AN INVESTIGATION OF MOBILIZED COLISTIN-RESISTANCE (MCR) GENE AND UNDERLYING DISSEMINATION MECHANISM USING CLINICAL, POULTRY AND ENVIRONMENTAL SAMPLES IN BANGLADESH

by

Sadia Tasnim

ID: 18376007

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Masters of Science in Biotechnology

Department of Matematics and Natural Sciences

Brac University

January, 2020

DECLARATION

It is hereby declared that

1. The thesis submitted is my own original work while completing 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 which has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all primary sources of help.

Student's Full Name & Signature:

_____

Sadia Tasnim

Student ID: 18376007

APPROVAL

The thesis/project titled “An Investigation of Mobilized Colistin-Resistance (mcr) Gene and Underlying Dissemination Mechanism Using Clinical, Poultry and Environmental Samples in Bangladesh” submitted by **Sadia Tasnim** (Student ID: 18376007) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology on January 2020.

EXAMINING COMMITTEE

Internal Supervisor:
(Member)

Iftekhar Bin Naser, PhD
Assistant Professor, MNS Department
BRAC University

External Supervisor:
(Member)

Dr. Kaiisser Mannoor
Head and Senior Scientist
institute for developing Science and Health initiatives (ideSHi)

Departmental Head:
(Chair)

A F M Yusuf Haider, PhD
Professor and Chairperson
MNS Department
BRAC University

ABSTRACT

Colistin, widely considered as the last-resort antibiotic for the treatment against multidrug-resistant (MDR) gram-negative bacteria worldwide, is being used indiscriminately in the poultry, livestock and fishery farms of Bangladesh as a growth promoter and an antimicrobial agent. Although the presence of mobilized colistin-resistance (mcr) gene in human, poultry and environmental samples has been reported in *E.coli* in Bangladesh recently, there is no report of mcr gene in other gram-negative bacteria. Moreover, the presence of colistin-resistant gene and their minimum inhibitory concentration was also analyzed and coherence was found. Therefore, a study was carried out to scrutinized mcr gene(s) from above mentioned all the sources in order to investigate those mcr possessing isolates which are intimately related to some hospital-acquired infections (HAIs). This study also aims to investigate clinical as well as poultry and environmental samples in order to identify the mechanism of dissemination of mcr genes in gram negative bacteria and assess the risk of transmitting resistant genes from poultry, livestock to human gut bacteria through the environment in Bangladesh. A total of 450 clinical isolates (acute respiratory infection, n=212; wound sample, n=150; diarrheal disease, n=88), Poultry (n=100), cow fecal (n=60) and water (n=65) were analyzed. All the isolates were cultured and confirmed using standard microbiological and biochemical tests. Kirby-Bauer disk diffusion method was used to screen colistin-resistant isolates and the DNA templates were subjected to PCR using mcr gene specific primers (mcr-1 to mcr-7). Furthermore, the minimum inhibitory concentration (MIC) was determined using broth micro-dilution method and MIC was found higher for those isolates which possess mcr-2 than those, possess mcr-1. Next, the plasmid DNA was extracted and the PCR was performed again in order to differentiate between plasmid-mediated and

chromosome-mediated resistance of colistin. Overall, 97 colistin-resistant (mcr-1 and mcr-2 only) bacteria were isolated, of which, 13.80% *E.coli*, 37.93% *Klebsiella pneumoniae*, 31.03% *Acinetobacter baumanii*, 3.45% *Pseudomonas aeruginosa*, 3.45% *Enterobacter spp*. Our study also revealed that mcr-1 gene was present in all type of sample and mcr-2 was detected in clinical and cow fecal samples only. We provide new evidence that besides *E.coli*, four other gram-negative bacteria in Bangladesh have become colistin resistant and also guidelines for relevant authorities to tackle the extensive and uncontrolled use of colistin in Bangladesh.

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Author, Sadia Tasnim (January 2020)

DEDICATION

To my greatest strength my beloved *husband*.

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CHAPTER 1

INTRODUCTION

1.1 **Background**

Antibiotics are the most loyal weapon against any diseases caused by bacteria though, medical authorities have already been confronting with infections for which no antibiotic is effective because of the possession of resistant gene(s) against certain drugs. Because of the extensive uses of last hope antibiotic named ‘Colistin’ in poultry, livestock and fishery, resistance also develops in human pathogens. Colistin belongs to the antimicrobial class designated polymyxins which originate from a gram-positive organism *Paenibacillus polymyxa*. This class consists of polymyxins A, B, C, D and E, of which only colistin (polymyxin E) and polymyxin B are used in clinical practice.

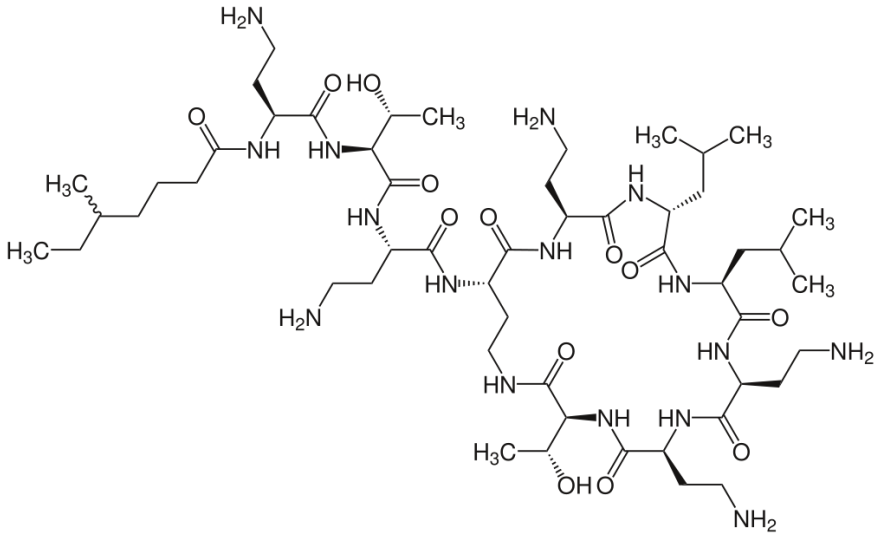


Figure 1: Structure of colistin

1.2 **Mechanism of action of colistin**

The bacterial cell membrane is the initial site of action for colistin. Colistin binds to lipopolysaccharide and phospholipids in the outer cell membrane of Gram-negative bacteria. It competitively displaces divalent ions (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids, which leads to disruption of the outer cell membrane, leakage of intracellular contents and bacterial death. Though the bactericidal action of colistin is still uncertain, some previous studies give some precise indications about the mechanism of action of colistin. One well-established assumption is that colistin molecules disrupt the physical integrity of the cytoplasmic membrane, which causes leakage of intracellular content [1]. The second hypothesis is that colistin causes the inner layer of the outer membrane and outer layer of the cytoplasmic membrane to come together, resulting in phospholipid exchange, and an osmotic imbalance. Additional possibilities, not necessarily mutually exclusive, are that colistin inhibits vital respiratory enzymes (type II NADH-quinone oxidoreductases) at the cytoplasmic membrane, that colistin induces the formation of reactive oxygen species when it crosses the cytoplasmic membrane or, in a similar manner to other cationic antimicrobial peptides, that colistin binds to bacterial DNA inhibiting replication and transcription [1].

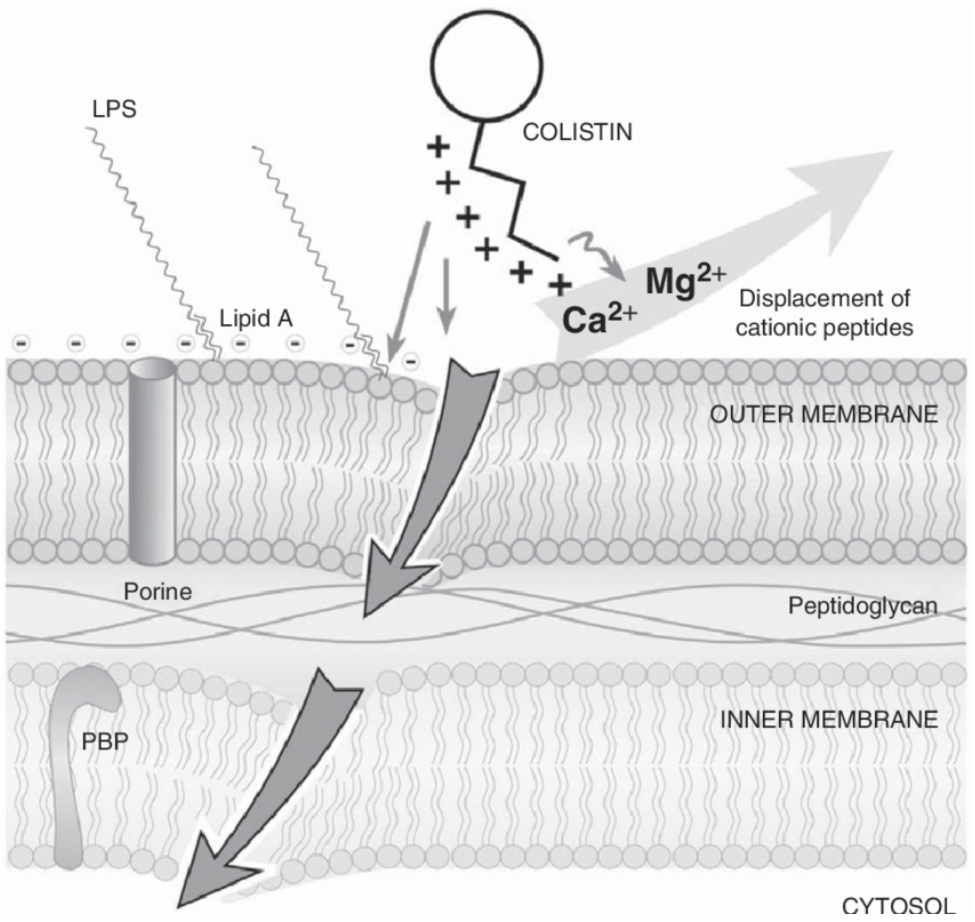


Figure 2: Mechanism of action of colistin

1.3 **Resistance mechanism of colistin**

The prevalence of resistance to colistin is relatively low and the data is scarce; probably, because the drug is rarely used for treatment. Ko as also quoted by Yahaf [2] identified the presence of resistance of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* against Colistin through gene analysis conducted in Korea; however, no further data was found. Many theories have been proposed about the general mechanism of developing resistance, such as through modification of LPS outer membrane structure, i.e. the bacteria change the negatively charged phosphate component of LPS into a neutral component, which weakens the binding of polymyxin with LPS wall. Moreover, the mechanism of efflux pump may also have a role in developing a mechanism of bacterial resistance against colistin. [3] It is assumed that the mechanism of resistance for Pseudomonas aeruginosa against polymyxin is involving protein on

LPS of the outer membrane (oprH) formed by the bacteria, which has polycationic characteristic and can substitute the position of calcium and magnesium ions as the stabilizer of LPS wall. The binding between oprH and LPS wall is so strong that polymyxin may not penetrate and cause damage to LPS [4]. Colistin must be administered according to its indication, based on the results of sensitivity test and should be closely monitored with adequate dose since inappropriate and irrational administration of colistin may induce cross-resistance with polymyxin B developing the emergence of a hypervirulent new strain in the future [5]. It is assumed that Acinetobacter baumannii develops mechanism of resistance since the bacteria has rapid and easy adaptation and can change into resistant phenotype when it is exposed to polymyxin. Another theory of polymyxin resistance to Acinetobacter baumannii is that there is a sub-population of polymyxin-resistant strain; therefore, initially, the detection using MIC method gives sensitive results, but the result will be resistant when we repeat the resistance culture procedure [6].

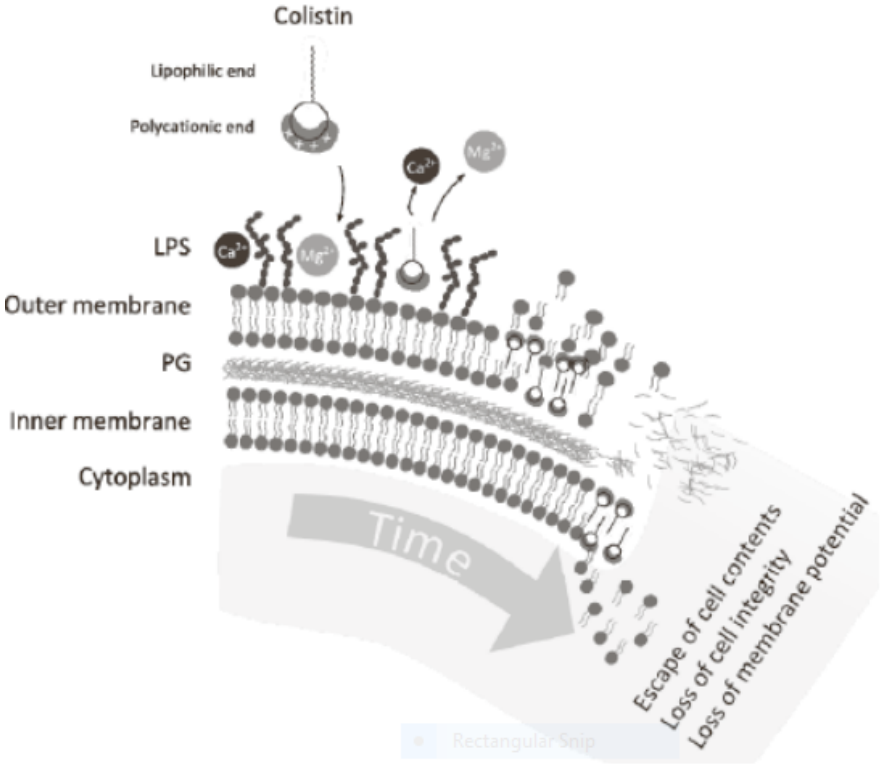


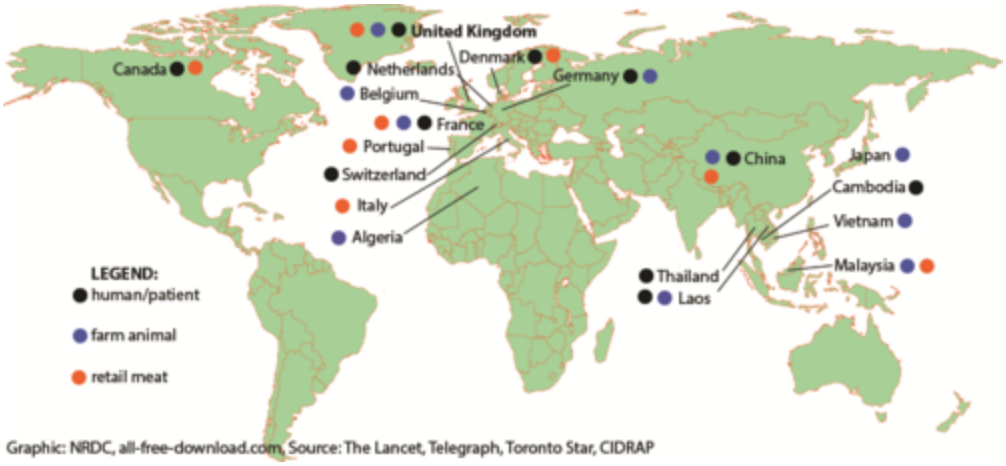
Figure 3: Resistance mechanism of colistin

