Isolation of Mobile Colistin-Resistance (*mcr*) Gene-Harboring Gram-Negative Bacteria from Chicken Droppings in Dhaka

By

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A thesis submitted to the Department of Biotechnology in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

Department of Biotechnology

School of Life Sciences

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Declaration

It is hereby declared that.

1. The thesis "Isolation of Mobile Colistin-Resistance (*mcr*) Gene-Harboring Gram-Negative Bacteria from Chicken Droppings in Dhaka" submitted is my original work while completing my degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

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Approval

The thesis titled "Isolation of Mobile colistin-Resistance (*mcr*) Gene-Harboring Gram Negative Bacteria from chicken droppings in Dhaka" submitted by Nayara Noor E Fatima, Student ID: 24176008, of Spring 2024, has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology.

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Ethics Statement

The study was conducted in accordance with all established rules for protecting animal welfare.

No animals were harmed in this study. All sources used in this paper are properly cited.

Dedication

This thesis is dedicated to my husband, Md. Mahmudul Hasan, without whom nothing would have been possible.

Abstract

The use of antibiotics in poultry industries across low and middle-income countries is increasing rapidly, contributing to the emergence of antibiotic resistance and leading to a public health threat. This study aimed to investigate the prevalence of Mobile Colistin Resistance (*mcr*) genes in *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* isolated from the fecal samples of broiler chickens in Dhaka city. A total of 100 fresh poultry droppings were collected from 8 different raw markets in Dhaka. The samples were processed and streaked onto HiCrome UTI Agar to presumptively isolate *E. coli* and *K. pneumoniae* and Leeds *Acinetobacter* Agar for *A. baumannii.* The isolates were further confirmed by polymerase chain reaction (PCR). Detection of *mcr* genes was performed by employing two sets of multiplex PCR targeting *mcr-1* to *mcr-5,* and *mcr-6* to *mcr-10*. A total of 84 *E. coli* and 14 *K. pneumoniae* isolates were confirmed by PCR. The *mcr-3* gene was the most abundant, being present in 83.7% of isolates, among which 74 were from *E. coli* and 8 from *K. pneumoniae*. The *mcr-8* gene was the second most prevalent, being present in 40 (40.8%) isolates, among which 39 were from *E. coli*, and 1 from *K. pneumoniae*. None of the isolates tested positive for *mcr-2, mcr-4, mcr-5* or *mcr-10.* Co-occurrence of two or more *mcr* gene variants occurred in 41.8% of *E. coli* isolates. Phenotypic colistin resistance was conducted on 15 *E. coli* isolates using the broth microdilution method to measure the minimum inhibitory concentration, and 14 isolates showed resistance to colistin. Discrepancies were observed between phenotypic and genotypic resistance to colistin. To our knowledge, this study marks the first report of *mcr*-3 in Bangladeshi poultry, and the first report of *mcr-6, mcr-8*, and *mcr*-9 in Bangladesh overall. The high prevalence of various *mcr* genes, notably *mcr*-3 and *mcr*-8, indicated industrial poultry as a potential source of multidrug-resistant pathogens.

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Table of contents

Contents

List of Tables

List of Figures

List of Acronyms

Abbreviation	Full Form
MIC	Minimum Inhibitory Concentration
mcr	Mobilized Colistin Resistance
ESBL	Extended-Spectrum Beta-Lactamase
PCR	Polymerase Chain Reaction
CFU	Colony-Forming Unit
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
bp	Base Pair
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
LB	Luria Bertani (Broth)
MHB	Mueller Hinton Broth
UTI	Urinary Tract Infection
TBE	Tris-Borate-EDTA
CAMHB	Cation-Adjusted Muller Hinton Broth
MDR	Multi-Drug Resistant
HGT	Horizontal Gene Transfer

Chapter 1

Introduction

1.1. Colistin: The Last Resort Antibiotic

Antibiotic resistance, a major global health problem often referred to as the silent pandemic, is caused by a plethora of heavily debated factors, with one of them being the overuse of antibiotics in food (Marshall & Levy, 2011). Exposure to antibiotics, often in the form of food, is considered by many to be a major driving force behind the increase in antibiotic resistance (Cantón & Morosini, 2011).

Colistin, or Polymyxin E, belongs to the polymyxin class of antibiotics, which includes Polymyxin A, B, C, D, and E (Kempf et al., 2016). Although historically primarily used as veterinary medicine, there has recently been an increase in its use in human medicine as a last-resort antibiotic to treat bacterial infections from highly resistant infectious agents (Marshall & Levy, 2011). A non-ribosomal cyclic cationic peptide, colistin, alongside Polymyxin B, is frequently used against Gram-Negative bacteria, particularly *Enterobacteriaceae*, and other ESKAPE pathogens. They are the only two Polymyxins used in clinical medicine (Mondal et al., 2024). First discovered in 1947 from the Gram-Positive bacterium *Paenibacillus polymyxa* subsp. *colistinus*, colistin use spread rapidly in the 1950s (Poirel et al., 2017). Widely used in veterinary medicine since its discovery to treat intestinal infections caused by Gram-Negative bacteria, the use of colistin in humans was restricted (Catry et al., 2015). The recent rise of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria has forced changes in the way clinicians prescribe and interact with medicine (Mondal et al., 2024). Due to a rise in such highly resistant Gram-Negative infectious bacteria, the global medical community has been forced to introduce colistin into patient treatment plans (Cantón & Morosini, 2011; Kempf et al., 2016; Mondal et al., 2024).

Colistin has a narrow spectrum of activity limited to a subset of Gram-Negative bacteria and is ineffective against Gram-Positive bacteria. The worldwide increase in infections caused by resistant Gram-Negative bacteria has been associated with an increase in the use of colistin.

Colistin demonstrates in vitro activity against most Gram-Negative bacteria; however, there are some *Enterobacteriaceae* that are intrinsically resistant to colistin. These comprise *Proteus spp, Serratia marcescens, Providencia spp,* and *Morganella morganii*. Although it is a necessary therapeutic option, its utilization carries various risks that need to be critically weighed.

As an indispensable therapeutic approach, the use of colistin is accompanied by many serious deleterious conditions that researchers and clinicians should focus on. Nephrotoxicity is a major problem with such use of colistin. Colistin can lead to acute kidney injury due to its toxic action on renal tubular cells. Higher doses and longer duration of colistin use increase the risks of those with pre-existing kidney disease. And concurrent use of other nephrotoxic drugs, such as vancomycin and aminoglycosides. Although less common, neurotoxicity also remains a significant risk. Some symptoms may include dizziness, confusion, neuromuscular blocking, and in severe cases, respiratory distress. People who take muscle relaxants or who have underlying neurologic disease are at increased risk. A further concern is the rise of resistance to colistin, especially in cases of inappropriate or prolonged use. In addition, altered pharmacokinetics and immune function among immunocompromised patients, e.g., patients undergoing chemotherapy or living with HIV/AIDS, will make side effects and less favorable treatment outcomes more likely to occur.

Worldwide, colistin had been used as a food additive for multiple purposes, including growth, development, and fighting off infections, before many countries started imposing regulations on it due to concern over antibiotic overuse (R. Wang et al., 2018). The indiscriminate use of colistin in animal feed in Asian and South American countries has been speculated to lead to the rise of colistin resistance in humans and animals (Fernandes et al., 2016), as well as the spread of the *mcr* gene, leading to the banning of colistin in China as a food additive. However, given that the exposure of animals to antibiotics, not just in food, but also as therapeutics, can cause the emergence of antibiotic resistance, residual antimicrobials in food, as well as overconsumption of antibiotics in animal husbandry, are human health concerns (Reig & Toldrá, 2008).

1.2. Plasmid-Mediated Resistance: The Mobile Colistin Resistance *(mcr*) Genes

Though recently, plasmid-mediated antibiotic resistance mechanisms in bacteria have been well documented, previously, mutations in bacterial chromosome only were though to give rise to antibacterial resistance (McPhee et al., 2003). The first plasmid-mediated mobile colistin-resistant (*mcr*) gene was discovered in an *Escherichia coli* strain from a pig farm in China (Liu et al., 2016), highlighting the urgent need for global action against the rapidly evolving resistance to the last-resort antibiotic. Subsequently, more variants of the *mcr* gene were found in succession; *mcr-2* was found in Belgium from porcine and bovine *E. coli* isolates (Xavier et al., 2016), *mcr-3* was reported in China from porcine *E. coli* (Yin et al., 2017), *mcr-4* was reported in Spain, Italy and Belgium in *E. coli* and *Salmonella* isolates (Roer et al., 2017), and *mcr-5* was isolated from *Salmonella* Paratyphi isolates from poultry in Germany (Borowiak et al., 2017).

