Isolation of Mobile Colistin-Resistance (*mcr*) Gene-Harboring *Escherichia coli* from Chicken Droppings in Dhaka

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing our 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. This thesis does not contain material that has been accepted or submitted for any other degree at a university or other institution.
4. We have acknowledged all the mail sources of help.

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Abstract

The use of antibiotics in poultry industries across the low and middle-income countries is increasing rapidly, contributing to emergence of resistance against the last line of drug like colistin causing public health threat. Therefore, this study aimed to investigate the prevalence of Mobile Colistin Resistance (*mcr*) genes in *E. coli* isolated from the fecal samples of broiler chickens in Dhaka city. A total of 50 fecal samples were collected from food markets and residential areas of Dhaka city. The samples were processed and streaked onto selective media to presumptively isolate *E. coli*. The isolates were further confirmed by polymerase chain reaction (PCR) analysis, targeting 16s rRNA regions. Detection of *mcr* genes was performed by employing multiplex PCR of *mcr-1* to *mcr-5* and *mcr-6* to *mcr-9*, while *mcr-10* gene was amplified by singleplex standard PCR. Lastly, phenotypic expression was assessed by the broth microdilution method (BMD) to determine the minimum inhibitory concentration (MIC). A total of 47 *E. coli* isolates were presumptively isolated from selective media and subsequently 41 confirmed by PCR followed by plasmid-mediated detection of *mcr* resistance genes. Among *E. coli* isolates, 3 carried *mcr-1* (7.3%), 33 *mcr-3* (80.5%), 12 *mcr-6* (29.3%), 2 *mcr-7* (4.9%) 28 *mcr-8* (68.3%) *and* 1 *mcr-9* (2.4%), *mcr-2*, *mcr-4*, and *mcr-10* were not detected in any of the isolates of *E. coli*. Phenotypic susceptibility testing of the isolates were carried out by using BMD method, revealing that most of the isolates harboring *mcr* genes are resistant to colistin at MIC up to 4 ug/mL. The high prevalence of various *mcr* genes, notably *mcr-3* and *mcr-8*, indicated that colistin resistance had been extensively disseminated in bacteria from chickens. Such findings indicate industrial poultry chicken as a potential source of multidrug-resistant pathogens.

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

Chapter 1

1.1 Introduction

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 are 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 (Van Boeckel et al., 2015).

Antibiotic overuse in poultry production and lack of control on its use have been a major reason for the rise of antibiotic-resistant bacteria. Among these, colistin has been increasingly used as a final line of treatment in case of multidrug-resistant Gram-negative infections. Unfortunately, colistin is increasingly applied in the poultry farms to prevent diseases and to ensure prolific and disease-free growth especially in the low- and middle- income countries like Bangladesh, where enforcement of regulations are rarely carried out. (Kempf et al., 2016). This misuse of colistin has accelerated the development and spread of colistin-resistant pathogens in the environment, creating serious public health concerns.

For the treatment of acute and chronic infections, colistin is a reliable antibiotic which is effective against gram-negative bacterial infections. It belongs to the polymyxin class of antibiotics and is the last line of defense against serious infections, including those caused by multidrug-resistant organisms. Colistin is the active agent of colistimethate sodium, which is given as an intravenous (IV) or intramuscular (IM) injection. Colistin attaches to fatty molecules present on the cell membranes of gram-negative bacteria and induces leakage of cell contents, which results in bacterial cell death (Mirjalili et al., 2022). 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 have

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*.

The resistance to colistin became more critical and concerning after the discovery of the mobilized colistin resistance (*mcr-1*) gene in China, which can be horizontally transferred between bacterial species, which can promote rapid spread of resistance (Liu et al., 2016). Bacteria resistant to colistin have subsequently been identified not only in clinical environments, such as intensive care units (ICUs), but also in animals, food and environmental samples in multiple countries including Bangladesh (Islam et al., 2020). Broiler chickens' feces are often left untreated in the environment, and can contaminate soil and water, and be a reservoir for bacteria resistant to antibiotics.

This research aims to examine the prevalence of colistin-resistant *Escherichia coli* in the feces of broiler chickens. Bacterial isolates will be isolated and presumptively identified on selective media. Species identification will be confirmed using PCR (polymerase chain reaction). ,The presence of *mcr* genes (*mcr1-mcr10*) will be detected using gene specific PCR. The minimum inhibitory concentration (MIC) of the *mcr* gene positive isolates will be measured by broth microdilution method (BMD). This study aims to bring about the environmental and public health risks of the misuse of antibiotics in the poultry industry and add to the increasingly accumulating evidence of antibiotic resistance in most developing nations, like Bangladesh.

1.2 Literature Review

The decades-old antibiotic colistin, sometimes referred to as polymyxin E, was formerly discontinued because of its nephrotoxicity. The advent of multidrug-resistant (MDR) bacteria, such as carbapenem-resistant Gram-negative bacteria including *Salmonella spp., E. coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and others, has led to its reintroduction (Sadek et al., 2021). Colistin lyses the cell by disrupting the outer

membrane of Gram-negative bacteria by attaching molecules to the lipid A structure of the lipopolysaccharides (Diani et al., 2024). Nevertheless, variations in the lipid A that decrease the affinity of colistin are the primary mechanism behind the development of resistance mechanisms. The modifications occurring due to the mobilized colistin resistance genes (*mcr*), which code a type of enzyme known as phosphoethanolamine transferase to attach a phosphoethanolamine moiety to lipid A, are most prevalent and cause colistin resistance (Gaballa et al., 2023).

In 2015, a breakthrough in our understanding of colistin resistance came when *mcr-1* was identified as the first gene in an *E. coli* strain found in a pig in China (Hassan & Kassem, 2020). In addition, the *mcr-1* gene was first identified in a human isolate in the United States; the patients do not travel abroad more than a year; they never contact cattle (Gaballa et al., 2023). Since then, other variations of *mcr* genes, including *mcr-2* to *mcr-10*, have been identified in diverse bacteria and in several geographical areas (Mondal et al., 2024). Such genes are often present on plasmids that facilitate their rapid spread worldwide as well as allow their vertical or horizontal transfer to the same or different bacterial species (Hassan & Kassem, 2020). The genetic sequences of these variations are different; nevertheless, they have the same objective, that is, to affect the bacterial lipid A, which reduces the ability of colistin to bind to and kill the bacterium. As a result of this difference, clinical and agricultural resistance can easily spread on a global scale (Mondal et al., 2024).

1.2.1 Mobilized Colistin Resistance (*mcr*) genes and Their Variants

Since this was the first mechanism of colistin resistance mediated by plasmids in Enterobacteriaceae, the successful discovery of the mobilized resistance to colistin (*mcr-1*) in 2015 was a paradigm shift in the war on antibiotic resistance. HGT of *mcr-1* rendered colistin, which is the last resort against multidrug-resistant Gram-negative infections, unreliable (Liu et al., 2016; Xavier et al., 2016). The phosphoethanolamine transferase coded by the *mcr-1* gene modifies the lipid A of bacterial lipopolysaccharide, lowering the affinity of colistin, and providing resistance (Xavier et al., 2016).

Since the discovery of *mcr-1*, other studies have revealed that *mcr* variations range across other species of bacteria and geographical locations. In 2016, it was found that *mcr-2* had nearly 76 nucleotide similarities with *mcr-1* in pigs and calves in Belgium (Xavier et al., 2016). Soon thereafter, *mcr-3* surfaced within human and pig *E. coli* isolates in China. These isolates were reported to harbor very large plasmids, usually of the IncHI2 type, with several other resistance genes (Yin et al., 2017). It is interesting to mention that based on the comparative analysis, at least six different variants of *mcr-3* were identified, meaning that the presence of antimicrobial pressure resulted in blistering evolution (Yin et al., 2017).

Globally, more varieties like *mcr-4* and *mcr-5* have also been found. While *mcr-5* was discovered in Salmonella isolates in Germany, *mcr-4* was initially identified in Salmonella enterica and *E. coli* isolates from animals and food products (Borowiak et al., 2017). These results highlighted the many plasmid backbones and genetic settings that promote the spread of *mcr genes* among bacterial populations (Borowiak et al., 2017).

Markedly, subvariants have also developed due to mutations in *mcr-1*. As an example, one enterotoxigenic *E. coli* (ETEC), which was isolated in a human stool sample, possessed the *mcr-1*.9. The difference was characterized as a single nucleotide polymorphism that introduced a change of amino acid in the catalytic active site of the enzyme. Its high mobility on an IncI2-type plasmid contributes to the potential of rapid spread in bacteria related to people (Zheng et al., 2018). Zheng et al. (2018) identify other point mutations, including novel variants like *mcr-1*-M6, as being associated with functional variances, including variation in level of resistance and additional amendments in the resistant mechanisms of the 6-strand resistant pathways.

The widespread occurrence of *metagenom*es in human, animal, and environmental samples is revealed as the result of metagenomic studies. In a large-scale experiment (N= 214,911 metagenomes across ecosystems), approximately 2 percent of the sequences encountered consisted of one or more of the nine known variants of *mcr*(Hendriksen et al., 2019). The findings show that *mcr* has stayed elastic and varied under antibiotic pressure.

1.2.2 Poultry as a Reservoir of Antimicrobial Resistance

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.

1.2.3 The use of Antibiotics in Poultry Production

Added to poultry water or their food, the antibiotics work as growth promoters and as a defense against bacterial infections. This is prevalent in LMICs, where there have been very minimal veterinary regulations and a lot of accessibility to over the counter (Khan et al., 2018). Farmers often use antibiotics (e.g., polymyxins, tetracyclines, sulfonamides, and fluoroquinolones) to enhance productivity. This improves flock health and productivity, but also subjects resistant strains of bacteria found in the gut microbiome of the chicken to a lot of pressure.

As the IntechOpen review states, extensive antibiotic application in food animals has facilitated horizontal transmission of the resistance genes, which increases the risk of humans being exposed to the resistant microorganisms through direct contact, environmental contamination, and the food system (Khan et al., 2018). This underlines the dual role of the poultry as an antimicrobial resistance source and carrier.

1.2.4 Emergence of Colistin Resistance in Poultry

Initially rejected in human medicine due to its toxicity, colistin has resurfaced as a final resort drug against Gram-negative microorganisms that are resistant to much more common antibiotics. The plasmid-mediated colistin resistance has become a serious problem due to its widespread application in chickens (*mcr* genes).

A Bangladeshi study established that the *mcr-1* gene was highly prevalent in chicken intestinal flora. The percentage of poultry isolates having the *mcr-1* gene was 36.4 percent, and 61.7

percent of *E. coli* isolates of 100 chicken samples were phenotypically resistant to colistin (Islam et al., 2020). What is indicative of this is that healthy hens carry pathogens carrying *mcr-1*, showing how the extensive agricultural use of colistin has created a reservoir of resistance genes that then may get introduced into human infections.

