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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 a PCR Detection Method for the Anti-colistin Gene *mcr-3***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
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APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
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«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Sun Xiaolin

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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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3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
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SUMMARY

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With the widespread use of antibiotics, bacterial resistance has become a growing concern, particularly the emergence of plasmid-mediated colistin resistance genes, which diminishes the efficacy of colistin, a "last-resort" antibiotic. This study designed and established a specific PCR detection method for the *mcr-3* gene to support rapid screening and resistance monitoring. A pair of primers was designed targeting a unique region of the *mcr-3* gene, located between the middle variable region and the catalytic active domain (1032-1287 bp), yielding a theoretical amplification product of 209 bp. The primers exhibited ideal physicochemical properties, with moderate GC content (55% and 60%) and similar Tm values (approximately 60 ℃). Experimental validation demonstrated high specificity, with only *mcr-3*-positive templates producing a 209 bp band, while negative controls showed no amplification. Sensitivity tests indicated that 1ng, 100 pg, and 10pg of *mcr-3* plasmid DNA could be detected, with band intensity decreasing with lower concentrations. Testing of 16 Salmonella strains (primarily from poultry, with a few from human sources) revealed no *mcr-3* gene, suggesting its rarity in the tested samples. This PCR method is simple, rapid, and suitable for screening *mcr-3* in livestock, human, and environmental samples, providing critical technical support for antimicrobial resistance monitoring and control strategies.

Key words: mcr-3, PCR detection, colistin resistance, Salmonella, antibiotic resistance

TABLE OF CONTENTS

INTRODUCTION

Importance of research question, research methods, research results and structure of the thesis, etc.

In this study, through sequence analysis, primer design and PCR reaction system optimization, we gained an in-depth understanding of the molecular characteristics and detection methods of the colistin resistance gene *mcr-3*, and found that the designed primers showed high specificity for *mcr-3* gene detection with approximately 100% accuracy. Sequence analysis revealed that the *mcr-3* gene shares most conserved regions with other mcr family members, with few specific sequences, showing a high degree of correlation in the middle variable region. In addition, in the analysis of PCR sensitivity testing, it was found that the established detection system can detect as low as 10 pg of *mcr-3* plasmid DNA, containing stable amplification capability, which is closely related to its detection accuracy and clinical applicability. It is worth noting that as a colistin resistance gene, *mcr-3* also exhibits transferability, which will adversely affect our treatment, and these resistance genes may be acquired through plasmid-mediated horizontal gene transfer. An in-depth study of this detection process will help us control the spread of resistant bacteria. Overall, as one of the important resistance mechanisms in bacterial communities, *mcr-3* as a colistin resistance gene may adapt to various clinical environments in multiple ways and play an important role in antimicrobial resistance surveillance.

The relevance of the topic is PCR detection method establishment and molecular analysis.

The purpose of the study is to establish a specific PCR detection method for the colistin resistance gene *mcr-3* provided basic technical support for further understanding and research on the prevalence, environmental distribution, resistance transmission, and infection prevention and control of *mcr-3*.

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understanding and research on the prevalence, environmental distribution, resistance transmission, and infection prevention and control of *mcr-3*.

The object of the study *mcr-3* gene.

The subject of the study *mcr-3* gene.

Research methods PCR detection method establishment.

The scientific novelty PCR detection method establishment.

The practical significance of the results obtained is An in-depth study of this detection process will help us control the spread of resistant bacteria.

Chapter I
LITERATURE REVIEW

1.1 Current Status of Bacterial Resistance

The discovery and application of antibiotics represent a milestone in medical history, significantly reducing mortality from infectious diseases. However, antibiotic misuse, particularly routine use in animal husbandry, has led to the rapid spread of resistant strains, with antimicrobial resistance (AMR) becoming a global public health crisis[1]. According to the World Health Organization (WHO), AMR causes millions of deaths annually and is projected to cause 10 million deaths by 2050[4]. The spread of resistant strains has made infection treatment more difficult, significantly increasing healthcare costs and patient mortality risk.

Certain bacteria exhibit severe resistance to key antibiotics[4]. Among Gram-negative bacteria, carbapenem-resistant Enterobacteriaceae (CRE) are typical examples of multidrug resistance. These strains demonstrate high-level phenotypic resistance to carbapenem antibiotics, with minimum inhibitory concentrations (MICs) typically exceeding 32 μg/mL, whereas sensitive strains have MICs below 2 μg/mL[4]. Genotypic analysis shows that CRE resistance is primarily mediated by β-lactamase genes (such as blaKPC and blaNDM) located on plasmids, which can spread between species like Escherichia coli and Klebsiella pneumoniae[7]. Pseudomonas aeruginosa is also a multidrug-resistant strain, exhibiting extensive resistance to aminoglycosides, quinolones, and β-lactam antibiotics. Phenotypic testing shows its resistance rate to quinolone antibiotics reaches 30%[7]. Genotypic studies have found that mutations in gyrA and parC genes are the primary resistance mechanisms, altering the structure of antibiotic targets[7].

