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DETECTION OF MOBILIZED COLISTIN RESISTANCE (*mcr*) GENE FROM LIVESTOCK WASTEWATER SAMPLES

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DETECTION OF MOBILIZED COLISTIN RESISTANCE (*mcr*) GENE FROM LIVESTOCK WASTEWATER SAMPLES

By

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A project report submitted to the Department of Biological Science

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ABSTRACT

DETECTION OF MOBILIZED COLISTIN RESISTANCE (*mcr*) GENE FROM LIVESTOCK WASTEWATER SAMPLES

Amy Chua Jia Xuan

The prolong extensive use of broad-spectrum antibiotics and misuse of antibiotics for numerous purposes have given rise to the rapid emergence of multi drug-resistance (MDR) bacteria. Colistin was once used as the last resort for treating infections caused by these MDR gram-negative bacteria, until the plasmid-mediated mobilized colistin resistance (*mcr*) gene which allowed the bacteria to resistant to colistin emerged. Given the significant roles of colistin in clinical use, the objectives of this research were to isolate, detect and determine the minimum inhibitory concentration (MIC) of colistin and antibiotics susceptibility of *mcr*-harbouring bacteria from livestock wastewaters. Livestock wastewater samples were collected from water contaminated with chicken faeces and water used to wash pork intestines and chicken intestines respectively. Gram-negative lactose-fermenting bacteria were isolated using MacConkey agar and the presence of *mcr* gene was detected using polymerase chain reaction (PCR). The MICs of *mcr*-positive isolates were studied using broth microdilution method and the antibiotic resistance profile was determined using Kirby-Bauer disc diffusion method. A total of 43 gram-negative lactose-fermenting bacteria were isolated from the samples. Among the isolates, *mcr-1* gene was detected in 7 out of 43 (16.28 %) isolates, *mcr-3* gene was detected in

1 out of 43 (2.33 %) isolates, and no *mcr-5* was detected in all the isolates. The results of broth microdilutions showed that the MIC of *mcr*-positive bacteria ranged between 1 μg/mL to 4 μg/mL. Besides, all the isolates harbouring *mcr-1* gene were found to co-resistant to 4 to 5 other antibiotics classes while the isolate harbouring *mcr-3* was found to co-resistant to another antibiotic class used in the study, suggesting that the *mcr-1* positive isolates were multi-drug resistance while the *mcr-3* positive isolate was not multi-drug resistance.

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Lastly, not forgetting my family members who always support me in my academic achievements even though they were not by my side. Their support and encouragement were the motivations which drove me forward throughout my academic study.

DECLARATION

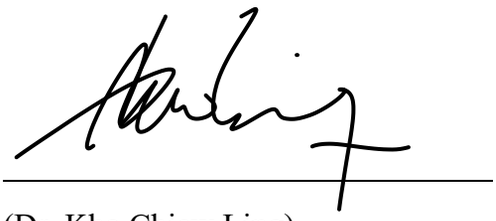
I hereby declare that this project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Amy Chua Jia Xuan

APPROVAL SHEET

This final year project report entitled "**DETECTION OF MOBILIZED COLISTIN RESISTANCE (*mcr*) GENE FROM LIVESTOCK WASTE WATER SAMPLES**" was prepared by AMY CHUA JIA XUAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:



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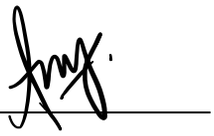
Date: 22/08/2022

PERMISSION SHEET

It is hereby certified that **AMY CHUA JIA XUAN** (ID No.: **19ADB06830**) has completed this final year project entitled "**DETECTION OF MOBILIZED COLISTIN RESISTANCE (*mcr*) GENE FROM LIVESTOCK WASTE WATER SAMPLES**" under the supervision of Dr. Kho Chiew Ling from the Department of Biological Sciences, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project thesis in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,



(AMY CHUA JIA XUAN)

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LIST OF ABBREVIATIONS

°	Degree
°C	Degree Celcius
µg	Microgram
µL	Microlitre
·OH	Hydroxyl radical
AMP	Ampicillin
$BaCl_2$	Barium Chloride
bp	Base Pair
Ca^{2+}	Calcium ion
CFU	Colony-forming Unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CM	Chloramphenicol
CPM	Cefepime
CRE	Carbepenem-resistance *Enterobacteriaceae*
CT	Colistin
CTR	Ceftriaxone
CTX	Cefotaxime
Dab	Diaminobutyric acid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
I	Intermediate
IPM	Imipenem
kb	Kilo base pair
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharide

mcg	Microgram
mcr	Mobilized Colistin Resistance
MDR	Multi-Drug Resistance
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
mL	Mililitre
mM	Millimolar
mm	Millimetre
N/A	Not applicable
NADH	Nicotinamide Adenine Dinucleotide
NI	No inhibition
O_2^-	Superoxide
PCR	Polymerase Chain Reaction
pEtN	Phosphoethanolamine
PRL	Piperacillin
R	Resistance
rpm	Revolutions per minutes
S	Susceptible
SDD	Susceptible-dose dependent
SOD	Superoxide Dismutase
spp.	Species
SXT	Sulfamethoxazole-trimethoprim
TAE	Tris-acetate EDTA
TE	Tetracycline
TNF	Tumour Necrosis Factor
TOB	Tobramycin
U	Unit
UV	Ultraviolet
V	Voltage

CHAPTER 1

INTRODUCTION

Colistin is a type of antibiotic which was discovered in *Paenibacillus polymyxa* in 1949 (Koyama, 1950). Historically, it was used in the intravenous formulation for the therapy of various infections caused by gram-negative bacteria. However, reports on the neurotoxicity and nephrotoxicity of colistin and the discovery of new antimicrobial agents which were less toxic made the usage of colistin gradually abandoned between the 1970s and 1990s (Lim et al., 2010).

Since the discovery of antibiotics, they have been widely used in treating the various types of infections caused by bacteria and have greatly reduced the mortality rate of infectious diseases. However, the prolong extensive use of broad-spectrum antibiotics and misuse of antibiotics for numerous purposes had created the selective pressure by eliminating susceptible bacteria, giving rise to the rapid emergence of drug-resistant bacteria (Prescott, 2014). The increase of antimicrobial resistance, especially carbapenem-resistant *Enterobacteriaceae* (CRE) presents a significant health care crisis worldwide. Limited or no effect of the currently available antimicrobial agents such as beta-lactams and fluoroquinolones which used to be effective in fighting the pathogens and slow discovery of new antibiotics left not many treatment options for treating bacterial infections. All these factors led to the reconsideration of the usage of colistin as the last resort in treating infections of multi-drug resistant (MDR) gram-negative bacteria (El-Sayed Ahmed et al., 2020).

Although the usage of colistin was shown to have a significant effect in combating multidrug gram-negative bacteria, its function was slowly depleted when colistin resistance started to develop in the bacteria. Initially, the resistance of bacteria to colistin was mainly caused by chromosomal mutations (McPhee, Lewenza and Hancock, 2003). However, it once again alarmed the health care system when discovery of the plasmid-mediated mobilized colistin resistance (*mcr*) gene was first reported in China in the year 2015 (Liu et al., 2016).

Mobilized colistin resistance gene was found to be plasmid-mediated which allowed colistin resistance gene to be horizontally transferred among the bacteria. The plasmids encoded for colistin resistance gene can be passed from the colistin resistant bacteria to non-colistin resistant bacteria through conjugation or transduction. Besides, colistin resistance genes can also be acquired by the bacteria from the environment through transformation (Burmeister, 2015). This transfer of genes can occur not only between bacteria of the same species but also between bacteria of different species. Therefore, the emergence of bacteria which are resistant to colistin rise dramatically in the recent year. When the colistin resistance gene is acquired by the MDR bacteria, they will no longer be susceptible to the colistin which acts as the last-line treatment and this might lead to untreatable bacterial infections.

When the antibiotics are not functioning anymore in combating the infections, it can result in many circumstances, such as prolong illness, increased medical visitation, more complex illness, usage of more expensive antibiotics and increased mortality rate in the worst situation.

Scientist have been putting in a lot of effort in discovering and developing new antibiotics but there were no new classes of antibiotics being found after 1980s. At the same time, research on disclosure and establishment of new antibiotics is found to be challenging as the research and development procedure for antibiotics development is difficult, high cost, time-consuming and often fails. Statistic showed that, over $1,000,000,000 is needed for developing a new antibiotic and the process can take up to 10-15 years (Wellcome, 2022). Furthermore, bringing a new antibiotic into the market is another difficult task as the public are usually fear of trying new drugs which are still lack of clinical trials.

Looking at the outpacing of the emergence of MDR bacteria to the development of new antibiotics, the only solution to cope with the crisis is to implement the regulations, prevention measures and surveillance on the proper usage of antibiotics (FDA, 2022). In this case, the minimum inhibitory concentration of antibiotics is important to evaluate the sufficient amount of antibiotics to be used to effectively cure the disease and antibiotic susceptibility profiles provide the health care professionals with an overview of the combination of antibiotics to prescribe.

Given the significant roles of colistin in the clinical use, the objectives of this research were:

i. to isolate lactose-fermenting bacteria resistant to colistin from livestock wastewater samples,

ii. to detect the presence of *mcr* gene in the isolates using polymerase chain reaction (PCR),

iii. to study the minimum inhibitory concentration (MIC) of colistin in *mcr*-positive isolates by broth microdilution method, and

iv. to study the susceptibility of the *mcr*-positive isolates to other antibiotics by Kirby-Bauer disc diffusion method.

CHAPTER 2

LITERATURE REVIEW

2.1 History and application of colistin

2.1.1 Discovery of colistin

The duration between 1950 to 1960 was once the golden era of antibiotic discovery, in which many of the antibiotics being widely used today were discovered at that time (Figure 2.1). The discovery of antibiotics was considered a big medical breakthrough. Due to the effective effect of antibiotics in treating various diseases and increasing the lifespan of humans, researchers had put in a lot of effort in the exploratory research in antibiotics (Kumazawa and Yagisawa, 2002). Polymyxins, a family of cationic cyclic lipodecapeptide antibiotics produced by spore-forming soil bacterium *P. polymyxa* were first discovered in late 1940s (Ainsworth, Brown and Brownlee, 1947). The family of polymyxin consists of 5 different compounds with chemically variations, namely polymyxin A, B, C, D and E. Colistin, also known as polymyxin E was isolated by Yasuo Komaya and co-workers in Japan in 1947 (Koyama, 1950). It was the first antibiotic with Japan origin which was extensively used globally (Kumazawa and Yagisawa, 2002).