A survey conducted on *E. Coli* isolates in poultry in Nepal showed that colistin resistance was also very high, and *mcr-1* occurs in some of these isolates (Tamrakar Lama et al., 2019). These findings confirm that the westward spread of colistin resistance in South Asia is directly linked with the raising of fowl in the region.

1.2.5 Zoonotic Transmission of Resistance

Zoonotic transmission of resistance is 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*, Trung and Matamoros (2020) offer two directions the gene can take a path: through the environment and through gastrointestinal infections. 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.

1.2.6 Global Distribution and Alarming Trends

It is also clear that resistance is common in North Africa. Colistin-resistant *Enterobacterales* were found in poultry farms in Algeria, according to a study. The resistant isolates had both chromosomal and plasmid-mediated resistance determinants (Chaalal & Touati, 2020). This demonstrates that resistance is a worldwide issue that spreads over various geographical areas with extensive chicken production, not just in South Asia.

Additionally, Khan et al. (2018) observed that ecological niches that support the resistance cycle are created by the persistence of resistant bacteria in the fowl gut and their excretion into

the environment. Poultry farm waste contaminates water and land, spreading resistance genes outside of farms and into nearby towns.

1.2.7 Human Health Implications

The swift development of antibiotic resistance in chickens has significant health consequences for people. The danger of incurable illnesses in humans is increased by the spread of resistant bacteria through contaminated meat, poultry handling, and environmental exposure. The presence of *mcr-1* in healthy poultry highlights the possibility of resistant infections sneaking into the human food chain undetected, according to Islam et al. (2020). Furthermore, Trung and Matamoros (2020) issued a warning, pointing out that the worldwide movement of poultry products promotes the spread of resistant genes across borders. Therefore, a worldwide health catastrophe can swiftly develop from what starts as regional antibiotic overuse in poultry.

1.2.8 Colistin Resistance in Bangladesh: Poultry and Clinical Settings

a. Poultry Context

Concern over the incidence of colistin-resistant Escherichia coli with the *mcr-1* gene in poultry production in Bangladesh is on the rise. 94% of isolates had colistin MICs ≥ 4 μg/mL, and 13.5% tested positive for *mcr-1* in a study examining 104 extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates from poultry environments, including live bird markets (LBMs), rural poultry farms (RPFs), and household backyard poultry (HBPs). According to Amin et al. (2020), 10 isolates came from LBMs, three from RPFs, and one from an HBP.

Multi-drug resistance (MDR) was present in every isolate that tested positive for *mcr-1*. The majority also showed resistance to fluoroquinolones and sulfamethoxazole (12 isolates), aminoglycosides (10 isolates), and nitrofurantoin (3 isolates), in addition to their universal resistance to tetracycline and third-generation cephalosporins. The presence of conjugative plasmids with sizes ranging from 23 to 55 MDa was discovered by genetic research. Interestingly, the 55 MDa plasmid, found in two isolates, had several resistance genes, such as qnrB, rmtB, blaCTX-M-group-1, and blaTEM-1 (ESBL). After HI1 and N replicons were found, it was further categorized as a member of the IncF family (Amin et al., 2020).

These results demonstrate the importance of poultry habitats as reservoirs for plasmid-mediated colistin resistance, particularly LBMs with high levels of human-animal contact. The danger of zoonotic transmission has significantly increased when mobile genetic factors and MDR characteristics are present.

b. Clinical Context

In Bangladesh, there have also been reports of colistin resistance mediated by *mcr genes* in a clinical setting. 31.6% of 225 clinical isolates from diarrheal patients (adults, children, and infants) in a surveillance investigation showed phenotypic resistance to colistin (MIC > 2 µg/mL) (Sarker et al., 2024).

According to molecular screening, 15.5% of isolates had one or more *mcr* genes: two isolates had combinations of these genes, while seven isolates had *mcr-1*, seventeen had *mcr-2*, and thirteen had *mcr-3*. Significantly, MICs ≥ 4 µg/mL were present in 91.3% of *mcr*-positive isolates. MDR characteristics were present in all colistin-resistant bacteria, and there was a statistically significant connection ($p = 0.000$) between resistance and *mcr*gene carriage (Sarker et al., 2024).

A variety of clinically relevant pathogens, including Escherichia spp., *Shigella flexneri, Citrobacter spp., Klebsiella pneumoniae,* and *Pseudomonas parafulva*, were found to have *mcr* genes, according to the study. The increasing likelihood of treatment failures in colistin therapy, a last-resort antibiotic against carbapenem-resistant Gram-negative bacteria, is reflected in this broad distribution across several taxa (Sarker et al., 2024).

In Bangladesh, *mcr*-mediated resistance is well-established in both clinical and poultry settings. Colistin-resistant *E. coli* that carry *mcr-1* are common in poultry and are frequently associated with MDR plasmids that promote horizontal gene transfer (Amin et al., 2020). The effectiveness of colistin in treating multidrug-resistant infections is severely compromised in clinical cases due to a wide variety of bacteria that contain *mcr-1*, *mcr-2*, and *mcr-3* (Sarker et al., 2024).

1.2.9 Research Gap

Longitudinal study in Spain has observed the trend of colistin resistance through *mcr-1* gene, highlighting the necessity of long-term observation to find out the effectiveness of the resistant gene over time (Miguela-Villoldo et al., 2022). In this study, longitudinal surveillance in both urban and rural poultry farms should be tracked to observe the *mcr* gene evolution trend. A study in Bangladesh has detected a high prevalence of the *mcr-1* gene among genetically diverse *E. coli* isolated from broiler chicken, indicating horizontal dissemination of *mcr-1* rather than clonal expansion (Ahmed et al., 2020). Another study has reported that the rare *mcr-1.26* gene in *E. coli* isolates from poultry suggested further genomic study to understand evolution of the *mcr* variants. These observations are very important in Bangladesh, as here, the poultry serves as a major protein source and the control over antibiotic use remains limited. The presence of *mcr* gene in food animals creates a direct public health risk factor through the food chain, environmental contamination, and direct human-animal contact. To diminish these risks, there is an urgent need for advanced genomic surveillance, including whole-genome sequencing and plasmid typing, to trace the genetic context of these resistance elements, map their transmission pathways, and understand the evolutionary mechanisms driving their persistence and diversification. The genetic context whole genome sequencing and plasmid typing should be applied. A study in Lebanon detected an overlap of sequence types of *mcr-1*-positive *E. coli* between isolates from chickens and chicken farm litter that suggests either the birds picked up the organisms from the litter or that they emanated from the animals(Anyanwu et al., 2023).

A study in Bangladesh has found out of 150 samples from poultry cloacal swabs, house flies (Musca domestica), and pond water in Mymensingh, 18% of *E. coli* isolates showed phenotypic colistin resistance, 8% tested positive for the *mcr-3* gene via PCR (Sobur et al., 2019). Environmental sampling should be included in the future studies to understand the transmission dynamic as the research suggests a contamination cycle between birds and their ecosystem. A comparison between human clinical isolates and poultry is needed to identify the relation and understand the resistance source. A study in Kathmandu explored the prevalence and co-existence of colistin resistance gene *mcr-1* along with carbapenemase resistance gene *OXA-48* in *E. coli* isolates from poultry farms and human specimens, highlighting the need for

studies on co-resistance patterns (Muktan et al., 2020). Another study in Bangladesh report out of 3,295 Enterobacterales isolates from Bangladeshi poultry and associated environments, 533 (16.2%) harbored *mcr* genes, including *mcr-1*, *mcr-2*, and *mcr-3* where some isolates co-expressed multiple *mcr* genes, which increased colistin minimum inhibitory concentrations (MIC), suggesting emergence of high-level resistance (Anyanwu et al., 2023). Other antibiotic resistance needs to be investigated such as carbapenemases, ESBL etc. which would provide a broader idea of multidrug resistance patterns because Bangladesh is often part of a multidrug resistance landscape which underscore the co-resistance patterns.

A study by Dhaka University researchers illustrates that lytic bacteriophages offer a cost-effective, sustainable approach to controlling MDR *Salmonella* in poultry, highlighting environmental and public health safety benefits (Khan & Rahman, 2022). Strategies like phage therapy, probiotics, vaccination may offer sustainable and non-antibiotic pressure-based solutions that will help replacing the antimicrobial growth promoters and limiting emergence of resistance.

1.3 Aims and Objective

The overall aim of this study was to investigate the prevalence and genetic basis of colistin resistance of *E. coli.* isolated from broiler chicken feces in local markets and small poultry shops of residential areas, applying microbiological, molecular, and antimicrobial susceptibility techniques. The specific objectives of this project were as follows:

1. To isolate *E. coli* species from the stool samples of broiler chicken using selective media.
2. To confirm the identity of the isolated bacteria through PCR by using species-specific primers.
3. To identify the exact mobile colistin resistance genes (*mcr*_1-*mcr*_10) in *E. coli* isolates using molecular analysis (PCR).
4. To investigate the prevalence of *mcr*1 to *mcr*10 gene in the isolated strains.

5. To determine the minimum inhibitory concentration (MIC) of colistin against the confirmed isolates by using the broth microdilution method (BMD).
6. To compare the genotypic and phenotypic resistance of *E. coli* to colistin.

Chapter 2

2.1 Methodology

2.1.1 Media and Reagents

Hicrome UTI:

According to HiMedia Laboratories (n.d), this media is mainly used for probable identification of microorganisms that have the potential to cause urinary tract infections and confirmation of the presence of this type of microorganism is several samples like food, water, environmental etc. This media performs as a general nutrient agar for several kinds of microorganisms. It is applied in the identification of some both gram-positive and gram-negative bacteria. UTI agar helps to identify and differentiate among the organisms by producing different color depending on the reactions caused by the specific enzymes with chromogenic substrates (HiMedia Laboratories, n.d.).

Hicrome UTI Agar is used as a purpose of the identification of some gram-positive and some gram-negative bacteria (HiMedia Laboratories, n.d.). HiMedia Laboratories (n.d.) describes that it identifies the bacteria based on the different colored colonies depending on the produced enzymes from the reaction based on specific species. According to the source, coliforms and *E. coli* are found to be able to cleave the chromogenic substrates present in the media. Besides, in Hicrome UTI Agar media *E. coli* mostly produces pink-purple colonies, whereas Enterococci form blue colonies. The pink-purple colonies on *E. coli* are mostly formed when the enzyme ß-D-galactosidase cleaves the other chromogenic substrates. On the other hand, coliforms mostly show purple color colonies when both of the chromogenic substrates are cleaved (HiMedia Laboratories, n.d.).

