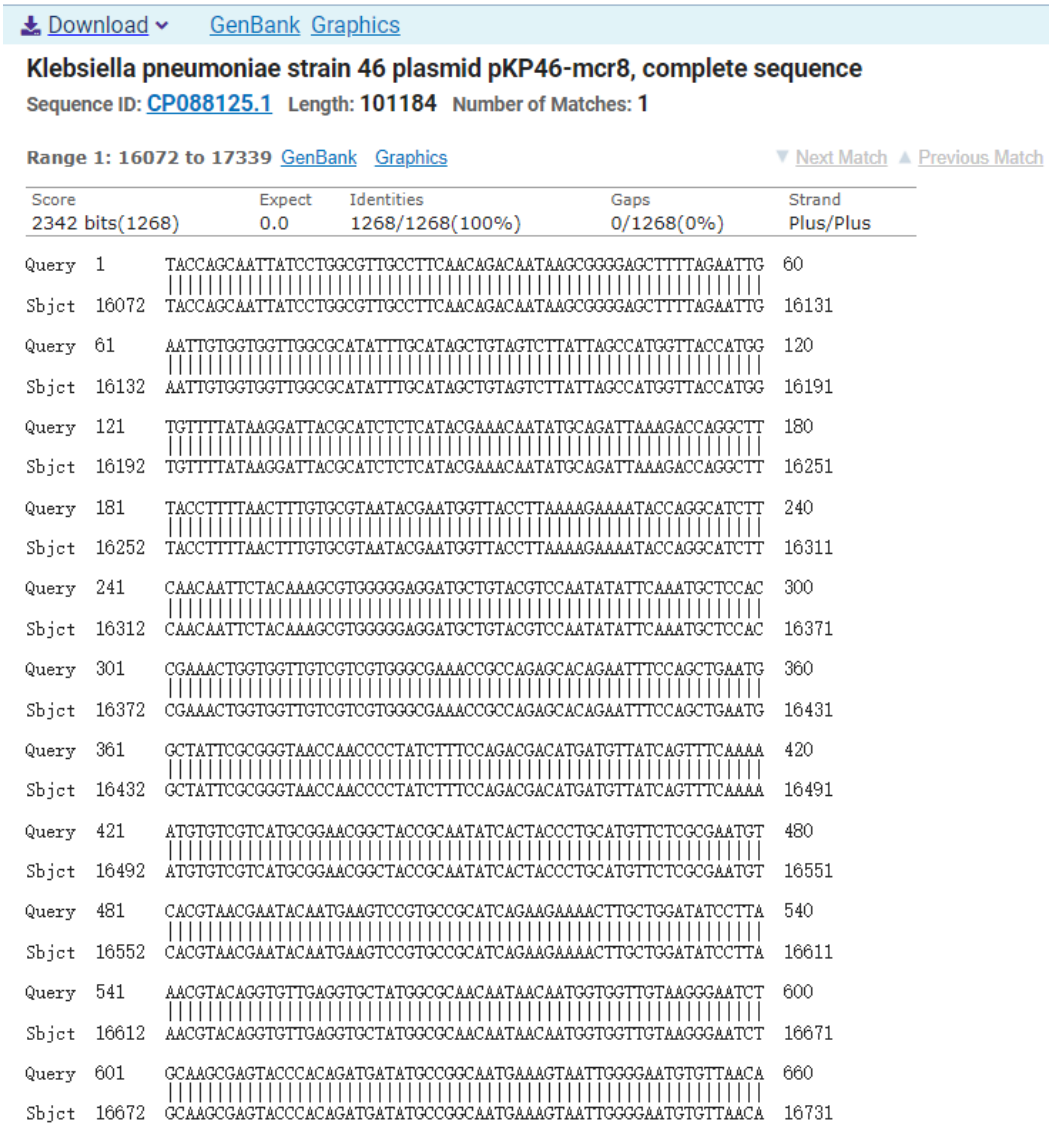

Figure 4 Results of positive product sequence comparison

3.3 Verification of PCR method sensitivity

The diluted plasmid solution carrying the *mcr-8* gene was detected using the now established PCR method. After PCR amplification and gel electrophoresis analysis, the gel imaging system was observed as shown in Figure 5. It was measured that the detection limit of *mcr-8* is less than 10 pg, indicating that the PCR method has high sensitivity and can significantly improve the detection rate and reduce missed diagnosis in the early stage of infection.