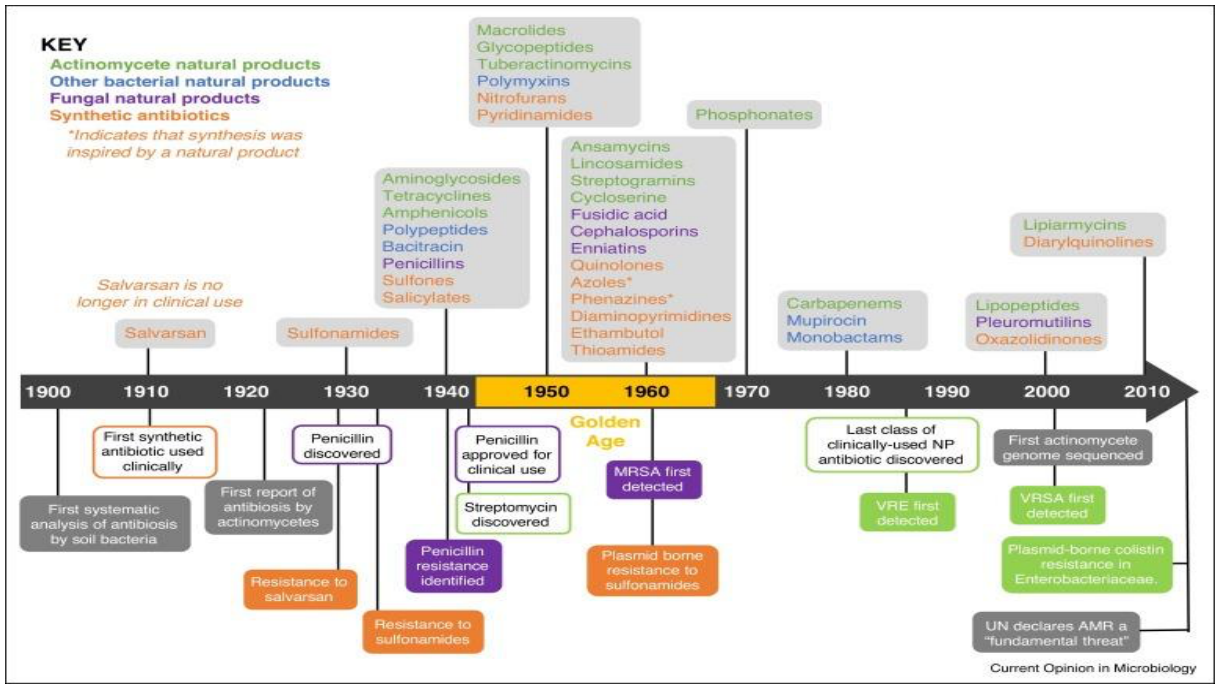

Figure 2.1 Timeline of antibiotic discovery (Hutchings, Truman and Wilkinson, 2019)**.**

2.1.2 Clinical usage of colistin

After the discovery, colistin was first introduced into clinical usage in 1952 as intravenous formulation. Owing to the effective bactericidal activity of colistin in different types of infection, colistin was approved as an antimicrobial agent in combating gram-negative bacteria by United States Food and Drug Administration (FDA) in 1959 (El-Sayed Ahmed et al., 2020). However, utilization of colistin was greatly reduced when it was found to possess significant neurotoxicity and nephrotoxicity in the patients after colistin treatment (Shrestha et al., 2014). Upon seeing the drawbacks of colistin in clinical use, the discovery of novel antibiotics which were less toxic led to the abandonment of clinical usage of colistin in the treatment of gram-negative bacterial infections in the 1980s. The usage of colistin was limited to ophthalmic, topical uses and also as nebulization for cystic fibrosis patients only (Poirel, Jayol and Nordmann, 2017).

With the popularity of MDR gram-negative bacteria, specifically *Pseudomonas aeruginosa*, *Actinetobacter baumannii* and *Klebsiella pneumonia* which are resistant to all the other available antibiotics, the usage of colistin was once again rekindled in mid-1990s. Colistin was clinically used in two forms, which were colistin sulphate for oral and topical use and colistinmethate sodium or colistin methanesulfonate for parenteral and nebulization use. Among these, colistin sulphate was the only form of colistin being approved to be used as one of the therapeutic measures for intestinal infections caused by gram-negative bacteria in the pig production (El-Sayed Ahmed et al., 2020).

2.2 Mode of Action of Colistin

2.2.1 Membrane Lysis Pathway

Gram-negative bacteria are characterized by the thin peptidoglycan layer which are surrounded by an outer membrane of lipopolysaccharide (LPS). The layer of LPS functions to restrict the passage of hydrophobic or large antibiotics (Vidaillac, Benichou and Duval, 2012). Besides, the negative charge of the lipopolysaccharide also contributes to the stability of the outer membrane of gram-negative bacteria.

The antibacterial activity of colistin is known as “self-promoted uptake” model in which the bacteria is killed by physical disruption of cell membrane by cationic peptides. The target of colistin is the lipid A on the LPS. The cationic diaminobutyric acid (Dab) of the colistin electrostatically interacts with the anionic phosphate group present on the lipid A of the LPS and competitively displaces the divalent cations, including calcium ions and magnesium ions from the phosphate group as shown in Figure 2.2 (El-Sayed Ahmed et al., 2020). Notably, affinity of colistin to the LPS is at least three times higher than the divalent cations as mentioned above. This leads to the destabilization of the LPS and destruction of the outer membrane of the bacteria (Hancock, 1997). The disruption of the outer membrane of bacteria affects the permeability of the outer membrane and causes the leakage of the cellular contents which directly kill the bacteria.

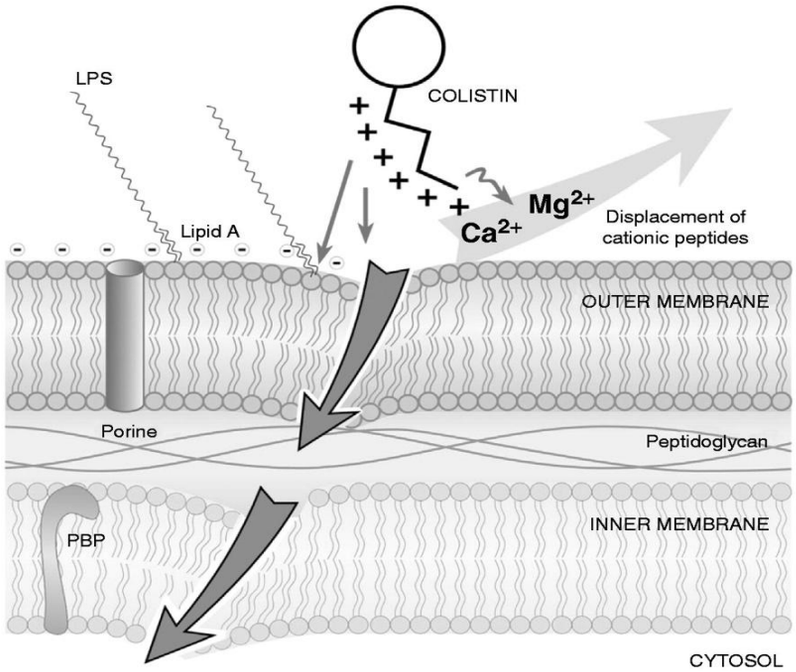

Figure 2.2 Action of colistin via membrane lysis pathway. Colistin binds to the divalent ions, Ca^{2+} and Mg^{2+} through electrostatic interactions and displaces the ions from lipid A on the LPS, leading to the destabilization of membrane (Bialvaei and Samadi Kafil, 2015).

2.2.2 Anti-endotoxin Pathway

Endotoxin is a predominant LPS that is found in the cell wall of gram-negative bacteria. Upon the lysis of the gram-negative bacteria due to disrupted cell membrane, the endotoxin of the bacteria is released. The endotoxin reacts by stimulating the inflammatory system of the host cell. In a serious case, the inflammatory response can lead to the dysfunction of the organ systems and induce lethal septic shock in the host (Gough, Hancock and Kelly, 1996). In this case, colistin exhibits antibacterial activities by binding and neutralizing the endotoxin produced when the gram-negative bacteria die (El-Sayed Ahmed et al., 2020). This prevents the interaction of the endotoxin to the host intact cells which can suppress the release of cytokines such as tumour necrosis factor (TNF). By doing so, septic shock which can cause death to the host can be prevented (Senturk, 2005).

2.2.3 Vesicle-vesicle Contact Pathway

Other than the above mechanisms, colistin can also perform antibacterial activity by stimulating the fusion of the periplasmic layer of outer membrane and inner membrane. Being cationic, colistin binds to the anionic phospholipid present in both inner membrane and outer membrane through electrostatic interactions (Yu et al., 2015). This encourages the exchange of phospholipids between the periplasmic layer of inner and outer membrane. The movement of phospholipids lead to the disruption of the membrane composition and destabilization of the cell membrane. Eventually, the bacteria die as a result of cell lysis due to the osmotic imbalance.

2.2.4 Hydroxyl Radical Death Pathway

Apart from that, colistin also carries out bactericidal activity by applying oxidative stress to the bacteria. Bacteria is killed by the accumulation of the hydroxyl radicals inside the bacterial cells (Yu et al., 2015). When the colistin first passes through the membrane of the bacteria, production of superoxide (O_2^-) is stimulated. Then, the O_2^- will be catalyzed by the superoxide dismutases (SOD) to undergo chemical changes to form hydrogen peroxide (H_2O_2). Both O_2^- and H_2O_2 damage the iron-sulfur cluster protein and lead to the release of iron. H_2O_2 subsequently oxidizes the ferrous ion (Fe^{2+}) into ferric iron (Fe^{3+}) through the Fenton reaction which produce hydroxyl radicals (·OH) (Imlay, 2013). These hydroxyl radicals play significant roles in damaging the DNA, proteins and lipids of the bacteria, leading to the death of bacterial cells.

2.2.5 Inhibition of Respiratory Enzymes

Even though the antibacterial activities of colistin against gram-negative bacteria are more commonly discussed, colistin can also react to gram-positive bacteria. The mode of action of colistin against gram-positive bacteria is believed to occur via the inhibition of the respiratory enzymes of the bacteria (El-Sayed Ahmed et al., 2020). There are three important energy transducing enzymes, quinones and reduced nicotinamide adenine dinucleotide (NADH) which serve pivotal roles in cellular respiration. Colistin reacts by inhibiting the respiratory enzymes in the bacteria. This mode of action of colistin was supported by the study showing the inhibition of the respiratory enzymes of NADH oxidase in gram-positive *Bacillus* spp. by colistin (Yu et al., 2015).

2.3 Types of Colistin Resistance

2.3.1 Intrinsic Resistance

There are some organisms which are intrinsically resistant to colistin. This means that the group of bacteria are naturally resistant to colistin, without the need of mutation or intake of foreign DNA which codes for colistin resistance gene as the resistance gene is chromosomally encoded. With this, all gram-positive bacteria are intrinsically resistant to colistin due to the absence of the outer membrane of bacteria which acts as the target for the antibacterial activity of colistin. Besides, some other gram-negative bacteria such as *Proteus* spp., *Providencia* spp., *Morganella morganii*, *Serratia* spp., *Brucella* spp., *Neisseria* spp., *Chromobacterium* spp. and *Burkholderia* spp. too are intrinsically resistance to colistin (WHO, 2018).

2.3.2 Acquired Resistance

Acquired resistance is the resistance of bacteria to the antibiotics in which the bacteria were previously susceptible to. Acquired resistance can occur through mutation or horizontal transfer of colistin resistance coded gene. Examples of bacteria which develop acquired resistance to colistin are *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* (Olaitan, Morand and Rolain, 2014).

In general, the emergence of acquired resistance to colistin in the species which are naturally susceptible to colistin is due to mutation. The bacteria undergo mutations in order for them to survive in the conditions which are unfavourable for their growth. The most common mechanisms of colistin resistance due to mutation are modifications of the lipopolysaccharide components of the outer membrane of the bacterial cell wall or loss of the lipopolysaccharide production (Aghapour et al., 2019). This type of acquired resistance allows the bacteria cell to pass on the mutated gene coded for colistin resistance to their offspring.

On the other hand, transferable colistin resistance is the resistance developed due to the presence of plasmid-mediated mobilized colistin resistance (*mcr*) gene. The *mcr* gene is present on the bacterial plasmid which gathers antibiotic resistance genes by transposition mechanism and site-specific recombination mechanism. Then, the plasmid are transferred from one bacterium to another (Bennett, 2008). This mechanism of plasmid transfer is known as horizontal gene transfer. When the plasmid coded for *mcr* gene is transferred to another bacterium which is susceptible to colistin, the bacterium can develop the resistance to colistin.

2.4 Mode of Colistin Resistance

2.4.1 Capsule formation

The bacteria can resist the antibacterial activities of colistin using several mechanisms. The role of capsular polysaccharide is shown to provide the bacteria with protection against the antibiotics by restricting the interactions of the cationic antimicrobial peptides to the bacteria (Campos et al., 2004). The biosynthesis of the capsule is upregulated by the Cpx and Rcs regulators in the colistin-resistant bacteria (Aghapour et al., 2019). Due to the presence of the capsule, colistin cannot reach the target site, lipid A which is present on the LPS to perform antibacterial activities. According to the previous study, almost all the members of *Enterobacteriaceae* are capable of synthesising capsules, which allows them to escape from the binding of colistin (Schembri et al., 2005). Besides, it was hypothesized that the number of capsular layers is actually related to the resistance level of the bacteria to colistin, in which more capsular layers contribute to higher levels of resistance to colistin (Formosa et al., 2015).

2.4.2 Lipopolysaccharide Modification

Many genes and operons are involved in the modification of the LPS of the colistin-resistant bacteria since it is the target for the binding of colistin. Among all, resistance of *Proteus* spp. to colistin is achieved by the expression of *arnBCADTEF* operon and *eptB* gene which stimulate the addition of phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the LPS. This regulation alters the cationic charges on the LPS which in return reduces the affinity of colistin to the LPS of the bacteria (Gogry et al., 2021).