The colonies that were found to be pink-purple on the Hicrome UTI Agar media after incubation are suspected to be *E. coli.* It was further isolated to be identified accurately.

Nutrient Agar:

Nutrient agar is known as an all-purpose media which provides an optimal condition for the growth of several kinds of microorganisms (TM Media, 2023). TM Media (2023) also explains that Nutrient Agar media is a basic type of media used for maintaining microorganisms. Besides, the source (TM Media, 2023) also explains that this media can be enriched using blood to cultivate fastidious microorganisms. Nutrient media has comparatively a simple formula and is mostly used for standard methods. HiMedia Laboratories Pvt. Ltd. (2024) describes that, this is basically a non-selective media used for the enumeration of bacteria which are not fastidious. This media is used to cultivate organisms retained from samples like feces, urine, water, food etc. (HiMedia Laboratories Pvt. Ltd., 2024). Nutrient Agar is mainly composed of yeast extract, beef extract, peptone along with nitrogen, carbon and vitamin compounds and sodium chloride helps in the case of osmotic balance (TM Media, 2023).

After identifying the target organism to isolate them Nutrient Agar Media has been used. The identified isolated colonies of *E. coli* are subcultured on the Nutrient Agar for them to be cultivated for stocking.

T1N1 Agar:

U.S. Food and Drug Administration (2017) describes that, T1N1 Agar is mainly known as the Tryptone Salt Agar where the concentration of NaCl is 1%. The basic of the ratio of the concentration of the NaCl, sodium Chloride and Agar is 1:1:2, which refer to that NaCl 10g, Sodium Chloride 10g and Agar 20g in 1L of distilled water (U.S. Food and Drug Administration, 2017).

After isolating the bacteria from the previous culture media and subcultured on the Nutrient Agar, pure colonies are identified and stocked by performing a stabbing method in T1N1 media for preserving the bacterial culture for future study.

Paraffin oil:

According to Khan (2024), Paraffin oil is a type of mineral oil which helps in the case of preservation of bacterial culture. When liquid paraffin oil is added to the top of the culture media

it helps to form a layer as a barrier between the bacterial colonies and environmental oxygen (Khan, 2024) For the preservation sterile paraffin oil is added over the stabbed culture grown under optimal temperature and kept at room temperature. Paraffin oil serves as a media for preventing dehydration (Bihar Animal Sciences University, 2020).

2.1.2 Buffers

TBE Buffer:

TBE or Tris Borate EDTA is one of the most common running buffers which is used in Gel Electrophoresis of nucleic acid (Kuntz, 1997). According to the *Nagoya Medical Journal* (n.d.), TBE buffer is more suitable for clear band of smaller DNA on Agarose Gel Electrophoresis. Moreover, this buffer is stable and has a higher buffering capacity which is important for several experiments (Nagoya Medical Journal, n.d.).

According to Gold Biotechnology (2022), in order to make 10x 1L TBE buffer:

Name of Reagent	Amount (g)
Tris Base	108 g
Boric Acid	55 g
EDTA	7.5 g

TE Buffer:

Tris-EDTA Buffer is often used to elute, protect and preserve DNA from any kind of degradation if kept for a long period of time (Kim et al., 2014). According to Verve Team (2016), this is a pH (8.0) buffer which chelates cations i.e. Mg2+. EDTA is known to inactive the enzyme nuclease as they bind to metal cation which are responsible to activate nuclease (Verve Team, 2016).

To prepare 500 mLTE buffer 10x stock solution Kasajima et al. (2004) –

Name of Reagent	Amount (g)
Tris-HCL	7.88
EDTA	1.46

PBS Buffer:

AT Bioquest, Inc. (2025) states that, PBS buffer is known as Phosphate Buffered Saline which is broadly used in biological applications. It also describes, PBS is capable of maintaining pH and ion concentration of human body. PBS buffer is also knowns to be non-toxic to cell and easy to prepare with good shelf life. Therefore, PBS buffer is mixed with the sample before plating them on the culture plates (AAT Bioquest, Inc., 2025).

According to AT Bioquest, Inc. (2025), in order to prepare 1x 1L of PBS Buffer:

Name of Reagent	Amount (g)
Sodium Chloride	8
Potassium Chloride	0.2
Sodium Phosphate Dibasic	1.44
Potassium Phosphate Monobasic	0.246

2.1.3 Other Media and Reagent

Agarose:

Agarose is known to be a subfraction mixture of agar which is extracted from red Algae. In case of gel electrophoresis, the flexibility and pore of the gel has the direct impact on the result (Grossman & Soane, 1995). Agarose is used for the separation of proteins, nucleic acids etc. (Grossman & Soane, 1995). By altering the concentration of the agar, the pore size of the gel can be transformed according to the sample size, i.e. larger pores are required if the sample size is large and vice versa (Grossman & Soane, 1995).

For this study 1.5% of agarose is used for the gel preparation in gel electrophoresis.

Luria Bertin Broth

According to the HiMedia Laboratories (2024), Luria Bertin Broth is a media with tryptone and vitamin B complex from yeast extract. It also contains sodium chloride which provides sodium ions. This helps in case of membrane transport and maintains osmotic balance. This helps in the case of isolated microorganisms (HiMedia Laboratories, 2024).

LB is used for preserving isolated specific bacterial culture with the addition of glycerol after incubation for 24 hours.

Glycerol:

Waksman (1956) describes that glycerol is used for glycerol stock of bacterial culture, which helps the bacterial culture under low temperature by preventing cell damages (Waksman, 1956). Various types of microorganisms can be preserved by using glycerol broth preservation method (Waksman, 1956).

For this 500µL of 50% glycerol and 500µL of bacterial culture grown in LB is taken. And further recommended to store at -20°C

Muller-Hinton Broth:

HiMedia Laboratories (2015) states that, MHB is a non-selective media which has a simple formula thus allows growth of a large variety of microorganisms. Muller Hinton Broth is often used for minimal inhibitory concentration through dilution. It is also used for Antimicrobial Susceptibility Test. This media contains beefs extract, casein acid, nitrogen and carbon source which are essential for microbial growth. The components present in this makes it suitable for MIC determination (HiMedia Laboratories, 2015).
MHB is prepared through heating until dissolved and autoclaved for sterilization. Then after it came to the room temperature, it us used for MIC Test.

2.2 Methods

2.2.1 Sample Collection

Broiler chickens' fresh fecal specimens were collected in clean and sterile containers using a sterile spatula. 6–8g of feces were obtained per sample. A total of 50 samples were collected for this study from various locations across Dhaka city, focusing primarily on food markets and small poultry shops situated in residential areas. The sampling sites included Merul Badda, Badda, DIT Project, Town Hall Bazar, Lalmatia residential area, Krishi Market, Kawran Bazar, and Mohammadpur.

Table 1: Sample Collection Location Coordinates

Location Name	Latitude	Longitude
Merul Badda	23.7806	90.4256
Badda	23.7810	90.4320
DIT Project	23.7830	90.4090
Town Hall Bazar	23.7940	90.4120
Lalmatia	23.7800	90.3760
Krishi Market	23.7835	90.4105
Kawran Bazar	23.7380	90.3980
Mohammadpur	23.7720	90.3660

These locations were selected to provide a representative overview of broiler chicken sources commonly accessed by the public in both commercial and residential settings. The sealed sample bottle was labeled properly and kept in an icebox to maintain temperature. So that the freshness of the samples are maintained till the samples are processed. The sample was transported to the laboratory within an hour of collection to preserve the integrity of the sample and minimize the overgrowth of the bacteria.

Fresh fecal samples from broiler chickens were collected to accurately assess the chickens' gut bacterial populations. A sterile spatula was used to collect fecal specimens in order to preserve the originality of the samples and prevent contamination. 6–8 g of feces were collected per sample to guarantee that we get enough material for the follow-up actions and microbiological analysis. Each sample was collected from a different cage. The samples were handled in aseptic conditions and placed in clean, sterile containers that were immediately sealed to avoid contamination or spillage before they were transported.

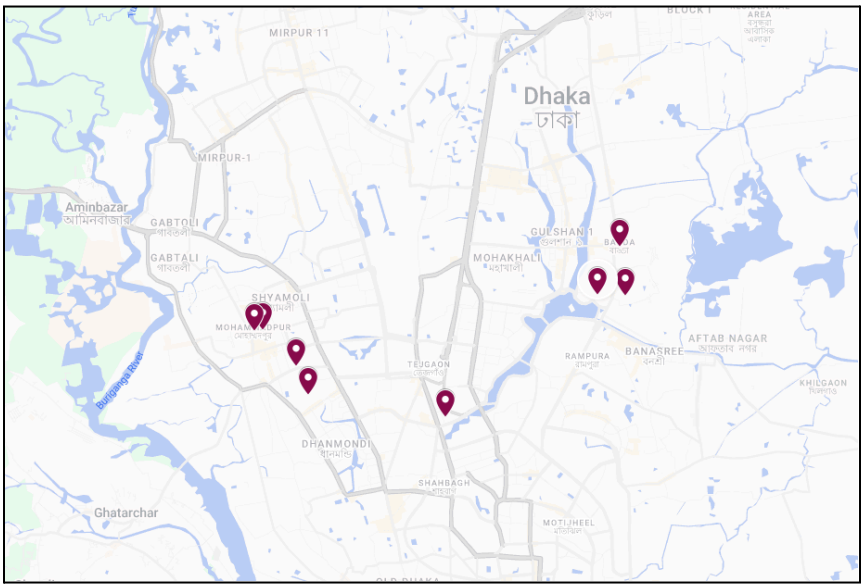

Figure 1: Sample Collection Locations (GIS)

2.2.2 Sample Processing

The sealed sample containers were stored in an icebox to stabilize the samples and prevent overgrowth of their bacterial microflora. It is essential since the fluctuations of temperature might cause a bloom in non-target bacteria, which can affect the results. It also helps in maintaining the cold temperature during transportation, which is crucial to preserve the viability and authenticity of the microbial flora present in the samples especially in case of antibiotic resistant bacteria (Zhao et al., 2015).

The sample bottles were well-labeled with IDs about which date of collection, time and source to ensure proper traceability in addition to avoiding mix-ups while dealing at a lab. All samples were transported to the laboratory within 1 hour after collection in order to prevent major alterations of their bacterial composition caused by environmental impacts and time storage (Serrano et al., 2018). Reduced delay between collection and laboratory analysis without treatment of samples over a longer time period preserves the integrity of the sample, especially

with respect to bacterial load and antibiotic resistance profiles that can degrade if samples are not treated in time. (Moussa et al., 2019).

All of the samples were processed on the day of arrival in the laboratory to ensure prompt isolation and identification of colistin-resistant *E. coli* and to minimize overgrowth or contamination with environmental strains.