Among Gram-positive bacteria, methicillin-resistant Staphylococcus aureus (MRSA) is resistant to β-lactam antibiotics due to the mecA gene, with MIC values typically exceeding 16 μg/mL[8]. According to the U.S. Centers for Disease Control

and Prevention (CDC), deaths directly attributable to MRSA increased from 57,200 in 1990 to 130,000 in 2021, highlighting its public health threat[9].

In treating multidrug-resistant Gram-negative bacterial infections, polymyxins (colistin) were once considered the last line of defense due to their unique mechanism of action[10]. Polymyxins exert bactericidal effects by binding to lipid A in bacterial outer membrane lipopolysaccharides, disrupting membrane integrity. Due to their different mechanism of action compared to other antibiotics, they were long considered unlikely to induce resistance[10]. However, in 2015, Chinese researchers discovered the plasmid-mediated colistin resistance gene mcr-1 in animal-derived *E. coli*, breaking this understanding. mcr-1 encodes phosphoethanolamine transferase, which modifies lipid A, reducing colistin affinity and conferring resistance to bacteria. Subsequently, genes such as mcr-2 to mcr-10 were discovered, all with similar functions but significant nucleotide sequence differences, indicating diverse origins and complex transmission routes.

The *mcr-3* gene was first identified in 2017 in porcine E. coli in China, with sequences significantly different from mcr-1 and mcr-2, suggesting a potentially independent evolutionary path. *mcr-3* is often located on plasmids such as IncHI2 and IncI2, which have efficient replication and transfer capabilities, allowing transmission between species such as Salmonella and Klebsiella. Additionally, truncated transposons (such as ΔTnAs2) often accompany upstream of *mcr-3*, increasing the risk of recombination and transmission in the environment. Phenotypic testing shows that strains carrying *mcr-3* have MIC values of 8 μg/mL or higher for polymyxins, while sensitive strains typically have values below 2 μg/mL. Genotypic detection relies on PCR technology, but existing methods have limitations in primer coverage and variant recognition ability. Studies have shown that *mcr-3* has been detected in animal, food, environmental, and human samples, suggesting its potential risk of transmission to humans through the food chain and water pollution. The preventive use of polymyxins in farming further exacerbates the spread of resistance genes.

The widespread transmission of *mcr-3* highlights the urgency of developing efficient and precise detection methods. Traditional phenotypic detection methods (such as MIC determination) are time-consuming, produce unstable results, and cannot identify resistance mechanisms, making it difficult to meet rapid screening needs. In comparison, polymerase chain reaction (PCR) technology has become the preferred tool for detecting resistance genes due to its high sensitivity, specificity, and operational simplicity. However, specific PCR methods for *mcr-3* are still imperfect, with existing techniques having limitations in variant coverage and detection efficiency. Therefore, developing a detection method for *mcr-3* that combines specificity and practicality is of great significance for resistance monitoring, epidemic prevention and control, and subsequent research.

1.2 The *mcr-3* Gene and Its Resistance Mechanism

The *mcr-3* gene is another plasmid-mediated colistin resistance gene discovered after mcr-1 and mcr-2. It was first found in porcine E. coli isolated in China in 2017[12]. As a member of the mcr gene family, *mcr-3* encodes phosphoethanolamine transferase, which modifies bacterial lipid A, reducing the cell membrane's affinity for colistin and conferring resistance to bacteria[11].

The *mcr-3* gene is approximately 1626 base pairs long and encodes a protein containing 541 amino acids. Compared to mcr-1 and mcr-2, *mcr-3* has relatively low nucleotide sequence homology, showing a certain independence in the evolutionary process. This difference provides a molecular basis for developing specific detection tools and indicates that it may have originated from different ecological environments.

From a protein structure perspective, the *mcr-3* protein includes two main functional regions: the N-terminal transmembrane domain, formed by five α-helices, which serves to fix the protein position; and the C-terminal catalytic domain, which is responsible for transferring phosphoethanolamine[12]. The catalytic domain of *mcr-3* resembles a phosphoethanolamine transferase in Neisseria in three-dimensional structure, suggesting functional conservation[13]. However, in the

middle part of the protein, *mcr-3* sequences differ significantly from other mcr members, a characteristic that can serve as a key target for typing and detection.

The transmission capability of *mcr-3* is influenced by both its structural characteristics and its carrier. *mcr-3* is often located on transferable plasmids such as IncI2 and IncHI2. These plasmids have efficient self-replication capabilities and can achieve horizontal transfer between many Gram-negative bacteria[14]. Mobile genetic elements are often present around the *mcr-3* gene, particularly truncated TnAs2 elements similar to transposons in aquatic bacteria, suggesting that the gene may spread across species through transposition and recombination, exacerbating its dissemination risk.

The expression level of *mcr-3* is influenced by both host regulatory systems and environmental factors. Environmental stimuli such as changes in pH and differences in metal ion concentrations, along with bacterial regulatory elements like two-component signaling systems, may regulate the expression of *mcr-3*. *mcr-3* often coexists with other resistance genes on the same plasmid, forming composite resistance carriers, further promoting bacteria to exhibit multidrug-resistant phenotypes, which significantly increases the difficulty of resistance control[15].