1.4 **Pharmacodynamics**

Pharmacodynamics is about the effectiveness of drug treatment, i.e. the inhibition effect or killing effect of bacteria, which is associated with the dose, drug interaction and the effect on the human body [7]. The measurement of drug effectiveness in vitro can be performed by several methods, such as the disc diffusion test according to Kirby Bauer method, or by measuring the minimum inhibitory concentration (MIC), i.e. the measurement of lowest drug concentration that can still inhibit bacterial growth after 18-24 hours of incubation. Another method that can be performed to evaluate the effectiveness of colistin is by performing the E test. [8] In 2005, the clinical laboratory standard institute (CLSI) determined that Colistin sulfate (10 ug disc of Colistin sulfate) instead of CMS, is used as sensitivity detection test of Kirby Bauer method to represent the Colistin, since CMS is the prodrug of Colistin, which is unstable and it will cause difficult interpretation on test results. [9] The sensitivity test method using disc diffusion test of Kirby Bauer through the utilization of 10 µg colistin disc for Pseudomonas aeruginosa isolates shows positive results when the inhibition zone around the disc is ≥11 mm and it is considered as resistant when the zone of inhibition is ≤10 mm. [10] However, there is no CLSI recommendation (2014) on the use of Kirby Bauer method for Acinetobacter baumannii isolates. [11] According to CLSI recommendation as quoted by Kwa, [12] the susceptibility testing for Colistin is better performed by minimum inhibitory concentration (MIC) method. The rational is that disc diffusion test by Kirby Bauer method for colistin has a very high level of interpretation error and therefore, MIC method is more preferable. [13] The CLSI recommendation for sensitivity test of P. aeruginosa with MIC method suggests that it is considered sensitive when the MIC is ≤2 ug/ mL and it is considered resistant when the MIC is ≥8 ug/mL; while for Acinetobacter baumannii isolates, the MIC of ≤2 ug/mL is considered sensitive and MIC of ≥4 ug/mL is considered as resistant. There has been no recommendation of CLSI for Enterobacteriaceae. [14] Based on its pharmacodynamics profile, the drug inhibits the activity of Gram-negative bacteria at an effective concentration or having concentration/dose dependent characteristics.

1.5 **Previous studies**

The recent discovery of Escherichia coli harboring plasmid-borne colistin resistance via the mcr-1 gene in 2015 provides a mechanism for rapid dissemination. The same resistant gene was found in the following year in *E. coli* (a 49-years old urinary tract infected female patient) in the USA [15]. Gradually, within 3 years other variants of mcr (mcr-1 to mcr-8) genes were found in Europe as well as in many Southeast Asian countries. Our neighboring countries: India and Pakistan have also coined mcr gene recently [16,17].

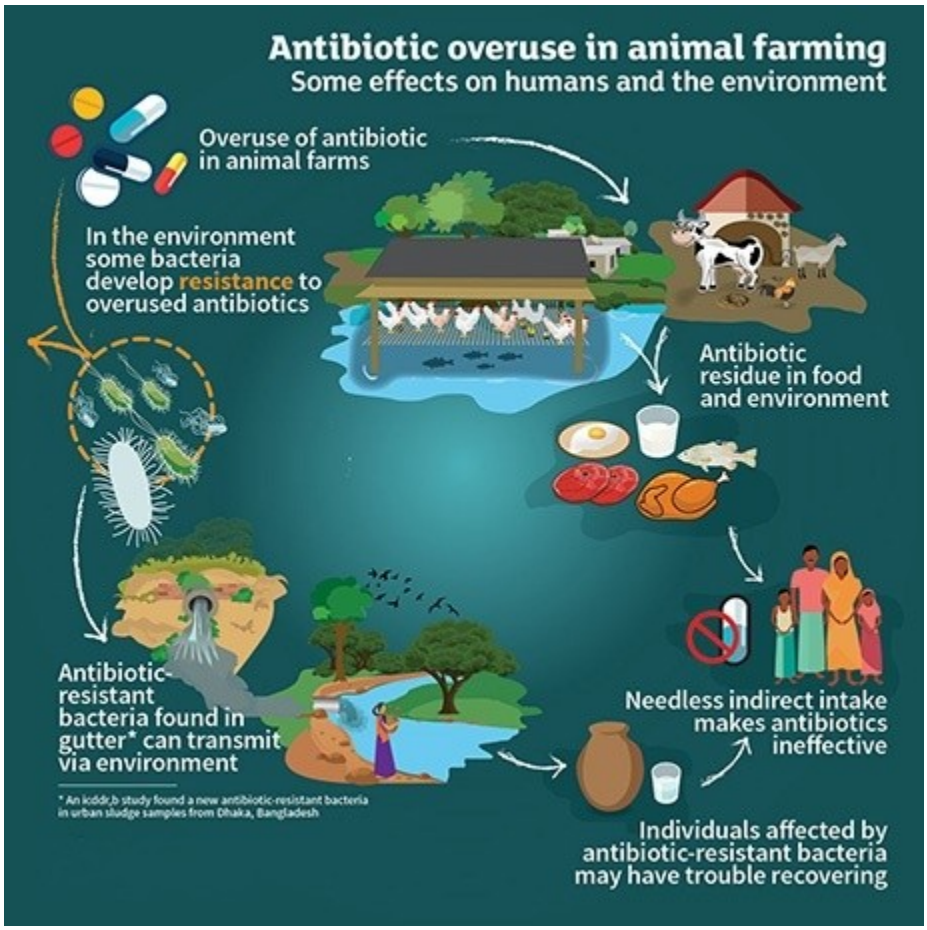


In our country, there are some studies performed in monitoring or surveillance of antibiotic resistance in poultry, livestock and fishery though coherence between antibiotic use in animals and the development of resistance in human pathogens has been described in several reports [18]. However, in Bangladesh mcr study was started in 2017 and till date, only *Escherichia coli* found resistant to colistin and possess resistance marker gene mcr-1 and mcr-2 only from poultry and mcr-1 gene from urban sludge, poultry and human [19]. Therefore, it can be more or less assumed there is a subtle coherence among farms, the environment and humans.

1.6 **Scheming up a vicious cycle**

Nowadays commercially available colistin- ‘ColistinPx’, ‘Colistin 4800 wsp’ etc. are being used in a large-scale as a growth promoter for poultry and livestock and becoming a probable hazard to human health in Bangladesh.[20] Moreover, people are unconsciously taking antibiotics.

However, indiscriminate use of this antibiotic in Bangladesh in different poultry, livestock and fish farm, increases the chance of the contamination of farm products by colistin residue. Above mentioned coherence is termed as ‘vicious cycle’ where transmission of resistant gene occurs in certain strain from poultry, livestock and fishery to humans as sludge from these sources goes to the nearby waterway. It is almost certain that people working in the poultry industry, veterinary and fishery are exposed to an unhygienic environment. They also have lack of knowledge about food safety, hygiene and sanitation and mainly depend on street foods of the nearby industries. Therefore, it is possible to transfer mobile colistin resistant (mcr) genes to food from industrial environment. The fishery is also notable to this phenomenon. Feces of chicken run to waterway through trellis which is also used as feed for fish and there is a high chance of transferring resistant gene to fish as well. On the other hand, in the urban areas, people mostly depend on restaurant food. Meat, fish and vegetables consist of the large portion of a day’s proper meal. Apart from very few restaurants, most do not concern about food safety. Poultry chicken meat is served in restaurants and sometimes the food remains even undercooked. As a result, the live bacteria in that food carrying resistant gene may transfer to human gut bacteria and finally, these gut bacteria spread the resistant gene through the environment. Consequently, people are being very silently trapped in this vicious cycle.



Source: icddr,b

1.7 **Resistance trends in bacteria**

Resistance trends are more prevalent among *Enterobacteriaceae* species as they are ubiquitous especially in the environment and animal system with enhanced propensity to acquire antibiotic resistance determinants through mobile genetic elements [21]. It is evident that antibiotic resistance in members of the family Enterobacteriaceae is on the rise. *Enterobacteriaceae* are Gram-negative bacteria of a large family that includes *Escherichia coli*, *Klebsiella*, Pseudomonas, Enterobacter *Salmonella*, *Shigella* and *Yersinia pestis*. They can cause a range of illnesses from bacteremia and endocarditis, to infections of the respiratory tract, skin, soft-tissues, urinary tract, joints, bones, eyes and CNS. Colistin and polymyxin B, however, do not have activity against *Proteus*, *Providencia*, *Serratia* species, *Pseudomonas mallei*, *Burkholderia*

cepacia, *Brucella* species, most gram-positive bacteria, gram-negative cocci, anaerobes, fungi, and parasites The resurgence of antibiotic resistant bacteria and the intimidating rate of antibiotic resistance genes' transmission around the globe has become a major concern in public health and prompting a wakeup call among the biomedical researchers and clinicians. Nosocomial infections are caused by a variety of organisms, including bacteria, fungi, viruses, parasites, and other agents. Infections can be derived from exogenous or endogenous sources and are transferred by either direct or indirect contact between patients, healthcare workers, contaminated objects, visitors, or even various environmental sources.

1.8 **Economic burden**

A survey of hospital-acquired infections (HAI) in the United States in 2011 reported a total of about 722,000 reported cases, with 75,000 deaths associated with nosocomial infections [22]. A second study conducted in 2002 estimated that when taking into account all types of bacterial infections, approximately 1.7 million patients suffered from HAIs, which contributed to the deaths of 99,000 patients per year [23].Therefore, the emerging issue of antibiotic resistance is amongst the CDC's "biggest threats" as pathogens rapidly evolve new ways to combat drug therapy. The evolutionary arms race between bacterial organisms and modern medicine is at a critical point as novel drugs are being developed at a slower rate than pathogens are evolving resistance. An infection caused by ESKAPE pathogens are prevalent in hospital settings, hospital-acquired infections (HAIs). The term ESKAPE pathogen was created to represent deadly bacterial pathogens with rapidly growing multi-drug resistant properties. The ESKAPE pathogen mnemonic stands for E for *Enterococcus faecium*, S for *Staphylococcus aureus,* K for *Klebsiella pneumoniae,* A for *Acinetobacter baumannii, P for Pseudomonas aeruginosa*, and E for *Enterobacter* species. Staphylococcus aureus is naturally susceptible to virtually every antibiotic that has ever been developed. Resistance is often acquired by horizontal transfer to genes from outside sources, although chromosomal mutation and antibiotic selection are also important. Infections caused by antibiotic-resistant strains of S. aureus have reached epidemic proportions globally7. The overall burden of staphylococcal disease, particularly that caused by methicillin resistant S. aureus strains (MRSA), is increasing in many countries in both healthcare and community settings. Skin and soft-tissue infections are the most common type of CA-MRSA

infection, accounting for approximately 90% of cases, of which 90% are abscesses and/or cellulitis with purulent drainage. MRSA strains also appear to be especially virulent with the capacity to cause fulminant, overwhelming infections, such as necrotizing fasciitis, necrotizing pneumonia, bone and joint infections accompanied by septic thromboembolic disease, purpura fulminans with or without Waterhouse-Friderichsen syndrome, orbital cellulitis and endophthalmitis, infections of the central nervous system, and bacteremia and endocarditis The CDC estimates antibiotic resistant ESKAPE pathogens cause over 2 million illnesses and approximately 23,000 deaths per year[24]. The growing numbers of antimicrobial-resistant pathogens, which are increasingly associated with nosocomial infection, place a significant burden on healthcare systems and have important global economic costs. Effects include high mortality and morbidity rates, increased treatment costs, diagnostic uncertainties, and lack of trust in orthodox medicine.

The resulting deterioration of the efficacy of our antibacterial armamentarium has come with substantial costs, which are expected to increase throughout the next decades. It has been estimated worldwide that at least 700,000 people die annually because of resistant infections, a figure that is predicted to increase to 10 million annual deaths by the year 2050 if the current trajectory is not altered. [25] Throughout the next few decades, the cumulative global cost of antibiotic resistance is predicted to exceed US$100 trillion [26]. Such a burden could be beyond measure in Bangladesh as empirical antibiotic treatment is more common here. There is evidence that resistance in some human enteric pathogens has arisen because of the transfer of resistant genes from animals to people via the food chain [27]. Furthermore, nephrotoxicity may occur due to high doses and prolonged courses of such drug, which will enhance risk for kidney injury via excessive exposure to the kidney, even in patients with minimal, or no underlying risk [28]. However, as persistent use of this antibiotic causes the spread of the last shot antibiotic- colistin resistant gene, it is now great concern to the researchers and policymaker. Although the presence of mobilized colistin-resistance (mcr) gene in human, poultry and environmental samples has already been reported from *E.coli* in Bangladesh recently, there was no report of mcr gene from other gram-negative bacteria and also from gram positive bacteria. Therefore, we carried out a study where we scrutinized mcr gene(s) from clinical, poultry and environmental samples and

tried to find out mcr gene in both gram-negative bacteria and gram-positive bacteria. This study aims to investigate clinical as well as poultry and environmental samples in order to identify the mechanism of dissemination of mcr genes in those bacteria which cause common infection to human in Bangladesh.