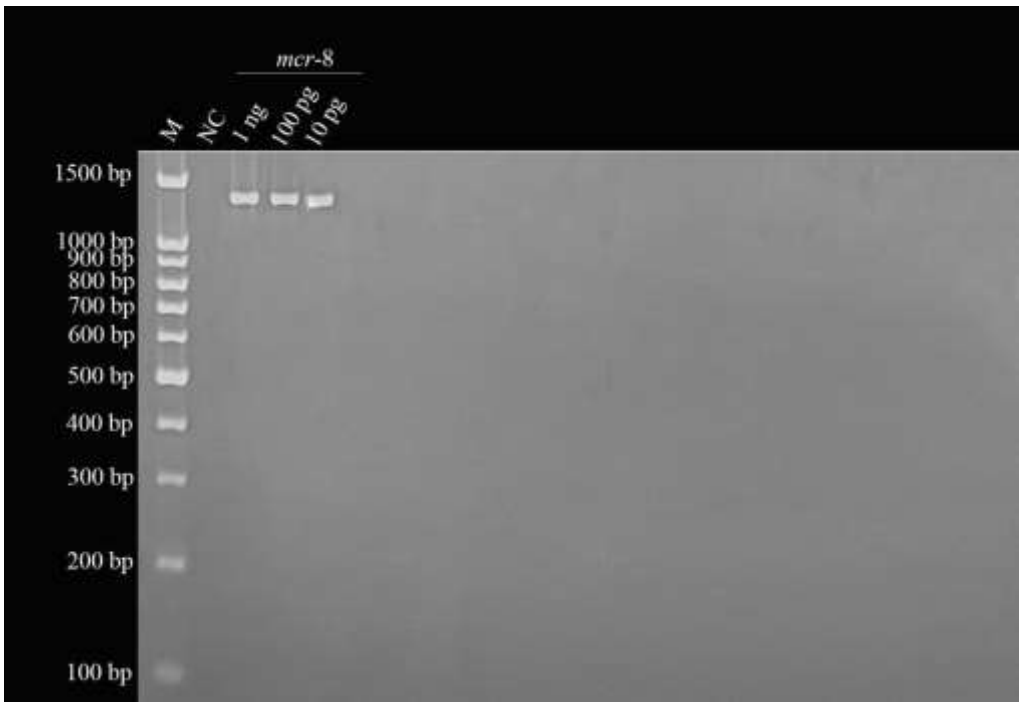

Figure 5 Detection limit results

3.4 Application of PCR method in detection of isolates

PCR was performed on *E. coli* strains (including 10 chicken-derived E100, E106, E118, E49, E63, E64, E65, E72, E85, E95) and *Salmonella* strains (including 5 human clinical samples isolated from *Salmonella* QLUF110, QLUF111, QLUF112, QLUF113, and QLUF114). After 2% agarose gel electrophoresis detection, the gel electrophoresis image was observed using a gel imaging system. As shown in Figure 6, all the amplification products of the tested strains showed no bands, indicating that none of the 15 strains contained the *mcr-8* gene.