From a molecular evolution perspective, sequence alignment results show that *mcr-3* has high homology with gene fragments from aquatic bacteria, suggesting it may have originated from microorganisms in the aquatic environment[16]. This provides new insights for tracing the ecological origin and developmental trajectory of *mcr-3* and further demonstrates the potential impact of environmental factors in the transmission of resistance.

1.3 Current Prevalence of the *mcr-3* Gene

Since its first report in 2017, the distribution range of the *mcr-3* gene has been continuously expanding, and it has now been detected in various bacteria from animals, environment, and humans in many countries and regions worldwide[17].

The ongoing spread of this gene both intensifies concerns about the clinical efficacy of colistin and demonstrates the risk of cross-species transmission.

The *mcr-3* gene has been detected with relatively high frequency in Asia, especially in China. This gene is predominantly found in E. coli and Salmonella from farmed animals, with samples from pigs and poultry being the most common[18]. *mcr-3*-carrying strains have also been continuously found in environmental samples such as wastewater, farm discharge water, and soil, indicating that it has penetrated from farming systems into the natural environment, creating a complex transmission network[19].

In European countries with developed animal husbandry, such as Germany and the Netherlands, there have been reports of *mcr-3*-positive strains, mostly from the intestines of livestock and poultry, meat products, and their surrounding environments. Some studies have detected *mcr-3* in wastewater treatment systems, suggesting that this gene may persist in urban sewage systems and further spread through sewer networks[20]. The overall detection rate in North America is not high, but there have been reports of individual human infection cases with *mcr-3*, which often coexists with other resistance genes such as β-lactamase genes and aminoglycoside resistance genes, forming multidrug-resistant phenotypes and further complicating treatment[20]. Research on Africa and South America is limited, but there have been reports of detecting the *mcr-3* gene in local food samples, water bodies, and bacteria from animals, reflecting its global transmission capability[20].

The distribution of *mcr-3* is closely linked to aquatic environments. This gene has high homology with gene fragments from some aquatic bacteria, suggesting it may have originated from natural bacterial communities in aquatic ecosystems. In rivers, lakes, aquaculture areas, and similar locations, the detection rate of *mcr-3*-positive strains is often higher than in soil or air samples, further confirming the "reservoir" function of water bodies in its transmission[12]. In terms of transmission modes, *mcr-3* can achieve horizontal gene transfer through transferable plasmids and often coexists with mobile genetic elements such as

integrons and transposons, forming complex genetic structures. *mcr-3* can be incorporated into multidrug-resistant plasmids, coexisting with other mcr genes, ESBLs, and fluoroquinolone resistance genes, forming a multi-gene resistance platform. This "integrated" resistance structure significantly enhances its ability to spread between different bacteria.

Despite some countries implementing restrictive policies, the detection rate of *mcr-3* has not significantly decreased under the continuous use of polymyxins in animal husbandry. This indicates that once this gene integrates into the microbial community, it may persist for a long time and is difficult to eliminate in the short term. In hospital infection cases, *mcr-3*-positive strains are mostly identified from urine, blood, and other samples, often associated with serious conditions such as sepsis and urinary tract infections, posing practical obstacles to clinical treatment.

1.4 Current Status of mcr Gene Detection Methods

1.4.1 Phenotypic Detection Methods

In conventional detection of polymyxin resistance, phenotypic methods remain the most essential and direct means. These methods indirectly determine resistance ability by evaluating bacterial growth under specific antibiotic concentrations. Currently, the broth microdilution method has a relatively high degree of standardization and stable results. However, it still has prominent limitations in practical application, such as lengthy operation time, equipment requirements, and relatively high costs, which are not conducive to high-throughput screening and emergency monitoring[22].

Besides the broth dilution method, agar dilution and disk diffusion methods are also commonly used for preliminary resistance determination. The disk diffusion method is frequently used for initial screening due to its simple operation. However, due to the uneven diffusion of polymyxins in agar, its detection sensitivity is relatively low, making it prone to false-negative results, so it has been recommended for cautious use by many standard guidelines. In recent years, some rapid phenotypic detection methods such as NP detection and the modified Hodge

test have emerged, attempting to achieve rapid identification based on changes in bacterial metabolism or inhibition zone characteristics. However, in polymyxin resistance detection, they still have issues such as low specificity and subjective interpretation, with poor applicability in environmental samples and complex bacterial communities[23].

Phenotypic detection remains an important part of polymyxin resistance research, but in mcr gene screening, its role is gradually being replaced by molecular detection techniques. More often, phenotypic detection is suitable for auxiliary verification and sensitivity confirmation[24].