CHAPTER 2

METHOD AND MATERIALS

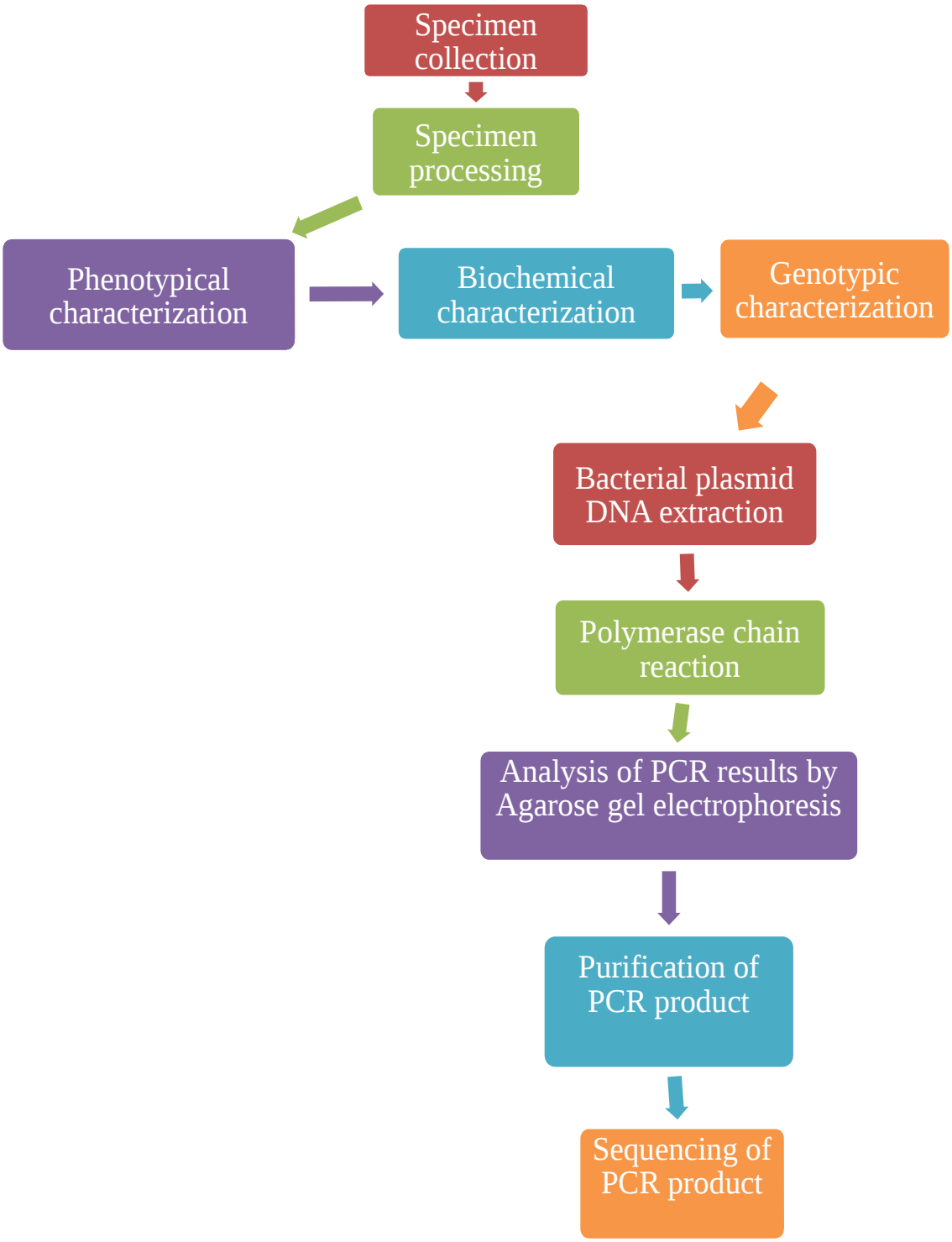
2.1 **Workplace**

All project tasks were executed in the laboratory of institute for developing Science and Health initiatives (ideSHi), 9 Mohakhali C/A (Amtoli), Hosaf High Tower, National Bank Building, 8th Floor, Dhaka-1212, Bangladesh from January 2018 to November 2019. Here bio-safety level 2 (BSL-2) is maintained and all the laboratory tasks were done inside biological safety cabinet.

2.2 **Sample collection, processing and preservation**

During the aforementioned interim period 450 clinical samples, 100 samples from poultry, 60 samples from cow fecal and 63 environmental samples (water samples) from nearby farms were collected. All the samples were first enriched in LB broth. For the long-term preservation purpose, samples were kept in cryo-tube contained skim milk tryptone glucose glycerol (STGG) media and storage temperature maintained around -70◦C.

2.3 **Flow diagram of study plan**



2.4 **Biochemical tests for confirmation**

At first all the samples were inoculated on MacConkey and blood agar plate and incubated at 37°C temperature. Next, in order to obtain pure culture of the isolate, colonies were carefully selected to perform subculture. Isolate identification was executed by different biochemical tests e.g. triple sugar iron (TSI), citrate, motility indole urease (MIU) tests, oxidase and catalase test. At least 2 suspected isolates per sample were selected. 100 clinical samples (55 Respiratory, 30 wound and 15 diarrheal samples), 50 poultry samples, 30 cows fecal and 30 environmental water samples, a total of 210 were collected.

2.5 **Gram stain**

Gram staining is a procedure which is used to differentiate between gram positive and gram negative organisms; hence it is differential stain. Gram positive and gram negative cells can be easily differentiated based on the structure of cell wall. In Gram staining procedure, as a result of having thick peptidoglycan layers, Gram positive bacteria retain the crystal violet dye, while Gram negative bacteria shows a pink/ red colour of safranin, a counterstain added after the crystal violet. Using sterile technique, a drop of saline was placed on the slide and a small amount of the bacteria were then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The fixed smear was flooded with crystal violet solution and allowed to remain for 1 minute. The crystal violet was rinsed off with distilled water. The slide was flooded with grams iodine solution mainly mordant and allowed to remain for one minute. After that, the smear was decolorized with 95% ethyl alcohol and gently washed with distilled water or tap water. Finally, it was counterstained with safranin for 45 seconds and gently washed with distilled water or tap water. The slide was then blot dried with bibulous paper and examined under oil immersion.

2.6 **Selective media**

Much of the study of microorganisms depends on its ability to grow in the laboratory, and this is possible only if suitable culture media are available for the growth of microorganism.

A culture medium is defined as a solid or liquid preparation used for the growth, transport, and storage of microorganisms. The effective culture medium must contain all the nutrients required for the growth of the microorganism. Selective media allows the growth of certain type of organisms, while inhibiting the growth of other organisms. These media contain antimicrobials, dyes, or alcohol to inhibit the growth of the organisms not targeted for study. For the identification and confirmation of *Enterobacteriace group and* gram-positive bacteria from the collected samples, two selective media were used MacConkey Agar (MAC) and Blood Agar.

2.6.1 MacConkey agar (MAC)

MacConkey Agar is a selective and differential culture media commonly used for the isolation of enteric Gram-negative bacteria. It is based on the bile salt-neutral redlactose agar of MacConkey. Crystal violet and bile salts in incorporated in MacConkey Agar to prevent the growth of gram-positive bacteria and fastidious gram-negative bacteria, such as *Neisseria* and *Pasteurella*. Gram-negative enteric bacteria can tolerate to bile salt because of their bile-resistant outer membrane. MacConkey Agar is selective for Gram negative organisms, and helps to differentiate lactose fermenting gram negative rods from Non lactose fermenting gram negative rods. It is primarily used for detection and isolation of members of family Enterobacteriaceae and *Pseudomonas* spp. On MAC, *Klebsiella* spp produce pink mucoid colonies.

2.6.2 Blood agar

Blood Agar is an enriched medium often used to grow fastidious organisms and to differentiate bacteria based on their hemolytic properties. It contains general nutrients and 5% sheep blood. It is useful for cultivating fastidious organisms and for determining the hemolytic capabilities of an organism. Some bacteria produce exoenzymes that lyse red blood cells and degrade hemoglobin; these are called hemolysins. Bacteria can produce different types of hemolysins. Beta-hemolysin

breaks down the red blood cells and hemoglobin completely. This leaves a clear zone around the bacterial growth. Such results are referred to as β-hemolysis (beta hemolysis). Alpha-hemolysin partially breaks down the red blood cells and leaves a greenish color behind. This is referred to as α-hemolysis (alpha hemolysis). The greenish color is caused by the presence of biliverdin, which is a by-product of the breakdown of hemoglobin. If the organism does not produce hemolysins and does not break down the blood cells, no clearing will occur. This is called γ-hemolysis (gamma hemolysis). The hemolysins produced by streptococci perform better in an anaerobic environment. Because of this, it is standard procedure to streak a blood plate and then stab the loop into the agar to provide an area of lower oxygen concentration where the streptolysins can more effectively break down the blood cells. *Streptococcus pneumoniae* does alpha haemolysis on blood agar.

2.7 **Biochemical characterization of the bacteria**

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual instructed by (Cappuccino and Sherman 2005). The biochemical tests performed were; Triple sugar iron agar test (TSI), IMViC test (Indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test and Motility test.

2.7.1 Triple Sugar Iron Agar test

Triple sugar iron (TSI) agar is a tubed differential medium used in determining carbohydrate fermentation and H2S production. Gas from carbohydrate metabolism can also be detected. Bacteria can metabolize carbohydrates aerobically (with oxygen) or fermentatively (without oxygen). TSI differentiates bacteria based on their fermentation of lactose, glucose and sucrose and on the production of hydrogen sulphide. Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121◦C. The inoculating needle was sterilized in the blue flame of the Bunsen burner till it turned into red hot and then allowed to cool. Using sterile technique, a small amount of the experimental bacteria from 24-hour pure culture was inoculated into the tubes by

means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37◦C.

2.7.2 Indole production test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. It is used as part of the IMViC procedures, a battery of tests designed to distinguish among members of the family Enterobacteriaceae. The presence of indole when a microbe is grown in a medium rich in tryptophan demonstrates that an organism has the capacity to degrade tryptophan. Detection of indole, a by-product of tryptophan metabolism, relies upon the chemical reaction between indole and p-dimethylaminobenzaldehyde (DMAB) under acidic conditions to produce the red dye rosindole. Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi,121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes and the tubes were incubated for 48 hours at 37◦C. Following incubation, five drops of Kovac's reagent were added. Then the colour of the cultures was examined, and the results were recorded. Formation of a rose red ring at the top of the liquid surface indicates a positive result. A negative result can have a yellow or brown layer.

2.7.3 Citrate utilization test

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source. In organisms capable of utilizing citrate as a carbon source, the enzyme citrasehydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and carbon dioxide. If carbon dioxide is produced, it reacts with components of the medium to produce an alkaline compound (e.g. sodium carbonate). The alkaline pH turns the pH indicator (bromthymol blue) from forest green to Prussian blue. Using sterile technique, small amount of the experimental bacteria from 24-hour pure culture was inoculated into the test tubes by means of a streak inoculation method with an inoculating needle and the tubes were incubated for 48 hours at 37◦C [14].

2.7.4 MIU (Motility- Indole- Urease) test

MIU Medium Base is used to detect motility, urease and indole production in single tube. Casein enzymatic hydrolysate provides amino acids and other nitrogenous substances. Sodium chloride maintains osmotic equilibrium. Dextrose is fermentable carbohydrate. Phenol red is the pH indicator which turns pink- red in alkaline conditions. Motility and urease reactions are read before testing Indole production. Motile organisms show either diffused growth or turbidity extending away from stab inoculation line while non-motile organisms grow along the stab line. Organisms that utilize urea produce ammonia which makes the medium alkaline, showing pink-red colour by the change in the phenol red indicator. Indole is produced from tryptophan present in Casein enzymatic hydrolysates. The indole produced combines with the aldehyde present in the Kovac's reagent to form a red complex.

Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C

2.7.5 Catalase test

The catalase test facilitates the detection of the enzyme catalase in bacteria. The function of this enzyme is to detoxify hydrogen peroxide (H_2O_2), which is formed from the superoxide radical by superoxide dismutase. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. A drop of hydrogen peroxide was taken on an autoclaved glass slide. A single colony of experimental bacteria was placed on the reagent drop and the result was observed immediately. Rapid production of O_2 bubbles indicates positive result; the absence of bubble formation is a negative catalase test.

2.7.6 Oxidase test

The oxidase test determines whether a microbe can oxidize certain aromatic amines, for example, p–aminodimethylaniline oxalate, to form coloured end products. When present, the cytochrome c oxidase oxidizes the reagent to purple colour end product. When the enzyme is not present, the reagent remains reduced and is colourless. One or two drops of oxidase reagent (p-aminodimethylaniline oxalate) were added on a filter paper. Using a sterile wooden toothpick, small amount of experimental bacteria was placed on the reagent. A development of pink, maroon, and violet to purple and finally black coloration within a few seconds indicate positive result. No colour change, or a light pink colouration on the colonies, is indicative of the absence of oxidase activity.

2.7.7 Analytical Profile Index (API 20E from bioMerieux, Inc.)

The Analytical Profile Index (API) system is one of the simplified test kits used for the identification of bacteria. Three API kits are available for identifying Enterobacteria-a screening kit of 10tests (10S), a basic set of 20 tests (20E) and for further characterization of an organism a kit of 50 tests (50E). The 20E kit contains all the tests of the 10S but only a few tests are in both the 20E and 50E. The individual tests consist of dehydrated chemicals in a set of plastic cupules (moulded to a strip of plastic) which are inoculated with a bacterial suspension. The development of this system of cupules has been described by Janin (1977). The inoculated whole API® 20E test kit strips were incubated overnight at 37°C in an aerobic incubator. After the incubation period, test results are used to construct a 7-digits profile. Using this profile, the identity of the bacterium was derived from the database with the relevant cumulative profile codebook or software

ESTS	**SUBSTRATE**	**REACTION TESTED**	**NEGATIVE RESULTS**	**POSITIVE RESULTS**
ONPG	ONPG	beta-galactosidase	colorless	yellow
ADH	arginine	arginine dihydrolase	yellow	red/orange

LDC	lysine	lysine decarboxylase	yellow	red/orange
ODC	ornithine	ornithine decarboxylase	yellow	red/orange
CIT	citrate	citrate utilization	pale green/yellow	blue-green/blue
H2S	Na thiosulfate	H2S production	colorless/gray	black deposit
URE	urea	urea hydrolysis	yellow	red/orange
TDA	tryptophan	deaminase	yellow	brown-red
IND	tryptophan	indole production	yellow	red (2 min.)
VP	Na pyruvate	acetoin production	colorless	pink/red(10 min.)
GEL	charcoal gelatin	Gelatinase	No diffusion of black	black diffuse
GLU	glucose	fermentation/oxidation	blue/blue-green	Yellow
MAN	mannitol	fermentation/oxidation	blue/blue-green	Yellow
INO	inositol	fermentation/oxidation	blue/blue-green	yellow
SOR	sorbitol	fermentation/oxidation	blue/blue-green	Yellow
RHA	rhamnose	fermentation/oxidation	blue/blue-green	Yellow
SAC	sucrose	fermentation/oxidation	blue/blue-green	Yellow
MEL	melibiose	fermentation/oxidation	blue/blue-green	Yellow
AMY	amygdalin	fermentation/oxidation	blue/blue-green	Yellow
ARA	arabinose	fermentation/oxidation	blue/blue-green	Yellow
OX	oxidase	oxidase	colorless/yellow	Violet

Table 1: API 20E (bioMerieux,Inc.) Reading Table

2.8 **Antibiotic Susceptibility test**

Since the disk diffusion assay is still in a routinely used method, isolates were chosen against colistin antibiotics based on the diameter of zone of inhibition by following the breakpoints (EUCAST,2019) of enterobacteria. MHA (Mueller-Hinton Agar, Luqiao, Beijing) plates were prepared and approximately 0.5 McFarland of strains were inoculated onto plates using temperature of 16–20 h in 35 °C ± 2 °C under ambient air condition.