There have been reports on the exact mechanisms of the *mcr* gene, with studies showing that the *mcr* gene encodes for a transferase that changes Lipid A present in the bacterial outer membrane, decreasing the attachment of colistin to the membrane, and thus, disallowing cell lysis (Rebelo et al., 2018). After the discovery of the first five variants of the *mcr* genes, the rest followed swiftly, increasing public health concern and highlighting the urgent need for increased *Enterobacteriaceae* screening (Borowiak et al., 2020). Further *mcr* variants emerged, *mcr-6*, *mcr-7, mcr-8, mcr-9*, and most recently, *mcr-10* (Carroll et al., 2019; X. Wang et al., 2018; Xedzro et al., 2025; Yang et al., 2018). All of the *mcr* variants, including *mcr-10,* were first reported in Asian and European countries, which, as previously mentioned, have historically had indiscriminate use of colistin in animal feed, and recent reports of emerging colistin resistance pose an alarming threat to global health. Given that multiple first reports of the *mcr* gene originated in farms, an interesting question is posed about the presence and prevalence of *mcr* genes in raw markets, from where most consumers obtain meat for daily consumption.

Zoonotic transmission of resistance is the transfer of resistance genes or resistant bacteria from animal to humans. Various studies attract attention to the zoonotic transmission tracks. Trying to predict the next step of *mcr-1*, researchers offered two directions the gene can take a path: through the environment and through gastrointestinal infections (Trung et al., 2017). They pointed out that chicken is one of the principal causes of resistant bacteria that may colonize or

infect human beings. Plasmids and transposons allow the transfer of *mcr* genes to other bacteria and environments, allowing them to multiply across bacterial species. The current effectiveness of last-resort antibiotics is threatened by such cross-species transfer, and the risk of resistant bacteria in poultry entering the clinical field could occur.

1.3. A Review of the Literature

A recent review analyzed the epidemiological implications and potential control strategies of *mcr*-gene-containing organisms isolated from low and middle-income countries (Anyanwu et al., 2023). The review concluded that, given the unregulated nature of colistin usage in low and middle-income countries, there has been a rapid spread of colistin-resistant bacteria, which often contain colistin-resistant genes along with other virulent genes, emphasizing the need for non-antimicrobial alternatives in animal feed. Lebanese researchers (Hmede & Kassem, 2018) found *mcr-1*-positive *E. coli* isolates from a poultry farm, where 88% of all tested isolates were *mcr-1*-positive. The *mcr-1* containing isolates were also resistant to multiple classes of antibiotics, and were classified as multidrug resistant (MDR). While *mcr*-harboring *E. coli* had previously been isolated in the Arabian Peninsula (Sonnevend et al., 2016), the resistance reported in the Lebanese research had the highest incidence of *mcr* genes in the region. Recent research has also suggested that food animals are a key reservoir of antibiotic-resistant (ABR) bacteria, due to the high antibiotic use in food production and preservation, as well as animal husbandry practices, posing a key challenge in the combat against increasing antimicrobial resistance (AMR) (Founou et al., 2016). The research concluded that this is especially true in developing countries with their large populations and looser regulations around antibiotic usage. However, this problem does not only persist in lower economically developed countries. A surveillance study conducted in the Netherlands and Belgium tested for the presence of colistin resistance from a one-health perspective (De Koster et al., 2024). The study tested hospitalized patients, long-term care-facility residents, children attending day care centers, pigs, and broilers for colistin resistance and found resistance across all One Health sectors. Despite these findings from developed countries, *mcr* variants are generally more prevalent in developing countries, according to research (Anyanwu et al., 2023). In Southeast Nigeria, a heavily poultry-dependent country, faecal swabs from poultry (n = 785) revealed the presence of *mcr-1* (Anyanwu et al.,

2021), proving that colistin resistance had spread to the birds in Southeast Nigeria. Given that research has shown developing countries to harbour more colistin-resistant bacteria compared to their developed counterparts, attention should be paid to the Global South, and especially to Bangladesh, an overpopulated developing country, heavily dependent on animal husbandry and poultry.

Chicken is the second most-consumed meat worldwide, and its demand continues to rise with population growth and urbanization (OECD/FAO, 2021). In Bangladesh, where a large portion of the population consumes poultry regularly, broiler chicken has become especially popular due to its affordability and accessibility. In response to growing demand, poultry farms, both large commercial production and small independent operations, are stepping up production. But broiler chickens, which is developed specifically to produce meat, have a weak immunity so they can easily be infected. To combat this vulnerability, farmers heavily rely on antibiotics, not only for treatment but also as growth promoters and preventive agents.

The poultry industry is one of the fastest-growing food production industries due to an increase in demand for affordable protein. There has been extensive use of antibiotics in the treatment of infections, growth stimulation, and prophylaxis to address this need. Nonetheless, in the long run, irresponsible consumption has contributed to the emergence and proliferation of antimicrobial resistance (AMR), which encompasses resistance to essential drugs of last resort such as colistin. There is a close relationship between antibiotic use in poultry and proliferation of resistant flora and mobile resistance genes, and this is especially true of the mobilized colistin resistance (*mcr)* genes.

There has been a lack of research into the presence of colistin resistance in Bangladeshi poultry and the presence of colistin in animal feed. According to the Animal Feed Act and Animal Feed Rule (THIS REPORT CONTAINS ASSESSMENTS OF COMMODITY AND TRADE ISSUES MADE BY USDA STAFF AND NOT NECESSARILY STATEMENTS OF OFFICIAL U.S. GOVERNMENT POLICY, 2022), the use of colistin and other antibiotics is prohibited in Bangladesh in animal feed. However, these regulations are difficult to implement, and there hasn’t been conclusive evidence that supports the proper enforcement of these guidelines.

Therefore, research into the presence of colistin-resistant genes in farm animals of Bangladesh is crucial.

1.4 Presence of *mcr* Gene in Bangladesh

Several studies have been conducted in Bangladesh, which showed the presence of Mobile Colistin Resistance (*mcr*) genes in clinical settings, but fewer studies have focused on the presence of *mcr* genes in environmental or veterinary settings. A study conducted in 2020 (Islam et al., 2020) found a high abundance of *mcr-1* in chicken droppings. This cross-sectional study was conducted from a number of districts across Bangladesh and analyzed fresh chicken droppings of poultry and native chickens (n=149). Though this study conducted a multiplex Polymerase Chain Reaction (PCR) to detect multiple *mcr* variants, the isolates yielded positive results for only *mcr-1*. Another recent study tested for the presence of *mcr* variants 1-5 in *E. coli* isolated from broiler meats and found the presence of *mcr-1* in all phenotypic colistin-resistant *E. coli* isolates (Tanzin et al., 2024). Though *mcr* variants have not yet been detected in farm animals in Bangladesh to our knowledge, they have been detected in environmental and clinical isolates. A study confirmed the emergence of the *mcr-5* gene through shotgun metagenomic analysis from hospital wastewater (Khan et al., 2024), cementing the need for immediate, effective action and urgent measures. Recently, a clinical study tested the presence of *mcr* variants 1-5 in bacteria isolated from diarrheal patients (n=179) (Sarker et al., 2024). This research found the presence of *mcr-1, mcr-2,* and *mcr-3,* with co-occurrence in two isolates. Given the clinical significance of colistin resistance, it is imperative to conduct thorough research into the presence of colistin resistance in farm animals and animal feed, given the recent rise in the dissemination of colistin resistance.

1.5 Hypothesis

The last study to examine the presence of *mcr* genes in poultry was conducted five years ago, and found a high abundance of *mcr-1* genes across different types of chicken. Therefore, the working hypothesis of this study is as follows: there will be an increase in the abundance of *mcr* genes, as well as the presence of *mcr-2* to *mcr-10* genes, which have previously not been isolated

from poultry, alongside *mcr-1* genes, due to the high usage of colistin as a food additive and therapeutic drug.

1.6. Aim of the Study

The main aim of this study was to investigate whether fresh broiler chicken droppings from local markets across Dhaka city contained Gram Negative bacteria harbored *mcr* genes, hence resistant to colistin. The specific objectives of this study included:

i. To isolate *E. coli, Klebsiella pneumoniae,* and *Acinetobacter baumannii* species from the stool samples of broiler chicken using selective media.
ii. To assess the potential transmission of colistin-resistant pathogens to poultry market workers in Dhaka markets.
iii. To confirm the identity of the isolated bacteria through PCR by using species-specific primers.
iv. To determine the susceptibility of the confirmed isolates to colistin by using the Microbroth Dilution method.
v. To identify the exact mobile colistin resistance genes (*mcr-1* to *mcr-10*) from the colistin-resistant isolates using molecular analysis (PCR).
vi. To investigate the prevalence of *mcr-1* to *mcr-10* gene-mediated colistin resistance in poultry-associated pathogens in Bangladesh.