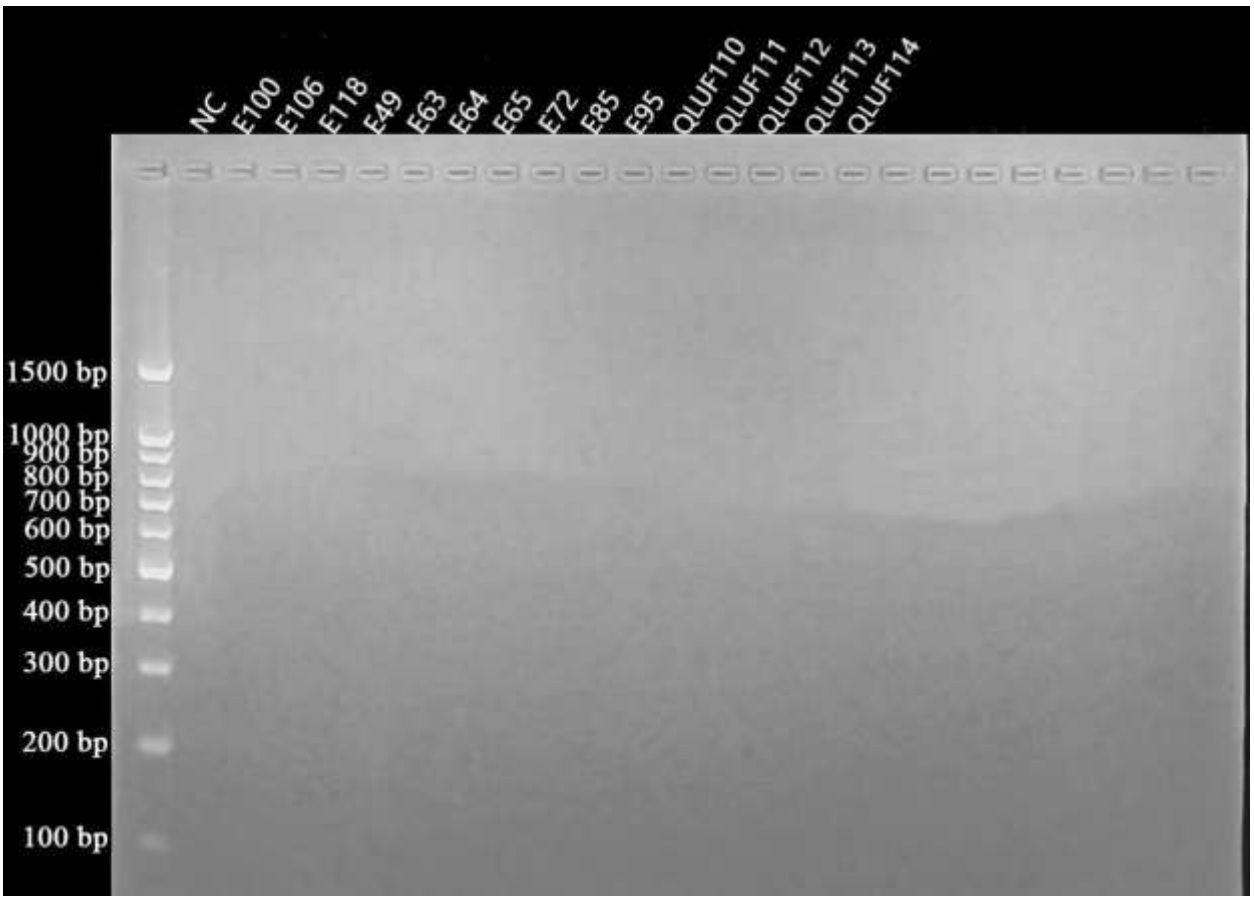

Figure 6 Agarose gel electrophoresis results detected by *E. coli* strain and *Salmonella* strain *mcr-8*

Summary of chapter 3

1. Specific verification: Positive control (*mcr-8* plasmid) amplified the target band of 1268 bp. Negative control (Escherichia coli ATCC 25922) and blank control (ddH_2O) had no bands, indicating no contamination and high specificity.

2. Annealing temperature optimization: Temperature gradient experiment (54-66℃) shows that the amplified band is clearest at 59.4℃ and is determined as the optimal annealing temperature.

3. Gene sequencing verification: After sequencing the positive control PCR product, the sequence of the *mcr-8* gene reached 100% by BLAST comparison, confirming the specificity of the method.