2.8.1 Reading the antibiotic disk test results

Using a 6 mm, 10 µg disk, a zone of inhibition of 11 mm or greater indicates sensitivity and allows for presumptive identification of colistin resistant isolate. The zone of inhibition should be measured in millimeters, including diameter of disk. In the case of an isolate completely resistant to colistin, the diameter of the disk (10 mm) should be recorded. A smaller zone of inhibition (less than 10 mm) or no zone of inhibition indicates resistant strain.

2.8.2 Minimal inhibitory concentration (MIC)

The resistant isolate was studied for the minimal inhibitory concentration of colistin, growth on selective agar plates in order to observe dose (4/8/16/32/64 mg/l) of the antibiotic for a particular isolate. The isolates which showed growth even at the highest concentration (64mg/l) were selected and subjected to plasmid DNA extraction and subsequent PCR.

2.9 **Enrichment Medium**

Luria-Bertani broth and alkaline peptone water (pH 8.4-9.0) was used as an enrichment media to support the growth of *Enterobacterace* group of isolates and gram-positive isolates from all the samples. Enrichment took place overnight in the shaker incubator at 37°C in an aerobic condition at 250 rpm (rotation per minute). Then plasmid DNA isolation was executed. Consequently, polymerase chain reaction and agarose gel electrophoresis were performed to get the desired results.

2.10 **Quality control**

It is important to maintain quality while doing any laboratory experiments. The procedures for quality control primarily monitor the accuracy of the work by checking the bias of data with the help of (certified) reference samples and control samples and the precision by means of replicate analyses of test samples as well as of reference and/or control samples.

2.11 **Quality control for confirmation tests of the organisms**

A negative control was set up for all confirmation tests that were performed. By doing this, we can check whether any contamination has occurred during the experiment or any external source is affecting the experiment or not.

2.12 **DNA extraction and purification**

As mcr genes are plasmid mediated, plasmid DNA was extracted carefully using kit (PureLink® Quick Plasmid Miniprep Kits) and tried to keep the concentration and purity of the DNA in a suitable range for a successful PCR.

2.12.1 Plasmid DNA extraction procedure

1. Harvest: 1–5 mL of the overnight LB-culture was centrifuged. (1–2 × 10^9 *E. coli* cells for each sample was used.) All medium was removed.
2. Resuspend: 250 µL Resuspension Buffer (R3) with RNase A was added to the cell pellet and the pellet was resuspended until it is homogeneous.
3. Lyse: 250 µL Lysis Buffer (L7) was added. The solution was mixed gently by inverting the capped tube until the mixture is homogeneous. Vortex was avoided. The tube was incubated at room temperature for 5 minutes.
4. Precipitate: 350 µL Precipitation Buffer (N4) was added. The solution was mixed immediately by inverting the tube, or for large pellets, the tube was vigorously shaken, until the mixture is homogeneous. Vortex was avoided. The lysate was centrifuged at >12,000 × g for 10 minutes.

5. Bind: The supernatant from step 4 was loaded onto a spin column in a 2-mL wash tube. The column was centrifuged at 12,000 × g for 1 minute. The flow-through was discarded and the column was placed back into the wash tube.
6. Wash and remove ethanol: 700 µL Wash Buffer (W9) with ethanol was added to the column. The column was centrifuged at 12,000 × g for 1 minute. The flow through was discarded and the column was placed into the wash tube. The column was centrifuged at 12,000 × g for 1 minute. The wash tube was discarded with the flow-through.
7. Elute: The Spin Column was placed in a clean 1.5-mL recovery tube. 75 µL of nuclease free water was added to the center of the column. The column was incubated for 1 minute at room temperature.
8. Recover: The column was centrifuged at 12,000 × g for 2 minutes. The recovery tube contains the purified plasmid DNA. The column was discarded. The plasmid DNA was stored at 4°C (short-term) or the DNA was stored in aliquots at −20°C (long-term).

2.13 **Measurement of DNA concentration and purity**

DNA concentration was measured with EON spectrophotometer (NanoDrop™, USA) using Take 3 plate. Approximately 2µl Nuclease Free water was used as blank and 2µl of DNA sample was loaded on the Take 3 plate and OD (optical density) was measured spectrophotometrically. The concentration was measured in ng/µl. The purity was checked using OD ratio at 260 nm/280 nm reading. The result was evaluated with Nano drop Software.

2.14 **Designing a PCR primer**

The DNA sequences of mcr genes were retrieved from the nucleotide database of the National center for Biotechnology Information (NCBI). The retrieved FASTA sequence was then used to design specific primers using Primer-BLAST.

2.15 **Software**

Primer-3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)

Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Primer-Blast is a widely used program for designing PCR primers. PCR is an essential and ubiquitous tool in genetics and molecular biology. Primer-Blast can also design hybridization probes and sequencing primers. PCR is used for many different goals. Consequently, primer blast has many different input parameters that can be controlled and that tell exactly what characteristics make good primers for certain goals.

2.15.1 Method

- ✓ http://www.ncbi.nlm.nih.gov/tools/primer-blast/ is browsed.
- ✓ Sequences are pasted.
- ✓ Parameters are changed if necessary.
- ✓ Get primers are clicked on and few minutes are required to get the result.

Primer Name	Sequence	Primer length	Product Size	TM	GC %
MCR-1	AGTCCGTTTGTTCTTGTGGC	20	320	58.98	50
	AGATCCTTGGTCTCGGCTTG	20		58.46	55
MCR-2	TGTTGCTTGTGCCGATTGGA	20	567	60.82	50
	AGATGGTATTGTTGGTTGCTG	21		56.5	42.86
MCR-3	TTGGCACTGTATTTTGCATTT	21	542	55.18	33.33
	TTAACGAAATTGGCTGGAACA	21		56.36	38.1
MCR-4	TCACTTTCATCACTGCGTTG	20	1116	56.67	45
	TTGGTCCATGACTACCAATG	20		55.03	45
MCR-5	TATCTCGACAAGGCCATGCTG	21	613	60.2	52.38
	GAATCTGGCGTTCGTCGTAGT	21		60.47	52.38
MCR-7	AGGGGATAAACCGACCCTGA	20	335	59.96	55
	TGATCTCGATGTTGGGCACC	20		60.11	55

Table 2: Primer list

2.16 **Polymerase Chain Reaction**

Polymerase chain reaction (PCR) is a common laboratory technique used to make millions or billions copies a particular region of DNA. This DNA region can be anything that is desired by the researcher. Typically, the goal of PCR is to make enough of the target DNA region so that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments. PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology. Here, in this study PCR was done to amplify DNA so that the desired band can be observed by gel electrophoresis.

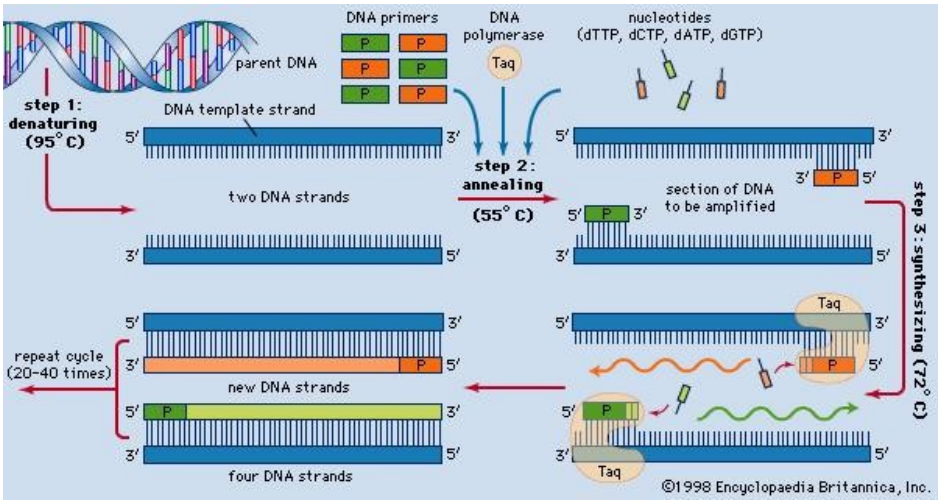


Figure 4: Principle of polymerase chain reaction

2.17 **Taq polymerase**

Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called Taq polymerase, after the heat-tolerant bacterium from which it was isolated (*Thermusaquaticus*). *T.aquaticus* lives in hot springs and hydrothermal vents. Its DNA polymerase is very heat-stable and is most active around 70°C (a temperature at which a human or *E. coli* DNA polymerase would be nonfunctional). This heat-stability makes Taq polymerase ideal for PCR.

2.18 **Conventional PCR**

PCR was performed using a T100 ™ thermal cycler (Bio-Rad, USA). The final reaction volume was 10µl which contains-

- 1 µl 10X PCR buffer with Mg (Clontech, code no:R001A),
- 0.2µl of dNTPs mixture (10 mM),
- 0.2µl of MgCl2 (50mM)
- 0.5µl forward and reverse primers,
- 0.05µl of Taq polymerase (Clontech, code no: R001A)
- 5.55 µl Nuclease Free Water
- 2 µl of template DNA.

Component	Amount
2.5 mM dNTPs	1µl
10X buffer(Mg^{2+})	1µl
Forward primer	0.5µl
Reverse primer	0.5µl
Taq polymerase	0.1µl
Nuclease free water	5.9µl
Template DNA	1µl
Total volume	10 µl

Table 3: Thermal cycling profile PCR master mix

- ⇨ The amount of template DNA was changed when it was necessary. Therefore, the amount of nuclease free water was also changed according to that.
- ⇨ Taq Polymerase was added right before loading the sample in the PCR machine (Infinigen, USA). All the steps were performed in the Eppendorf PCR cooler rack.

PCR condition	Temperature	Time	
Initial denaturation	94°C	3 minutes	
Denaturation	94°C	30 seconds	35 cycles
Annealing	58°C	30 seconds	
Extension	72°C	60 seconds	
Final extension	72°C	10 minutes	
	10°C	∞	

2.19 **The steps of PCR**

Table 4: Thermal cycling profile

The key ingredients of a PCR reaction are Taq polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

The basic steps are:

Denaturation (94°C): This step heats the reaction strongly to separate, or denature, the DNA strands. This provides single-stranded template for the next step.

Annealing (58°C): This step cools the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.

Extension (72°C): This step raises the reaction temperatures so Taq polymerase extends the primers, synthesizing new strands of DNA.

Cycle repeated 35 times in this PCR reaction, which generally took maximum 2 hours.

2.20 **Gel Electrophoresis**

The results of a PCR reaction are usually visualized using gel electrophoresis. Gel electrophoresis is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. Amplified DNA is loaded in wells in the gel close to the negative electrode. In the presence of an electrical field, negatively charged DNA moves toward the positive pole through the small holes that make up the gel matrix. These holes allow the shorter fragments of DNA to migrate faster than their longer counterparts. Once the reaction is complete, the length of the amplified DNA can be accurately determined by comparing with a DNA ladder. Amplified PCR products were analyzed by electrophoresis on 1%, 1.5% and 2% agarose gel and a definite amount of agarose powder (Ultrapure, Invitrogen, USA) was dissolved in definite amount of 1X TBE buffer. Then it was heated to dissolve in a microwave oven for about 1-2 minutes. The mixture was then allowed to cool down at room temperature. To the cooled agarose gel, 2µl of Gel red (Biotium, Cat no: 41003, USA) was added. The gel was then poured on the gel casting tray previously set with the comb and allowed to solidify. While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

2.20.1 Detection of PCR product by Agarose Gel Electrophoresis

4/5µl of the PCR product was mixed with 2µl of loading dye and was loaded into the individual wells of the gel. A ladder of size 1Kb plus (Invitrogen, USA) was used to ensure amplification of the desired gene and measure the exact product size which was estimated to be within 1,500bp. Amplified PCR products were electrophoresed at 130 volts for 1 hour. The separated DNA bands were observed on a Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

2.21 **PCR Product purification**

MinElute PCR Purification Kit Protocol (using a microcentrifuge)

This protocol is designed to purify double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA. Fragments ranging from 70bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge.

Important points before starting

- Ethanol (96–100%) to Buffer PE before use, should be added.
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- pH indicator I volume of 1:250 to Buffer PB (i.e.,120 µl pH indicator I to 30 ml Buffer PB or 600 µl pH indicator I to 150 ml Buffer PB) should be added. The yellow color of Buffer PB with pH indicator I indicates a pH of 7.5.
- pH indicator I to entire buffer contents should be added. pH indicator I to buffer aliquots should not be added.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

2.21.1 Procedure

1. Buffer PB volume of 5 should be added to 1 volume of the PCR reaction and should be mixed. It is not necessary to remove mineral oil or kerosene.

For example, 250 µl of Buffer PB should be added to 50 µl PCR reaction (not including oil).

2. When pH indicator I has been added to Buffer PB, color should be checked that the mixture is yellow. If the color of the mixture is orange or violet, then 10 µl of 3 M sodium acetate (pH 5.0) should be added and should be mixed. Then color of the mixture will turn to yellow.

3. A MinElute column in a provided 2 ml collection tube in a suitable rack should be placed.

4. To bind DNA, the sample to the MinElute column should be applied and centrifugation for 1 min should be done.

For maximum recovery, all traces of the sample to the column should be transferred.

5. Flow-through should be discarded. The MinElute column back into the same tube should be placed.

6. To wash, 750 µl Buffer PE was added to the MinElute column and centrifuged for 1 min.

7. Flow-through was discarded and placed the MinElute column back in the same tube. Then the column was centrifuged for an additional 1 min at maximum speed.

8. The MinElute column was placed in a clean 1.5 ml microcentrifuge tube.

9. To elute DNA, 10 µl Buffer EB (10 mMTris·Cl, pH 8.5) or water was added to the center of the membrane, then the column was kept stand for 1 min, and then centrifuge was done for 1 min.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA.

2.22 **DNA sequencing (Sanger sequencing method)**

DNA sequencing is a laboratory method used to determine the sequence of a DNA molecule. It is also called Sanger Sequencing as the method was developed by Frederick Sanger. In Sanger sequencing, the DNA to be sequenced serves as a template for DNA synthesis. A DNA primer is designed to be a starting point for DNA synthesis on the strand of DNA to be sequenced. Four

individual DNA synthesis reactions are performed. The four reactions include normal A, G, C, and T deoxynucleotide triphosphates (dNTPs), and each contains a low level of one of four dideoxynucleotide triphosphates (ddNTPs): ddATP, ddGTP, ddCTP, or ddTTP. The four reactions can be named A, G, C and T, according to which of the four ddNTPs was included. When a ddNTP is incorporated into a chain of nucleotides, synthesis terminates. This is because the ddNTP molecule lacks a 3' hydroxyl group, which is required to form a link with the next nucleotide in the chain. Since the ddNTPs are randomly incorporated, synthesis terminates at many different positions for each reaction.