Chapter 2

2. Methods and Materials

2.1. Study Design and Specimen Collection

This cross-sectional study was conducted to assess the presence and prevalence of the mobile colistin-resistance (*mcr*) genes in Gram-Negative bacterial isolates present in chicken feces. Fresh poultry droppings (n = 100) were collected from eight different raw markets (bazaars) in Dhaka city, as demonstrated in Figure 1. Each dropping originated from a different broiler chicken in a unique shop within the raw markets (**Table 2.1**). Therefore, all one hundred samples belonged to different chickens spread across a hundred different shops. All other types of chicken were excluded from the study. The specimens were collected between February to June 2025.

Table 2.1: Sampling Points for Poultry Droppings.

Sampling Point	Number of Samples Collected
Merul Badda	15
Badda	10
DIT Project	10
Town Hall Bazar	10
Lalmatia	10
Krishi Market	10
Kawran Bazar	20
Mohammadpur	15

2.2. Bacterial Isolation and Identification

The poultry droppings were collected following the protocol described by Islam et al (Islam et al., 2020). To summarize, one gram of faeces was mixed with 400 µL of Phosphate Buffered Saline (PBS). Subsequently, one loopful of the diluted chicken faeces was streaked onto the desired selective media. In this study, HiCrome UTI Agar was used for the isolation of both *E. coli* and *K. pneumoniae,* and interpreted according to the technical sheet of HiCrome UTI Agar

(HiCrome UTI Agar M1353, n.d.). Additionally, Leeds *Acinetobacter* Medium was used for the presumptive identification and isolation of *A. baumannii.*

Following overnight incubation at 37℃, isolated colonies that were suspected to be *E. coli* and *K. pneumoniae* were selected and streaked on Nutrient Agar plates, after which they were inoculated into Tryptone Salt (T1N1) Agar, for preparation of a pure culture repository, which was then stored at room temperature with paraffin oil. The pure isolates were then processed for DNA extraction.

2.3. Molecular Characterization of Bacterial Isolates

The suspected isolates were confirmed using polymerase chain reaction (PCR).

2.3.1. DNA Extraction

Bacterial DNA was extracted using a modified version of the protocol described in (Dashti & Dashti, 2009). In short, single colonies of suspected *E. coli*, *K. pneumoniae* and *A. baumannii* that were previously streaked on Nutrient Agar were suspended in a microcentrifuge tube containing 200 µL of Tris-EDTA (TE) buffer, and mixed through vortexing. Then the mixtures were centrifuged at 13,000 RPM for 10 minutes, after which the supernatant was discarded. The remaining pellet was resuspended with 200 µL of TE buffer and boiled in a 100℃-water bath for 10 minutes. This step was followed by another centrifugation at 13,000 RPM for 10 minutes, after which the supernatant was transferred to an empty, sterile microcentrifuge tube.

2.3.2. Identification of *E. coli*

PCR was performed based on the identification of the*16SrDNA* for *E. coli*. The primer pair ECO-F (5'-GACCTCGGTTTAGTTCACAGA-3') and ECO-R (5'-CACCACGCTGACGCTGACCA-3') was used as described in an original study (Punom et al., 2020). PCR mixtures were made in a 13 µL volume comprising 6.5 µL 2X Master Mix

(TakaraBio), 1 µL of each primer (10 µM), 2.5 µL of nuclease-free water, and 2 µL of extracted DNA template. The PCR was conducted in an Applied Biosystem thermal cycler from ThermoFischer Scientific, and followed the following temperatures: 95°C for 5 minutes, followed by 30 cycles of 94°C for 45 seconds, 52°C for 45 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 min. The expected size of the*16SrDNA* for *E. coli* is 585 bp.

Table 1.2: PCR condition for *E. coli* identification

Step	Temperature	Time
Initial Denaturation	95°C	5 min
Denaturation	94°C	45 sec
Annealing	52°C	45 sec
Extension	72°C	1 min
Final Extension	72°C	5 min

2.3.3. Identification of *Klebsiella pneumoniae*

For the identification of *Klebsiella pneumoniae*, the primer pair Pf (5′-ATT TGA AGA GGT TGC AAA CGA T3′)/Pr1 (5′-TTC ACT CTG AAG TTT TCT TGT GTT C-3′) and Pf/Pr2 (5′-CCG AAG ATG TTT CAC TTC TGA TT-3′) were used (Mahmudunnabi et al., 2018). PCR mixtures were made in a 13 µL volume comprising 6.5 µL 2X Master Mix (TakaraBio), 1 µL of each primer (10 µM), 2.5 µL of nuclease-free water, and 2 µL of extracted DNA template. The PCR was conducted in an Applied Biosystem thermal cycler from ThermoFischer Scientific, and followed the following temperatures: 94°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, 57°C for 20 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 min. The expected size of the product is 130 bp.

Table 2.2: PCR condition for *K. pneumoniae* identification

Step	Temperature	Time
Initial Denaturation	94°C	10 min
Denaturation	94°C	30 sec

Annealing	57°C	20 sec
Extension	72°C	30 sec
Final Extension	72°C	10 min

2.3.4. Identification of *Acinetobacter baumannii*

PCR was performed based on the identification of the $bla_{\text{OXA-51}}$ gene for *A. baumannii* (primers: OXA-51-F: 5'TAATGCTTTGATCGGCCTTG-3' and OXA-51-R: 5'-TGGATTGCACTTCATCTTGG-3'.

PCR mixtures were made in a 15-µL volume comprising 4.9 µl Nuclease free water, 7.5 µL 2 × PCR Master Mix (TakaraBio) 0.3 µL of forward and reverse primers (10 µM), and 2 µL of DNA template. The PCR program was set up in an Applied Biosystem (BioRad) thermal cycler as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The expected size for the $bla_{\text{OXA-51}}$ gene is 353 bp.

Table 2.4: PCR condition for *Acinetobacter baumannii* identification

Step	Temperature	Time
Initial Denaturation	94°C	5 min
Denaturation	94°C	1 min
Annealing	55°C	1 min
Extension	72°C	1 min
Final Extension	72°C	10 min

2.3.5 Agarose Gel Electrophoresis

The PCR products were visualized using electrophoresis. For *E. coli* and *Klebsiella pneumoniae*, 1.2% and 2% agarose gels were used, respectively. The products were subjected to

electrophoresis in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH of 8.0) containing 0.5 μg/mL DNA ethidium bromide dye, at 110V for 50 minutes, and the gels were examined under ultraviolet light.

2.4. Detection of the Mobile Colistin Resistance (*mcr*) Genes

The *mcr* genes were detected using 2 sets of multiplex polymerase chain reactions. The first multiplex PCR was performed to detect *mcr-1* to *mcr*-5, and the second multiplex PCR was performed to detect *mcr-6* to *mcr*-10.

2.4.1 Detection of *mcr*-1 to *mcr*-5

A multiplex PCR was performed to determine the presence of *mcr* genes as described in an original study. The primer details are given below (**Table 2.3**). To summarize the procedure, PCR mixtures were made in a 30 μL volume comprising 15 μL 2X Master Mix (TakaraBio), 1 μL of each primer (10 μM), 2 μL of nuclease-free water, and 2 μL of extracted DNA template. The PCR was conducted in an Applied Biosystem thermal cycler from ThermoFischer Scientific, USA, and followed the following temperatures: 94°C for 15 minutes, followed by 25 cycles of 94°C for 30 seconds, 55°C for 90 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 min (Islam et al., 2020).

Table 2.3: PCR condition for *mcr 1-5* detection

Step	Temperature	Time
Initial Denaturation	94°C	15 min
Denaturation	94°C	30 sec
Annealing	55°C	90 sec
Extension	72°C	1 min
Final Extension	72°C	10 min

2.4.2. Detection of *mcr* 6 to *mcr 10*

A multiplex PCR was performed to determine the presence of *mcr* genes as described in an original study. The primer details are given below (**Table 2.6**). Briefly, PCR mixtures were made in a 25 µL volume comprising 12.5 µL 2X Master Mix (TakaraBio), 0.5 µL of each primer (10 µM), 6.5 µL of nuclease-free water, and 2 µL of extracted DNA template. The PCR was conducted in an Applied Biosystem thermal cycler from BioRad Scientific, USA, and followed the following temperatures: 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C` for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 min

Table 2.6: PCR condition for *mcr 6-10* detection

Step	Temperature	Time
Initial Denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	55°C	30 sec
Extension	72°C	1 min
Final Extension	72°C	10 min

2.4.3. Agarose Gel Electrophoresis

The PCR products were visualized using electrophoresis using 1.5% agarose gels for each multiplex PCR. The products were subjected to electrophoresis in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH of 8.0) containing 0.5 µg/mL DNA ethidium bromide dye, at 110V for 50 minutes, and the gels were examined under ultraviolet light.