2.4.3 Role of Efflux pump

There were studies that revealed the involvement of the efflux pump system in the colistin resistance mechanism. Efflux pumps are transport proteins which serve the role of getting rid of toxic substances including antibiotics from the cell to the external environment. KpnEF, AcrAB and Sap proteins are the efflux pumps being found in *Enterobacteriaceae* which contribute to the colistin resistance (Aghapour et al., 2019). The level of resistance to colistin is increased with the activation of these pumps. KpnEF efflux pump is an important component of the bacteria which involves in the removal of antibiotics, including colistin from the cells, thus allowing the bacteria to become resistant to antimicrobial agents. For such, an antimicrobial susceptibility test of mutant KpnEF was conducted to verify the function of efflux pump in causing antibiotics resistance and the results revealed that *K. penumoniae* with mutant KpnEF showed significant reduction of the resistance to the antibiotics (Srinivasan and Rajamohan, 2013). Therefore, it implied the remarkable role of efflux pump in causing colistin resistance in the bacteria.

2.4.4 Plasmid Mediated Resistance

Plasmid-mediated *mcr* genes encode for phosphoethanolamine transferase which modifies lipid A through addition of the phosphoethanolamine moiety to lipid A (Liu et al., 2017). Due to the modification of lipid A structure, the affinity of colistin to the lipid A is greatly reduced and hence the antibacterial activities of colistin are affected. As a result, this increases the MIC of colistin 4-fold to 8-fold in *Escherichia coli* (Poirel, Jayol and Nordmann, 2017). It indicates that higher concentration of colistin is required to kill the bacteria with *mcr* gene.

2.5 Background of *mcr* Genes

2.5.1 Discovery of *mcr* Genes of Different Variants

Up to now, there are a total of 10 slightly different variants of *mcr* genes being detected and identified from various sources, including foods, humans, animals, farms and environment. The first plasmid-mediated colistin resistance gene, *mcr-1* was isolated from *E. coli* in 2015 in China (Liu et al., 2016). Data showed that *mcr* genes existed for more than three decades and most of the *mcr*-2 to *mcr-9* were actually detected in the last decade (Luo, Wang and Xiao, 2020). The timeline of the *mcr* gene discovery is shown in Figure 2.3 in which *mcr-2* was first detected from *E. coli* in Belgium (Xavier et al., 2016) while *mcr-3* gene was characterized from *E. coli*, *Aeromonas* spp. and *Proteus* spp. from animal and human in South Afrika, Europe and Asia (Yin et al., 2017). Besides, *mcr-4* was first detected in *Salmonella Typhimurium* from pig in 2013 in Italy and *mcr-5* was detected in *Salmonella enterica* serovar *Paratyphi* B from poultry in Germany (Borowiak et al., 2017; Carattoli et al., 2017). The gene of *mcr-6* was found in *Moraxella pluranimalium* from animal, in Europe (AbuOun et al., 2017). Moreover, *mcr-7* was discovered in *K. pneumoniae* from chicken source and *mcr-8* in *K. pneuomoniae* from pig and human source in China (Yang et al., 2018; Wang et al., 2018). Till 2018, *mcr-9* was isolated from MDR *S. enterica* serovar *Typhimurium* from human patient in Washington and subsequently *mcr-10* genes which shares similar nucleotide identity with mcr-9 was isolated from *Enterobacter roggenkampii* (Carroll et al., 2019; Wang et al., 2020). Among all, *mcr-1* has 30 variants, *mcr-2* has 7 variants, *mcr-3* has 40 variants, *mcr-4* has 6

variants, *mcr-5* has 4 variants, *mcr-8* has 3 variants and *mcr-9* has 3 variants (Mentasti et al., 2021).

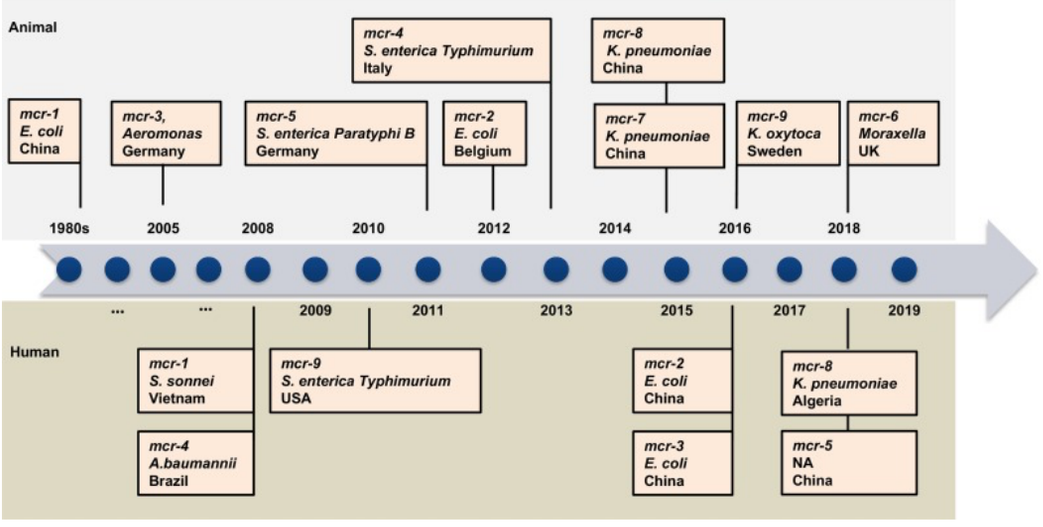

Figure 2.3 Timeline of *mcr* gene discovery (Luo, Wang and Xiao, 2020)**.**

2.5.2 Prevalence of *mcr* Genes

The *mcr* genes can now be found distributed all around the world, in which the genes have been detected in 57 countries where most of the *mcr* genes were isolated from livestock and followed by human, meats and other food products. China, being the first country in which the *mcr* gene was detected, contributes the highest prevalence of *mcr* genes. It is due to the reason that China utilized the largest amount of colistin in agricultural purposes as compared to other countries (Luo, Wang and Xiao, 2020). Studies has shown that *mcr* genes was detected in various species of bacteria, including *E. coli*, *K. pneumoniae*, *S. enterica*, *C. sakazakii*, *Raoultella ornithinolytica* and *Aeromonas* spp., where *Enterobacteriaceae* constitutes the dominant group of bacteria which carry *mcr* genes isolated from human and animal source (Elbediwi et al., 2019).

CHAPTER 3

MATERIALS AND METHODS

3.1 List of Materials and Apparatus

All apparatuses, equipments and consumables used were tabulated in Table 3.1 while all chemicals used were tabulated in Table 3.2.

Table 3.1 Apparatuses, equipments and consumables used throughout the experiment with their respective manufacturers

Apparatuses / Equipments / Consumables	Manufacturers
96 well culture plates	Nest Biotechnology
Analytical balance	Copens Scientific
Autoclave machine	Hirayama
Benchtop mini centrifuge	Sartorius
Biosafety cabinet	myLab™
Collection swab	Biomedia
Electrophoresis tank	Major Science
Falcon tube (15 mL)	Nest Biotechnology
Falcon tube (50 mL)	TPP
Freezer	Liebherr Medline
Heat block	Bioscience
Incubator	Memmert
Laminar flow hood	ESCO
Light board	Major Science
Microcentrifuge tube (1.5 mL)	Premier Diagnostics

Table 3.1 (Continued)

Apparatuses / Equipments / Consumables	Manufacturers
Microfuge 16 centrifuge	Beckman Coulter
Micropipette tips	Ratiolab, Eppendorf, NEST scientific
Micropipettes	Eppendorf
Microplate reader	Thermo Scientific
Microwave oven	Samsung
Nanodrop spectrophotometer	Thermo Scientific
PCR thermal cycler	Bio-Rad
PCR tubes (0.2 mL)	Orioner
Petri dishes	Nest Biotechnology
Power supply	Major Science
Quick-spin mini centrifuge	Straits Scientific
Refrigerator	REMI Scientific
Schott's bottles	Schott Duran
Shaking incubator	N-Biotek
Spectrophotometer	Thermo Scientific
UV illuminator	Bio-Rad
Vortex machine	Biocote Ltd

Table 3.2 Chemicals used throughout the experiment with their respective manufacturers

Purpose	Chemical	Manufacturers
Isolation of lactose fermenters & Total DNA extraction	MacConkey Agar	Himedia
	LB agar	Condalab
	Colistin	Gold Biotechnology
	LB broth	Condalab
mcr genes detection by PCR	Ultrapure distilled water	-
	PCR buffer	1st Base
	$MgCl_2$	1st Base
	dNTPs	1st Base
	Primers pairs	1st Base
PCR products detection by agarose gel electrophoresis	Agarose powder	1st Base
	100 bp ladder	Smobio
	1 kb ladder	Vivantis
	Novel juice	Simply™
	TAE buffer	-
MIC determination using broth microdilution & Kirby-Bauer disc diffusion test	0.85% saline	Kollin Chemicals
	1% H_2SO_4	R&M Chemicals
	Barium chloride	Systerm
	Mueller-Hinton agar	Conda Lab
	Mueller-Hinton broth	Himedia

3.2 Media Preparation

Different types of media were prepared for the purposes of isolating and culturing of bacteria. Medium preparation was carried out according to the instructions of the manufacturers as tabulated in Table 3.3.

The media were prepared by first calculating the mass of dehydrated medium required for the preparation of desired volume of media. Then, the dehydrated medium was dissolved using distilled water and poured into a clean Schott bottle. The volume of medium was topped up using distilled water to the desired volume. After that, the media were autoclaved at 121°C for 15 minutes for sterilization before use. Autoclaved broth was left in room temperature to cool down before use. Molten agar was allowed to cool to 45-50°C after autoclaving. The agar was then poured into Petri dishes inside the laminar flow hood and left for 30 minutes for the agar to solidify and dry. All the media were kept at room temperature and the conditions of the media were checked from time to time for any contamination.

For the isolation of colistin-resistance bacteria, 2 μg/mL of colistin was supplemented to the MacConkey agar. The colistin was added into the molten agar when the agar has cooled to 45-50°C after being autoclaved. The molten agar was swirled properly before being poured into Petri dishes.

Table 3.3 Instructions of medium preparation

Media	Ingredients	Amount	Autoclave conditions
MacConkey Agar	Dehydrated medium Distilled water	51.53 g Top up to 1000 mL	121°C (15 min)
LB agar	Dehydrated medium Distilled water	35 g Top up to 1000 mL	121°C (15 min)
LB broth	Dehydrated medium Distilled water	20 g Top up to 1000 mL	121°C (15 min)
MH agar	Dehydrated medium Distilled water	38 g Top up to 1000 mL	121°C (15 min)
MH broth	Dehydrated medium Distilled water	21 g Top up to 1000 mL	121°C (15 min)
0.85% (w/v) saline	NaCl Distilled water	0.85 g 100 mL	121°C (15 min)

3.3 Water Samples Collection

Livestock wastewater samples were collected using 50 mL sterile Falcon tubes from the wet market of Kampar on 28th February 2022. Samples collected for this research were (i) water contaminated with chicken faeces, (ii) water used to wash pork intestines and (iii) water used to wash chicken organs.

3.4 Isolation of Lactose Fermenters

3.4.1 Serial Dilution of Water Samples

The livestock wastewater samples collected were serially diluted to 10^{-1}, 10^{-2} and 10^{-3} dilutions using sterile distilled water. By using pipette, 1 mL of sample was transferred into 9 mL of distilled water in a clean Falcon tube to obtain 10^{-1} dilution. The subsequent dilutions were made from a previous dilution to obtain 10^{-2} and 10^{-3} dilutions of the sample.

3.4.2 Inoculation onto MacConkey Agar

The samples were inoculated onto MacConkey agar supplemented with 2 μg/mL of colistin using spread plate method. By using a micropipette, 100 μL of each dilution was transferred onto a separate MacConkey agar plate and spread evenly using a spreader until the sample dried. The spreader was dipped into absolute ethanol and flamed for sterilization each time before using. The inoculated agar plates were incubated at 37°C for 18 hours in the incubator.