4. Sensitivity verification: Plasmid DNA gradient dilution (10^{-9} to 10^{-11}) showed a lower detection limit of 10 pg.

5. Isolate detection results: PCR amplification of 15 clinical isolates (10 *E. coli* and 5 *Salmonella* strains) had no target bands, indicating that the *mcr-8* gene was not detected in the detection bacterial population.

CONCLUSION

At present, there are many detection methods for other *mcr* genes, but standardized detection solutions for *mcr-8* are lacking. This study is the first time based on ordinary PCR technology, and successfully established a PCR detection method for the drug-resistant gene *mcr-8* in *Salmonella*, and its specificity and sensitivity were verified through experiments. Experimental results show that the designed primers *mcr-8*-F (TACCAGCAATTATCCTGGCGT) and *mcr-8*-R (AACTGCGGAAGACAGTGGT) can specifically amplify target bands that are consistent with the expected size at annealing temperature of 59.4℃. The sequencing comparison confirmed that the amplified product had a 100% identity with the *mcr-8* gene sequence. Through sensitivity verification, it was determined that the detection limit of *mcr-8* genes was less than 10 pg and showed good stability in repeated experiments. The establishment of this method provides technical support for the rapid screening of *mcr-8* genes, and does not require complex instruments and is easy to obtain reagents and consumables. It is suitable for routine testing in ordinary laboratories, and has the advantages of simple operation, low cost and high specificity. The research results show that this method can provide a reference for the early clinical identification of polymyxin-resistant strains, and also provide technical means for monitoring the prevalence trend of *mcr-8* gene in strains.

The experimental samples of this study were synthesized by Shanghai Shenggong Biotechnology Company and the corresponding gene fragments were integrated into the plasmid. PCR inhibitors may exist in the actual sample, which will affect the DNA extraction efficiency and amplification effect. The sample range can be expanded in the future to verify the universality of the method, and optimize the DNA extraction steps for complex samples, reduce inhibitor interference, and further improve detection efficiency and stability. Moreover, the functional mechanism of the *mcr-8* gene is not yet clear. In the future, the specific molecular mechanism of *mcr-8* gene mediated drug resistance can be analyzed

through gene knockout, transcriptomics and ligation transfer experiments, integrated detection data and epidemiological information, and dynamically monitor the spread trend of *mcr-8*, providing a scientific basis for antibiotic use policies and drug resistance prevention and control strategies. It can also be combined with metagenomics, real-time fluorescence quantitative PCR (qPCR) and other technologies to further improve the throughput and accuracy of *mcr-8* gene detection, and provide more comprehensive data support for drug resistance research. At the same time, strengthening international drug resistance monitoring cooperation and establishing a global unified *mcr-8* gene database will help early warning of drug resistance crises and jointly protect antibiotic resources and public health security.

1. At present, the *mcr-8* gene lacks a standardized detection scheme. This study is the first time based on ordinary PCR technology to establish a specific detection method for *mcr-8* gene in *Salmonella*. The designed primer *mcr-8*-F/R specifically amplified the target band at an annealing temperature of 59.4°C, and the PCR product sequencing results were 100% consistent with the *mcr-8* gene. The detection limit is <10 pg at the same time, with good repeatability and high stability. This method is easy to operate, low cost, and does not require complex instruments. It is suitable for routine testing in ordinary laboratories. Provide technical support for early screening of clinical drug-resistant strains and monitoring of *mcr-8* epidemic trends.

2. The experimental sample is a synthetic plasmid, and the interference of PCR inhibitors in the actual sample on DNA extraction and amplification was not considered. The sample range can be expanded to verify universality and optimized DNA extraction steps to reduce the effect of inhibitors.

3. Future research directions can further study the function of *mcr-8* gene and explore its drug-resistant molecular mechanism. Integrate testing data and epidemiological information, dynamically monitor transmission trends, and guide antibiotic use and drug resistance prevention and control policies.

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