Following synthesis, the products of the A, G, C, and T reactions are individually loaded into four lanes of a single gel and separated using gel electrophoresis, a method that separates DNA fragments by their sizes. The bands of the gel are detected, and then the sequence is read from the bottom of the gel to the top, including bands in all four lanes. For instance, if the lowest band across all four lanes appears in the A reaction lane, then the first nucleotide in the sequence is A. Then if the next band from bottom to the top appears in the T lane, the second nucleotide in the sequence is T, and so on. Due to the use of dideoxynucleotides in the reactions, Sanger sequencing is also called "dideoxy" sequencing.

The DNA samples were sent at IEDCR (Institute of Epidemiology, Disease Control and Research) for sequencing. The calculation of cycle sequencing was obtained according to the measured template concentration.

Component	Amount
5X PCR sequencing buffer	2.0µl
Big dye(2.5X)	0.50µl
Primers	0.2 µl
Template	1-10 ng
Water	up to 10 µL

Table 5: Master cycle components with amount

The tubes containing the template were spun, and 10-20 ng/µL (depending on the concentration) of each of the purified PCR products were added to the 8-tube PCR strip. Then nuclease free water was added to the mixture to make the total volume 10 µL. The PCR tubes were centrifuged at 4000 rpm for 3 minutes. Then, the PCR strip was placed in the Mastercycler® gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to following thermal cycling profile: pre-denaturation at 95°C for 10 minutes; 25 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 4 minutes; and a final extension at 72°C for 6 minutes.

PCR condition	Temperature	Duration	
Initial denaturation	95˚C	10 minutes	
Denaturation	95 ˚C	10 seconds	**25 cycles**
Annealing	55 ˚C	5 seconds	
Extension	72 ˚C	4 minutes	
Final extension	72 ˚C	6 minutes	
	10 ˚C	∞	

Table 6: Thermal cycling profile for cycle sequencing

After completion of cycle sequencing, the reaction plate was centrifuged at 4100 rpm for 2 minutes. Then, 45µl of solution and 10 µl of X-terminator (Applied Biosystems, USA) were added per 10µl volume. Both of the solution aid in removal of impurities by desalting as salt interferes with electro-kinetic injection and elimination of remaining labeled ddNTPs, thus, minimizing background noise produced by dye blobs in the sequencing results. Before addition, the X-terminator solution was vortexed properly at maximum speed for at least 30 seconds, until it became homogenous. As it was difficult to pipette the highly dense X-terminator solution out from the bottom of its container, wide bore micropipette tips were used. Later, the reaction plate was sealed and vortexed for half an hour. The mixture was then centrifuged at 4100g for 2 minutes and the supernatant collected for capillary electrophoresis. 10 µl of supernatant was transferred to a fresh sequencing tube. Before

placing the sequencing tubes into the capillary electrophoresis instrument, it was covered with Septa mat. The rest of the supernatant was stored at +4°C for later use.

2.23 **Sequence analysis**

Sequencing data were analyzed by Chromas Lite 2.4 software to identify the sequence alignments for showing identity and detecting mutations. The obtained sequence was subjected to further analysis using the Basic Local Alignment Search Tool (BLAST) for finding sequence similarity with sequences already reported in online databases.

Basic Local Alignment Search Tool (BLAST): https://blast.ncbi.nlm.nih.gov/Blast.cgi

2.23.1 BLAST (Basic Local Alignment Search Tool)

BLAST is an algorithm that can compare and align a query nucleotide or protein sequence with a number of sequences contained in its database. It finds regions of local similarity between the sequences by calculating the statistical significance of matches. It is both rapid and sensitive and hence is used by millions of biologists. It is available online at the National Center for Biotechnology Information (NCBI) website (Lobo, 2008).

Method:

- https://blast.ncbi.nlm.nih.gov/Blast.cgi is browsed
- Sequences are pasted
- Parameters are changed if necessary
- BLAST is clicked on and few minutes are required to get the result.

CHAPTER 3

RESULTS

3.1 **Microbiological culture and biochemical tests**

Samples were cultured on MacConkey in order to identify gram-negative bacteria. Next, biochemical tests were performed to identify the specific isolates. Table 10 indicates the number of isolates and their name collected from different sources.

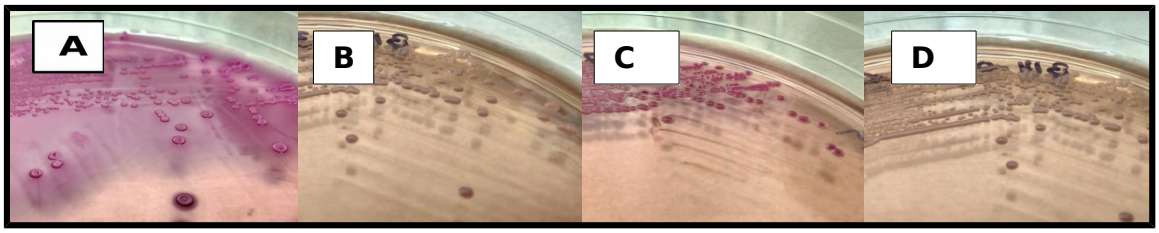


Figure 5: Colonies of A. *E. coli*, B. *P. aeruginosa*, C. *A. baumannii* and D. *K. pneumoniae* on MacConkey agar plate

Agar Plate	Bacterial Colony Morphology						
	Size	Form	Elevation	Margin	Colour	Texture	Appearance
MacConkey	Medium	Circular	Convex	Entire	Pink	Mucoid	Shiny

Table 7: Colony Morphology on MacConkey

Bacterial isolates	Colony characteristics of Gram positive organisms					
	Size	Margin	Elevation	Pigment	Form	Consistency
S. aureus	Medium	Entire	Convex	Golden yellow or white	Circular	Smooth
Enterococcus spp.	Small	Entire	Raised	Watery	Circular	Smooth
S. pneumoniae	Small	Entire	Raised	Greenish	Circular	Smooth

Table 8: Colony morphology on blood agar

3.2 **Gram staining**

Gram staining result of the strains were observed under microscopic oil emersion lens for the confirmation of cellular morphology and arrangement. Some colonies were purple in color and spherical, remained in chain or pairs denoting that they were gram positive bacteria and some are pink under microscope denotes gram negative bacteria.

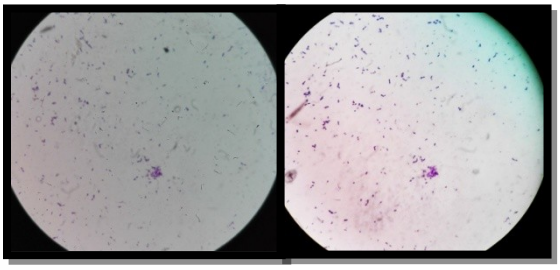


Figure 6: Microscopic image of Gram staining. Image at the left side was the result from wound swab samples and indicates gram-positive bacteria and the right side image indicates the results from poultry samples which is gram negative bacteria (pink in color)

3.3 **Biochemical identification**

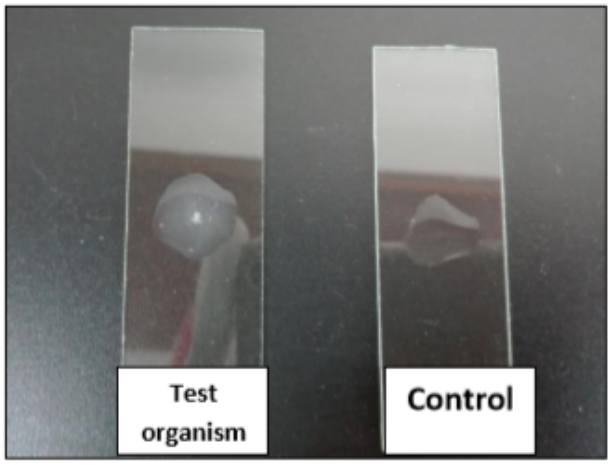
Experimental bacterial strains were subjected to several Biochemical Tests and all gave standard results of gram-positive and gram-negative bacteria. The results are as follows:

IMVic				Nitrate Reduction Test	Catalase Test	Oxidase Test	MIU Test		TSI (Triple Sugar Iron Test)				
Indole Test	Methyl Red Test	Voges-Proskaur Test	Citrate Utilization Test				Motility	Urease	Slant	Butt	Carbohydrate Fermentation	H_2S	CO_2 or Gas
(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)	Yellow Acidic	Yellow Acidic	(+)	(-)	(+)

Here, (+) indicates positive result and (-) indicates negative test result

Table 9: Biochemical Identification Results

Figure 7: Results of biochemical tests of gram-negative bacteria



Organism shows positive catalase activity by the rapid formation of O2 bubbles

Figure 8: Catalase test result

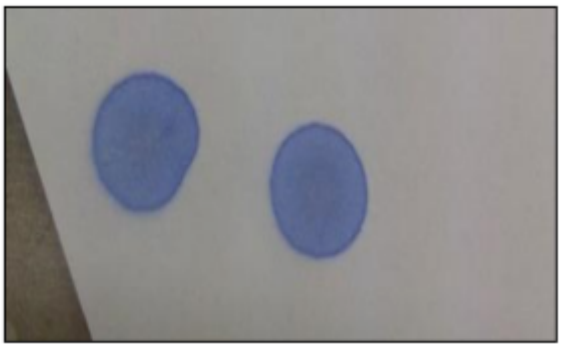


Figure 9: Oxidase test result

Source	Type	Organism, n (%)						
		ECO	KPN	ACB	PSA	ENTB	MRSA	ENTC

Clinical	Respiratory (n=212)	39 (18.40%)	52 (24.53%)	28 (13.21%)	11 (5.19%)	17 (8.02%)	22 (10.38%)	43 (20.28%)
	Wound (n=150)	13 (8.67%)	23 (15.33%)	25(16.67 %)	9 (6%)	8 (5.33%)	37 (24.67%)	35 (23.33%)
	Diarrheal (n=88)	26 (29.55%)	24 (27.27%)	4 (4.55%)	18 (20.45%)	8 (9.09%)	5 (5.68%)	3 (3.41%)
Total	N=450	78 (17.33%)	99 (22%)	57 (12.67%)	38 (8.44%)	33(7.33%)	64 (14.22%)	81 (18%)

Table 10: Number of clinical isolates and their source

*ECO= *E.coli*, KPN=*Klebsiealla pneumoniae*, ACB=*Acinetobacter baumanii*, PSA= *Pseudomonas aeruginosa*, ENTB=*Enterobacter spp*., MRSA= Methicillin-resistant *Staphylococcus aureus*, ENTC= *Enterococcus spp.*

Source	Type	Organism						
		ECO	KPN	ACB	PSA	ENTB	MRSA	ENTC

Poultry	Meat (n=23)	6 (26.09%)	4 (17.39%)	3 (13.04%)	1 (4.35%)	9 (39.13%)	-	-
	Lung (n=30)	9 (30%)	5 (16.67%)	3 (10%)	2 (6.67%)	11 (36.67%)	-	-
	Digestive tract (n=34)	8 (23.53%)	5 (14.71%)	4 (11.76%)	3 (8.82%)	14 (41.18%)	-	-
	Liver (n=19)	8 (42.11%)	3 (15.79%)	2 (10.53%)	1 (5.26%)	5 (26.32%)	-	-
	Cloacae (n=29)	7 (24.14%)	5 (17.24%)	4 (13.80%)	2 (6.90%)	11 (37.93%)	-	-
	Oropharangeal (n=20)	7 (35%)	4 (20%)	3 (15%)	1 (5%)	5 (25%)	-	-
Total	N= 155	45 (29.03%)	26 (16.77%)	19 (12.26%)	10 (6.45%)	55 (35.48%)	-	-

Table 11: Number of isolates from poultry and their source

*ECO= *E.coli*, KPN=*Klebsiealla pneumoniae*, ACB=*Acinetobacter baumanii*, PSA= *Pseudomonas aeruginosa*, ENTB=*Enterobacter spp*., MRSA= Methicillin-resistant *Staphylococcus aureus*, ENTC= *Enterococcus spp.*

Source	Type	Organism						
		ECO	KPN	ACB	PSA	ENTB	MRSA	ENTC
Environment	Water, n=63	11 (17.46%	9 (14.29%	7 (11.11%)	19 (30.16%	17 (27%)	-	-

		)	)		)			
Livestock	Cow fecal, n=60	14 (23.33%)	10 (16.67%)	17 (28.33%)	11 (18.33%)	8 (13.33%)	-	-
Total	N= 123	148 (20.33%)	141 (19.37%)	100 (13.80%)	78 (10.71%)	113 (15.52%)	-	-

Table 12: Number of isolates from environment and livestock and their source

*ECO= *E.coli*, KPN=*Klebsiealla pneumoniae*, ACB=*Acinetobacter baumanii*, PSA= *Pseudomonas aeruginosa*, ENTB=*Enterobacter spp*., MRSA= Methicillin-resistant *Staphylococcus aureus*, ENTC= *Enterococcus spp.*

Among 728 isolates collected from different sources (clinical, n= 450, poultry, n=155, Environmental water, n=63 and cow fecal, n=60), were as follows: *E. coli* (n=148), *Klebsiealla pneumoniae* (n=144), *Acinetobacter baumanii* (n=100),*Pseudomonas aeruginosa* (n=78), *Enterobacter spp.* (n=113), *MRSA* (n=64) and *Enterococcus spp.* (n=81). However, gram-positive bacteria were isolated from clinical samples only and MRSA was found to be high in number; followed by *Enterococcus spp.*, *Klebsiealla pneumoniae, Acinetobacter baumanii, E.coli, Psudomonas aeruginosa and Enterobacter spp*. MRSA also showed highest existence in wound sample. Furthermore, in respiratory samples, number of *Klebsiealla pneumoniae* was high and *E.coli* showed highest range in samples collected from diarrheal patient. In the case of poultry samples, *Enterobacter spp*. was the most prevalent in meat, lung and cloacae. Furthermore, *E.coli* was found in liver, digestive tract and oropharyngeal. Moreover, *Acinetobacter baumanii* and *Pseudomoans aeruginosa* was found in the highest percentage isolated from cow fecal and environmental water sample respectively.

3.4 **Antibiotic susceptibility tests**

Both Kirby-Baur disk diffusion method and minimum inhibitory concentration (EUCAST guidelines, 2018) was measured in order to observe antibiotic resistance pattern. Table 14 and Table 17 indicates resistance pattern based on Kirby-Baur disk diffusion method and minimum inhibitory concentration (MIC) respectively.