3.4.3 Obtaining Pure Culture of Lactose Fermenters

For each sample, pink colonies indicating the lactose fermenters from the master agar plates with countable number of colonies were streaked onto a new MacConkey agar using an inoculation loop. The inoculation loop was flamed after each quadrant streaking to dilute the culture so that single colonies of bacteria can be obtained. The newly inoculated plates were incubated at 37°C for another 18 hours in the incubator. Observations of the colony morphology were recorded.

3.4.4 Maintaining Bacterial Cultures

The pure isolates from MacConkey agar were streaked onto LB agar for short storage. The inoculated plates were incubated at 37°C for another 18 hours inside the incubator. The LB agar plates with the growth of cultures were then sealed using Parafilm and kept in the refrigerator at 4°C.

3.5 Total DNA Extraction using Boiling Method

Cultures from LB agar were inoculated into LB broth and incubated at 37°C for 18 hours inside the shaking incubator. After the incubation, 1 mL of each culture broth was transferred into a new and sterile microcentrifuge tube. The cultures were centrifuged at 13000 rpm for 5 minutes. The supernatant of the centrifuged cultures was discarded and 1 mL of distilled water was added. The distilled water was pipetted up and down several times to totally resuspend the pellet. Then, the bacterial suspensions were incubated using a pre-heated heat block at 100°C for 10 minutes and cooled to room temperature. The suspensions were then centrifuged at 13000 rpm for another 5 minutes. The supernatant containing the total DNA of the bacteria was pipetted into another new microcentrifuge tube and stored in the freezer at -20°C for PCR use.

3.6 *mcr* Gene Amplification using PCR

3.6.1 PCR reagents

PCR reagents were prepared as master mix in a microcentrifuge tube. The volume needed for one reaction was then distributed into each PCR tube and DNA templates were added to make up a 25 µL reaction mixture. For each round of PCR, negative control using ultrapure distilled water to replace DNA templates and positive control using sample W7 were prepared. The PCR reagents used to prepare 1 reaction mixture were listed in Table 3.4. Then, PCR for *mcr-1*, *mcr-3* and *mcr-5* genes was performed using Bio-rad PCR thermal cycler.

Table 3.4 Concentration and volume of PCR reagents to make up a 25 µL reaction mixture

Reagent	Stock concentration	Final concentration	Volume (µL)
Ultrapure distilled water	-	-	13.8
PCR buffer	10 ×	1 ×	2.5
$MgCl_2$	25 mM	2 mM	2.0
dNTPs	10 mM	0.2 mM	0.5
Forward primer	10 mM	0.4 mM	1.0
Reversed primer	10 mM	0.4 mM	1.0
Taq polymerase	5 U/µL	1 U	0.2
DNA	-	-	4.0

For different types of *mcr* genes being targeted, different primer pairs were used. The sequences of the primer pairs used for amplification of *mcr-1*, *mcr-3* and *mcr-5* genes were listed in Table 3.5.

Table 3.5 Nucleotide sequence for the primers used for *mcr* genes amplification

Target gene	Primer	Nucleotide sequence (5' to 3')	Amplicon size (bp)	Source
mcr-1	Forward	GCTCGGTCAGTCCGTTTGTTCTTG	1497	(Zhang et
	Reverse	GGATGAATGCGGTGCGGTCTT		al., 2018b)
mcr-3	Forward	CGCTTATGTTCTTTTTGGCACTGTATT	1063	(Zhang et
	Reverse	TGAGCAATTTCACTATCGAGGTCTTG		al., 2018b)
mcr-5	Forward	GTGAAACAGGTGATCGTGACTTACCG	271	(Zhang et
	Reverse	CGTGCTTTACACCGATCATGTGCT		al., 2018a)

3.6.2 PCR Profiles

The PCR profile used for amplification of *mcr-1*, *mcr-3* and *mcr-5* genes was one denaturation cycle at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 70 seconds and finally followed by one extension cycle at 72°C for 5 minutes. After the PCR was completed, the PCR products were immediately kept in the freezer at -20°C to minimize the degradation of the DNA.

3.7 Analysis of PCR Products using Agarose Gel Electrophoresis

After gene amplification using PCR, the presence of the amplified genes were determined using agarose gel electrophoresis. The TAE buffer was used as the running buffer. For the PCR products of *mcr-1* and *mcr-3* amplification, 1% (w/v) agarose gel and 1 kb DNA ladder were used. Meanwhile, for the PCR products

of *mcr-5* amplification, 1.5% (w/v) agarose gel and 100 bp DNA ladder was used. For DNA ladder, 4 µL of DNA ladder was mixed with 1 µL of novel juice before loading into the well while for PCR products, 5 µL of PCR product was mixed with 1 µL of novel juice before loading into the well. Then, the gel was run at 90V for 40 – 45 minutes. After the gel electrophoresis has completed, the gel was viewed under UV transilluminator to observe the presence of the amplicons. The targeted amplicon for *mcr-1* gene is 1479 bp, *mcr-3* gene is 1067 bp and *mcr-5* gene is 271 bp.

3.8 MIC Determination by Broth Microdilution

3.8.1 Preparation of 0.5 McFarland Standard

Preparation of 0.5 McFarland standard was done by mixing 99.5 mL of 1% (v/v) of H_2SO_4 with 0.5 mL of 1.175% (w/v) of $BaCl_2$. The absorbance of the standard at 600 nm was measured using a spectrophotometer to ensure the reading is within 0.08 – 0.13, which is equivalent to bacterial density of approximately $1\text{-}2\times10^8$ CFU/mL (Hudzicki, 2012). The prepared 0.5 McFarland standard was wrapped using aluminium foil and kept away from light.

3.8.2 Preparation of Bacterial Suspension

For the isolates with positive *mcr* genes, they were subcultured onto a new LB agar and incubated at 37°C for 18 hours. The colonies were inoculated into 0.85% saline and compared with 0.5 McFarland to obtain similar turbidity. The bacterial suspension was diluted using saline if the suspension was too heavy or more colonies were inoculated into the suspension if the suspension was too light.

Then, the bacterial suspension was further diluted 150 × using MH broth to achieve 1×10^6 CFU/mL (Tankeshwar, 2022).

3.8.3 Preparation of Colistin Solution

Stock colistin solution with concentration of 10 mg/mL was diluted to 512 µg/mL using autoclaved distilled water.

3.8.4 Broth Microdilutions using 96-well Plate

Serial dilution of colistin solution was carried out in the wells of the 96-well plate from the 512 µg/mL colistin solution until a concentration of 0.25 µg/mL was obtained. One well was reserved for medium control which only contained medium and another well for growth control which did not contain antibiotics in each row of well. After different concentrations of colistin solution were prepared, 100 µL of bacterial suspension was inoculated into each well. A new pipette tip was used each time after transferring the bacterial suspension into one well. Three replicates were performed for each *mcr*-positive isolate. Then, the inoculated microtitre plates were incubated at 37°C for 18 hours.

3.8.5 Determination of MIC value

The turbidity of the broth was observed under light. The MIC value was first determined qualitatively by identifying the lowest concentration of colistin in which the broth remained clear using naked eyes. Then, the absorbance reading of the broth in each well was measured quantitatively using microplate readers. The absorbance reading of the broth cultures in each well was compared to the absorbance reading of the medium control and the MIC was defined as the lowest concentration of colistin which gave similar absorbance reading to the medium

control. The test was repeated when three replicates did not show consistent MIC values.

3.9 Antibiotic Susceptibility Determination via Kirby-Bauer Test

3.9.1 Preparation of Mueller-Hinton (MH) Agar

Molten MH agar was dispensed into Petri dishes and allowed to solidify at room temperature. The volume of the molten agar in each Petri dish was maintained consistent to prepare agar of similar thickness.

3.9.2 Preparation of Bacterial Suspension

For the isolates with positive *mcr* genes, they were subcultured onto a new LB agar and incubated at 37°C for 18 hours. Then, the colonies from the agar plate were inoculated into 0.85% saline and compared with 0.5 McFarland to obtain similar turbidity. The bacterial suspension was diluted using saline if the suspension was too heavy or more colonies were inoculated into the suspension if the suspension was too light.

3.9.3 Inoculation of Bacteria onto MH agar

A sterile swab was immersed into the bacterial suspension. Then, the swab was rotated against the wall of the bottles containing bacterial suspension to remove excess fluid. The bacterial suspension was inoculated onto the MH agar by streaking the swab over the entire plates. The plate was rotated 60° and streaked again two times. The inoculated plates were left to dry at room temperature for 3 to 5 minutes.

3.9.4 Placement of antibiotic discs

By using sterile forceps which were flamed over Bunsen burner and cooled down, antibiotic discs were placed onto the MH agar plate inoculated with bacterial suspension. Four types of antibiotic discs were placed in each agar plate with centre-to-centre distance of at least 24 mm to each other (Hudzicki, 2012). The discs were pressed down softly to ensure the contact of the disc to the surface of the agar. A total of 12 antibiotic discs were used to determine the antibiotic susceptibility profiles for each of the *mcr*-positive isolates. The list of the antibiotic discs used was tabulated in Table 3.6. Then, the plates were incubated at 37°C for 18 hours.

Table 3.6 Types of antibiotic disc with their respective dose and brand

Antibiotics Disc	Dose	Brand
Ampicillin	10 mcg	Himedia
Cefepime	30 mcg	Himedia
Cefotaxime	30 mcg	Himedia
Ceftriaxone	30 mcg	Himedia
Chloramphenicol	30 mcg	Himedia
Ciprofloxacin	5 mcg	Himedia
Colistin	10 mcg	Oxoid
Imipenem	10 mcg	Oxoid
Piperacillin	30 mcg	Oxoid
Sulfamethoxazole-trimethoprim	25 mcg	Oxoid
Tetracycline	30 mcg	Himedia
Tobramycin	10 mcg	Himedia

3.9.5 Antibiotic Susceptibility Determination

The plates were held a few inches above a black surface to observe the presence of a zone of inhibition clearly. The sizes of the zone which form around the antibiotic discs were measured using a ruler to the nearest mm. The susceptibility of the isolates to the antibiotics was determined according to the published CLSI guidelines (CLSI, 2022).

CHAPTER 4

RESULTS

4.1 Isolation of Lactose-fermenting Gram-negative Bacteria

4.1.1 Lactose Fermenters on MacConkey Agar

MacConkey agar was used to select for gram-negative bacteria and differentiate lactose-fermenting bacteria from non-lactose-fermenting bacteria. The growth of bacteria was observed on MacConkey agar after incubation at 37°C for 18 hours. Lactose-fermenting bacteria grew as pink colonies while non-lactose fermenting bacteria grew as colourless colonies on MacConkey agar. Master agar plates showing the mixture of growth of lactose-fermenting bacteria and non-lactose fermenting bacteria from water contaminated with chicken faeces were shown in Figure 4.1. Subculturing of the single pink colony from the master agar plate with the countable number of pink colonies onto a new MacConkey agar was performed until a pure single colony was obtained.