1.4.2 Molecular Detection Methods

Molecular detection technologies have become the primary means of identifying and monitoring mcr genes due to their high sensitivity, rapidity, and specificity. Polymerase chain reaction (PCR) is widely used in laboratory detection because of its simple operation and intuitive results. For example, conventional PCR primers for mcr-1 and mcr-2 developed by Rebelo et al. can accurately detect target genes in clinical, food safety, and animal-derived samples[25]. Real-time quantitative PCR (qPCR) further improves efficiency by real-time monitoring of amplification progress through fluorescent signals, supporting qualitative and quantitative analysis, particularly suitable for complex samples such as wastewater and livestock feces. For instance, Xavier et al. successfully quantified the expression level of mcr-2 in environmental samples using qPCR, although it has higher requirements for instruments and operation[26]. Multiplex PCR detects multiple mcr genes in a single reaction, significantly improving the efficiency of large-scale screening. The multiplex PCR system for mcr-6 to mcr-9 established by Borowiak et al. is an example, though its primer design and system optimization are relatively complex[27]. Additionally, whole-genome sequencing (WGS) serves as a comprehensive genetic analysis tool that not only identifies known mcr genes but can also discover new variants and their genetic environments. For example, Roer et al. analyzed the distribution of *mcr-3* variants in bloodstream infections through

WGS[28]. However, its high cost and complex data analysis limit its routine application. Metagenomic sequencing can analyze resistance genes in environmental samples without cultivation. Quesada et al. used this technology to detect mcr-1 in poultry and livestock samples, though challenges exist in sequencing depth and data interpretation[29]. In recent years, the CRISPR-Cas system has gained prominence due to its high sensitivity and rapidity. The mcr-1-targeted CRISPR detection system designed by Shen et al. can complete gene identification in a short time[30]. Although molecular detection methods for *mcr-3* are still being perfected, particularly with limitations in variant coverage and adaptation to complex samples, the continuous optimization of these technologies provides important support for research and application of resistance genes.

1.5 Research Purpose and Significance

With the misuse of antibiotics, bacterial resistance to antibiotics has become increasingly severe, making colistin one of the last resorts for treating multidrug-resistant Gram-negative bacteria. However, the emergence of mcr genes allows bacteria to acquire resistance to colistin. Among these, the *mcr-3* gene has been frequently detected in samples from animals, humans, and the environment, continuously spreading globally. Although PCR detection methods for *mcr-3* already exist, they have issues such as insufficient specificity and incomplete variant coverage. Therefore, a stable, reliable, and simple molecular detection method for the *mcr-3* gene is of significant importance.

This study designs specific primers based on the conserved sequences of the *mcr-3* gene to establish a highly stable and accurate PCR detection method. Through primer screening, reaction condition optimization, and amplification effect verification, a technical system suitable for routine laboratory screening has been developed. The established PCR method can be applied to screening livestock, food, and environmental samples, contributing to global monitoring of *mcr-3* and laying a foundation for risk assessment and control strategy development for the *mcr-3* gene.

Summary of the chapter I

1.This chapter provides a comprehensive introduction to bacterial antibiotic resistance, with particular focus on the emergence of plasmid-mediated colistin resistance genes that threaten the effectiveness of "last-resort" antibiotics like colistin.

2.The *mcr-3* gene, first discovered in Chinese porcine E. coli in 2017, encodes phosphoethanolamine transferase that modifies bacterial lipid A, conferring colistin resistance with MIC values ≥8 μg/mL compared to <2 μg/mL in sensitive strains.

3.Current detection methods include phenotypic approaches (broth microdilution, disk diffusion) which are time-consuming and equipment-intensive, and molecular methods (PCR, qPCR, WGS) which offer higher sensitivity and specificity but have limitations in variant coverage.

4.The research aims to establish a stable, reliable PCR detection method for *mcr-3* gene screening in livestock, food, and environmental samples to support global resistance monitoring and control strategy development.

Chapter II

OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Materials

2.1.1 Strains and Samples

The strains used in this study included positive control strains, negative control strains, and environmental sample isolates. The positive control was a laboratory-preserved, artificially synthesized *mcr-3* plasmid (carrying the *mcr-3*.1 variant); negative control strains included Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603, and Salmonella enterica ATCC 14028, all confirmed by sequencing not to carry mcr series resistance genes.

To simulate actual sample detection scenarios, some naturally sourced strains were collected, with Salmonella strains mainly isolated from chicken sources and a few from human infection cases. All strains were stored short-term in a 4°C refrigerator and resuscitated in LB medium at 37°C before use.

2.1.2 Main Reagents

Tryptone, yeast extract, sodium chloride (culture medium components); 2× Taq Master Mix (Novizan Company, Suzhou, China); nucleic acid dye; agarose and 50× TAE buffer (for electrophoresis); DL2000 DNA Marker; FastPure Gel DNA Extraction Mini Kit (Vazyme, China); EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, China); FastPure Plasmid Mini Kit (Vazyme, China), etc.

2.1.3 Main Equipment

Table 2-1 Main Equipment

Equipment Name	Model	Company
PCR Amplifier	Mycycler	Bio-Rad, USA
Nucleic Acid Protein Analyzer	NanoDrop2000	ThermoFisher, USA
Constant Temperature Incubator	DHP-9082	Shanghai Yiheng Technology Co., Ltd.

Clean Bench	SW-CJ-2F	Suzhou Purification Group, China
Electronic Analytical Balance	SL601	Shanghai Minqiao Electronic Instrument Factory
Gel Imaging System	2020D	Beijing New Technology Company
Autoclave	Agaro-PowerTMe	Beijing Liuyi Biotechnology Co., Ltd.
Autoclave	LDZX-50KBS	Shanghai Shenan Medical Technology Co., Ltd.