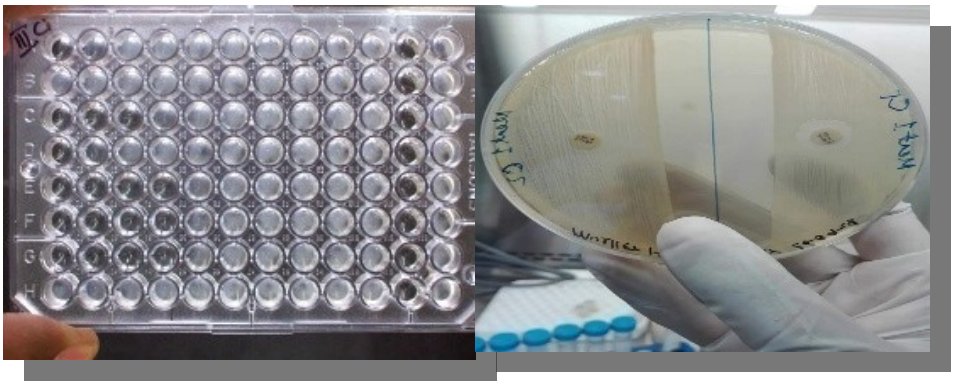


Figure 10: Antibiotic susceptibility results

Organisms	Clinical (n=70)	Poultry (n=38)	Environmental water sample (n=22)	Cow fecal (n=12)	Total (N=142)
ECO	7 (10%)	14 (38.84%)	3 (13.64%)	3 (25%)	27 (19.01%)
KPN	5 (6.58%)	9 (26.68%)	2 (9.09%)	2 (16.67%)	18 (12.68%)
ACB	2 (2.63%)	1 (2.63%)	5 (22.73%)	5 (41.67%)	13 (9.15%)
PSA	2 (2.63%)	3 (7.89%)	9 (40.91%)	2 (16.67%)	16

					(11.27%)
ENTB	3 (3.95%)	11 (28.95%)	3 (13.64%)	-	17 (11.97%)
MRSA	42 (55.26%)	-	-	-	42 (55.26%)
ENTC	9 (11.84%)	-	-	-	9 (11.84%)

Table 13: Antibiotic susceptibility results of isolated bacterial strains using Kirby-Baur disk diffusion method

*ECO= *E.coli*, KPN=*Klebsiealla pneumoniae*, ACB=*Acinetobacter baumanii,* PSA= *Pseudomonas aeruginosa*, ENTB=*Enterobacter spp.*, MRSA= Methicillin-resistant *Staphylococcus aureus*, ENTC= *Enterococcus spp.*

Total 142 isolates showed resistant to colisitin disk. *E.coli*, *Klebsiealla pneumoniae* and *Enterobacter spp*. expressed highest resistance to colistin but percentage is higher for *Acinetobacter baumanii* in cow fecal. However, among gram positive bacteria 55.26% *MRSA* and 11.84% *Enterococcus spp.* expressed resistance to colistin disk.

3.5 **Molecular detection**

Polymerase chain reaction (PCR) was performed in order to confirm genes in those isolates which were the phenotypically resistant. Figure 11 indicates percentages of mcr positive isolates.

After execution of polymerase chain reaction (PCR) mcr positive isolates were found from four different sources. The highest percentage of mcr positive strains found in both clinical and poultry samples. In clinical sample the number of mcr positive isolates is higher for *Klebsiealla pneumoniae* followed by *Acinetobacter baumanii*, *MRSA*, *E.coli* and *Pseudomonans aeruginosa*. However, percentage is higher for *Enterobacter spp.* in poultry samples. Furthermore,

Acinetobacter baumanii and *Pseudomonas aeruginosa* showed the highest resistance to colistin found in cow fecal and environmental water samples respectively.

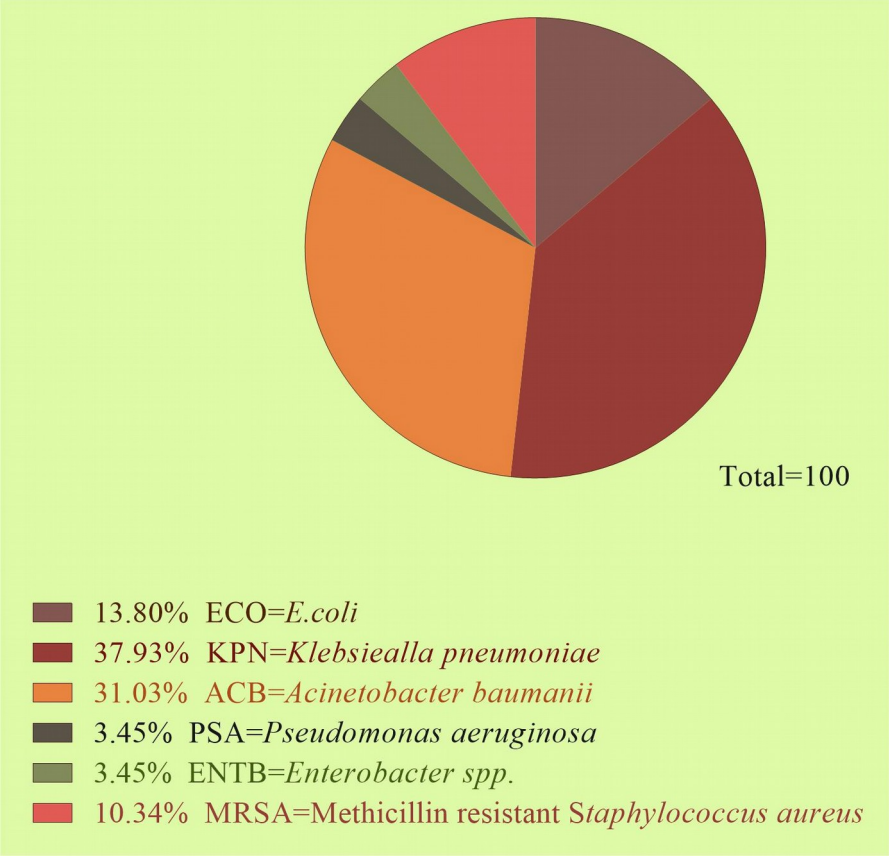


Figure 11: Percentages of mcr positive isolates

Type of mcr	Type of Clinical isolates			Total
mcr-1	Respiratory	Wound	Diarrheal	24 (5.33%)
	4(2%)	19(12.67%)	1(1.14%)	
mcr-2	2(0.94%)	3(2%)	-	5(1.11%)
mcr-1 and mcr-2	-	3(2%)	-	3 (2%)

Table 14: Percentages of mcr-1 and mcr-2 positive clinical isolates

Clinical isolates were sub divided into three parts, e.g. respiratory, wound and diarrheal samples. Highest percentage of mcr-1 positive strain found in wound samples yet mcr-2 was only found in respiratory and wound sample. There was also co-existence of mcr-1 and mcr-2 in wound and diarrheal samples.

Type of mcr	Poultry
mcr-1	39 (25.16%)
mcr-2	-
Co-existence	-

Table 15: Percentages of mcr-1 positive isolates from poultry

Analyzing mcr positivity from poultry, it was observed that *Enterobabcter spp.* showed highest resistance found in all parts of chicken apart from liver swab. In liver swab *E.coli* showed the highest resistance. However, there was no co-existence of mcr-1 mcr-2 from poultry samples.

Type of mcr	Cow fecal n=60	Environmental water sample n=63

mcr-1	2 (3.33%)	5 (7.94%)
mcr-2	6 (10%)	-
Co-existence	2 (3.33%)	-

Table 16: Percentages of mcr-1 and mcr-2 positive isolates from cow fecal and environmental water sample

In cow fecal the percentage of resistance was higher for mcr-2 than mcr-1. Co-existence of these two genes found in cow samples and mcr-1 found from environmental water samples only.

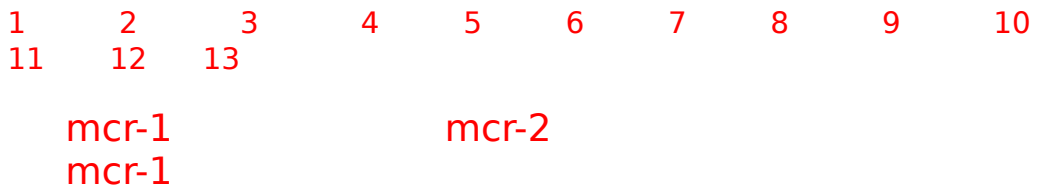
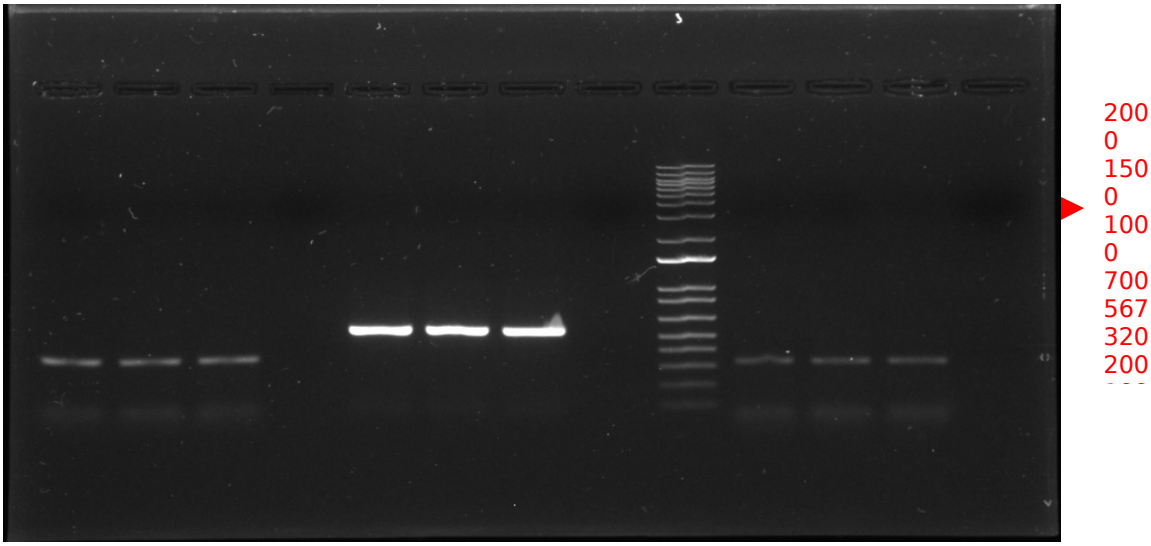


Figure 12: Gel electrophoresis results of mcr-1 and mcr-2. Lane 1, lane2 and lane 3 indicates mcr-1 gene found from clinical, poultry and environmental water samples. Lane 5, lane 6 and lane 7 indicates mcr-2 gene detected from clinical and cow samples. Lastly, lane 10, lane 11 and lane 12 indicates mcr-1 gene from cow fecal water sample. Lane 4, lane 8 and lane 13 indicates negative control.



Bacterial isolate	Source	mcr-1 (mg/ml)	mcr-2 (mg/ml)
Acinetobacter baumanii	Clinical	64	16
Acinetobacter baumanii	Clinical	16	16
Pseudomonas aeruginosa	Clinical	16	16
E.coli	Clinical	4	32
Klebsiealla pneumoniae	Clinical	4	16
E.coli	Chicken	4	32
Enterobacter spp.	Chicken	32	64
Klebsiealla pneumoniae	Chicken	16	64
E.coli	Environment	4	64
Pseudomonas aeruginosa	Environment	16	32
Acinetobacter baumanii	Cow fecal	4	16

Acinetobacter baumanii	Cow fecal	16	16
E.coli	Cow fecal	4	4
Pseudomonas aeruginosa	Cow fecal	32	64

Table 17: Correlation between mcr gene and minimum inhibitory concentration (MIC)

Ten isolates were randomly analyzed in order to observe minimum inhibitory concentration (MIC) at six different concentrations 2mg/ml, 4mg/ml, 8mg/ml, 16mg/ml, 32mg/ml, 64mg/ml. Among above mentioned six concentrations, 64mg/ml was the highest concentration at which analyzed isolates' growth was observed. However, it was perceived that MIC was higher for mcr-2. Therefore, it is clearly understandable that average concentration is higher for mcr-2 than mcr-1.

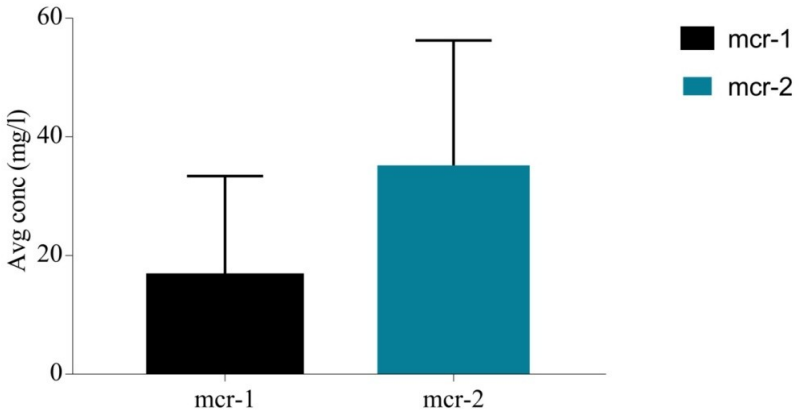


Figure 13: Correlation between mcr gene and average minimum inhibitory concentration (MIC)

3.6 **Resistance type:**

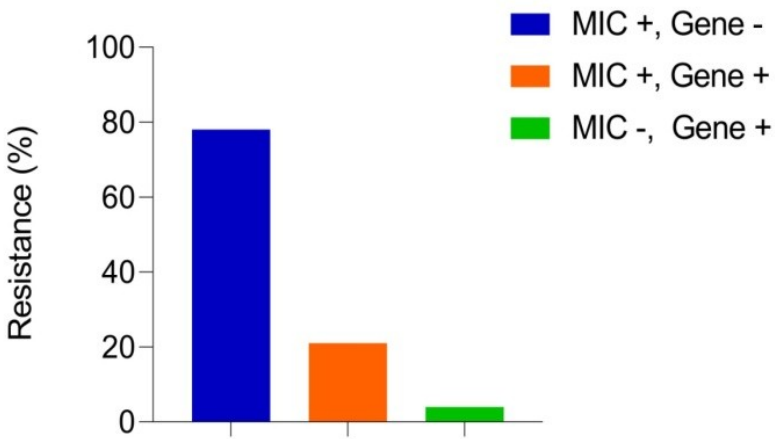


Figure 14: Resistance type of bacteria isolates

Taking consideration of minimum inhibitory concentration, three types of resistance were found. The first type showed growth at high MIC but still no gene was detected. The second type grew at highest MIC and also mcr gene was found. Finally, the last type was MIC sensitive but still those isolates possessed mcr gene. The percentage was higher for first type and very few isolates followed last type of resistance.