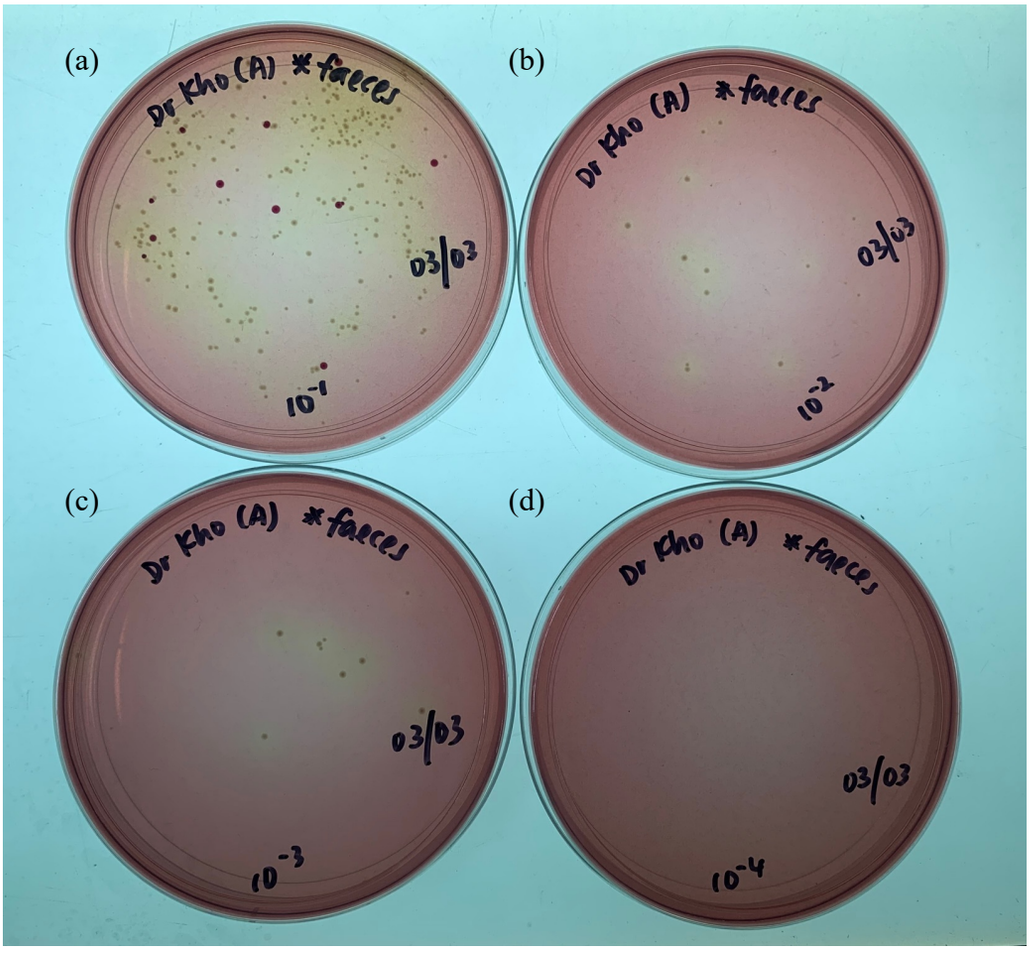

Figure 4.1 Observations of growth of lactose-fermenting and non-lactose-fermenting bacteria on MacConkey agar. Water contaminated with chicken faeces samples of (a) 10^{-1} dilution; (b) 10^{-2} dilution; (c) 10^{-3} dilution; (d) 10^{-4} dilution.

4.1.2 Colony Morphology of Pure Culture

A total of 43 pink colonies with 9 from water used to wash chicken organs, 12 from water contaminated with chicken faeces and 22 from water used to wash pork intestines were randomly selected and isolated by subculturing onto a new MacConkey agar each. The isolates were streaked until a single colony was obtained as shown in the representative agar plate in Figure 4.2. The morphology of a single colony of each isolate was observed with respect to the shape, size, surface, colour, opacity, elevation and margin. Observations of colony morphology for all the 43 bacterial isolates were listed in Table 4.1.

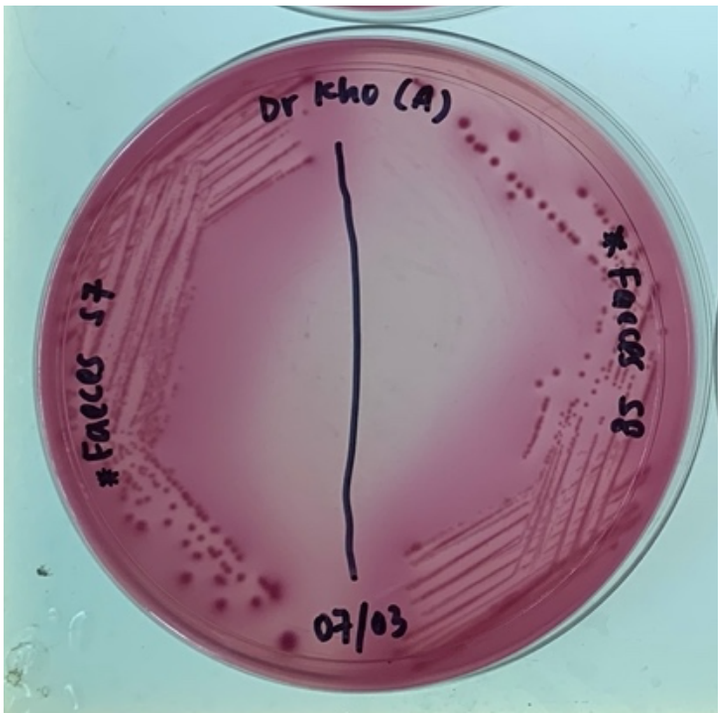

Figure 4.2 Isolation of colony until a single colony can be obtained

Table 4.1 Morphology of pure culture on MacConkey agar

Sample source	Isolates	Morphology						
		Shape	**Size**	**Surface**	**Colour**	**Opacity**	**Elevation**	**Margin**
Water used to wash chicken organs	CS1	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS2	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS4	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS5	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS8	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS9	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS10	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS11	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	CS13	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
Water contaminated by chicken faeces	FS1	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS2	Circular	Small	Smooth	Pink	Opaque	Flat	Even
	FS3	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS4	Circular	Small	Smooth	Pink	Opaque	Flat	Even
	FS5	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS6	Circular	Medium	Smooth	Pink	Opaque	Flat	Even

Table 4.1 (Continued).

Sample source	Isolates	Morphology						
		Shape	**Size**	**Surface**	**Colour**	**Opacity**	**Elevation**	**Margin**
Water contaminated by chicken faeces (cont.)	FS7	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS8	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS9	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS10	Circular	Small	Smooth	Pink	Opaque	Flat	Even
	FS11	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	FS12	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
Water used to wash pork intestines	PS1	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	PS7	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS9	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	PS10	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	PS35	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS36	Circular	Medium	Smooth	Pink	Opaque	Flat	Even
	PS39	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS40	Circular	Small	Smooth	Pink	Opaque	Convex	Even
	PS45	Circular	Medium	Smooth	Pink	Opaque	Flat	Even

Table 4.1 (Continued).

Sample source	Isolates	Morphology						
		Shape	**Size**	**Surface**	**Colour**	**Opacity**	**Elevation**	**Margin**
Water used to wash pork intestines (cont.)	PS46	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS47	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS48	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS55	Circular	Small	Smooth	Pink	Opaque	Convex	Even
	PS56	Circular	Large	Smooth	Pale pink	Opaque	Convex	Even
	PS59	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS61	Circular	Medium	Smooth	Pale pink	Opaque	Convex	Even
	PS63	Circular	Small	Smooth	Pink	Opaque	Convex	Even
	PS65	Circular	Large	Smooth	Pale pink	Opaque	Convex	Even
	PS66	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS67	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS69	Circular	Medium	Smooth	Pink	Opaque	Convex	Even
	PS70	Circular	Medium	Smooth	Pink	Opaque	Flat	Even

4.2 Amplification of *mcr* Genes

The amplification of *mcr* genes for all isolates were done via PCR using different primer pairs as mentioned in Table 3.5 and the PCR products were electrophoresed. The presence of *mcr* genes in the isolates was determined by observing the presence of amplicons at a specific size on agarose gel. The number of isolates that harboured the positive *mcr* gene was tabulated in Table 4.2. Out of 43 isolates, *mcr* gene was detected in 8 isolates, in which *mcr-1* gene was detected in 7 out of 43 (16.27%) isolates, *mcr-3* gene was detected in 1 out of 43 (2.33%) isolates and *mcr-5* gene was not detected in all the isolates. As shown in Figure 4.3, *mcr-1* gene was only detected from isolates from water contaminated with chicken faeces, including FS1, FS2, FS3, FS5, FS6, FS11 and FS12 as specific bands of 1479 bp which is close to 1500 bp were observed in comparison to the 1 kb DNA ladder. For amplification of DNA using *mcr-3* primer pairs, only 1 isolate, which is PS47 showed a specific band of 1067 bp which is close to 1000 bp in comparison to the 1 kb DNA ladder as shown in Figure 4.4. On the other hand, none of the isolate carried *mcr-5* gene as no isolate showed a specific band of 271 bp between 200 bp and 300 bp in comparison to the 100 bp DNA ladder as shown in Figure 4.5.

Table 4.2 Number of isolates with positive *mcr* gene for each sample.

Sample	Number of isolates with positive *mcr* gene		
	mcr-1	***mcr-3***	***mcr-5***
FS	7/12 (58.3%)	0/12 (0%)	0/12 (0%)
CS	0/9 (0%)	0/9 (0%)	0/9 (0%)
PS	0/22 (0%)	1/22 (4.5%)	0/22 (0%)
Total	7/43 (16.27%)	1/43 (2.33%)	0/43 (0%)

FS: water contaminated with faeces sample; CS: water used to wash chicken organs; PS: water used to wash pork intestines

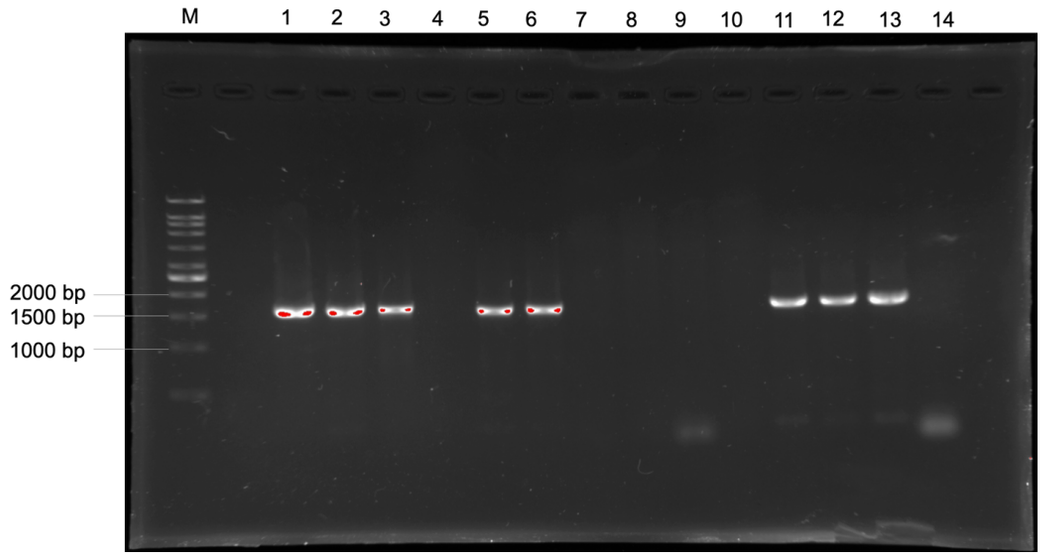

Figure 4.3 Agarose gel image of *mcr-1* gene amplification. Lane M is 1kb DNA ladder; Lanes 1-12 are isolates FS1-FS12; Lane 13 is the positive control using sample W7; Lane 14 is the negative control without DNA template.

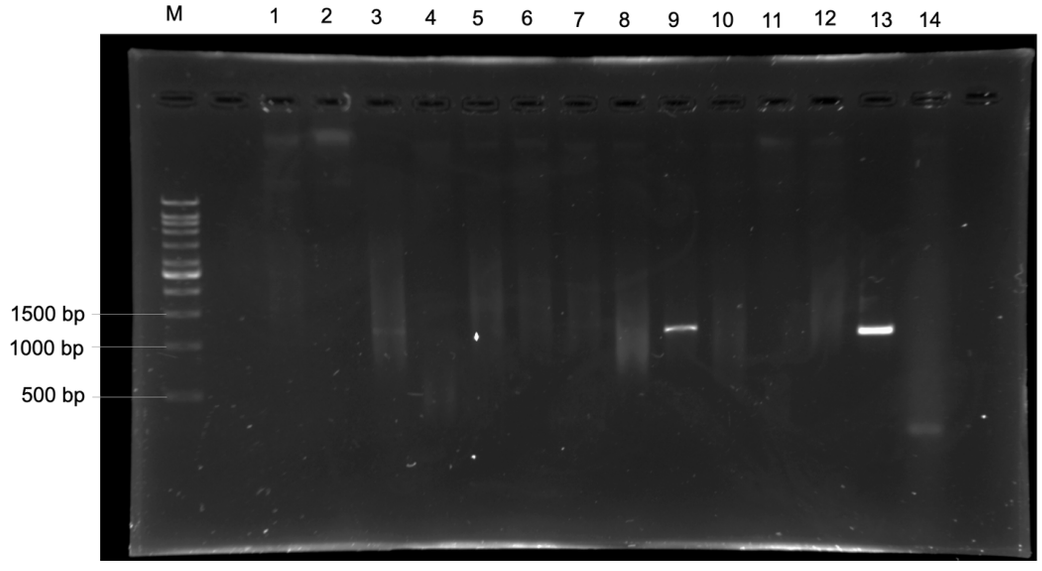

Figure 4.4 Agarose gel image of *mcr-3* gene amplification. Lane M is 1kb DNA ladder; Lanes 1-12 are isolates PS9, PS10, PS35, PS36, PS39, PS40, PS45, PS46, PS47, PS48, PS55 and PS56 respectively; Lane 13 is positive control using sample W7; Lane 14 is the negative control without DNA template.