2.2 Methods

2.2.1 *mcr-3* Gene Sequence Analysis and Primer Design

To establish a specific PCR detection method for the *mcr-3* gene, this study downloaded the *mcr-3* reference sequence (GenBank accession number: NG_055505.1) from the NCBI database (https://www.ncbi.nlm.nih.gov) and collected various reported *mcr-3* variant sequences for conserved region analysis for primer design.

Through multiple sequence alignment, it was found that the *mcr-3* gene has relatively high conservation in the middle region of the coding area, while also showing significant differences from other mcr family genes (such as mcr-1, mcr-2, mcr-4, etc.). Therefore, this region was selected as the target for primer design.

Primer design was completed using the NCBI Primer-BLAST tool. Design parameters were set as follows: primer length 18-22 bp, GC content 50%-60%, Tm values 58-62°C, theoretical amplification fragment length controlled between 200-300 bp, avoiding obvious hairpin structures or dimers.

The final determined primer sequences are shown in Table 2-2, corresponding to an amplification product size of 209 bp:

Table 2-2 *mcr-3* Primer Sequences and Amplification Information

Primer Name	**Primer Sequence**（5′→3′）	**Product Length**（bp）	**Tm Value**（℃）	**GC Content**（%）
*mcr-3*_209F	CATGCTATGACGAGGTTGTCCTT	209	60.1	52.2
*mcr-3*_209R	GTTGTCATAGGTGTTGGTGAGC		59.8	47.6

The designed primers were verified by Primer-BLAST, showing complete match with the *mcr-3* reference sequence and no non-specific binding to other mcr family members or common bacteria, demonstrating good specificity and compatibility. The primers were synthesized by a commercial company, purified by HPLC, and prepared as 100 μM stock solutions, stored at -20°C for later use.

2.2.2 Strain Activation and DNA Extraction

All strains required activation before use. Frozen strains were removed from glycerol tubes, streak-inoculated onto LB agar plates, and cultured at 37°C for 12-16 hours until individual colonies formed. For environmental samples such as water and fecal samples, they were similarly inoculated onto LB agar plates for cultivation and subsequent detection.

Bacterial genomic DNA extraction was performed using the EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, China). The specific steps were as follows: 1-2 single colonies were inoculated into 5 mL LB liquid medium, cultured overnight at 37°C and 200 rpm on a shaker, then 1 mL of bacterial culture was taken for DNA extraction according to the kit instructions. After DNA extraction, its concentration and purity were detected using a NanoDrop 2000 (Thermo Fisher Scientific, USA) nucleic acid analyzer, and the DNA solution was diluted to a concentration suitable for PCR amplification and stored at -20°C for later use.

For positive control plasmid DNA, extraction was performed using the FastPure Plasmid Mini Kit (Vazyme, China) according to instructions, followed by concentration determination and dilution for storage.

2.2.3 Establishment of PCR Reaction System

To establish a PCR reaction system suitable for *mcr-3* gene detection, the following reaction system with a total volume of 25 μL was used: 12.5 μL of 2× Taq Master Mix, 0.5 μL of upstream primer (*mcr-3*_209F, 10 μM), 0.5 μL of downstream primer (*mcr-3*_209R, 10 μM), 1 μL of template DNA, and nuclease-free water to make up to 25 μL.

The amplification program was set as follows: pre-denaturation at 95°C for 10 min, followed by 35 cycles, each cycle including denaturation at 95°C for 15 s, annealing for 60 s, extension at 72°C for 60 s, and a final extension at 72°C for 5 min.

For annealing temperature, a gradient of 55-65°C was set, with 12 gradients (55.0°C, 55.3°C, 55.9°C, 56.8°C, 58.1°C, 59.4°C, 60.6°C, 61.9°C, 63.2°C, 64.1°C, 64.7°C, 65.0°C) for PCR amplification and gel electrophoresis detection.

2.2.4 Detection and Specificity Verification of PCR Products

After PCR amplification, 5 μL of amplification product was thoroughly mixed with 1 μL of 6× DNA Loading Buffer and separated by electrophoresis in a 3% agarose gel. The buffer used for electrophoresis was 1× TAE, with the electrophoresis voltage set at 120 V for approximately 90 min. After electrophoresis, the gel was observed and recorded under a UV gel imaging system. To facilitate the determination of amplification product size, DL2000 DNA Marker was added as a molecular weight standard in each electrophoresis run.

To verify the specificity of the PCR reaction system, artificially synthesized *mcr-3* plasmid DNA (positive control) and Escherichia coli ATCC 25922 genomic DNA (negative control) were used as templates for amplification.

2.2.5 PCR Sensitivity Verification

To evaluate the sensitivity of the PCR system, amplification was performed using different concentrations of *mcr-3* plasmid DNA templates (1 ng, 100 pg, 10

pg), with all reaction components and amplification conditions remaining consistent.

2.2.6 Sample Testing

To detect the presence of the *mcr-3* gene in environmental source strains, this study conducted PCR detection on 16 Salmonella strains (QLULN1, QLUY5, QLUY930, QLUY102, QLUY105, QLULD4, QLUY408, QLUY412, QLUY419, QLULM7, QLUY703, QLULL1, QLULR4, QLUY404, QLUY201, QLUY203). These strains were mainly isolated from chicken sources, with a few from human infection cases.