2.5. Detection of Phenotypic Colistin Resistance

The phenotypic colistin resistance was measured by determining the minimum inhibitory concentration via the broth microdilution method of 15 *E. coli* isolates, due to resource constraints. Minimum inhibitory concentrations (MICs) are considered the ‘gold standard’ for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the

performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate, for example when determining the susceptibility of coagulase- negative staphylococci to teicoplanin. The range of antibiotic concentrations used for deter- mining MICs is universally accepted to be in doubling dilution steps up and down from 1 mg/L as required. The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation (this period is extended for organisms such as anaerobes, which require prolonged incubation for growth). The method described below is an amended version of the procedure described in the BSAC Guide to Sensitivity Testing[1] and can be adapted for determining the minimum bactericidal concentration (MBC) of an antimicrobial for an organism by substituting IsoSensitest agar (ISA; Oxoid, Basingstoke, UK) with IsoSensitest broth (ISTB; Oxoid) and then subculturing to drug-free media or can be truncated for use as a 'breakpoint' method.

According to CLSI guidelines, 2024, one of the approved methods for colistin susceptibility testing is broth microdilution (BMD). By which the minimum inhibitory concentration (MIC) is determined. This method facilitates the determination of the lowest concentration by which bacterial growth is inhibited.

2.5.1. Master Stock Preparation:

0.1344 g of colistin sulfate powder was dissolved in a MCT with 1 mL distilled H_2O. After weighing, the colistin stock had a concentration of 134,400 µg/mL colistin sulfate. This substance has a molecular weight of 1743 g/mol, and the molecular weight of its active colistin is 1155 g/mol. Consequently, the stock contained 66.3% pure colistin. Therefore, the final concentration of colistin in the stock solution was 89,100 µg/mL.

2.5.2. Intermediate Stock Preparation:

In another Falcon tube an intermediate stock with a colistin concentration of 256 µg/mL was prepared.

2.5.3 Preparation of Cation-Adjusted Muller-Hinton Broth:

The subsequent step was the preparation of Cation-Adjusted Mueller-Hinton Broth, or CAMHB. CAMHB is a growth medium that is used in susceptibility testing for bacteria. The process began by creating 2X Mueller-Hinton Broth (MHB) and then adding calcium (Ca^{2+}) and magnesium (Mg^{2+}) salts. The salts were prepared by dissolving 2.77 g of $CaCl_2$ in 100 mL of dH_2O and 8.47 g of $MgCl_2$ in another 100 mL of dH_2O. This step was followed by the addition of 2 mL of the calcium solution and 1 mL of the magnesium solution to 100 mL of MHB. This then constituted the finished CAMHB.

2.5.4 Preparation of the Working Stock Solutions:

The working stock solutions of colistin was prepared by serial dilution. In this process, 10 microcentrifuge tubes (MCTs) were labeled with a different concentration of colistin and 750 µL of CAMHB were added to the aforementioned tubes. From the intermediate stock (256 µg/mL), 750 µL was added to MCT 1, giving a final concentration in MCT 1 of 128 µg/mL. All other MCTs are then obtained by the procedure of 2-fold serial dilution.

Table 2.7: Concentration of colistin in each MCT after serial dilution.

Microcentrifuge Tube (MCT)	Colistin Concentration (µg/mL)	Volume of Colistin (µL)	Volume of CAMHB (µL)
MCT 1	128	750	750
MCT 2	64	750	750
MCT 3	32	750	750
MCT 4	16	750	750
MCT 5	8	750	750
MCT 6	4	750	750
MCT 7	2	750	750
MCT 8	1	750	750
MCT 9	0.5	750	750
MCT 10	0.25	750	750

Following the 2x serial dilution process, 750 µL was transferred and the following final concentrations were reached: MCT 2 (64 µg/mL), MCT 3 (32 µg/mL), MCT 4 (16 µg/mL), MCT 5 (8 µg/mL), MCT 6 (4 µg/mL), MCT 7 (2 µg/mL), MCT 8 (1 µg/mL), MCT 9 (0.5 µg/mL), and MCT 10 (0.25 µg/mL). Finally, in MCT 10, 750 µL was removed after the last dilution.

2.5.5. Preparation of Bacterial Culture:

An isolated colony from a Mueller-Hinton Agar (MHA) plate, that been previously revived, was inoculated in Mueller-Hinton Broth (MHB). The broth was incubated in a shaking incubator at 37°C for 18-24 hours. Then, 100 µL of bacterial culture was taken and added to CAMHB to adjust the OD to 0.5 McFarland.

2.5.6. Microtiter Plate Layout:

The bacterial suspension was inoculated into the microtiter plate:100 µL of the inoculum was added to each well containing media with colistin. The final concentrations of colistin in each well ranged from 64 µg/ml to 0.125 µg/ml.

Well 1 had a concentration of 64 ug/mL and well 10 had a concentration of 0.125 ug/mL, and the 11^{th} and 12^{th} wells were for positive control with no antibiotic, negative control with no bacterial culture respectively.

Table 2.8: Microtiter plate layout for MIC

	1	2	3	4	5	6	7	8	9	10	11	12
A (Bacteria 1)	100 ul (128ug /mL) WS + 100 uL Bacterial culture	100 ul (64ug/ mL) WS + 100 uL Bacterial culture	100 ul (32ug/m L) WS + 100 uL Bacterial culture	100 ul (16ug/m L) WS + 100 uL Bacterial culture	100 ul (8ug/mL) WS + 100 uL Bacterial culture	100 ul (4ug/m L) WS + 100 uL Bacterial culture	100 ul (2ug/mL) WS + 100 uL Bacteria l culture	100 ul (1ug/m L) WS + 100 uL Bacteria l culture	100 ul (0.5ug/ mL) WS + 100 uL Bacteria l culture	100 ul (0.25ug/ mL) WS + 100 uL Bacteria l culture	100 ul CAMHB + 100 uL Bacterial culture (Positive Control)	200uL CAMH B (Negati ve Control)
B (Bacteria 1 Duplicate)	100 ul (128ug /mL) WS + 100 uL Bacterial culture	100 ul (64ug/ mL) WS + 100 uL Bacterial culture	100 ul (64ug/m L) WS + 100 uL Bacterial culture	100 ul (16ug/m L) WS + 100 uL Bacterial culture	100 ul (8ug/mL) WS + 100 uL Bacterial culture	100 ul (4ug/m L) WS + 100 uL Bacterial culture	100 ul (2ug/mL) WS + 100 uL Bacteria l culture	100 ul (1ug/m L) WS + 100 uL Bacteria l culture	100 ul (0.5ug/ mL) WS + 100 uL Bacteria l culture	100 ul (0.25ug/ mL) WS + 100 uL Bacteria l culture	100 ul CAMHB + 100 uL Bacterial culture (Positive Control)	200uL CAMH B (Negati ve Control)
C												
D (Bacteria 2)	100 ul (128ug /mL) WS + 100 uL Bacterial culture	100 ul (64ug/ mL) WS + 100 uL Bacterial culture	100 ul (64ug/m L) WS + 100 uL Bacterial culture	100 ul (16ug/m L) WS + 100 uL Bacterial culture	100 ul (8ug/mL) WS + 100 uL Bacterial culture	100 ul (4ug/m L) WS + 100 uL Bacterial culture	100 ul (2ug/mL) WS + 100 uL Bacteria l culture	100 ul (1ug/m L) WS + 100 uL Bacteria l culture	100 ul (0.5ug/ mL) WS + 100 uL Bacteria l culture	100 ul (0.25ug/ mL) WS + 100 uL Bacteria l culture	100 ul CAMHB + 100 uL Bacterial culture (Positive Control)	200uL CAMH B (Negati ve Control)
E (Bacteria 2 duplicate)	100 ul (128ug /mL) WS + 100 uL Bacterial culture	100 ul (64ug/ mL) WS + 100 uL Bacterial culture	100 ul (64ug/m L) WS + 100 uL Bacterial culture	100 ul (16ug/m L) WS + 100 uL Bacterial culture	100 ul (8ug/mL) WS + 100 uL Bacterial culture	100 ul (4ug/m L) WS + 100 uL Bacterial culture	100 ul (2ug/mL) WS + 100 uL Bacteria l culture	100 ul (1ug/m L) WS + 100 uL Bacteria l culture	100 ul (0.5ug/ mL) WS + 100 uL Bacteria l culture	100 ul (0.25ug/ mL) WS + 100 uL Bacteria l culture	100 ul CAMHB + 100 uL Bacterial culture (Positive Control)	200uL CAMH B (Negati ve Control)
F												
G (Bacteria 3)	100 ul (128ug /mL) WS +	100 ul (64ug/ mL) WS +	100 ul (64ug/m L) WS + 100 uL	100 ul (16ug/m L) WS + 100 uL	100 ul (8ug/mL) WS + 100 uL	100 ul (4ug/m L) WS + 100	100 ul (2ug/mL) WS + 100 uL	100 ul (1ug/m L) WS + 100	100 ul (0.5ug/ mL) WS + 100	100 ul (0.25ug/ mL) WS + 100	100 ul CAMHB + 100 uL Bacterial	200uL CAMH B (Negati