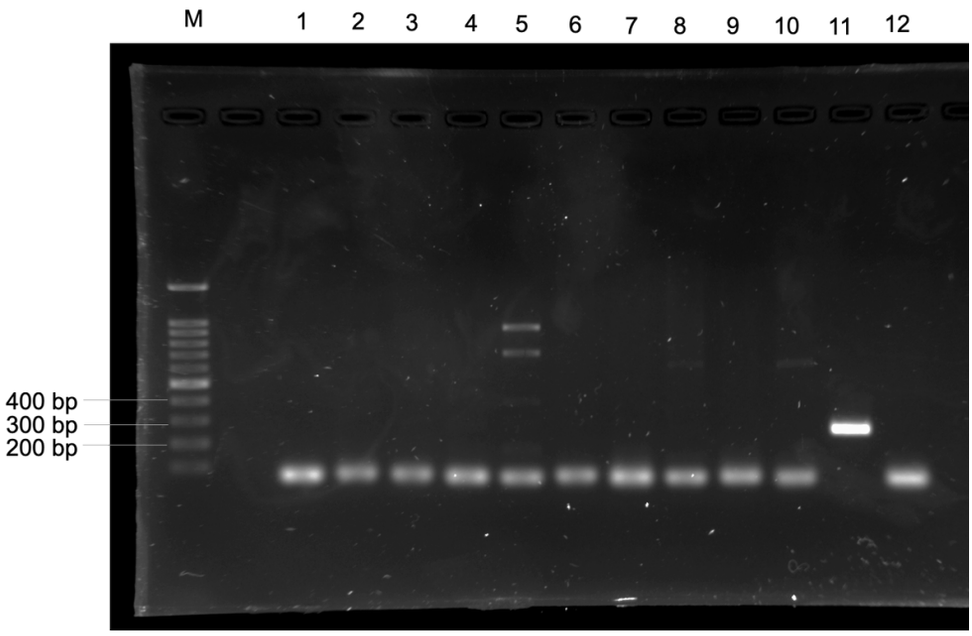

Figure 4.5 Agarose gel image of *mcr-5* gene amplification. Lane M was 100 bp DNA ladder; Lanes 1-10 are isolate FS6, FS7, FS8, FS9, FS10, FS11, FS12, PS1, PS7 and PS9 respectively; Lane 11 is positive control using sample W7; Lane 12 is the negative control without DNA template.

4.3 MIC of Colistin Determination

4.3.1 Qualitative analysis of MIC

MIC of colistin for all the *mcr*-positive isolates was determined via broth microdilution method. After incubation at 37°C for 18 hours, the growth of bacteria was determined qualitatively by observing the turbidity of the broth and quantitatively by measuring the absorbance reading of broth cultures at 600 nm. The MIC of colistin for the bacteria is indicated by the lowest concentration of colistin required to inhibit the growth of bacteria. A 96-well microtitre plate showing the turbidity of the broth cultures in different concentrations of colistin is shown in Figure 4.6. The MICs of colistin for two tested isolates, FS3 and FS5 are 4 μg/mL because the growth of bacteria which is indicated by the turbidity of the broth culture was not observed starting from 4 μg/mL onwards.

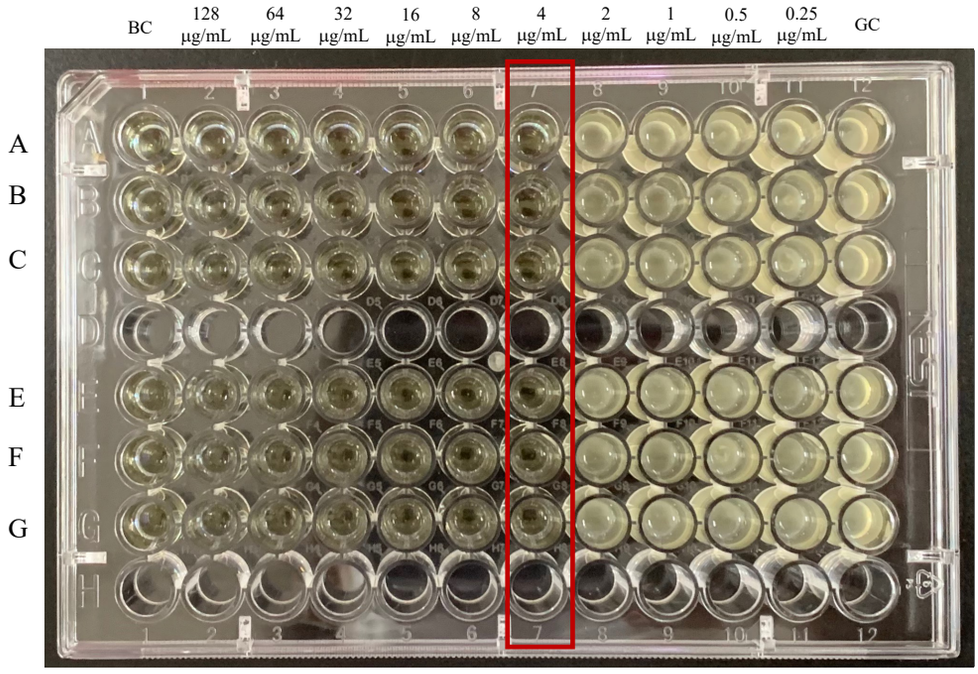

Figure 4.6 MICs determination for isolates FS3 and FS5 in a 96-well microtitre plate. Row A – C are triplicates of isolate FS3 while Row E – G are triplicates of isolate FS5. BC indicates broth control, GC indicates growth control and the concentrations labelled above the 96-well plate indicates the concentration of colistin in the respective column of well. The MICs for both samples are 4 µg/mL as no turbidity was observed in the well containing 4 µg/mL colistin and onwards for both samples.

4.3.2 Quantitative analysis of MIC

The absorbance readings of the broth culture at 600 nm for all 9 isolates including a positive control, W7 were shown in Appendix Table A. The means and standard deviations of absorbance reading for three replicates of each isolate were listed in Table 4.3. The MIC of colistin is defined as the lowest concentration of colistin which did not give distinct difference of absorbance reading in comparison to broth control. The MICs were translated into categories of resistant, intermediate and susceptible according to the published CLSI guidelines (CLSI, 2022). The MIC of colistin for the *mcr*-positive isolates with respect to their categories were summarized in Table 4.4.

Table 4.3 Means and standard deviations of absorbance reading of bacterial cultures with different concentrations of colistin at 600 nm.

Isolates	Absorbance reading at 600 nm (mean ± SD) [1]										MIC (μg/mL)
	0.25 μg/mL	0.5 μg/mL	1 μg/mL	2 μg/mL	4 μg/mL	8 μg/mL	16 μg/mL	32 μg/mL	64 μg/mL	128 μg/mL	
FS1	0.464 ± 0.018	0.454 ± 0.038	0.423 ± 0.056	0.057 ± 0.003	0.065 ± 0.016	0.055 ± 0.001	0.055 ± 0.001	0.055 ± 0.002	0.055 ± 0.001	0.053 ± 0.001	2
FS2	0.480 ± 0.069	0.445 ± 0.038	0.215 ± 0.027	0.055 ± 0	0.053 ± 0.001	0.058 ± 0.003	0.054 ± 0.002	0.052 ± 0	0.052 ± 0.001	0.051 ± 0.002	2
FS3	0.709 ± 0.030	0.691 ± 0.051	0.497 ± 0.133	0.475 ± 0.021	0.048 ± 0.008	0.074 ± 0.031	0.054 ± 0.002	0.053 ± 0.001	0.054 ± 0.003	0.051 ± 0.001	4
FS5	0.533 ± 0.049	0.579 ± 0.060	0.531 ± 0.082	0.496 ± 0.067	0.054 ± 0.001	0.053 ± 0.003	0.052 ± 0.002	0.055 ± 0.005	0.051 ± 0	0.051 ± 0.001	4
FS6	0.618 ± 0.053	0.584 ± 0.027	0.574 ± 0.028	0.050 ± 0.003	0.051 ± 0.011	0.050 ± 0.001	0.050 ± 0.001	0.050 ± 0.002	0.048 ± 0.002	0.049 ± 0.001	2
FS11	0.517 ± 0.064	0.366 ± 0.025	0.049 ± 0.001	0.050 ± 0.002	0.051 ± 0.002	0.054 ± 0.009	0.050 ± 0.001	0.075 ± 0.046	0.048 ± 0.002	0.050 ± 0.002	1
FS12	0.504 ± 0.041	0.417 ± 0.028	0.437 ± 0.052	0.426 ± 0.035	0.051 ± 0.003	0.052 ± 0.001	0.052 ± 0.002	0.050 ± 0.002	0.049 ± 0.001	0.049 ± 0	4
PS47	0.427 ± 0.092	0.333 ± 0.082	0.307 ± 0.085	0.274 ± 0.084	0.059 ± 0.002	0.053 ± 0.001	0.052 ± 0.001	0.052 ± 0.001	0.052 ± 0.001	0.051 ± 0.001	4
*W7	0.429 ± 0.065	0.408 ± 0.053	0.402 ± 0.042	0.369 ± 0.097	0.054 ± 0.004	0.052 ± 0.001	0.051 ± 0.002	0.054 ± 0.003	0.052 ± 0.001	0.050 ± 0.001	4

[1] mean ± standard deviation (SD) (n=3) was repeated for one time
Shading indicates the means absorbance readings from which the MIC of the respective isolates was determined
* indicates positive control

Table 4.4 MIC and interpretive category of *mcr*-positive isolates.

Isolates	MIC of colistin (μg/mL)	Interpretive Category
FS1	2	Intermediate
FS2	2	Intermediate
FS3	4	Resistant
FS5	4	Resistant
FS6	2	Intermediate
FS11	1	Intermediate
FS12	4	Resistant
PS47	4	Resistant
*W7	4	Resistant

* indicates positive control

4.4 Antibiotic Resistance Determination by Kirby-Bauer Test

All the 9 *mcr*-positive isolates were further tested for their susceptibility towards another 11 types of antibiotics. The diameters of the zone of inhibition after incubation at 37°C for 18 hours with the different antibiotic discs were measured. A MH agar showing the zones of inhibition of different antibiotics was shown in Figure 4.7. The diameters of the inhibition zone were listed in Appendix Table B. The inhibition zone diameters of each antibiotic were interpreted according to the published CLSI guidelines and recorded in Table 4.5.

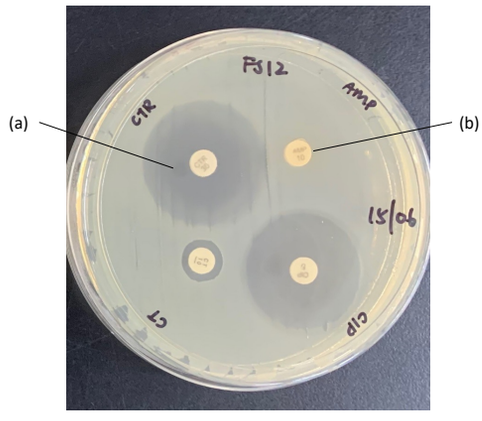

Figure 4.7 The inhibition zone of antibiotics discs in a MH agar plate.
(a) showed inhibition zone while (b) showed no inhibition zone.

Table 4.5 Interpretative categories of resistance to different antibiotics.