3.7 **Year wise resistance**

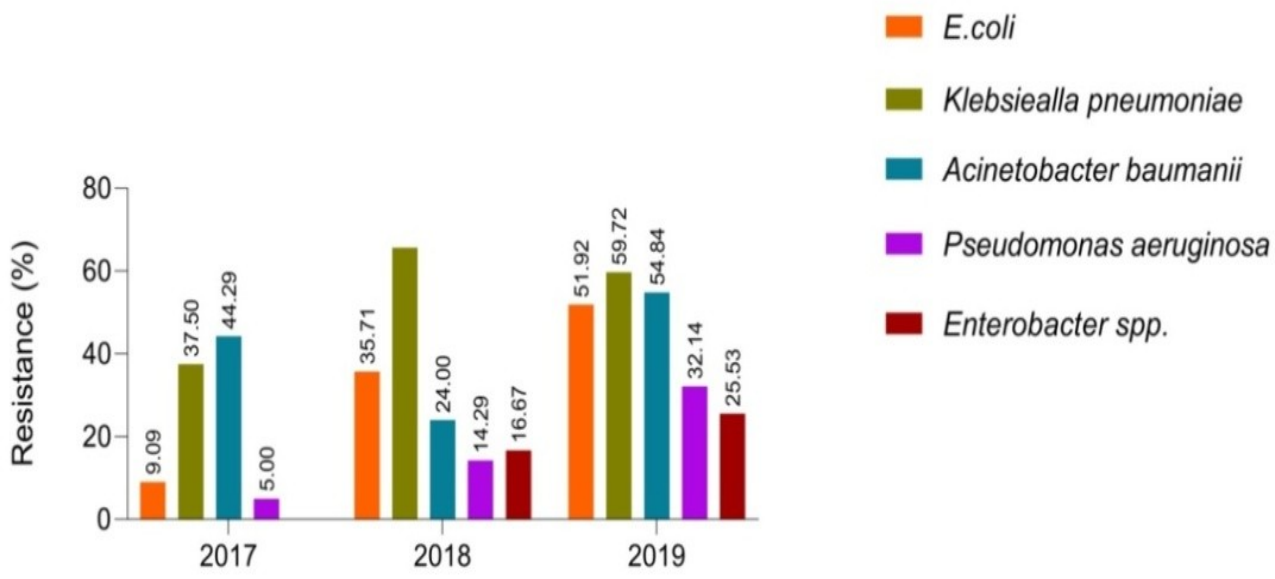


Figure 15: Year wise resistance

Some isolates were preserved at -80ºC at the lab since 2017 and sample collection continued in 2018 and 2019. Therefore, considering three years' isolates resistance pattern was analyzed. No doubt the resistance trend was the highest in 2019. Resistance trend is higher for *Klebsiealla pneumoniae* in all the three years.

3.8 **Template preparation from culture plate (Sequencing of PCR product)**

For sequencing purpose, two templates were prepared from two different strains. After done with PCR and PCR purification, they were analyzed by sequencing. The sequencing data were analyzed by Chromas Lite 2.1 software. This tool generates a four colors chromatogram showing the result of sequencing run. Different bases are represented in different colors that are defined below:

1.Adenosine=Green

2.Guanine=Black

3.Cytosine=Blue

4. Thymine=Red

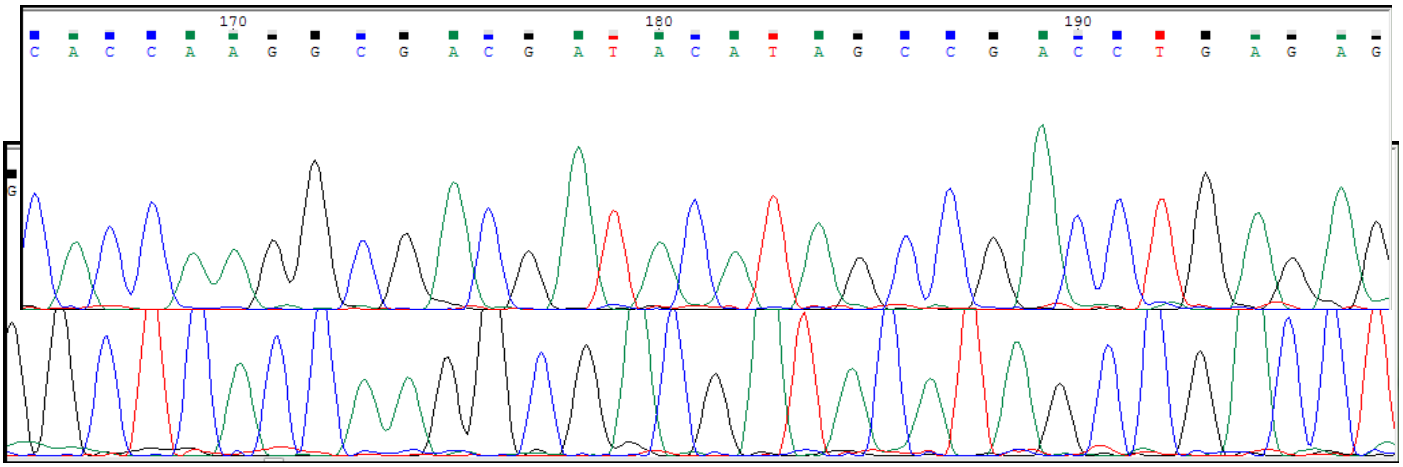


Figure 16: Diagrammatic representation of a part of the chromatogram of sequence of two strains. Here, the peaks are well-formed which are shown by different color. There is no background messiness, which indicates, the refined sequence had proper concentration o of template and primer. In the chromatogram, the area under the peak is considered as the measure of component concentration.

>L3c1p5-21-19-6-57 PM.ab1

TTCTTTCGCGTGATAAGCCGCTGCGTAGCTATGTCAATCCGATCATGCCAATCTACTCGGTGGGTAAGCTTGCCAGTATT

GAGTATAAAAAAGCCAGTGCGCCAAAAGATACCATTTATCACGCCAAAGACGCGGTACAAGCAACCAAGCCTGATATGCG

TAAGCCACGCCTAGTGGTGTTCGTCGTCGGTGAGACGGCACGCGCCGATCATGTCAGCTTCAATGGCTATGAGCGCGATA

CTTTCCCACAGCTTGCCAAGATCGATGGCGTGACCAATTTTAGCAATGTCACATCGTGCGGCACATCGACGGCGTATTCT

GTGCCGTGTATGTTCAGCTATCTGGGCGCGGATGAGTATGATGTCGATACCGCCAAATACCAAGAAAATGTGCTGGATAC

GCTGGATCGCTTGGGCGTAAGTATCTTGTGGCGTGATAATAATTCGGACTCAAAAGGCGTGATGGATAAGCTGCCAAAAG

CGCAATTTGCCGATTATAAATCCGCGACCAACAACGCCATCTGCAACACCAATCCTTATAACGAATGCCGCGATGTCGAT

GCCCCCTTTGGGCTTAAA

Sequencing data of clinical sample

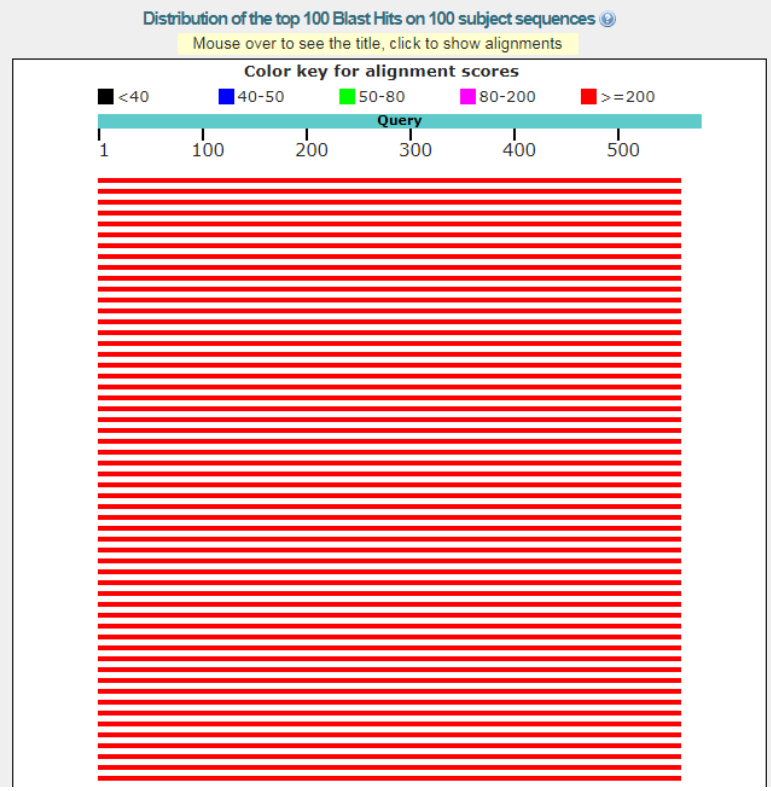
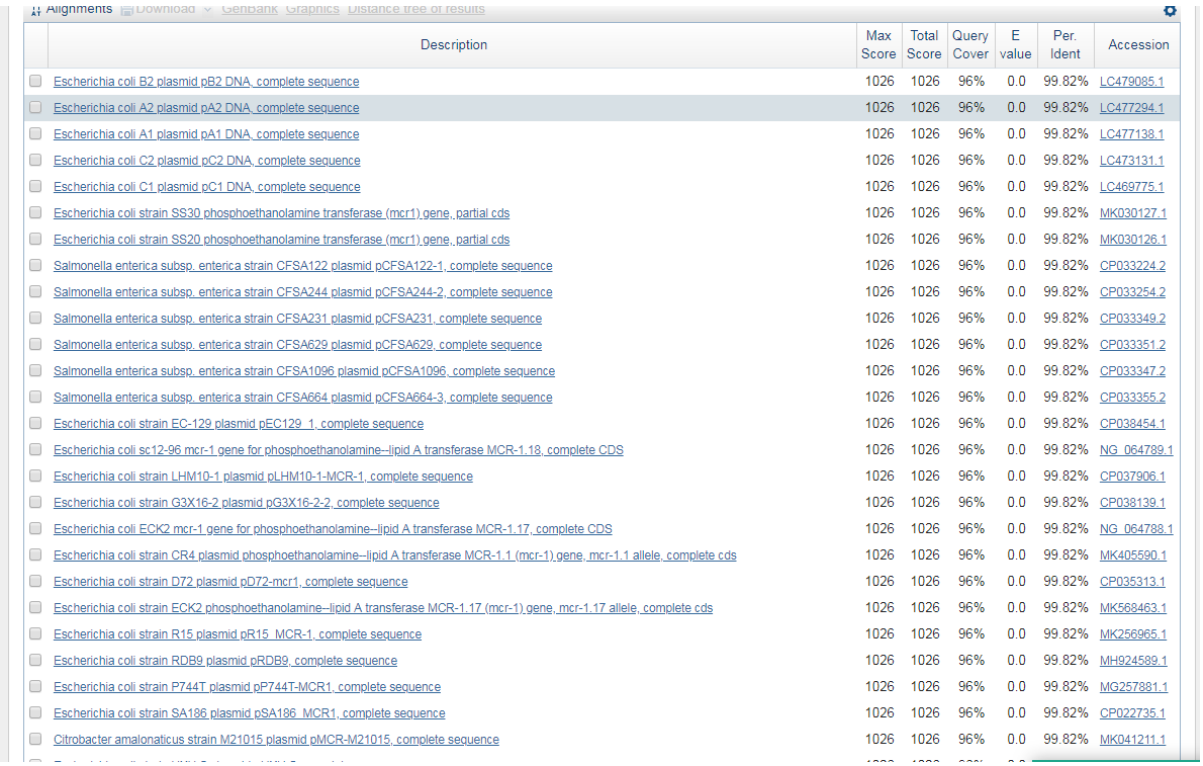


Figure 17: Graphic Summary of BLASTn result using the sequenced PCR product of clinical sample



Alignments Download GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Escherichia coli B2 plasmid pB2 DNA, complete sequence	1026	1026	96%	0.0	99.82%	LC479085.1
Escherichia coli A2 plasmid pA2 DNA, complete sequence	1026	1026	96%	0.0	99.82%	LC477294.1
Escherichia coli A1 plasmid pA1 DNA, complete sequence	1026	1026	96%	0.0	99.82%	LC477138.1
Escherichia coli C2 plasmid pC2 DNA, complete sequence	1026	1026	96%	0.0	99.82%	LC473131.1
Escherichia coli C1 plasmid pC1 DNA, complete sequence	1026	1026	96%	0.0	99.82%	LC469775.1
Escherichia coli strain SS30 phosphoethanolamine transferase (mcr1) gene, partial cds	1026	1026	96%	0.0	99.82%	MK030127.1
Escherichia coli strain SS20 phosphoethanolamine transferase (mcr1) gene, partial cds	1026	1026	96%	0.0	99.82%	MK030126.1
Salmonella enterica subsp. enterica strain CFSA122 plasmid pCFSA122-1, complete sequence	1026	1026	96%	0.0	99.82%	CP033224.2
Salmonella enterica subsp. enterica strain CFSA244 plasmid pCFSA244-2, complete sequence	1026	1026	96%	0.0	99.82%	CP033254.2
Salmonella enterica subsp. enterica strain CFSA231 plasmid pCFSA231, complete sequence	1026	1026	96%	0.0	99.82%	CP033349.2
Salmonella enterica subsp. enterica strain CFSA629 plasmid pCFSA629, complete sequence	1026	1026	96%	0.0	99.82%	CP033351.2
Salmonella enterica subsp. enterica strain CFSA1096 plasmid pCFSA1096, complete sequence	1026	1026	96%	0.0	99.82%	CP033347.2
Salmonella enterica subsp. enterica strain CFSA664 plasmid pCFSA664-3, complete sequence	1026	1026	96%	0.0	99.82%	CP033355.2
Escherichia coli strain EC-129 plasmid pEC129_1, complete sequence	1026	1026	96%	0.0	99.82%	CP038454.1
Escherichia coli sc12-96 mcr-1 gene for phosphoethanolamine--lipid A transferase MCR-1.18, complete CDS	1026	1026	96%	0.0	99.82%	NG_064789.1
Escherichia coli strain LHM10-1 plasmid pLHM10-1-MCR-1, complete sequence	1026	1026	96%	0.0	99.82%	CP037906.1
Escherichia coli strain G3X16-2 plasmid pG3X16-2-2, complete sequence	1026	1026	96%	0.0	99.82%	CP038139.1
Escherichia coli ECK2 mcr-1 gene for phosphoethanolamine--lipid A transferase MCR-1.17, complete CDS	1026	1026	96%	0.0	99.82%	NG_064788.1
Escherichia coli strain CR4 plasmid phosphoethanolamine--lipid A transferase MCR-1.1 (mcr-1) gene, mcr-1.1 allele, complete cds	1026	1026	96%	0.0	99.82%	MK405590.1
Escherichia coli strain D72 plasmid pD72-mcr1, complete sequence	1026	1026	96%	0.0	99.82%	CP035313.1
Escherichia coli strain ECK2 phosphoethanolamine--lipid A transferase MCR-1.17 (mcr-1) gene, mcr-1.17 allele, complete cds	1026	1026	96%	0.0	99.82%	MK568463.1
Escherichia coli strain R15 plasmid pR15_MCR-1, complete sequence	1026	1026	96%	0.0	99.82%	MK256965.1
Escherichia coli strain RDB9 plasmid pRDB9, complete sequence	1026	1026	96%	0.0	99.82%	MH924589.1
Escherichia coli strain P744T plasmid pP744T-MCR1, complete sequence	1026	1026	96%	0.0	99.82%	MG257881.1
Escherichia coli strain SA186 plasmid pSA186_MCR1, complete sequence	1026	1026	96%	0.0	99.82%	CP022735.1
Citrobacter amalonaticus strain M21015 plasmid pMCR-M21015, complete sequence	1026	1026	96%	0.0	99.82%	MK041211.1