2.2.3 Sample Preparation and Primary Culture

From each sample, 1 g of fecal sample was mixed with 400 µL of sterile Phosphate Buffered Saline (PBS) into falcon tubes. The falcon tubes were vortexed well to form a homogeneous suspension. The suspension was then streaked on HiCrome UTI Agar and incubated at 37°C for 18–24 hours for selective bacterial growth, as per guidelines. (*Islam* et al., 2020) After incubation, examination of HiCrome UTI Agar (HiMedia, India) plates for typical colonies was performed. A chromogenic media, HiCrome UTI Agar, is the most famous selective differential medium and contains various chromogenic substrates thus enabling identification based on colony color and morphology (Ramya et al., 2020).

In this study, colonies showing chromogenic patterns of Escherichia coli (usually pink to red due to β-D-galactosidase activity) and Klebsiella pneumoniae (typically metallic blue or green depending on enzyme activity. (Ramya et al., 2020). The selected colonies were considered presumptive isolates. An isolated colony was aseptically inoculated using a wire loop streaking onto Nutrient Agar slants for encouragement of pure cultures. Inoculated plates were then incubated between 18 to 24 h. at a temperature of 37°C to ensure adequate time for colony growth and isolation. Nutrient Agar was a non-selective medium, and thus chromogenic agar did not allow the isolates to obtain an inhibition-free zone to further undergo biochemical and molecular characterization, including PCR-based species confirmation and antibiotic susceptibility testing.

2.2.4 Bacterial Stocking

Preservation Using Tryptone-Salt (T1N1) Agar

Pure bacterial colonies isolated from Nutrient Agar plates were carefully picked using sterile inoculating loops and streaked onto Tryptone-Salt (T1N1) agar plates. The plates were incubated at 37°C for 18–24 hours to allow for optimal colony growth. T1N1 agar is a nutrient-rich medium that provides essential salts and amino acids, maintaining the viability and stability of the bacterial isolates over time (Atlas, 2010). After incubation, the plates with well-isolated colonies were sealed with parafilm to prevent desiccation and contamination and were stored at 4°C for short- to medium-term preservation. This method ensures that the phenotypic characteristics of the bacteria remain stable and allows for repeated use of the isolates in downstream microbiological and molecular assays without significant changes in morphology or growth patterns.

Preservation Using Glycerol Stocks

For long-term storage, pure bacterial isolates were first cultured in Luria-Bertani (LB) broth and incubated at 37°C with shaking at 150 rpm until they reached the logarithmic phase of growth, ensuring that actively dividing and healthy cells were available for cryopreservation. Once the cultures achieved sufficient turbidity, an equal volume of sterile 50% glycerol solution was added to the broth culture, resulting in a final glycerol concentration of approximately 25%. This mixture was gently homogenized and aliquoted into sterile cryovials, which were then stored at −20°C. Glycerol acts as a cryoprotectant by preventing ice crystal formation that could damage cell membranes during freezing, thus preserving the viability of the bacterial cells for extended periods (Sambrook & Russell, 2001). These glycerol stocks can be revived by thawing a small aliquot and inoculating it onto fresh Nutrient Agar or LB plates, providing a reliable source of bacterial isolates for molecular studies such as PCR detection of colistin resistance genes and virulence determinants.

2.2.5 DNA Extraction using Modified Heat Shock Method

DNA extraction was performed using a modified heat shock method. This procedure was adapted from the procedure described by Dashti et al. (2009).

Steps of the method:

1. Picked a single colony from Nutrient Agar plate, then inoculated and suspended in TE buffer:

Initially, a single colony was selected by making sure there were no other bacterial colonies or contaminants on the Nutrient Agar plate to obtain a good strain and not a mixed culture that can affect any molecular study downstream. The bacterial colony was then suspended in Tris-EDTA (TE) buffer, where the presence of tris could maintain a constant pH and chelate divalent cations such as Mg^{2+}, which would inhibit DNAase activity to protect DNA integrity.

2. The MCT tubes were incubated in dry heat bath at 95°C for 20 minutes:

The bacterial suspension is then incubated at 95°C in a dry heat bath for 20 min. It ruptured the cell wall and membrane of the bacteria, ultimately inhibiting it and releasing intracellular materials like DNA. This temperature will denature proteins and membranes, but the DNA is still intact enough to use for molecular applications like PCR.

3. It was then centrifuged at 10,000 rpm for 10 minutes:

Cell samples suspended in TE buffer after the lysis were then centrifuged at 10,000 rpm for 10 minutes to pellet cell debris. Then the DNA was left in the supernatant, and denatured proteins plus membrane fragments formed a pellet at the base of the tube.

4. The supernatant, containing the DNA was collected and stored at -20°C for further molecular analysis. (Downstream Analysis):

The supernatant containing crude DNA was gently separated and transferred to an MCT and stored in -20 °C until further use in downstream molecular analysis. As identified by Dashti et al., this approach is highly inefficient. Methods innovative and cost-effective DNA purification

method (Marchini et al., 2009) was assessed for purity using both quantitative PCR and with standard molecular genetics techniques that include both genotyping to confirm individual identity and amplification of mitochondrial control region samples.

2.3 PCR and Gel Electrophoresis

2.3.1 Molecular Identification and Confirmation of Escherichia coli Isolates

The identification and confirmation of *Escherichia coli* (*E. coli*) isolates were performed using a specific PCR assay targeting the ecotin precursor gene. This method is widely used due to its specificity and reliability in detecting *E. coli* strains in various samples, including poultry and clinical samples. (Punom et al., 2020)

The primers used in this study were:

ECO-F: 5'-GACCTCGGTTTAGTTCACAGA-3'

ECO-R: 5'-CACCACGCTGACGCTGACCA-3'

These primers are designed to amplify a specific region of the ecotin precursor gene, which is present in *E. coli* but absent in most other bacteria, thereby ensuring the specificity of the assay. (Punom et al., 2020)

PCR Reagents and Conditions

1. PCR Master Mix**:** Takara EmeraldAmp® PCR Master Mix (2X), (Takara Bio Inc., Japan) was used for the reactions to occur. This master mix contains DNA polymerase, dNTPs, $MgCl_2$, and a reaction buffer, providing optimum set-up for amplification with minimal error rates.
2. Nuclease-Free Water**:** Takara Nuclease-Free dH_2O (Takara Bio Inc., Japan) was used to dilute the PCR mix, ensuring a nuclease-free set-up that could degrade the DNA or primers.

3. DNA Template**:** Thermally lysed DNA extracted from presumptive *E. coli* isolates was used as the template.

The Amplification was performed using a Bio-Rad T100 Thermal cycler. (Bio-Rad Laboratories, n.d.) It consisted of initial denaturation at 95 °C for 5 minutes, amplification for 30 cycles which consisted of denaturation at 94 °C for 45 seconds, annealing at 52 °C for 45 seconds, and extension at 72 °C for 60 seconds. Following the amplification cycles, an additional extension was performed at 72°C for 5 minutes. The conditions were optimized and used for the PCR for confirmation of *E. coli*. The approximate size of the expected product size of PCR with ECO-F and ECO-R primers is 582 bp. The size of this amplicon is in agreement with previous work using these primers for the detection of *E. coli.* (Punom et al., 2020)

2.3.2 Detection of *mcr* Genes with PCR

Mobile Colistin Resistance (*mcr*) genes associated with colistin resistance in bacteria were evaluated using PCR-based detection methods in this study. A dire emergence of colistin resistance across the globe, both within hospitals and farms, limits the value of a last-line-of-defence antibiotic. At least 10 *mcr* genes (*mcr*1-*mcr*10) are characterized, and in this study, primers targeting all *mcr* genes were applied to guarantee full coverage of *mcr*-mediated colistin resistance. Of these, *mcr*1 to *mcr*5 genes were targeted by Multiplex PCR, while *mcr-6* to *mcr-9* genes were also analysed using a separate multiplex PCR. For the individual analysis of each *mcr-10* gene, a single PCR reaction was performed.

Multiplex PCR conditions for *mcr*1 to *mcr*5 were performed in 15 µL reaction mixtures containing 15 µL EmeraldAmp® PCR Master Mix (2X) (Takara Bio Inc., Japan), high-fidelity DNA polymerase, dNTPs, $MgCl_2$, and reaction buffer. Two microliters of thermal lysed DNA was added as the DNA template along with 1 µL of each forward and reverse primer specific for *mcr*1 to *mcr*5, and 3 µL of nuclease-free water in this reaction. The PCR was started with denaturation at 94 °C for 15 minutes, so as to obtain full denaturation of the DNA. This was then followed by 25 cycles of 30 s denaturation at 94 °C, 90 s annealing at 55 °C and 60 s extension

at 72 °C. After amplification cycles, a final elongation step was conducted at 72°C for 10 minutes for completion of all remaining DNA synthesis and kept at 4°C until analysis.

Multiplex PCR was also done for the detection of *mcr*6 to *mcr* 9 genes. For the multiplex PCR (*mcr*6 to *mcr*9), the reaction components included 12.5 µL of EmeraldAmp® PCR Master Mix (2X), 1 µL of each *mcr*6 to *mcr*9 forward and reverse primer, 2.5 µL of nuclease-free water, and 2 µL of thermal lysed DNA. The amplification began with an initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification. The cycles included denaturation at 95°C for 30 seconds, annealing of the primers at 55°C for 30 seconds, and extension at 72°C for 60 seconds. Lastly, a final extension step was performed at 72°C for 10 minutes and the reaction was maintained at 4°C.

Finally, the PCR corresponding to the detection of the gene *mcr*10 was performed with a single set of primers. The reaction mixture consisted of 6·5 µL of EmeraldAmp® PCR Master Mix (2X), 1 µL each of the forward and reverse primer (*mcr*10_fw, *mcr*10_rev), 2·5 µL of nuclease-free water, and 2 µL of the thermally lysed DNA. For *mcr*10, amplification was performed as follows: an initial denaturation step at 95°C 3 min, 30 cycles at 95°C 30 sec, 55°C 30 sec, 72°C 60 sec. Subsequently, a final elongation was performed at 72°C for 10 minutes and the reaction kept at 4°C.

The PCR products for all the *mcr* genes were analyzed using gel electrophoresis, after amplification, to confirm the presence of the target genes. Ethidium bromide-stained 1.2% agarose gel using TBE buffer was visualized under UV light. PCR products sizes were determined with a 100 bp DNA ladder used as a molecular size marker. The agarose gel was run at 110V for 50 min to separate PCR products based on their size.The gel was visualized under UV transilluminator and the bands corresponding to the expected amplicon sizes of each of the *mcr* gene were confirmed. The sizes of the amplified bands were then verified by comparing the amplification products to the DNA ladder.