Antibiotics	Diamteter of Clear Zone (mm)				Category									Percentage		
	S	SDD	I	R	FS1	FS2	FS3	FS5	FS6	FS11	FS12	PS47	W7	R	S	I / SDD
AMP	≥ 17	-	14-16	≤ 13	R	R	R	R	R	R	R	R	R	100%	0%	0%
CPM	≥ 25	19-24	-	≤ 18	SDD	SDD	SDD	SDD	SDD	SDD	SDD	S	S	0%	12.5%	87.5%
CTX	≥ 26	-	23-25	≤ 22	R	I	R	R	R	R	R	S	I	75%	12.5%	12.5%
CTR	≥ 23	-	20-22	≤ 19	I	S	R	R	I	R	I	S	R	37.5%	25%	37.5%
CM	≥ 18	-	13-17	≤ 12	R	R	R	R	R	R	R	S	R	87.5%	12.5%	0%
CIP	≥ 26	-	22-25	≤ 21	R	R	R	R	R	R	R	I	I	87.5%	0%	12.5%
CT	-	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-	-	-
IPM	≥ 23	-	20-22	≤ 19	S	S	I	I	I	R	S	R	S	25%	37.5%	37.5%
PRL	-	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-	-	-
SXT	≥ 16	-	11-15	≤ 10	R	R	R	R	R	R	R	S	R	87.5%	12.5%	0%
TE	≥ 15	-	12-14	≤ 11	R	R	R	R	R	R	R	I	R	87.5%	0%	12.5%
TOB	≥ 15	-	13-14	≤ 12	S	R	S	S	S	S	S	S	S	12.5%	87.5%	0%

AMP: Ampicillin; CPM: Cefepime; CTX: Cefotaxime; CTR: Ceftriaxone; CM: Chloramphenicol; CIP: Ciprofloxacin; CT: colistin; IPM: Imipenem; PRL: Piperacillin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; TOB: Tobramycin; S: Susceptible; SDD: Susceptible-dose dependent; I: Intermediate: R: Resistance; N/A: Not applicable

CHAPTER 5

DISCUSSION

5.1 Isolation of Lactose Fermenters

Determining the sampling source is the utmost important process for the detection of bacteria carrying colistin resistance gene. In this research, the samples were collected from livestock wastewater, which included water contaminated with chicken faeces, water used to wash pork intestines and water used to wash chicken organs. This is because *mcr* genes were shown to be widely spread in *Enterobacterales* that commonly grow in the gut of poultry and livestock which act as the reservoir hosts of the bacteria carrying *mcr* genes (Liu et al., 2016; Javed et al., 2020).

Primary screening of the colistin resistance strain of *Enterobacterales* in the sample collected was done by culturing the bacteria on MacConkey agar supplemented with 2 μg/mL of colistin. MacConkey agar contains crystal violet and bile salts which inhibit the growth of gram-positive bacteria and lactose which is used as carbohydrate source for fermentation. Lactose fermentation produces acid which changes the colour of pH indicator in the agar into red (Aryal, 2021). In this case, gram-negative bacteria were selected for and lactose fermenters were differentiated as pink colonies. At the meantime, 2 μg/mL colistin was supplemented in the agar to eliminate bacteria which are susceptible to colistin.

The pink colonies on the MacConkey agar were then randomly chosen and subcultured onto a new agar plate to obtain a pure culture of isolates. Single colonies must be obtained as each distinct colony represents an individual bacterium with specific characteristics. After the characterization of isolates, the single colony was subcultured onto Luria Betani (LB) agar which consisted of non-selective nutrient components to support the general routine growth of the isolates (Quinlan, 2018).

5.2 Total DNA extraction

DNA extraction is a crucial step to obtain DNA in sufficient quantity for PCR analysis (Gupta, 2019). In order to study the presence of *mcr* genes in the isolates, total DNA extraction of 43 isolates from 3 different sample sources were performed using boiling method. According to study by other researchers, boiling method was proven to be efficient in extracting total DNA of bacteria as it resulted satisfactory purity and concentration of DNA for downstream molecular biology techniques (Dimitrakopoulou et al., 2020). Furthermore, the results of gene amplification using DNA extracted via boiling method were shown to be reproducible when DNA extracted via commercial kit was used instead (Dashti et al., 2009). Not only that, the procedure of DNA extraction using boiling method is easy, rapid and economic.

5.3 Amplification of *mcr* gene

Screening of *mcr* genes was conducted by amplifying the *mcr* genes through PCR using specific primer pairs. In this study, only *mcr-1*, *mcr-3* and *mcr-5* genes were targeted in this research as other *mcr* gene variants were found to be less prevalent worldwide (Bastidas-Caldes et al., 2022). Gene is a specific sequence of nucleotides which encodes for particular functional proteins. This sequence of DNA can be amplified by polymerase chain reaction (PCR) using a specific set of forward and reverse primers. The primers used anneal to the specific sequences of the *mcr* gene and the sequence being flanked by the primers would be elongated by Taq polymerase, generating PCR product with specific size. In this case, the amplification using *mcr-1* primer pairs gave amplicons of 1497 bp, amplification using *mcr-3* primer pairs gave amplicons of 1063 bp and amplification using *mcr-5* primer pairs gave amplicons of 271 bp.

Although all the isolates tested can grow on the agar plate supplemented with 2 μg/mL of colistin as mentioned in Chapter 5.1, *mcr* genes were only detected in a few of those isolates. In these *mcr*-negative isolates, their colistin resistance might arise from the intrinsic resistance instead of plasmid-mediated *mcr* genes.

5.3.1 *mcr-1*

The bacteria harbouring *mcr-1* gene was found to be the most prevalent as compared to all the other variants of *mcr* genes (Javed et al., 2020). Successful amplification of *mcr-1* gene was observed in 7 out of 12 (58.3%) isolates from chicken faeces. Although the identity of the species harbouring the *mcr* gene was

not studies in this research, study from other researchers showed that *mcr-1* was most commonly found in *Enterobacterales*, where *E. coli* was the predominant one (Lentz et al., 2021). Not only that, the results are also corresponded to the studies which reported poultry faeces as the predominant source of *mcr* gene due to the colonization of the *mcr*-gene harbouring bacteria in the intestines of poultry (Javed et al., 2020). The prevalence of *mcr* gene in the poultry is mostly caused by the unguarded use of colistin as veterinary medicine for poultry especially for the preventive measures, treatment of infections caused by *Enterobacteriaceae* and acts as growth factors. The extensive utilization of colistin create selective pressure to select for *mcr* gene harbouring bacteria in the poultry and these genes are rapidly spread between inter-species and intra-species through horizontal transfer of plasmid.

5.3.2 *mcr-3*

So far, *mcr-3* gene was most frequently being isolated from *Enterobacterales*, especially *E. coli* and the most common variant of *mcr* gene in poultry and pigs is known to be *mcr-1* and followed by *mcr-3* (Duggett et al., 2018; Lentz et al., 2021). However, *mcr-3* gene was only successfully being detected in 1 out of 22 (4.5%) isolate from water used to wash pork intestines in this study. This might due to the reason that *mcr-3* was more prevalent in sick pigs as compared to healthy pigs (Fukuda et al., 2018).

5.3.3 *mcr-5*

There was no successful amplification of *mcr-5* gene being observed in all the isolates. Besides due to the higher prevalence of *mcr-5* gene in diseased pig,

mcr-5 was also found to be more frequently being isolated from the nasal swab as compared to anal swab in both poultry and swine (Chen et al., 2018; Fukuda et al., 2018). Not only that, studies showed that *mcr-5* gene was more commonly found in *Salmonella enterica* which was not being targeted and isolated during the primary screening due to its non-lactose fermenting properties (Lentz et al., 2021).

5.3.4 Co-occurrence of *mcr* Genes

The presence of more than one variant of *mcr* gene or more than one plasmid which carry same variant of *mcr* gene was observed in a colistin-resistance bacterium (Duggett et al., 2018). Although it is common to isolate bacteria which harbour *mcr* gene, the case of co-existance of different variants of *mcr* gene in the bacterium is very rare (Duggett et al., 2018). In this research, none of the *mcr*-positive isolates showed the co-occurrence of different *mcr* variants.

5.4 Minimum Inhibitory Concentration of Colistin

Broth microdilution was the gold standard used to study the minimum inhibitory concentration (MIC) of colistin. MIC of colistin plays a significant role in providing the precise concentration of colistin required to inhibit the growth of pathogens so that sufficient amounts of colistin can be used in treatment and the effect of colistin at high dosage can be minimized. MIC of the *mcr*-positive isolates was determined by culturing the isolates in different concentrations of colistin and determining the lowest concentration of colistin in the broth in which growth of microorganisms were not observed.

Due to the lack of clinical and pharmacokinetic/ pharmacodynamic modelling data as a result of the abandon of colistin usage for many years, the breakpoints of colistin susceptibility and susceptible interpretive categories were still not established (CLSI, 2020). According to CLSI, 2022, when the MIC is equal to or more than 4 μg/mL, the microorganisms are considered as colistin-resistant, while any MIC equal to or smaller than 2 μg/mL are classified as intermediate. In this study, both qualitative and quantitative analysis showed that out of the 8 *mcr*-positive isolates, 4 were categorized as colistin-resistant and 4 were categorized as intermediate according to the published CLSI guidelines.

Phosphoethanolamine transferase family protein is synthesized by the *mcr*-positive bacteria as a result of *mcr* gene expression (Luo et al., 2017). The production of phosphoethanolamine transferase allow the cells to modify the lipid A structure on the lipopolysaccharide and eliminate the colistin binding site, causing the bacterial cell to resist to colistin antibacterial activity. However, the MIC of colistin for *mcr*-positive bacteria was normally found to range from 4 to 8 mg/L, which is low as compared to the MIC value for the bacteria which are intrinsically resistant to colistin (Luo et al., 2017).

For the bacteria classified as resistant to colistin, at least 4 μg/mL of colistin is needed to inhibit their growth. On the other hand, for the bacteria with MIC of 2 μg/mL or less, their response rate towards colistin was low as compared to the resistant phenotype (Humphries, 2021). However, they were shown to have no insertions and deletions in their promoter and expected to express the gene normally (Chew et al., 2017). The possible reason of the low response rate of

bacteria towards colistin even though they harbour the *mcr* genes is due to the varying degrees of the *mcr* gene expression in different isolates. Gene expression of individual is depending on the physiological state of the cells and the DNA transcription factors with other specific regulators (Berthoumieux et al., 2013). Due to this difference in the expression of the *mcr* gene, the isolates might not be able to produce enough phosphoethanolamine transferase to modify their lipid A component and make themselves resistant to colistin rapidly. This causes the bacteria to be killed by colistin and thus no growth of bacteria was observed at high concentrations of colistin even though *mcr* genes which code for the synthesis of phosphoethanolamine transferase are harboured by those cells.

In order to get the accurate reading of the MIC of colistin, fresh cultures of isolate were used for the study of MIC of colistin. The usage of fresh culture is an important key in determining the MIC of colistin as the growth rate of cells is closely related to the protein synthesis rate (Shahrezaei and Marguerat, 2015). Besides, the broth microdilution protocol in 96-well plates was carried out in a careful manner. Serial dilutions of the antibiotic in the broth culture were prone to contaminations due to the repetitive transfer step involved in each sequential serial dilution (Gomez et al., 2007). Thus, the micropipetter was handled carefully without touching the pipette tips to anywhere except the experimental media throughout the procedure. Moreover, proper mixing of the colistin was done by pipetting up and down the broth for a few times before transferring to the following well. Furthermore, triplicates of broth microdilution were done for each isolate to ensure accurate and reliable readings of MIC of colistin were obtained.

5.5 Antibiotic Susceptibility Test

Bacteria can carry more than one plasmid with each plasmid acting as the scaffold to assemble various antibiotic resistance genes by transposition and site-specific recombination mechanisms (Bennett, 2008). Therefore, bacteria can be resistant to one antibiotic or many antibiotics simultaneously. Co-existence of *mcr* gene and other antibiotic resistance gene was observed in previous studies (Ćwiek et al., 2021). Disc diffusion method was used to test the susceptibility of the *mcr*-positive bacteria to other antibiotics. The susceptibility of the bacteria to specific antibiotics was evaluated by measuring the diameter of the clear zone around the antibiotics disc which indicates the inhibition of bacterial growth and comparing the diameter of the clear zone with published CLSI guidelines (Hudzicki, 2012).