Figure 18: Top BLAST hits that produced significant alignments with query sequence

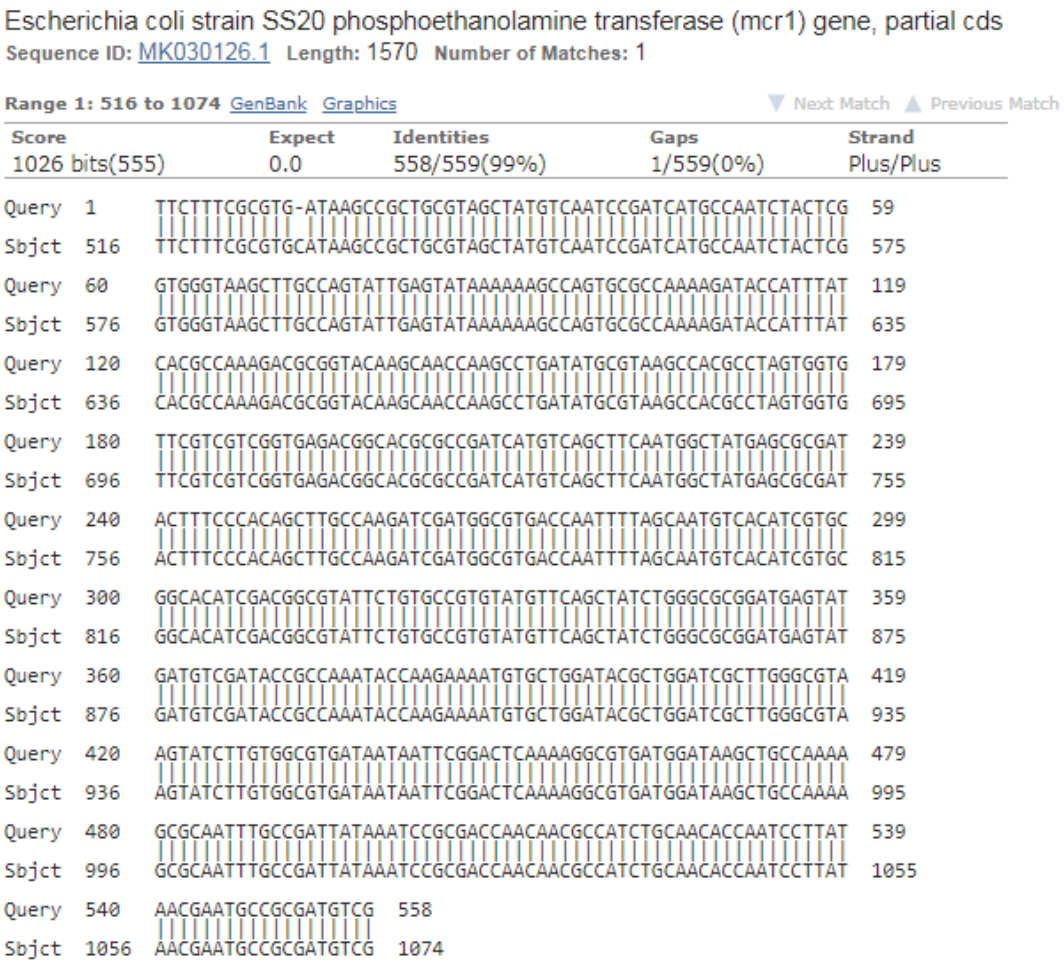


Figure 19: One of the matched sequence with MCR-1 gene

CHAPTER 4

DISCUSSION

Indiscriminate use of colistin in poultry, livestock and agriculture enables human gut bacteria to acquire resistant gene due to horizontal transfer. In this study a vicious cycle of transmitting mcr gene was depicted between human and poultry, livestock through the environment. However, a few studies have developed in Bangladesh recently. A study which was conducted by Islam, et al., 2017 reported *E.coli* possessing mcr-1 gene from the sludge. [30] Moreover, Dutta, et al., 2019 and Farzana, et al., 2019 also reported mcr-1 gene from *E.coli* from poultry and human respectively. [31] Analyzing these studies it was quite clear a subtle coherence of transmitting colistin resistant gene exists between poultry livestock and human. [32] Till date only mcr-1 gene has been detected and reported in *E.coli* only. However, in this study apart from *E.coli* four other gram negative bacteria which were *Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter spp*. was reported possessing both mcr-1 and mcr-2 gene. Our neighbouring country India, Pakistan and Mayanmar also reported mcr genes from clinical, poultry, food and environment samples. For instance, Kumar, et al., 2018 reported mcr gene in *Klebsiealla pneumoniae* which was chromosome mediated resistant gene. [33] Moreover, Aggarwal, et.,al 2018 reported Colistin-resistant *Klebsiella pneumoniae* in Surgical Polytrauma Intensive Care Unit of Level-1 Trauma Center which was first Case Series from Trauma Patients in India. [34] Furthermore, another study reported mcr gene in food in *Klebsiealla pneumoniae*. [34] In Pakistan, mcr gene was found in *Pseudomonas aeruginosa* and San, et al., 2019 reported mcr-1 gene in *E.coli* in Mayanmar. [35] Comparing our results with aforementioned reported bacteria reported in our neighbouring country, it is quite clear that all type of bacteria which cause some common hospital-acquired infections (HAIs) to human are acquiring resistant gene in Bangladesh.

However, among all the isolates highest percentages of mcr gene possessed by *Klebsiealla penumoniae* (37.93%) meaning prevalence is high in neighbouring country as well as our

country and the lowest percentages was *Pseudomonas aeruginosa* and *Enterobacter spp.* *Klebsiella penumoniae* is one of the most common infectious agents in hospital settings and causes hospital-acquired infections (HAIs) including pneumonia, bloodstream infections, wound or surgical site infections, and meningitis. [36] *Klebsiella* bacteria are normally found in the human intestines (where they do not cause disease). They are also found in human stool (feces). In healthcare settings, *Klebsiella* infections commonly occur among sick patients who are receiving treatment for other conditions. [37] Patients whose care requires devices like ventilators (breathing machines) or intravenous (vein) catheters, and patients who are taking long courses of certain antibiotics are most at risk for *Klebsiella* infections. [38] Healthy people usually do not get *Klebsiella* infections. In healthcare settings, *Klebsiella* bacteria can be spread through person-to-person contact (for example, from patient to patient via the contaminated hands of healthcare personnel, or other persons) or, less commonly, by contamination of the environment. [39] The bacteria are not spread through the air. Patients in healthcare settings also may be exposed to *Klebsiella* when they are on ventilators (breathing machines), or have intravenous (vein) catheters or wounds (caused by injury or surgery). Unfortunately, these medical tools and conditions may allow *Klebsiella* to enter the body and cause infection. This is how resistant gene is also spreading at a high rate. [40]

Moreover, *Acinetobacter baumanii* (31.01%) also showed higher resistance to colistin. *Acinetobacter* is a group of bacteria (germs) commonly found in the environment, like in soil and water. While there are many types, the most common cause of infections is *Acinetobacter baumannii*, which accounts for most *Acinetobacter* infections in humans. *Acinetobacter baumannii* can cause infections in the blood, urinary tract, and lungs (pneumonia), or in wounds in other parts of the body. It can also “colonize” or live in a patient without causing infections or symptoms, especially in respiratory secretions (sputum) or open wounds. [41] Furthermore, *Pseudomonas aeruginosa* and *Enterobacter spp.* both have the same resistance percentages 3.45%. These two organisms are a type of bacteria (germ) that is found commonly in the environment, like in soil and in water. [42] Of the many different types of *Pseudomonas*, the one that most often causes infections in humans is called *Pseudomonas aeruginosa*, which can cause infections in the blood, lungs (pneumonia), or other parts of the body after surgery.

Enterobacter infections can include bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, CNS infections, and ophthalmic infections. *Enterobacter* infections can necessitate prolonged hospitalization, multiple and varied imaging studies and laboratory tests, various surgical and nonsurgical procedures, and powerful and expensive antimicrobial agents [42].

According to EUCAST (>2mg/l; resistant and ≤2mg/l; sensitive) recommendation the susceptibility testing for Colistin is better performed by minimum inhibitory concentration (MIC) method. [43] The rational is that disc diffusion test by Kirby Bauer method for colistin has a very high level of interpretation error and therefore, MIC method is more preferable.[44] Moreover, analyzing our study results, it was observed that minimum inhibitory concentration has a great influence on the presence of resistant gene. The concentration is higher for those isolates which possess mcr-2 gene as it is the variant of mcr-1 and also concentration is higher for those isolates which have the coexistence of mcr-1 and mcr-2. To the best of our knowledge this is the first time where the correlation between MIC and exposure of gene has been showed.

However, in poultry samples, the percentage of mcr resistant isolate is 21.90% and only mcr-1 gene found from this source. The highest resistance was found from the digestive tract, followed by lung, cloacae, meat, oropharyngeal and liver. As food safety is a paramount concern in Bangladesh from the public health point of view and a remarkable number of people suffer from foodborne illnesses each year in our country, the main item as a day's meal, chicken was chosen to investigate mcr genes and it is observed that resistant gene has been spread in almost every part of chicken. It is almost certain that people working in the poultry industry, veterinary and fishery are exposed to an unhygienic environment. They also have a lack of knowledge about food safety, hygiene and sanitation and mainly depend on street foods of the nearby industries. Therefore, it is possible to transfer mobile colistin resistant (mcr) genes to food from industrial environment. The fishery is also notable to this phenomenon. Feces of chicken run to waterway through trellis which is also used as feed for fish and there is a high chance of transferring resistant gene to fish as well. On the other hand, in urban areas, people mostly depend on restaurant food. Meat, fish and vegetables consist of the large portion of a day's proper meal.

Apart from a very few restaurants, most do not concern about food safety. Poultry chicken meat is served in restaurants and sometimes the food remains even undercooked. As a result, the live bacteria in that food carrying resistant gene may transfer to human gut bacteria and finally these gut bacteria spread the resistant gene through environment. Consequently, people are being very silently trapped in this vicious cycle. It is very alarming as 14% and 12% mcr resistance found in meat and liver respectively. Besides, digestive tract of chicken plays a crucial role to spread mcr genes in the environment. Analyzing cow samples, both mcr-1 and mcr-2 found and mcr-1 found in environmental water samples only. However, *Acinetobacter baumanii* showed the highest percentage of resistance detected from cow samples and only *E.coli* found from the environmental samples which possess mcr genes. As human health is at present a main concern, a subtle coherence between environment and human was observed. Moreover, mcr-1 found in all types of samples yet mcr-2 found only in clinical and cow samples and within same isolate named *Acinetobacter baumanii*. However, how the condition is deteriorating day by day could be clearly understood if this study is more scrutinized. There were some isolates which expressed resistance gene, but they were not resistant phenotypically. On the other hand, some isolates showed resistant to colistin disk or their MIC was high yet no gene found after polymerase chain reaction (PCR) performed. The most interesting thing is very few isolates found sensitive when MIC was assessed or they found sensitive to colsitin disk yet still resistant gene found in them. Such isolate is *Acinetobacter baumanii*. It is assumed that *Acinetobacter baumannii* develops mechanism of resistance since the bacteria has rapid and easy adaptation and can change into resistant phenotype when it is exposed to polymyxin. Another theory of polymyxin resistance to *Acinetobacter baumannii* is that there is a sub-population of polymyxin-resistant strain; therefore, initially the detection using MIC method gives sensitive results, but the result will be resistant when we repeat the resistance culture procedure.[45] These results give an alarming indication of how the resistant gene is spreading at a high rate. The isolates which are phenotypically sensitive, yet gene found might be possessing pseudogene or unexpressed gene. On the other hand, those isolate which are phenotypically resistant still no gene detected, can be other resistant genes which have not detected yet. However, it was also observed that, with time the resistance trend is also increasing. Preserved isolates in our lab was analyzed and also new samples collected in 2018 and 2019 and it was perceived that the percentage is higher in 2019

than in 2018 and 2017. Furthermore, the isolates which was collected in 2017, was only phenotypically resistant to colistin but isolates collected in 2018 and 2019 are both phenotypically and genotypically resistant. Moreover, apart from mcr-1, its variant mcr-2 is also found in human for the first time in our country.

However, there are some limitations in this study. Sanger sequencing is performed here where next generation sequencing (NGS) could have given more information like whether other than mcr-1 to 7 any novel genes are involved for colistin resistance in Bangladesh. Finally, information about resistance spectrum of the isolates for other antibiotics would have given more useful information. However, due to limitations in budget and resources these investigations wasn’t performed in this study.

CHAPTER 5

CONCLUSION

Treatment with polymyxin, particularly colistin has been reintroduced to clinical practice against multi-resistant Gram-negative bacteria (MDRO). For therapy, colistin is recommended for severe infection cases caused by Gram-negative MDRO, but it should be given in combination treatment with other antibiotics and it is not recommended for empirical treatment and monotherapy as it has severe toxic effects on human health. The recommended duration of treatment for pneumonia or bacteremia is 14 days. Physicians should be aware of the possible side effects of Colistin, particularly nephrotoxicity and neurotoxicity. It is recommended to have an evaluation of kidney function, i.e. creatinine serum level every other day to provide close monitoring on the possibility of nephrotoxic effect. The rational treatment of Colistin must be closely monitored so that pan-resistant strains will not be developed. Continuous studies and further knowledge on the structure and activity of colistin, its pharmacokinetics, pharmacodynamics and optimal combination treatment have made colistin as a promising alternative antibiotic treatment against Gram-negative bacteria MDRO in the future but indiscriminate use of this antibiotic recently in poultry and livestock making the condition worse as resistant gene is spreading at a high rate and also resistant genes are found in almost all common gram-negative bacteria. Therefore, the necessary strategies should be taken immediately to stop the overuse of this antibiotic.

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