	100 uL Bacterial culture	100 uL Bacterial culture	Bacterial culture	Bacterial culture	Bacterial culture	uL Bacterial culture	Bacterial culture	uL Bacterial culture	uL Bacterial culture	uL Bacterial culture	culture (Positive Control)	ve Control)
H (Bacteria 3 Duplicate)	100 ul (128ug /mL) WS + 100 uL Bacterial culture	100 ul (64ug/ mL) WS + 100 uL Bacterial culture	100 ul (64ug/mL) WS + 100 uL Bacterial culture	100 ul (16ug/mL) WS + 100 uL Bacterial culture	100 ul (8ug/mL) WS + 100 uL Bacterial culture	100 ul (4ug/mL) WS + 100 uL Bacterial culture	100 ul (2ug/mL) WS + 100 uL Bacterial culture	100 ul (1ug/mL) WS + 100 uL Bacterial culture	100 ul (0.5ug/ mL) WS + 100 uL Bacterial culture	100 ul (0.25ug/ mL) WS + 100 uL Bacterial culture	100 ul CAMHB + 100 uL Bacterial culture (Positive Control)	200uL CAMHB (Negative Control)

After inoculation, the microtiter plate was incubated at 37°C for 18-24 hours.

Chapter 3

3. Results

3.1. Sampling Sites

The purpose was to determine the presence and prevalence of colisin resistance genes across broiler chickens in raw markets in Dhaka City. A total of 100 faecal samples were collected from 100 different shops from 8 major raw markets. Each sample originated from a unique broiler chicken. The shops were selected due to being major trade points in Dhaka city; most of the broiler chickens sent to restaurants, residential shops, and smaller bazaars originate from one of the eight sampling sites covered in this study (Figure 3.1).

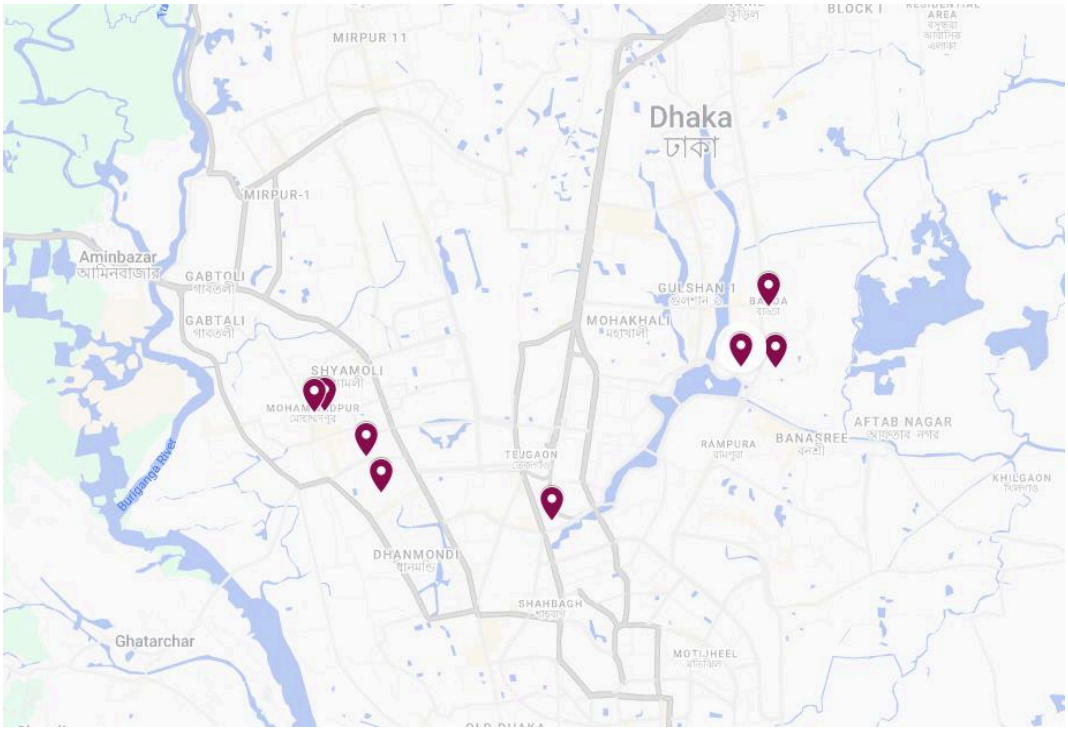
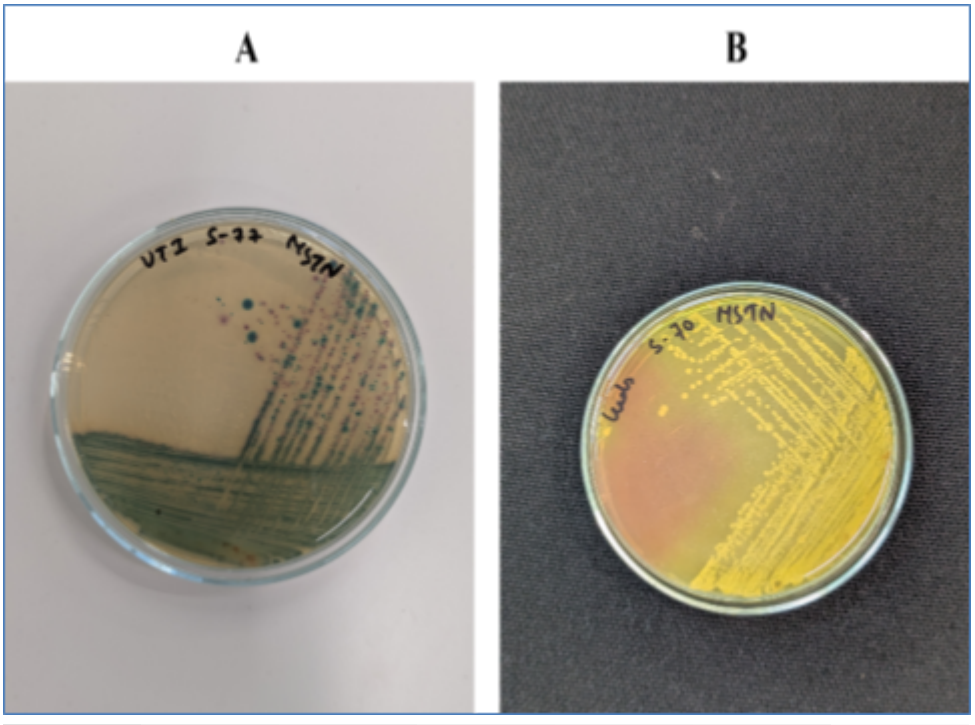


Figure 3.1: The Sample Collection Sites Pointed on QGIS Using GPS Coordinates

3.2. Bacterial Isolation and Identification

From the 100 samples, 6 samples yielded no growth on UTI and therefore were eliminated. From the remaining 94 samples, 120 isolates were recovered, and 100 and 20 were analyzed for the presence of *E. coli* and *Klebsiella pneumoniae* due to their purple and blue appearance on HiCrome UTI agar, respectively (Figure 2).

Furthermore, from the remaining 94 samples, 50 additional isolates were presumptively suspected to be *Acinetobacter baumannii* due to their yellow appearance on Leeds Acinetobacter Agar.



3.2.1. Detection of *E. coli*

Out of the suspected 100 of *E. coli* isolates, 84 of them yielded the expected band size, which is 585 bp, after agarose gel electrophoresis, yielding a percentage of 84% (Figure 3.3).