The genomic DNA of each strain was extracted according to the method described in section 2.2.2 and used as a template in the PCR reaction system established in section 2.2.3. The amplification products were detected by 3% agarose gel electrophoresis as described in section 2.2.4, with DL2000 DNA Marker as the molecular weight standard, and observed and recorded under a UV gel imaging system.

Summary of chapter II

1.Materials included positive control (synthesized *mcr-3* plasmid), negative controls (E. coli ATCC 25922, K. pneumoniae ATCC 700603, S. enterica ATCC 14028), and 16 Salmonella environmental isolates primarily from chicken sources.

2.Primer design was based on sequence analysis of the *mcr-3* gene using NCBI database, targeting the conserved region between 1032-1287 bp to generate a 209 bp amplification product with primers having optimal GC content (52.2% and 47.6%) and Tm values (~60°C).

3.The established PCR system used 25 μL total volume containing 2× Taq Master Mix, specific primers, template DNA, with optimized amplification conditions including pre-denaturation at 95° C for 10 min, 35 cycles of 95° C/15s, annealing/60s, 72° C/60s, and final extension at 72° C for 5 min.

4.Methods included annealing temperature optimization (55-65°C gradient), specificity verification using positive/negative controls, sensitivity testing with serial dilutions (1 ng to 10 pg), and sample screening of environmental Salmonella strains.

Chapter III
EXPERIMENTAL PART

3.1 Effect of Annealing Temperature on PCR Results

Annealing temperature tests were conducted at 55.0 °C, 55.3 °C, 55.9°C, 56.8°C, 58.1°C, 59.4°C, 60.6°C, 61.9°C, 63.2°C, 64.1°C, 64.7°C, and 65.0°C. The band was most distinct at an annealing temperature of 60.6°C, thus the optimal annealing temperature was determined to be 60.6°C.

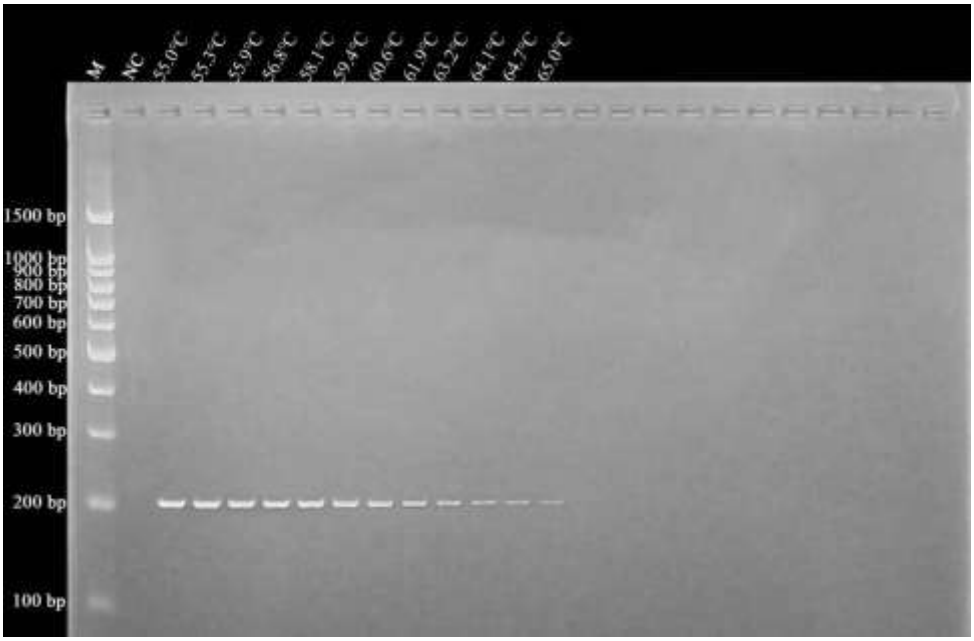

Figure 3.1 Agarose gel electrophoresis image for annealing temperature optimization

3.2 Primer Specificity Verification

To verify the specificity of the *mcr-3* primers (*mcr-3*_209F and *mcr-3*_209R), artificially synthesized *mcr-3* plasmid DNA (positive control) and Escherichia coli ATCC 25922 genomic DNA (negative control) were used as templates for amplification according to the PCR reaction system described in section 2.2.3, and the amplification products were detected by 3% agarose gel electrophoresis as described in section 2.2.4. The electrophoresis results are shown in Figure 3-2,

with the positive control group showing a clear band at 209 bp and the negative control group showing no bands.

Lane 1: DL1500 DNA Marker; Lane 2: *mcr-3* plasmid DNA (positive control, 209 bp band); Lane 3: E. coli ATCC 25922 DNA (negative control, no band).