Primer list of *mcr* genes:

Table 2: List of *mcr* gene detection primers and their sequences with amplification size

Gene	Primer Name	Primer Sequence	Amplification Size
mcr-1	*mcr*1_320bp_fw	5'-AGTCCGTTTGTTCTTGTGGC-3'	320 bp
	*mcr*1_320bp_rev	5'-AGATCCTTGGTCTCGGCTTG-3	
mcr-2	*mcr*2_700bp_fw	5'-CAAGTGTGTTGGTCGCAGTT-3'	715 bp
	*mcr*2_700bp_rev	5'-TCTAGCCCGACAAGCATACC-3'	
mcr-3	*mcr*3_900bp_fw	5'-AAATAAAAATTGTTCCGCTTATG -3'	929 bp
	*mcr*3_900bp_rev	5'-AATGGAGATCCCCGTTTTT-3'	
mcr-4	*mcr*4_1100bp_fw	5'-TCACTTTCATCACTGCGTTG-3'	1116 bp
	*mcr*4_1100bp_fw	5'-TTGGTCCATGACTACCAATG-3'	
mcr-5	*mcr*5_fw	5'-ATGCGGTTGTCTGCATTTATC-3'	1644 bp
	*mcr*5_rev	5'-TCATTGTGGTTGTCCTTTTCTG-3'	
mcr-6	*mcr*6_fw	5'-AGCTATGTCAATCCCGTGAT-3'	252 bp
	*mcr*6_rev	5'-ATTGGCTAGGTTGTCAATC-3'	
mcr-7	*mcr*7_fw	5'-GCCCTTCTTTTCGTTGTT-3'	551 bp
	*mcr*7_rev	5'-GGTTGGTCTCTTTCTCGT-3'	
mcr-8	*mcr*8_fw	5'-TCAACAATTCTACAAAGCGTG-3'	856 bp
	*mcr*8_rev	5'-AATGCTGCGCGAATGAAG-3'	
mcr-9	*mcr*9_fw	5'-TTCCCTTTGTTCTGGTTG-3'	1011 bp
	*mcr*9_rev	5'-GCAGGTAATAAGTCGGTC-3'	
mcr-10	*mcr*10_fw	5'-TATCCTGAGCCGTCTTGAAC-3'	386 bp
	*mcr*10_rev	5'-GGATCAGCGAAGCGAGCA-3'	

The sequences of *mcr*1 to *mcr*5 primers were obtained from Rebelo et al. (2018), while primer sequences for *mcr*6 to mc10 are adapted from Borowiak et al. (2020). In order to detect *mcr* genes, multiplex PCR assays were performed using these primers.

2.3.3 Gel Electrophoresis and Visualization

PCR products were run on a 1.5% agarose gel with ethidium bromide staining. Molecular size marker was a 50 bp DNA ladder. The gel was electrophoresed at 110 V for 50 min and bands were visualized on a UV transilluminator. The presence of a single band of approximately 582 bp was interpreted as a positive result for *E. coli.* Appropriate controls were included for the validation of the PCR assay. Positive Control, DNA of a known *E. coli* strain was added to the positive control to ensure concentrations of PCR reagents and amplification conditions were valid. Negative Control**,** A PCR reaction mixture without ANY DNA template was included to check for contamination or non-specific amplification. By using these controls, we could validate the results and ensure the accuracy of the PCR assay.

2.4 Colistin Susceptibility Testing with Microbroth Dilution 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.

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 has 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 contains 66.3% pure colistin. Therefore, the final concentration of colistin in the stock solution was 89,100 µg/mL.

Intermediate Stock Preparation :

In another Falcon tube, put 57.5 µL of your master stock solution and add water to make 20 ml total. This new solution will have a colistin concentration of 256 µg/mL. According to the formula $C_1V_1=C_2V_2$

$V_1 = (C_2 \times V_2) / C_1$

$V_1 = (256\ \mu g/mL \times 20{,}000\ \mu L) / 89{,}100\ \mu g/mL$

$V_1 \approx 57.5$ µL

Therefore, $V_2 = 57.5$ µL

C1 is the initial concentration (89,100 µg/mL),

V1 is the volume of the initial concentration used here, 57.5 µL,

C_2 is the desired concentration of the final solution

V_2 is the desired final volume (20 mL).

Preparation of Cation-Adjusted Muller-Hinton Broth:

The next step is to prepare Cation-Adjusted Mueller-Hinton Broth, or CAMHB. CAMHB is a growth medium that is used in susceptibility testing for bacteria. The process begins by creating 2X Mueller-Hinton Broth (MHB) and then adding calcium (Ca^{2+}) and magnesium (Mg^{2+}) salts. The salts are 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 is followed by adding 2 mL of the calcium solution and 1 mL of the magnesium solution to 100 mL of MHB. This then constitutes the finished CAMHB. (Islam et al., 2020). Adding these cations enhances bacterial growth and is essential to accurate antimicrobial susceptibility testing, especially for Gram-negative organisms.

Preparation of the Working Stock Solutions:

The working stock solutions of colistin must be made by serial dilution. In this process, 10 microcentrifuge tubes (MCTs) will each be labeled with a different concentration of colistin and 750 µL of CAMHB added to it. From the intermediate stock (256 µg/mL), 750 µL is 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 serial dilution.

Table 3: 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

Connecting to the last MCT, 750 µL is transferred and the following final concentrations are thus 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 is removed after the last dilution to prevent contamination between tubes.

Preparation of Bacterial Culture:

Take an isolated colony from a Mueller-Hinton Agar (MHA) plate, and inoculate it in Mueller-Hinton Broth (MHB). Incubate the broth in a shaking incubator at 37°C for 18-24 hours. Culture the bacteria in Muller-Hinton Agar (MHA) by streaking incubate the culture plate for 18-24 hours at 37C. Select an isolated colony from the plate and inoculate in MHB and incubate it at 37 °C overnight in a shaking incubator. Then, take 100 µL of bacterial culture and add it to CAMHB to adjust the OD to 0.5 McFarland.

Microtiter Plate Layout

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

Well 1 will have the concentration of 64 ug/mL and well 10 will have 0.125 ug/mL concentration, and the 11^{th} well is for positive control with no antibiotic, and the 12^{th} well is for negative control with no bacterial culture.

	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/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)
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/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)
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/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)
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/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)
F												
G (Bacteria 3)	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)
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)

Figure 2: Microtiter Plate layout for MIC of Colistin in *E. coli*

After inoculation, the microtiter plate is incubated at 37°C for 18-24 hours. Following incubation, the wells are visually checked for bacterial growth. The MIC is considered to be the

lowest concentration of colistin in which no visible growth is detected. This concentration is the effective inhibitory concentration of colistin against the bacterial isolate.

Chapter 3

3.1 Results

3.1.1 Selective-Media based Identification of *E. coli*

The isolates were sub cultured on HiCrome UTI Agar, a selective media for differentiating *E. coli* as well as other Gram-negative bacteria, after processing the 50 broiler chicken samples and inoculation. When incubated, different colorful colonies on the agar ranged in color from purple to pink, which were *E. coli* colonies. A total of 47 suspected *E. coli* colonies were picked up. Pure cultures were prepared after streaking the suspected *E. coli* colonies on Nutrient Agar plates. These pure cultures were subsequently preserved for more detailed investigation, including further phenotype and genotype examinations. The direct inoculation on HiCrome UTI Agar for *E. coli* from the 50 broiler-chicken stool samples successfully isolated and differentiated for *E. coli,* and it also helped for accurate characterization of colony appearance.

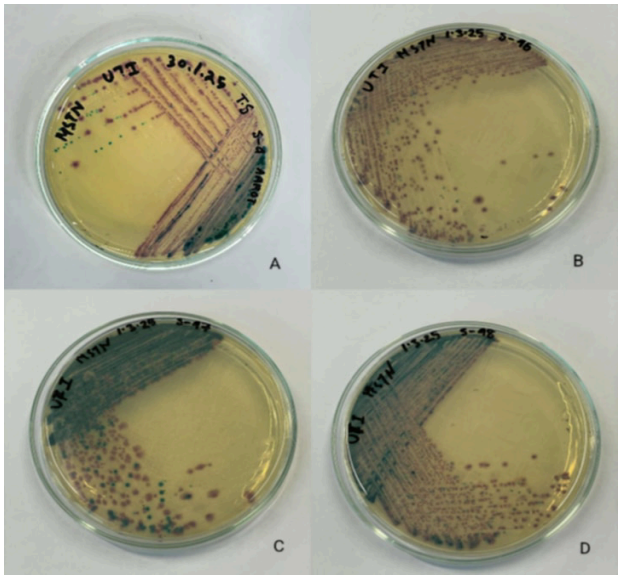

Figure 3: HiCrome UTI Agar (HiMedia, India) after Sample Inoculation A, B, C, & D UTI agar plates exhibiting colonies of different morphologies

3.1.2 PCR-Based Identification of *E. coli*

A total of 50 samples were collected out of the 50 samples 47 were suspected *E. coli.* The suspected *E. coli* were determined based on their morphology and chromogenic characteristics on HiChrome UTI agar media (HiMedia, India). After the identification PCR 41 *E. coli* was confirmed using ECO-F and ECO-R primers, which amplifies the 16s rRNA region. A positive control from A known *E. coli* was used to verify the results. All the *E. coli* positive samples showed bands at 585 bp similar to the positive control.

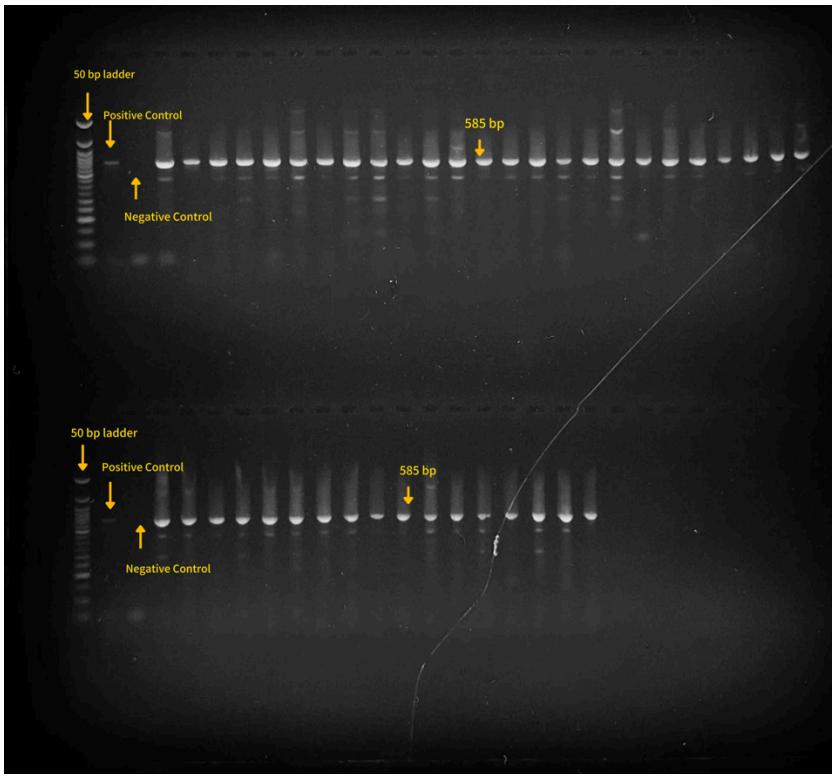

Figure 4: Gel electrophoresis result of *E. coli* PCR products

A positive control was used in each PCR experiment (i.e., a known strain of *E. coli*). This control was employed to check that PCR results were truthful and reliable. The well from the *E. coli* positive control sample showed a sharp, bright band of expected size 585bp for positive amplification and no band for negative amplification. The detected *E. coli* strains were

subsequently examined, and all *E. coli* positive strains also showed a band of 585 bp, the same in size with the band of the positive control.