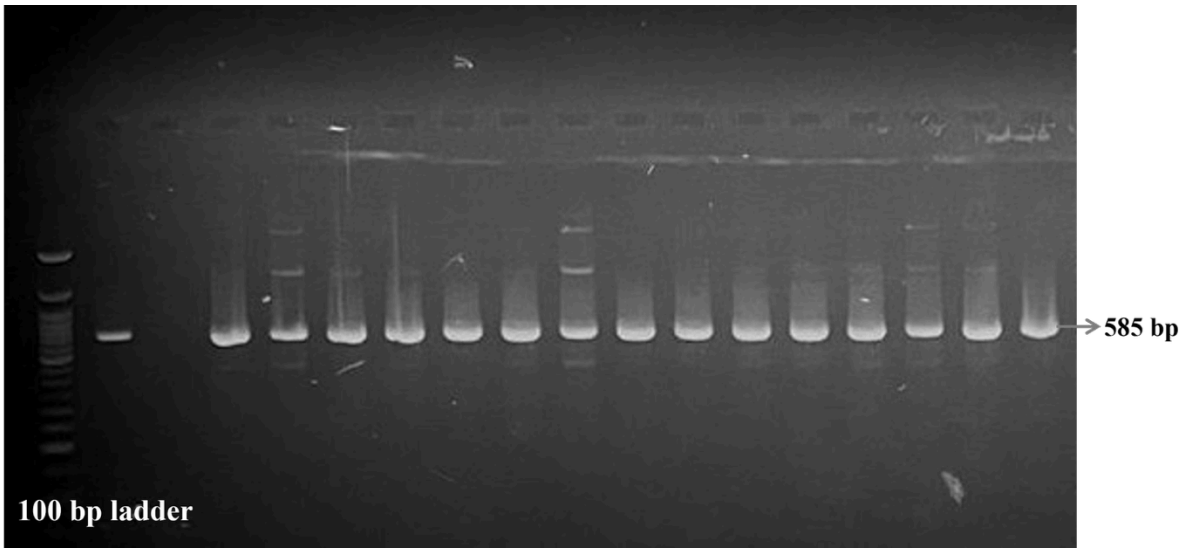


Figure 3.3: Detection of *E. coli* (585bp) by PCR

3.2.2. Detection of *Klebsiella pneumoniae*

Of the suspected 20 *Klebsiella pneumoniae* isolates, 14 of them yielded the expected band size, which is 130 bp, after agarose gel electrophoresis, yielding a percentage of 70%.

Figure 3.4: Detection of *K. pneumoniae* (130bp) by PCR

3.2.3. Detection of *Acinetobacter baumannii*

Of the suspected 50 *Acinetobacter baumannii* isolates, none of them yielded the expected band size (353 bp) after agarose gel electrophoresis.

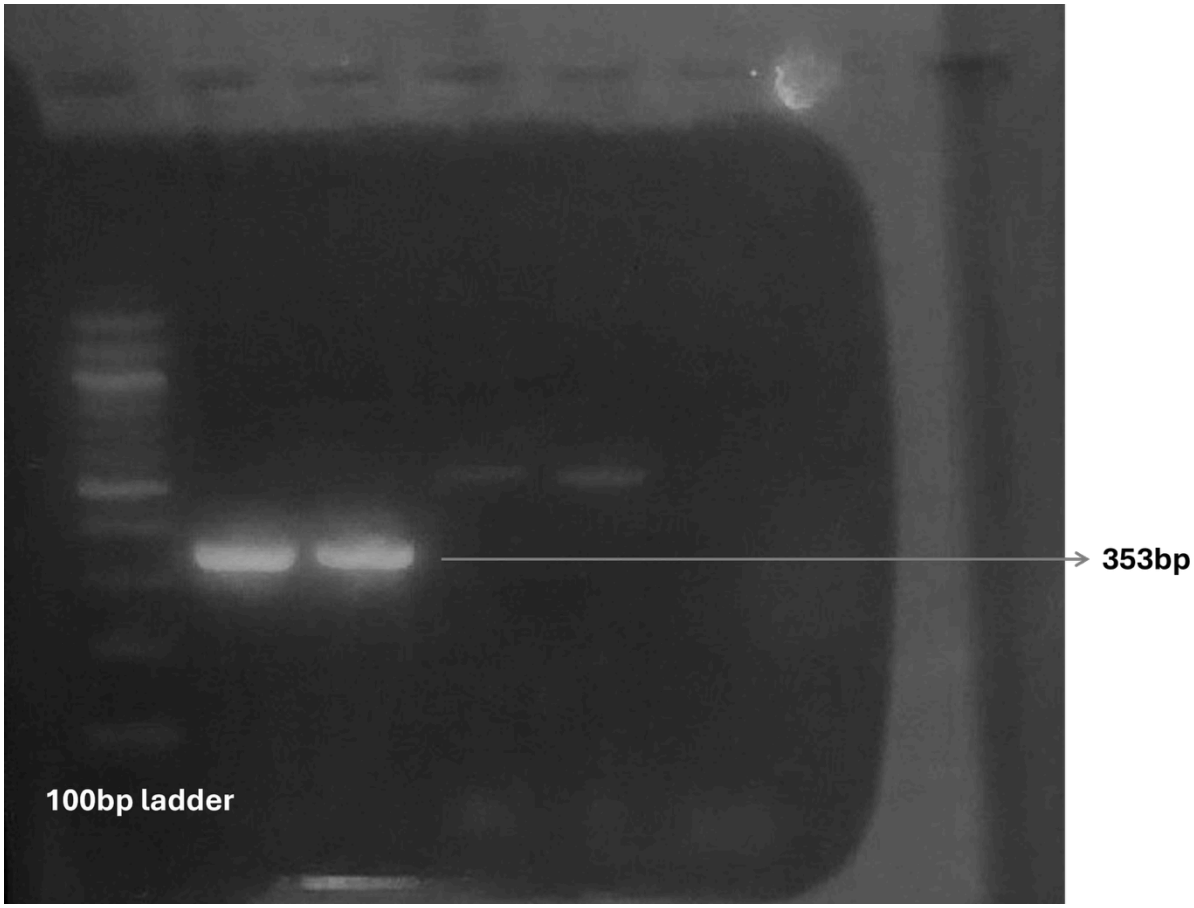


Figure 3.5: Gel Run of *Acinetobacter baumannii* which yielded no positive results

3.3. Detection of *mcr* genes

3.3.1. Detection of *mcr-1 to mcr-5*

The presence of *mcr*-1 to *mcr*-5 was assessed in all 98 isolates through multiplex PCR analysis. No isolate showed the presence of all five *mcr* genes. The *mcr-3* gene was the most abundant, being present in 82 (83.7%) isolates, among which 74 were from *E. coli* and 8 from *K. pneumoniae*. None of the isolates showed the presence of *mcr-2, mcr-4,* or *mcr-5* genes. The second highest yield from this multiplex PCR was the *mcr-1* gene, present in 8 (8.2%) isolates, with 6 coming from *E. coli* and 2 from *K. pneumoniae.*