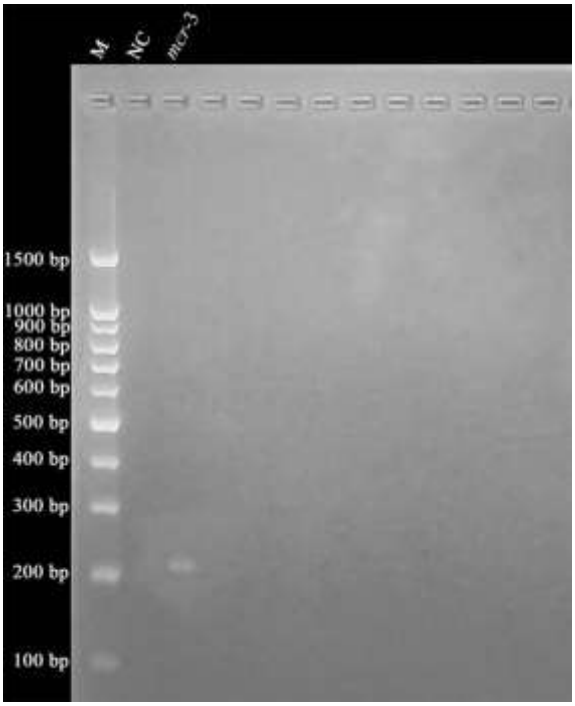

Figure 3.2 Agarose gel electrophoresis results for *mcr-3* primer specificity verification

3.3 Sensitivity Analysis of PCR Reaction System

To assess the sensitivity of the PCR reaction system, different concentrations of *mcr-3* plasmid DNA (1 ng, 100 pg, 10 pg) were used as templates for amplification according to the PCR reaction system described in section 2.2.3, and the amplification products were detected by 3% agarose gel electrophoresis as described in section 2.2.4. The electrophoresis results are shown in Figure 3-3, with 1 ng, 100 pg, and 10 pg templates all producing bands at 209 bp, with band intensity decreasing sequentially with decreasing template concentration.

Lane 1: DL1500 DNA Marker; Lane 2: 1 ng *mcr-3* plasmid DNA; Lane 3: 100 pg *mcr-3* plasmid DNA; Lane 4: 10 pg *mcr-3* plasmid DNA.

Figure 3.3 Agarose gel electrophoresis results for sensitivity analysis of the *mcr-3* PCR reaction system

3.4 Detection Results of *mcr-3* in Isolated Strains

To detect the presence of the *mcr-3* gene in environmental source strains, PCR detection was performed on 16 Salmonella strains (QLULN1, QLUY5, QLUY930, QLUY102, QLUY105, QLULD4, QLUY408, QLUY412, QLUY419, QLULM7, QLUY703, QLULL1, QLULR4, QLUY404, QLUY201, QLUY203). These strains were mainly isolated from chicken sources, with a few from human infection cases. The genomic DNA of each strain was extracted according to section 2.2.2, amplified using the PCR reaction system from section 2.2.3, and the amplification products were detected by 3% agarose gel electrophoresis as described in section 2.2.4. The electrophoresis results are shown in Figure 3-4, with none of the 16 Salmonella strains showing a 209 bp band.

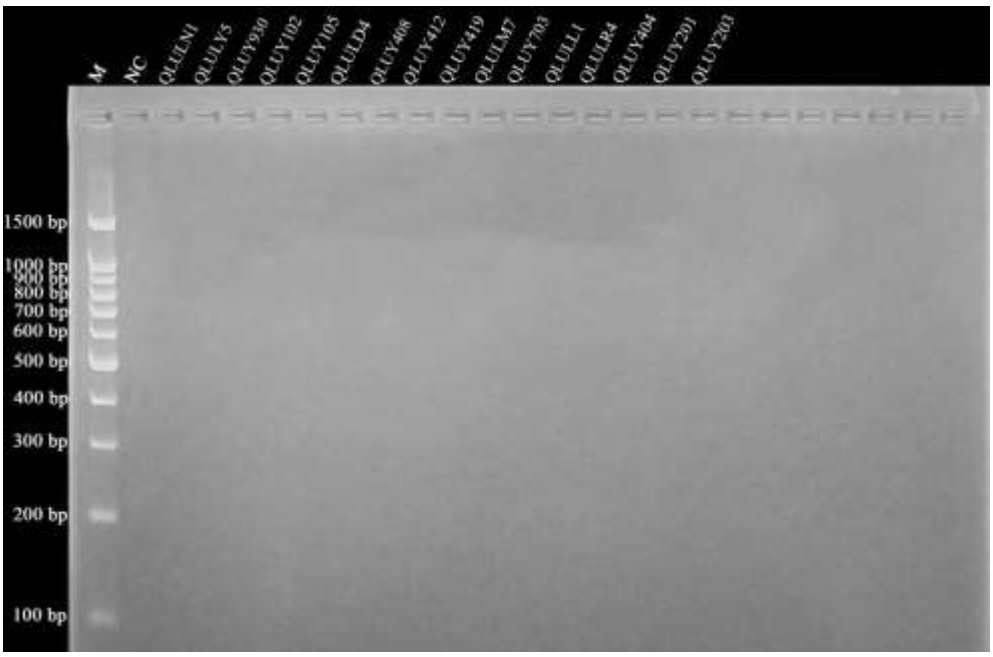

Figure 3.4 Agarose gel electrophoresis results for *mcr-3* detection in Salmonella strains

Lane 1: DL1500 DNA Marker; Lane 2: *mcr-3* plasmid DNA (positive control, 209 bp band); Lanes 3-18: Salmonella strains QLULN1, QLUY5, QLUY930, QLUY102, QLUY105, QLULD4, QLUY408, QLUY412, QLUY419, QLULM7, QLUY703, QLULL1, QLULR4, QLUY404, QLUY201, QLUY203 (all with no bands).