Of the 47 presumptive *E. coli* isolates, 41 were identified as *E. coli* by PCR amplification of the 16s rRNA gene and had intense bands at 585 bp. The remaining 6 samples with no amplification were *E. coli* PCR negative. This PCR-based confirmation confirmed the presence of *E. coli* in most of the presumptive samples, and the application of HiChrome UTI Agar for the preliminary screening could be a trusted tool.

3.1.3 PCR-based Detection of *mcr* genes Detection Result

For *mcr-1* to *mcr-5* detection, multiplex PCR was performed. Then multiplex of *mcr-6* to *mcr-9* was performed, and PCR of *mcr-10* was performed only with the *mcr-10* gene-specific primers. A total of 79 *mcr* genes were detected in this PCR-based *mcr* gene detection. 3 *mcr-1* (7.3%), 33 *mcr-3*(80.5%), 12 *mcr-6*(29.3%), 2 *mcr-7* (4.9%), 28 *mcr-8* (68.3%) and 1 *mcr-9* (2.4%).

The detection of *mcr* determinants, two different multiplex PCR assays, for *mcr-1–mcr-5* and for *mcr-6–mcr-9*, were employed in the present study. Furthermore, *mcr-10* was detected by PCR using *mcr-10*-specific primers. This method yielded colistin resistance of 41 PCR-confirmed Escherichia coli isolates, and 79 *mcr* genes in all. Prevalence of each *mcr* gene was measured, and diverse *mcr*-resistance model was observed in the bacterial population.

These findings indicates that there is a diverse distribution of colistin resistance genes in *E. coli* found in broiler chicken gut. Among them *mcr-3* gene is the most prevalent. In *mcr-1* to *mcr-5* multiplex PCR *mcr-1* and *mcr-3* genes are detected 3 *mcr-1* Genes were detected, where 1 isolate had both the *mcr-1* and *mcr-3* Genes.

First, specific primers for *mcr-1* to *mcr-5* genes were used for the multiplex PCR reaction to detect *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5*. Three isolates harbored *mcr-1* gene, the first resistance plasmid gene for colistin. Though the rate of *mcr-1* was low in this study, its detection tones the catching of colistin resistance, although it was a lower *mcr* type than the other *mcr*

genes. *mcr-3* gene, which we connected with moderate levels of resistance to colistin, and occurred most frequently, in 33 isolates. This high incidence indicated that *mcr-3* contributed greatly to the dissemination of colistin resistance, which is of particular importance in clinics and environments.

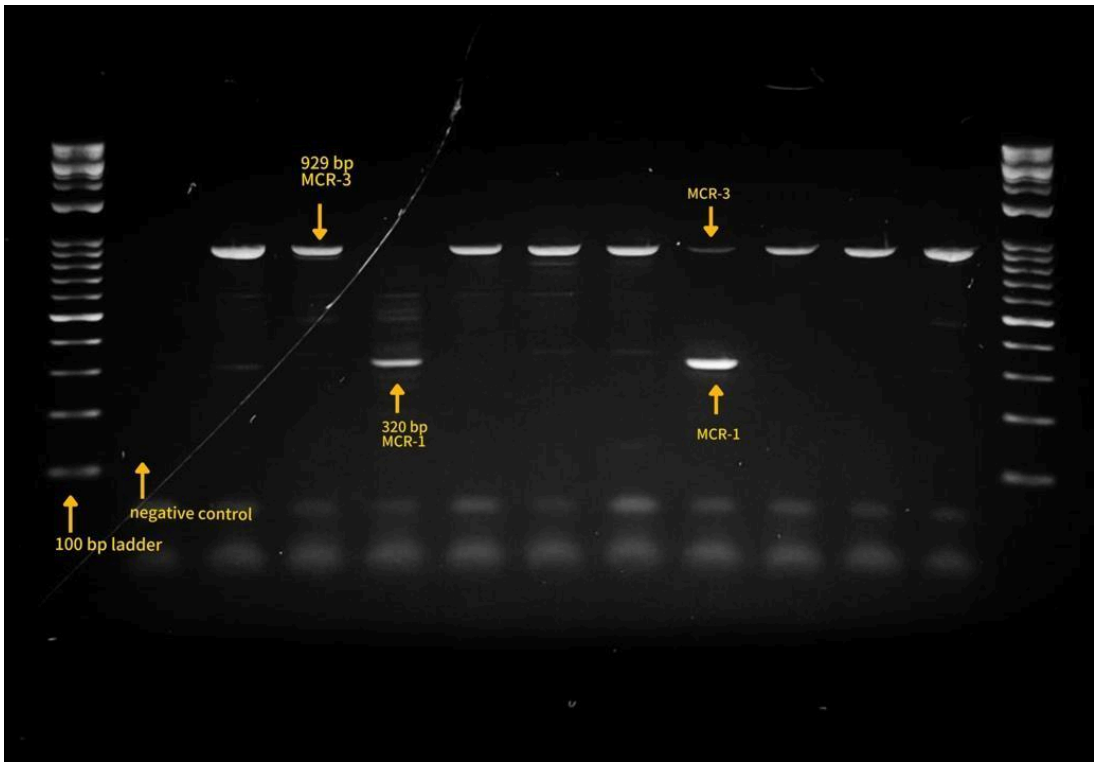

Figure 5: *mcr-1* to *mcr-5* Multiplex PCR, Gel Electrophoresis Result. Two *mcr-1* showed positive result, showing bands at 320 bp compared to the 100bp DAN ladder and *mcr-3* showed positive results for 9 out of 10 samples at 929 bp compared to the 100 bp DNA ladder.

12 isolates were found to carry the *mcr-6* gene, which indicates a moderate prevalence of the *mcr-6* gene among the *E. coli* isolates. *mcr-7* gene was found in 2 isolates, corresponding to a relatively low prevalence in the study population. Although the rate of *mcr-7* detection was lower, *mcr-7* is an important gene as it is involved in high-level resistance to colistin, and its ditection in this study also suggested that it is a crucial gene. *mcr-8*, as another notable gene, was harbored by 28 isolates, and it was the second most common *mcr* gene after *mcr-3*. *mcr-8* was predominantly detected in *E. coli* and its predominant occurrence, as found in this study, indicates that this gene could play an important role in colistin resistance. (Lemlem, et al,. 2023)

E. coli isolates that harbored *mcr-6* genes also possessed *mcr-8* genes in 11 out of 12 cases. The *mcr-8* gene is 2nd most prevalent right after the *mcr-3* gene.

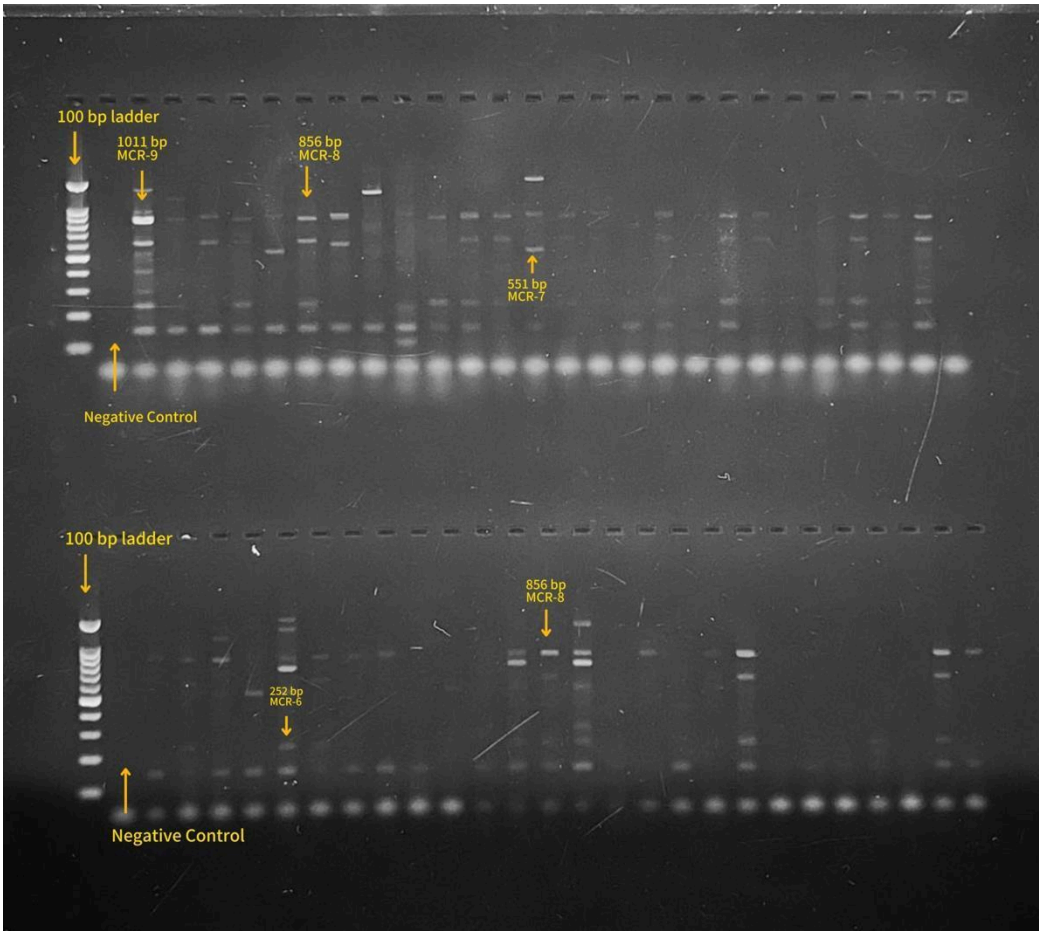

Figure 6: *mcr-6* to *mcr-9* Multiplex PCR, Gel Electrophoresis Result

One isolate was observed to be positive for the *mcr-9* gene, which highlights its modest frequency in the present study. Nonetheless, *mcr-9* is relevant because colistin resistance and additionally contributes to the reservoir of *mcr*-encoded resistance elements in Gram-negative bacteria. Meanwhile, none of the 41 PCR-confirmed *E. coli* isolates were found to have *mcr-10* gene. The absence of *mcr-10* indicates that it is either a rare or not common in the *E. coli* isolates of this study. Although *mcr-10* was not found in China, the existence of other *mcr* (such as *mcr-3* and *mcr-8*) raises a considerable challenge for the treatment of colistin resistance infection. The following table shows the PCR results of *mcr-1* to *mcr-10* genes.