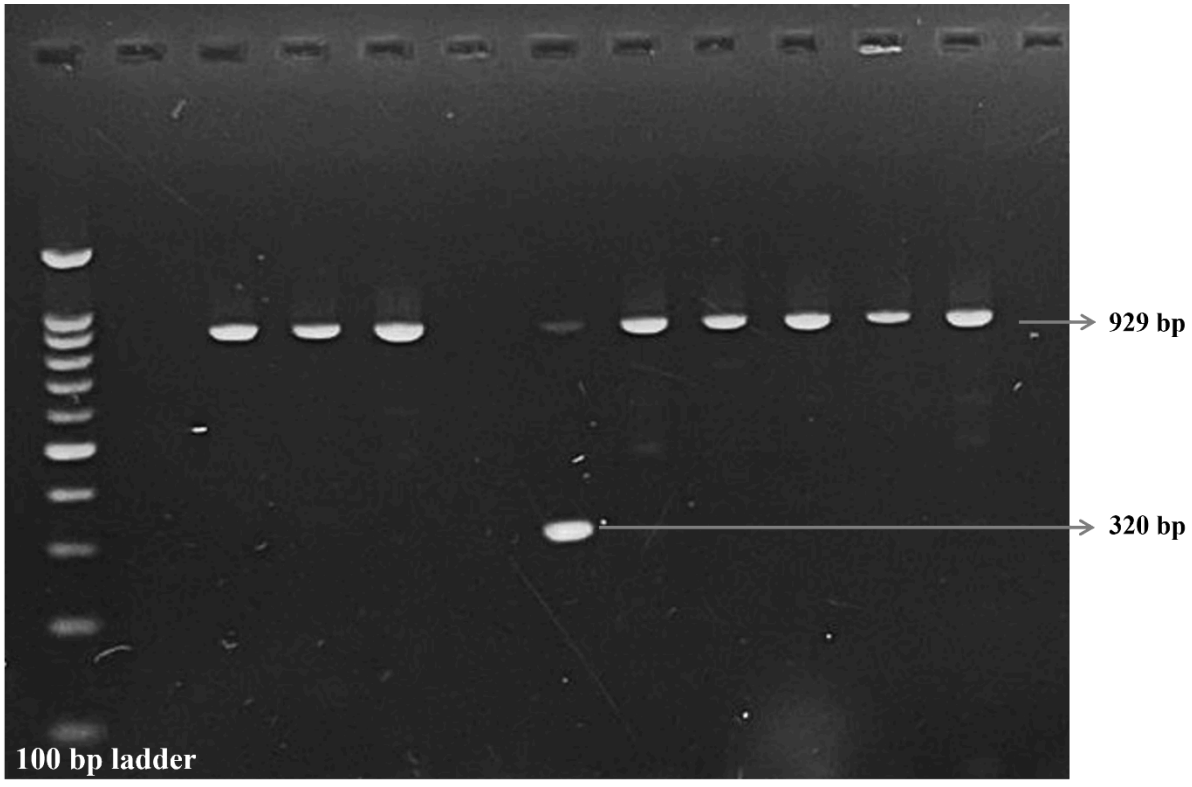


Figure 3.6: Detection of *mcr* variants 1-5 by PCR

Figure 3.7: *mcr-1* to *mcr-5* frequency in *E. coli* isolates

Figure 3.8: *mcr-1* to *mcr-5* frequency in *K. pneumoniae* isolates

3.3.2. Detection of *mcr*-6 to *mcr-10*

The presence of *mcr*-6 to *mcr*-10 was assessed in all 98 isolates through multiplex PCR analysis (Figure 6). No isolate showed the presence of all five *mcr* genes, and none of the isolates were positive for the *mcr-10* gene. The *mcr-8* gene was the most prevalent, being present in 40 (40.8%) isolates, among which 39 were from *E. coli*, and 1 from *K. pneumoniae*. The number of isolates that showed the presence of *mcr-6*, *mcr-7*, and *mcr-9* was 25 (25.5%), 3 (3.1%), and 2 (2%), respectively. None of the *K. pneumoniae* isolates showed the presence of *mcr-7* or *mcr-9*, and four were positive for *mcr-6.*

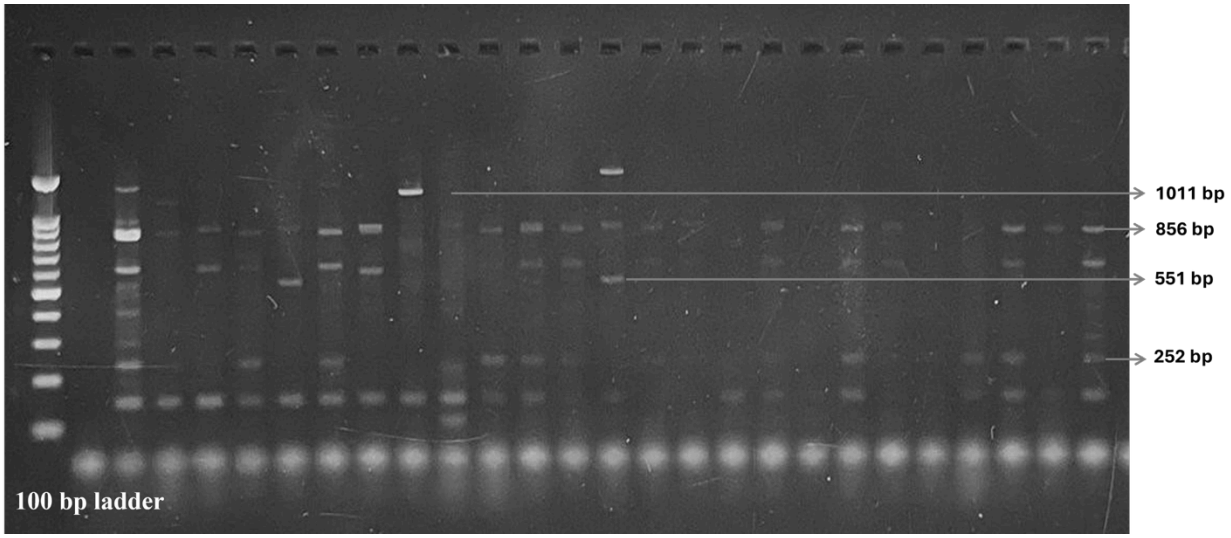


Figure 3.9: Detection of *mcr* variants 6-9 by PCR

Figure 3.10: *mcr* 6 *to mcr-10* frequency in *E. coli* isolates

Figure 3.11: *mcr-6* to *mcr-10 frequency in K. pneumoniae isolates*

Interestingly, a few isolates showed the coexistence of multiple *mcr* genes, with 3 *K. pneumoniae* being positive for three different *mcr* genes, and 41 *E. coli* showing the presence of two or more *mcr* genes. Additionally, two *E. coli* isolates yielded the *mcr-3, mcr-6, mcr-8,* and *mcr-9* genes simultaneously. Interestingly, 83 (98.8%) *E. coli* isolates yielded at least one *mcr* gene variant, and 9 (35.7%) of *K. pneumoniae* isolates showed positive results for at least one *mcr* gene variant (Figure 7).

3.4. Detection of Phenotypic Resistance to Colistin

The phenotypic colistin susceptibility of 15 *Escherichia coli* isolates as determined by the MIC values (in μg/mL) of colistin (ranging from 2 to 16 μg/mL) is given below.

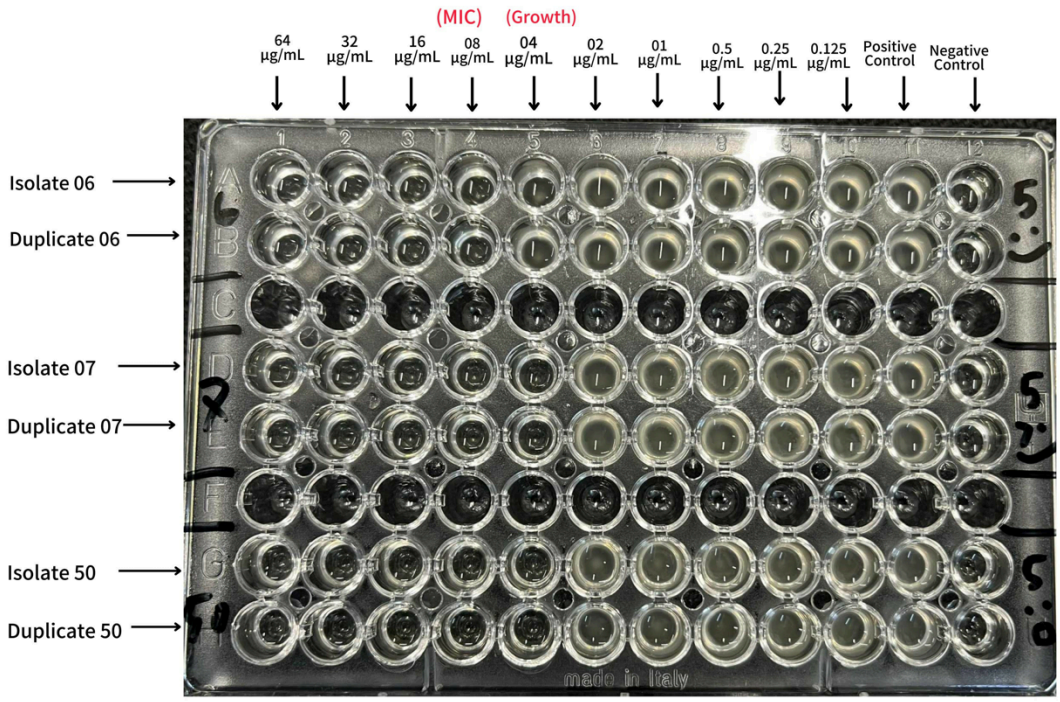


Figure 3.12: MIC determination by observing growth in different concentrations of colistin in a microtiter plate by the BDM method.

Table 3.1: MIC results of *E. coli* isolates using the BMD method

Sample no	MIC (µg/mL)	*mcr* Present	Susceptibility status
1	4	3, 6, 8, 9	Resistant
6	8	3, 7, 8	Resistant
7	4	3, 7, 8	Resistant
11	16	1, 3	Resistant
14	4	3, 6, 8	Resistant
22	4	6,8	Resistant
23	4	3, 8	Resistant
24	2	3	Susceptible
25	4	3	Resistant
28	4	3, 6, 8	Resistant

31	8	6, 8	Resistant
37	4	3, 7	Resistant
40	4	3, 8	Resistant
45	4	none	Resistant
50	4	3	Resistant

These isolates were selected based on the distribution of *mcr* genes, where some samples had various types and numbers of *mcr* genes, ranging from no *mcr* genes to 4 *mcr* genes in one isolate. Samples no. 6 and 31 showed MIC at 8 μg/mL, and sample no. 11 was resistant up to 8 μg/mL and MIC of it being 16 μg/mL. Only one sample displayed susceptibility against colistin. Additionally, 14 out of 15 isolates were resistant to colistin. All of them harbored different variations of *mcr* genes except for sample no. 45, which did not possess any of the ten *mcr* genes.