Summary of chapter III

1.Annealing temperature optimization revealed that 60.6°C produced the most distinct amplification bands among the 12 temperature gradients tested (55.0-65.0°C), establishing this as the optimal reaction condition.

2.Specificity verification demonstrated that only *mcr-3*-positive plasmid DNA produced the expected 209 bp band, while negative control (E. coli ATCC 25922) showed no amplification, confirming high specificity of the designed primers.

3.Sensitivity analysis showed successful detection at concentrations of 1 ng, 100 pg, and 10 pg of *mcr-3* plasmid DNA, with band intensity decreasing proportionally with template concentration, indicating good sensitivity for low-concentration samples.

4.Sample testing of 16 Salmonella strains (mainly chicken-derived) revealed no *mcr-3*-positive isolates, suggesting the gene may be uncommon in the tested sample population, though the PCR method proved reliable for screening purposes.

CONCLUSION

This study designed and established a specific PCR detection method for the colistin resistance gene *mcr-3*, providing technical support for rapid screening of resistant strains. By analyzing the sequence characteristics of *mcr-3* through the NCBI database, specific primers were designed, a stable PCR reaction system was constructed, and its specificity and sensitivity were verified. The experiment used artificially synthesized *mcr-3* plasmid as a positive control and Escherichia coli ATCC 25922 as a negative control, and tested 16 Salmonella strains primarily from chicken sources. This method is simple to operate, has a short detection cycle, and can be used for *mcr-3* screening in livestock and human samples, contributing to global antimicrobial resistance monitoring and the development of control strategies, providing technical guarantees for public health safety.

Sequence analysis showed that the *mcr-3* gene has a highly specific fragment in the 1032-1287 bp region, with less than 40% homology to other family members such as mcr-1 and mcr-2, laying a molecular foundation for primer design. Based on this, the designed primer pair (forward: 5'-CATGCTATGACGAGGTTGTCCTT-3', reverse: 5'-GTTGTCATAGGTGTTGGTGAGC-3') can amplify a 209 bp fragment, theoretically covering variants from *mcr-3*.1 to *mcr-3*.30, with bioinformatic verification confirming no cross-reaction with non-target genes.

The PCR reaction system used a total volume of 25 μL, including 12.5 μL of 2× Taq Master Mix, 0.075 μL of upstream primer (*mcr-3*_209F, 10 μM), 0.075 μL of downstream primer (*mcr-3*_209R, 10 μM), 1 μL of template DNA, and nuclease-free water to make up the rest. The amplification program was 95°C pre-denaturation for 10 min, 35 cycles (95°C for 15 s, 62°C for 60 s, 72°C for 60 s), and 72°C extension for 5 min. The amplification products were analyzed by 3% agarose gel electrophoresis, with band sizes consistent with expectations.

Experimental validation showed that the method has good specificity and sensitivity. In the specificity test, only *mcr-3*-positive plasmid DNA produced a

clear band at 209 bp, while the negative control (Escherichia coli ATCC 25922) showed no amplification product. Sensitivity analysis showed that 1 ng, 100 pg, and 10 pg of *mcr-3* plasmid DNA templates all produced 209 bp bands, with band intensity decreasing sequentially with decreasing concentration, demonstrating suitability for low-concentration template detection. Sequencing results showed high match with the *mcr-3* gene sequence, further verifying the accuracy and specificity of the PCR method. Additionally, PCR detection for the *mcr-3* gene was performed on laboratory-isolated strains, but no *mcr-3* gene-positive strains were found.

In summary, a PCR method for detecting the *mcr-3* gene has been established. This PCR method is simple to operate, has a short detection cycle, and provides a reliable technical means for laboratory screening of the *mcr-3* gene. It can be applied to screening livestock samples, food samples, and environmental samples, contributing to global monitoring of *mcr-3* and laying a foundation for risk assessment and control strategy development for the *mcr-3* gene.

Summary of Conclusion

1.The study successfully established a specific PCR detection method for *mcr-3* gene with designed primers targeting the highly specific 1032-1287 bp region, showing <40% homology to other mcr family members and theoretically covering variants *mcr-3*.1 to *mcr-3*.30.

2.The optimized PCR system demonstrated excellent performance characteristics including high specificity (only *mcr-3*-positive samples produced 209 bp bands), good sensitivity (detection limit of 10 pg template DNA), and reliable reproducibility confirmed by sequencing verification.

3.Although no *mcr-3*-positive strains were detected among the tested Salmonella isolates, the method provides a simple, rapid, and reliable technical approach for *mcr-3* screening in livestock, food, and environmental samples.

4.This PCR method contributes to global antimicrobial resistance monitoring by providing technical support for rapid screening of resistant strains, risk assessment, and development of control strategies for public health safety

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