Table 4: PCR results of *E. coli* with *mcr*-(1-10) Primers

Sample no.	Primer Name.										
	Eco.	*mcr*1 (320 bp)	*mcr*2 (700 bp)	*mcr*3 (900 bp)	*mcr*4 (1100 bp)	*mcr*5 (1600)	*mcr*6 (252 bp)	*mcr*7 (551 bp)	*mcr*8 (856 bp)	*mcr*9 (1011 bp)	*mcr*10
E. coli (UTI)(S#E)											
1	P	N	N	P	N	N	P	N	P	P	N
2	P	N	N	N	N	N	N	N	P	N	N
3											
4	P	N	N	P	N	N	N	N	P	N	N
5	P	N	N	N	N	N	P	N	P	N	N
6	P	N	N	P	N	N	N	P	P	N	N
7	P	N	N	P	N	N	P	N	P	N	N
8	N										
9	N										
10	P	N	N	N	N	N	N	N	P	N	N
11	P	P	N	P	N	N	N	N	N	N	N
12	N										
13	P	N	N	P	N	N	N	N	N	N	N
14	P	N	N	P	N	N	P	N	P	N	N
15	P	N	N	P	N	N	P	N	P	N	N
16	P	N	N	P	N	N	P	N	P	N	N
17	P	N	N	P	N	N	N	N	P	N	N
18	P	N	N	P	N	N	N	N	P	N	N
19	P	N	N	P	N	N	N	N	N	N	N
20	P	N	N	P	N	N	N	N	P	N	N
21	P	N	N	P	N	N	N	N	N	N	N
22	P	N	N	N	N	N	P	N	P	N	N
23	P	N	N	P	N	N	N	N	P	N	N
24	P	N	N	P	N	N	N	N	N	N	N
25	P	N	N	P	N	N	N	N	N	N	N
26	P	N	N	P	N	N	P	N	N	N	N
27											
28	P	N	N	P	N	N	P	N	P	N	N
29	P	N	N	P	N	N	N	N	P	N	N
30	N										
31	P	N	N	N	N	N	P	N	P	N	N

32	N										
33	P	N	N	P	N	N	N	N	N	N	N
34	P	N	N	P	N	N	N	N	N	N	N
35	P	N	N	P	N	N	N	N	P	N	N
36	P	N	N	P	N	N	N	N	P	N	N
37	P	N	N	P	N	N	N	P	N	N	N
38											
39	P	P	N	N	N	N	P	N	P	N	N
40	P	N	N	P	N	N	N	N	P	N	N
41	P	N	N	P	N	N	N	N	P	N	N
42	N										
43	P	N	N	P	N	N	N	N	N	N	N
44	P	N	N	P	N	N	N	N	N	N	N
45	P	N	N	N	N	N	N	N	N	N	N
46	P	N	N	P	N	N	N	N	N	N	N
47	P	P	N	N	N	N	N	N	P	N	N
48	P	N	N	P	N	N	N	N	P	N	N
49	P	N	N	N	N	N	P	N	P	N	N
50	P	N	N	P	N	N	N	N	N	N	N
Total	41	3	0	33	0	0	12	2	28	1	0

P: Positive result with band size of expected amplification size

N: Negative result, no bands were observed of the expected amplification size.

Some of the PCR reactions showed non-specific bands, which were unidentified and considered a negative result. A few of the PCR reactions displayed some non-specific bands that did not reflect the expected product sizes of the target genes. These were regarded as untypable and interpreted as a negative finding of *mcr* genes. Unspecific bands can have several reasons such as primer-dimer formation, annealing at a temperature where it is not in the optimum range or amplification of a sequence that was not in the target. These non-specific amplifications are not the target gene, therefore were removed from continued analysis, so the valid, specific results could be used to determine the presence or absence of *mcr* genes in the samples.

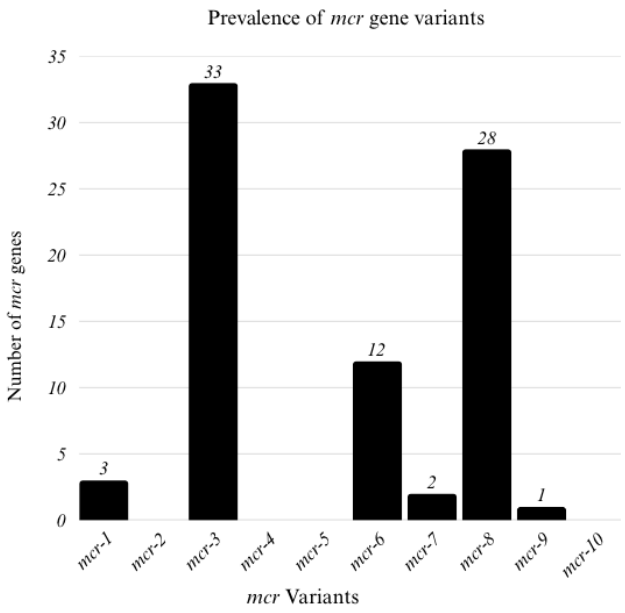

Figure 7: *mcr* gene variation and frequency.

Overall, 79 *mcr* genes were found among 41 PCR-confirmed *E. coli* isolates (Table 4). The distribution of the prevalence of each gene is: *mcr-1* (3 isolates), *mcr-3* (33 isolates), *mcr-6* (12 isolates), *mcr-7* (2 isolates), *mcr-8* (28 isolates), *mcr-9* (1 isolate) and *mcr-10* (0 isolates). The most frequent genes were *mcr-3* and *mcr-8*, which were present in the majority of the isolates, highlighting their strong contribution in terms of colistin resistance. Only 1 *E. coli* isolate did not harbour any of the 10 *mcr* genes. The lack of *mcr-10* in the tested population indicated that it is not as common to *E. coli*, but it should be monitored as a new resistance mechanism has emerged continuously.

3.1.4 BMD results (MIC of Colistin in *E. coli*) :

Colistin susceptibility of 15 *E. coli* isolates as determined by the MIC values (in µg/mL) of colistin (ranging from 2 to 16 µg/mL). MIC with the BMD method was determined for 15 *E. coli* isolates.

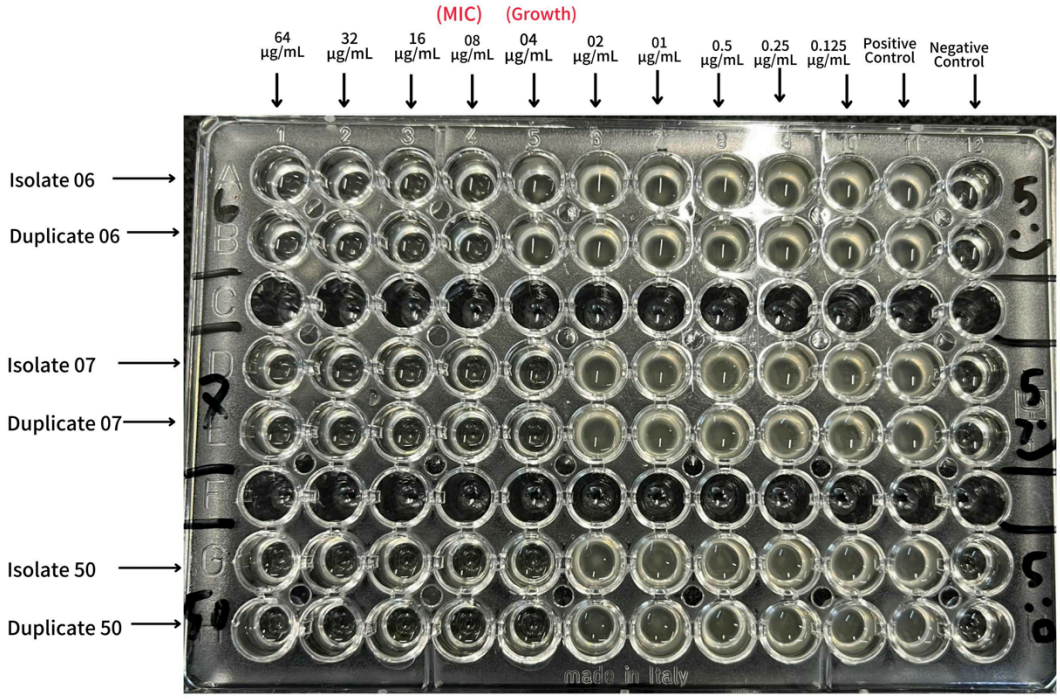

Figure 8: MIC determination by observing growth in different concentrations of colistin in a microtiter plate by the BDM method.

Table 5: 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 picked 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. 11 out of 15 isolates' resistance breakpoint was intermediate MIC of them being at 4 μg/mL. All of them harbored different variations of *mcr* genes except for sample no. 45, which did not possess any of the ten *mcr* genes.

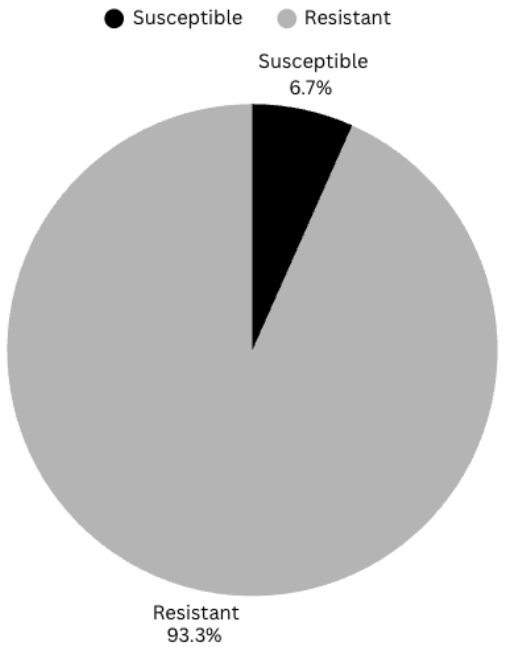

Figure 9: Pie chart of Resistance breakpoints of *E. coli* isolates based on MIC values

Chapter 4

4.1 Discussion

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 samples collection location that the probability of cross-contamination of food is very much likely.