Chapter 4:

4. Discussions

This study investigated the gut microbiome of broiler chickens for the prevalence of ten *mcr* variants, *mcr-1* to *mcr-10*, which are linked to phenotypic colistin resistance in Bangladesh in both clinical and environmental settings (Islam et al., 2020; Nisa et al., 2024; Sarker et al., 2024; Tanzin et al., 2024). While collecting the samples from different locations in Dhaka city, it was observed that the broiler chickens were kept in small cages in groups and the cages were overcrowded with chickens with little to no air flow or ventilation systems and the cages were not cleaned very frequently. These conditions are very suitable for the breeding ground of harmful bacteria. As these chickens are also raised in similar conditions in industrial farms, the use of antibiotics can be a common practice for raising these animals. The poultry shops and fresh produce shops are situated very close to each other in almost every sample collection location that the probability of cross-contamination of food is very much likely.

The study showed a high abundance of the hazardous *mcr-3* and *mcr-8* genes, which were present in 83.7% and 40.8% of all tested isolates. Beyond the aforementioned variants, there was also a large percentage of *mcr-6* carrying bacteria (25.5%). None of the isolates yielded positive results for *mcr-2, mcr-4,* or *mcr-5.* To the best of our knowledge, this study marks the first report of the presence of *mcr* variants beyond *mcr-1* in Bangladeshi livestock and the first report of the presence of *mcr-6, mcr-8,* and *mcr-9* in Bangladesh overall. Contrary to previous studies that had found a large number of *mcr-1* harboring isolates (Islam et al., 2020), this study only found the *mcr-1* gene in 8% of the isolates. Research has shown that the abundance of *mcr* variants has changed largely over the years, with the abundance of *mcr-9* increasing (Martiny et al., 2022). This is reflected in our study compared to previous studies, given the higher amount of *mcr-3, mcr-6,* and *mcr-8* recorded.

Co-occurrence of two or more *mcr* variants was observed in 44.9% of the isolates, and further experimentation, such as broth dilution, is needed to determine whether single or co-occurrence in these isolates is associated with increased phenotypic colistin-resistance, as reported by multiple previous studies (dos Santos et al., 2020; García et al., 2018). Although this study did not identify colistin-resistant isolates across all Dhaka bazaars, the results were obtained across the eight major bazaars and therefore are likely to be generalizable.

There were some noticeable discrepancies between the genotypic and phenotypic susceptibility to colistin, suggesting that factors other than the presence or absence of *mcr* genes may be responsible for phenotypic colistin-resistance. *E. coli* isolate 4 for instance, showed resistance to colistin under the broth micro-dilution method, whereas that isolate was one of the only one of two in the present study, which did not harbor any *mcr* genes. Additionally, isolates 24 and 25 were both carriers of *mcr-3*, but only one of them (24) was susceptible to colistin, while the other was resistant. Contrary to assumption, the isolate which harbored 4 different *mcr* genes showed resistance to colistin, but . These findings pose questions about the presence of *mcr* genes and their direct relationship in harboring resistance. The discrepancies indicate that other factors, such as genes present in the bacterial chromosome, may also be responsible for colistin resistance or susceptibility.

Antibiotics used in animal feed are a human health concern due to the potential for horizontal gene transfer (HGT) from resistant to sensitive bacteria, or the potential for acquiring multiple *mcr* variants in the context of colistin. There is also a risk of zoonotic transmission of resistant bacteria. Therefore, the findings of this study and the presence of *mcr* genes in the chicken gut microbiome are of global importance, especially with previously unreported *mcr* variants. Previous studies have shown that exposure to antibiotics leads to acquired antibiotic resistance (Cantón & Morosini, 2011; Kempf et al., 2016), but due to resource constraints, the presence of colistin and other antibiotics in poultry feed could not be detected. Previous studies have shown a high abundance of *mcr* gene variants in *E. coli* (Olaitan et al., 2014), and this study found similar results, with 98.8% of *E. coli* isolates having single or co-occurrence of *mcr* genes.

Detection of *mcr* gene harboring *E. coli* is a possible reason for overuse of colistin, not only as a treatment for infection but also as a growth promoter. As *E. coli* is part of the broiler chicken's gut microbiome, its survival is necessary for metabolism and digestion. Exposure to colistin for a long time can be a primary cause of the development of *mcr* genes.

The rapid spread of colistin resistance in *Escherichia coli* is a public health concern as colistin is the last-line antibiotic for nosocomial multidrug-resistant pathogens. In the present study, 84 PCR-confirmed *E. coli* were screened for the presence and prevalence of Mobilized Colistin Resistance genes (*mcr*) with multiplex PCR assays targeting *mcr-1* to *mcr-9* and a specific assay for *mcr-10*. The results demonstrated that there was a great abundance of diversity of *mcr* genes and that *mcr-3* and *mcr-8* were predominant, which are important elements of colistin resistance dissemination among clinical and environmental sources.

The high number of colistin-resistant strains is alarming, given the use of colistin as a last-resort antibiotic (Marshall & Levy, 2011) and the increase in antibiotic resistance across the globe. Studies forecast that by 2050, South Asia, Latin America, and the Caribbean will have the highest antibiotic resistance-related mortality rate (Naghavi et al., 2024), and the way to avert this scenario would be to develop a Gram-Negative drug pipeline. The increase in AMR in Gram-Negative bacteria is one of the reasons this study is consequential. It underscores the importance of establishing proper antibiotic surveillance systems to reduce AMR rates, alongside implementing proper stewardship programs. Additionally, this study highlights the importance of maintaining transparency about the presence of antibiotics in animal feed. Previously, implementing a complete ban on colistin in animal feed had shown results (Catry et al., 2015), and similar protocols could be strictly implemented in Bangladesh.

This study acts as the first report of *mcr* gene variants beyond *mcr-1* in poultry. Additionally, this study also marks the first instance of *mcr-6*, *mcr-8*, and *mcr-9* in Bangladesh, to the best of our knowledge. Additionally, this study covered the major bazaars in Dhaka, which are centres from which poultry is supplied to restaurants, shops, and residential areas.

It is noteworthy to discuss the limitations of this study. Due to resource constraints, only eight of the major raw markets of Dhaka city could be targeted; Dhaka is a densely populated city with many major bazaars scattered throughout. Additionally, the presence of colistin in poultry feed could not be detected; the laboratory where this experiment was conducted lacked the proper equipment to detect antibiotics in poultry feed, and additionally, permission to check the feed for antibiotics could not be obtained from the proper authorities. It was also not possible to discern whether the bacteria detected are natural reservoirs of colistin resistance or are presenting newly acquired traits. However, despite these limitations, this study's findings show an urgent need for establishing proper antibiotic stewardship and surveillance programs.

Chapter 5

5. Conclusion

This study showed, contrary to previous studies conducted in Bangladesh, that the *mcr-3* variant predominates in the gut microbiome of broiler chickens in Dhaka raw markets, and acts as the first report of *mcr-3, mcr-6, mcr-8,* and *mcr-9* from the poultry gut microbiome, and the first report of *mcr-6, mcr-8,* and *mcr-9* overall in Bangladesh.

5.4 Future Prospects

Future studies could significantly increase the scope of sample collection by targeting other major cities of Bangladesh or major raw markets across all 64 districts. This would yield a larger sample size, making way for more statistically significant findings, given that it would mark the first study of its kind. Additionally, as noted in the limitations section, there was an inability to assess the amount of colistin present in the chicken feed, so direct conclusions could not be drawn about where the poultry gut bacteria had accumulated the colistin resistance from. Therefore, proper funding and resources can be allocated to conducting research into poultry feed, to investigate whether large amounts of antibiotics are being added to the feed, and whether this extends beyond poultry into other animals as well. Lastly, epidemiological studies can be conducted to investigate whether the *mcr*-harboring bacteria directly transfer from the chickens to the people in the raw markets who handle the poultry, and the consumers who buy the meat. The scope of this study can also be increased by investigating more colistin-resistant Gram-Negative bacteria present in poultry droppings, such as *Salmonella*.

Chapter 6

6. References

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