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. (Rama et al., 2023) 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, 41 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 results of this study highlighted significant heterogeneity in the distribution of *mcr* genes across the *E. coli* isolates. 79 *mcr* genes were identified among the 41 isolates, and *mcr-3* and *mcr-8* were the most frequently encountered genes. *mcr-3* was the most prevalent, found in 33

(80.5%) isolates, and *mcr-8* followed, 28 isolates (68.3%). These results were striking because recent studies in the authors' region show a lack of *mcr-3* among *E. coli* isolates obtained from the chicken gut microbiota, although *mcr-1* and 2 have been reported. The high prevalence of the *mcr-3* gene in this study indicates that *mcr-3* may play a role in the dissemination of colistin resistance among *E. coli*, particularly within clinical settings, where colistin is one of the few remaining drugs available as a treatment option for multidrug-resistant infections. (Liu et al., 2016). This disparity in the past and present reports reflects the change in scope over time of antimicrobial resistance and the importance of ongoing surveillance to track the emergence of resistance genes such as *mcr-3* across the ecosystem, including animal agriculture.

The *mcr-6* gene was also detected from 12 isolates, again representing a moderate occurrence of this gene within the *E. coli* species. *mcr-6* gene has also been detected in other Gram-negative bacteria such as *Klebsiella pneumoniae* and *E. coli*. Its relatively low frequency in this study underpins the notion that, although less frequent than *mcr-3* and *mcr-8*, *mcr-6* is also an important contributor to colistin resistance. The occurrence of *mcr-6* from *E. coli* isolates indicates the increased diversity of *mcr* genes and the complexity of colistin resistance in Gram-negative bacteria. In addition, the identification of *mcr-6* suggests that colistin resistance extends beyond few *mcr* variants to a large diversity of resistance genes.

Even though the prevalence of *mcr-3* and *mcr-8* was high only two isolates (4.9%) hardboard the *mcr-7* gene, suggesting a low occurrence of *mcr-7* in poultry. Nevertheless, *mcr-7* is an important gene because it is a high-level colistin-resistance gene, even though it is less prevalent. The detection of *mcr-7* in this study, although it is in low prevalence, is alarming and suggests that this gene can significantly contribute to high-level colistin resistance in clinical practice, especially nosocomial infections. The low occurrence of *mcr-7* in our data could mirror differences at the regional level, as differences in the occurrence of *mcr-7* in *E. coli* populations have been observed worldwide. As *mcr-7* is subject to high-level resistance, its spread and appearance should be monitored.

Likewise, *mcr-9* was detected in only one isolate (2.4%) and its low occurrence during the study was established. Although *mcr-9* was less common than other *mcr* genes, its detection needs

attention due to the role it could play in the reservoir of colistin resistance in Gram-negative bacteria. This study indicated the detection of *mcr-9* which possibly mediate dissemination of resistance at less frequency. It is important to keep monitoring *mcr-9*, as the spread of these *mcr*-like genes of low prevalence could assist the expansion of a resistance reservoir in clinical environments.

Interestingly, none of the *E. coli* isolates harbored the *mcr-10* gene in the present study. The loss of *mcr-10* indicates that it may be rare or not observed in the *E. coli* population in the region. This result is consistent with observations from other areas where *mcr-10* is rare or absent. Although *mcr-10* was not identified in this work, its eventual appearance in the future cannot be ruled out. With the continued detection of new *mcr* genes, it is important to remain watchful for its prevalence in clinical isolates given that *mcr-10* and other new forms could represent a critical problem for treating colistin resistant infections.

Thus, this study reveals a very high prevalence of *mcr-3* and *mcr-8*, which is undoubtedly important for transmission as well as persistence of colistin resistance in populations of *E. coli* and other Gram-negative bacteria, particularly in hospital settings, where colistin was used as last resort therapy. The finding of these additional genes, including *mcr-6*, *mcr-7*, and *mcr-9*, highlights the heterogeneity of colistin resistance mechanisms. That the *mcr-10* is not present also demonstrates the changeability in the distribution of *mcr* genes.

The MICs of 15 *E. coli* isolates were highly resistant to colistin, and *mcr* genes carried by 12 of these isolates were investigated. Minimum inhibitory concentration (MIC) values predominantly intermediate (4 μg/mL; range, 2 μg/mL to 16 μg/mL). *mcr-3* was carried by Sample 24, which was fully susceptible, and 10 isolates with other combinations of *mcr* genes exhibited intermediate resistance. Out of these, three isolates (samples 6, 11, 31) showed resistance with a minimum inhibitory concentration (MIC) value ≥ 8μg/mL and the presence of *mcr-1*, *mcr-3*, *mcr-6*, *mcr-7*, and *mcr-8* genes was associated with higher levels of resistance. The most prevalent gene was *mcr-3* (73%); *mcr-8* (40% but only 2 strains) was other, whereas *mcr-6*, *mcr-7* and *mcr-9* were less common. In fact, this suggested that *mcr-10* was quite rare in the population, which was even more interesting, as no *mcr-10* was found. There was also an

inconsistency inheritance of all three *mcr*s genotypes and phenotype, where some of the isolates with multiple *mcr* genes presented with intermediate resistance, suggesting that the presence of *mcr* genes does not predict the level of resistance. The work adds another twist to the narrative of colistin resistance and highlights the need for a combined molecular and phenotypic surveillance to monitor and control resistance to the last resort antibiotic. Therefore, continuous surveillance for further epidemiological studies are needed for preventing the dissemination of colistin-resistant *E. coli* from one setting to another in clinical or agricultural environments.

The MICs of 15 *E. coli* isolates were highly resistant to colistin, and *mcr* genes carried by 14 of these isolates were investigated. Minimum inhibitory concentration (MIC) values predominantly resistant (4 μg/mL; range, 2 μg/mL to 16 μg/mL). *mcr-3* was carried by Sample 24, which was fully susceptible, and 10 isolates with other combinations of *mcr* genes exhibited resistance. Out of these, three isolates (samples 6, 11, 31) showed resistance with a minimum inhibitory concentration (MIC) value ≥ 8μg/mL and the presence of *mcr-1*, *mcr-3*, *mcr-6*, *mcr-7*, and *mcr-8* genes was associated with higher levels of resistance. The most prevalent gene was *mcr-3* (73%); *mcr-8* (40% but only 2 strains) was other, whereas *mcr-6*, *mcr-7* and *mcr-9* were less common. In fact, this suggested that *mcr-10* was quite rare in the population, which was even more interesting, as no *mcr-10* was found. There was also an inconsistency inheritance of all three *mcr* genotypes and phenotypes, where some of the isolates with multiple *mcr* genes presented with intermediate resistance, suggesting that the presence of *mcr* genes does not predict the level of resistance. The work adds another twist to the narrative of colistin resistance and highlights the need for a combined molecular and phenotypic surveillance to monitor and control resistance to the last resort antibiotic. Therefore, continuous surveillance for further epidemiological studies are needed for preventing the dissemination of colistin-resistant *E. coli* from one setting to another in clinical or agricultural environments.

4.2 Limitations

This study was conducted with only 50 samples, which is a small sample size and limited to only *E. coli.* The sampling area was restricted to Dhaka city only and poultry farms were not included. The virulence genes of *E. coli* were not assessed in this study. Extended-Spectrum Beta-Lactamase (ESBL) detection has not been performed yet. As well as the complete assessment of broiler chicken gut microbiome was not conducted in this study. Genome sequencing of the *mcr* was not performed to verify our findings of *mcr* genes. Lastly, the chicken feed and water ware not tested for confirm the presence of colistin.

4.3 Strength

This study combines genotypical and phenotypical surveillance that helps to detect the reliability of colistin resistance and the correlation between genotype and phenotype. Targeting the full range of *mcr* genes represents a complete perspective which established a strong basis for future whole genomic studies. The detection of *mcr* genes gives insight about region specific data about colistin resistance. The study ranged from sampling procedure to PCR and broth microdilution procedure which signifies the transparency and reproducibility. The plasmid mediated gene transfer, this highlights the potential for horizontal gene transfer which will raise awareness in controlling infection. Moreover, this study bridges agricultural study and public health implications, which is qualified for awareness raising activities. This study gives a complete visual on broiler chicken colistin resistance in Bangladesh which will serve as a standard for future study.

Chapter 5

Conclusion

This research is about detecting the prevalence and distribution of mobile colistin resistance (*mcr*) genes in the bacteria *E. coli* isolates in chicken fecal samples. In this study, confirmed isolates of *E. coli* were 84% which showed resistance to colistin and they harbored the *mcr* genes from *mcr-1* to *mcr-9*. In *E. coli* isolates *mcr-3* (88.1%) and *mcr-8* (46.4%) showed dominance and lower significance of *mcr-1* (7.1%), *mcr-6* (25%), *mcr-7* (3.6%), and *mcr9* (2.4%). Significantly, *mcr-10*, as well as *mcr-2* and *mcr-4* were not detected. All the resistant samples of *E. coli* were carrying the *mcr-3* gene. This emphasizes the broiler chicken in Dhaka as a potent reservoir of antibiotic resistant pathogens. Moreover, the spread of *mcr-3* and *mcr-8* suggest a shift in resistance dynamics.

A previous study reported that 1200 *E. coli* strains yet did not detect diverse variants of *mcr* genes only reported *mcr-1* through *mcr-5.* While our study shows a diverse variant of *mcr* genes from *mcr-1* to *mcr-9* (Ahmed et al., 2020). In other low-income and middle-income countries the report shows the detection level of *mcr* genes in poultry are up to as high as 50% whereas, our study shows very high rates for *mcr-3* and *mcr-8* (Lemlem et al., 2023). Detection of *mcr-1* through *mcr-9* in *E. coli* and high abundance of *mcr-3* and *mcr-8*, gives a clear understanding of colistin resistance diversity in Bangladesh. The plasmid mediated gene and the diverse *mcr* gene present in the chicken stool sample indicates the risk of transmission to humans through food chain or other type of contamination. (Umair et al., 2022).

Based on our findings of *mcr-3* and *mcr-8* genes and the resistance of the gene in colistin antibiotics, we conclude that we should take strict actions against the use of antibiotics in poultry farms and animal feeds since the environment plays a crucial role in the transmission of antibiotic resistant bacteria. The findings of this research plays a vital role in establishing the need of preserving the need of colistin efficiency and stepping in for the policy makers to protect the public health in Bangladesh and for global well-being.

Chapter 6

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