Bacteria resistant to three or more out of seven antimicrobial classes are defined as multidrug resistant (MDR). The seven classes of antimicrobial drugs include β-lactams, aminoglycosides, tetracyclines, quinolones, sulfonamides, phenicols and polymyxin. The antibiotic discs used for antibiotic susceptibility test in this study can be classified into these seven group, in which ampicillin, cefotaxime, ceftriaxone, cefepime, imipenem and piperacillin is classified as β-lactams; tobramycin is classified as aminoglycosides; tetracycline is grouped under tetracyclines; ciprofloxacin is categorized as quinolones; trimethoprim-sulfamethoxazole is grouped under sulfonamides; chloramphenicol being classified as phenicols; colistin as polymyxin (Dominguez et al., 2018).

There was study showed that high percentage of bacteria isolated from chicken was resistant to ciprofloxacin, ampicillin, sulfamethoxazole, tetracycline and chloramphenicol and multi-drug resistance was observed in most of the *mcr-1* positive *E. coli* isolates (Ćwiek et al., 2021). This was in agreement with the results obtained in the present study in which resistance to multiple antimicrobial classes was observed for the *mcr-1* positive isolates from the chicken faeces sample. The *mcr-1* harbouring isolates were resistant to 5 to 6 classes of antimicrobial drug, including colistin resistance simultaneously. While for *mcr-3* positive isolates, the isolate was only co-resistant to ampicillin and imipenem from β-lactams class, so the isolate was not considered to be multi-drug resistant.

Tetracycline resistance was one of the most common resistance being observed in animal isolates as tetracycline was the predominantly used in veterinary and animal production systems. The selection of tetracycline resistance brought about by the long-term feeding of tetracycline was not reversed even with the long-term withdrawal of the usage of tetracycline in feeding and treatment in animal production (Langlois et al., 1983). In this research, all the isolates from chicken faeces were found to be resistant to tetracycline but isolate from water used to wash pork intestines was susceptible to tetracycline. Studies have shown that highest amounts of tetracycline resistance were found in the bacteria isolated from poultry litter as they excreted up to 90% of their metabolites in faeces (Tadesse et al., 2012). Not only that, the bacteria resistant to tetracycline were commonly found to be co-resistant with ampicillin, streptomycin, chloramphenicol and sulfonamide (Tadesse et al., 2012). The statement

corresponded to the results obtained in this research in which all the isolates resistant to tetracycline were also resistant to ampicillin and chloramphenicol simultaneously.

On the other hand, all the *mcr* positive isolates were found to be resistant to β-lactams. The highest percentage of ampicillin resistance (100%) was observed, followed by cefotaxime resistance (75%), ceftriaxone resistance (37.5%) and imipenem resistance (25%). All the *mcr-1* harbouring isolates from chicken faeces was categorized as cefepime susceptible-dose dependent (SDD). The isolates of SDD to cefepime are known to show the identical clinical response if higher concentration of cefepime is used by giving higher dosage or more frequent doses, or both without exceeding the approved maximum dosing regimens (CLSI, 2022).

In order to get consistent results for the disc diffusion method, Meuller-Hinton (MH) agar was used as a standard medium to culture the bacteria for antibiotic susceptibility test. This is because MH agar is a non-selective and non-differential medium which supports the growth of the bacteria, thus antibiotics become the only factor that can inhibit the growth of bacteria in this case. Besides, MH agar is a loose agar which allows a better diffusion of antibiotics into the agar and provides a more accurate diameter of clear zone. Furthermore, MH agar is supplemented with starch which absorbs the toxins produced by the bacteria so that the toxins do not interfere with antibiotics (Aryal, 2018).

5.5.1 Limitation of Disk Diffusion Method

The zone diameter breakpoint for determination of categories of bacterial colistin resistance is not being reported until now. This is due to the highly amphipathic properties of colistin causing a poor diffusion of colistin into the agar, resulting in a very small clear zone to be observed on the agar (Mitton et al., 2019). Therefore, it is difficult to determine the categories of colistin susceptibility in which the isolates belong to. Even by increasing the concentration of the colistin in the antibiotic disc, the accuracy of the disc diffusion assay does not appear to improve (Tan, 2006). However, it was found that by using modified composition with the reduction of agar concentration in the commercial MH agar by 30%, the diffusion of the colistin can be improved, resulting in a more reliable diameter reading for colistin susceptibility test (Uwizeyimana et al., 2020).

Apart from that, since the adjustment of the MIC breakpoint for piperacillin from ≤ 64 μg/mL to ≤ 16 μg/mL based on the pharmacokinetic-pharmacodynamic modelling, the disc diffusion breakpoints are not established due to the absence of correlate data of disc diffusion method to the revised piperacillin MIC breakpoint (Tamma et al., 2012). Therefore, the susceptibility of the isolates to piperacillin was not evaluated through the disc diffusion method in this research.

CHAPTER 6

CONCLUSION

In conclusion, a total of 43 colistin resistant gram-negative lactose-fermenting bacteria were randomly being isolated from 3 livestock wastewater sample using MacConkey agar supplemented with colistin. Secondary screening of the *mcr* gene using PCR found that prevalence of *mcr-1* was the highest as it was detected with the highest percentage (16.27%) among the isolates, followed by *mcr-3* (2.33%) and no *mcr-5* gene was detected. Besides, most of the isolates from water contaminated with chicken faeces (58.3%) were found to harbour *mcr-1* gene, while only one isolate from water used to wash pork intestine (4.5%) was found to carry *mcr-3* gene, and none of the isolates from water used to wash chicken organs was detected for any variants of the *mcr* genes. The MIC of colistin for different *mcr*-positive isolates was ranged from 1 μg/mL to 4 μg/mL which was found to be much lower than those bacteria which are intrinsically resistant to colistin. According to the published CLSI guidelines, 4 out of 8 isolates were categorized as resistant to colistin and 4 were classified as intermediate according to their MICs. Besides colistin resistance, *mcr*-positive isolates were found to co-resistant to other antimicrobial agents. For *mcr-1* harbouring isolates from the water contaminated with chicken faeces, they were resistant to at least 5 classes of antimicrobial agent and thus were considered as MDR bacteria. While for *mcr-3* harbouring isolates from water used to wash pork intestine, the isolate was only resistant to two antimicrobial agent classes including polymyxin, hence the isolate was not classified as MDR bacteria.

REFERENCES

AbuOun, M., Stubberfield, E.J., Duggett, N.A., Kirchner, M., Dormer, L., Nunez-Garcia, J., Randall, L.P., Lemma, F., Crook, D.W., Teale, C., Smith, R.P. and Anjum, M.F., 2017. mcr-1 and mcr-2 (mcr-6.1) variant genes identified in Moraxella species isolated from pigs in Great Britain from 2014 to 2015. *Journal of Antimicrobial Chemotherapy*, 72(10), pp.2745–2749.

Aghapour, Z., Gholizadeh, P., Ganbarov, K., Bialvaei, A.Z., Mahmood, S.S., Tanomand, A., Yousefi, M., Asgharzadeh, M., Yousefi, B. and Samadi Kafil, H., 2019. Molecular mechanisms related to colistin resistance in Enterobacteriaceae. *Infection and Drug Resistance*, Volume 12, pp.965–975.

Ainsworth, G.C., Brown, A.M. and Brownlee, G., 1947. 'Aerosporin', an Antibiotic Produced by Bacillus aerosporus Greer. *Nature*, 160(4060), pp.263–263.

Aryal, S., 2018. *Mueller Hinton Agar (MHA) – Composition, Principle, Uses and Preparation*. [online] Microbiologyinfo.com. Available at: <https://microbiologyinfo.com/mueller-hinton-agar-mha-composition-principle-uses-and-preparation/> [Accessed 21 August 2022].

Aryal, S., 2021. *MacConkey Agar- Composition, Principle, Uses, Preparation and Colony Morphology*. [online] Microbiologyinfo.com. Available at: <https://microbiologyinfo.com/macconkey-agar-composition-principle-uses-preparation-and-colony-morphology/#:~:text=of MacConkey Agar-,MacConkey agar is used for the isolation of gram-negative,dairy products and biological specimens.> [Accessed 21 August 2022].

Bastidas-Caldes, C., de Waard, J.H., Salgado, M.S., Villacís, M.J., Coral-Almeida, M., Yamamoto, Y. and Calvopiña, M., 2022. Worldwide Prevalence of mcr-mediated Colistin-Resistance Escherichia coli in Isolates of Clinical Samples, Healthy Humans, and Livestock—A Systematic Review and Meta-Analysis. *Pathogens*, 11(6), p.659.

Bennett, P.M., 2008. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, 153(S1), pp.S347–S357.

Berthoumieux, S., de Jong, H., Baptist, G., Pinel, C., Ranquet, C., Ropers, D. and Geiselmann, J., 2013. Shared control of gene expression in bacteria by transcription factors and global physiology of the cell. *Molecular Systems Biology*, 9(1), p.634.

Bialvaei, A.Z. and Samadi Kafil, H., 2015. Colistin, mechanisms and prevalence of resistance. *Current Medical Research and Opinion*, 31(4), pp.707–721.

Borowiak, M., Fischer, J., Hammerl, J.A., Hendriksen, R.S., Szabo, I. and Malorny, B., 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella enterica subsp. enterica serovar Paratyphi B. *Journal of Antimicrobial Chemotherapy*, 72(12), pp.3317–3324.

Burmeister, A.R., 2015. Horizontal Gene Transfer. *Evolution, Medicine, and Public Health*, 2015(1), pp.193–194.

Campos, M.A., Vargas, M.A., Regueiro, V., Llompart, C.M., Albertí, S. and Bengoechea, J.A., 2004. Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides. *Infection and Immunity*, 72(12), pp.7107–7114.

Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., Pezzotti, G. and Magistrali, C.F., 2017. Novel plasmid-mediated colistin resistance mcr-4 gene in Salmonella and Escherichia coli, Italy 2013, Spain and Belgium, 2015 to 2016. *Eurosurveillance*, 22(31), p.30589.

Carroll, L.M., Gaballa, A., Guldimann, C., Sullivan, G., Henderson, L.O. and Wiedmann, M., 2019. Identification of Novel Mobilized Colistin Resistance Gene mcr-9 in a Multidrug-Resistant, Colistin-Susceptible Salmonella enterica Serotype Typhimurium Isolate. *mBio*, 10(3), pp.e00853-19.

Chen, L., Zhang, J., Wang, J., Butaye, P., Kelly, P., Li, M., Yang, F., Gong, J., Yassin, A.K., Guo, W., Li, J., Song, C. and Wang, C., 2018. Newly identified colistin resistance genes, mcr-4 and mcr-5, from upper and lower alimentary tract of pigs and poultry in China. *PLOS ONE*, 13(3), p.e0193957.

Chew, K.L., La, M.-V., Lin, R.T.P. and Teo, J.W.P., 2017. Colistin and Polymyxin B Susceptibility Testing for Carbapenem-Resistant and mcr -Positive Enterobacteriaceae: Comparison of Sensititre, MicroScan, Vitek 2, and Etest with Broth Microdilution. *Journal of Clinical Microbiology*, 55(9), pp.2609–2616.

CLSI, 2020. Polymyxin Breakpints for Enterobacterales, pseudomonas aeruginosa, and Acinetobacter spp. (MR01), pp.1–3.

CLSI, 2022. Performance Standards for Antimicrobial Susceptibility Testing. *CLSI supplement M100*, 32, pp.32–41.

Ćwiek, K., Woźniak-Biel, A., Karwańska, M., Siedlecka, M., Lammens, C., Rebelo, A.R., Hendriksen, R.S., Kuczkowski, M., Chmielewska-Władyka, M. and Wieliczko, A., 2021. Phenotypic and genotypic characterization of mcr-1-positive multidrug-resistant Escherichia coli ST93, ST117, ST156, ST10, and ST744 isolated from poultry in Poland. *Brazilian Journal of Microbiology*, 52(3), pp.1597–1609.

Dashti, A.A., Jadaon, M.M., Abdulsamad, A.M. and Dashti, H.M., 2009. Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. *Kuwait Medical Journal*, 41(2), pp.117–122.

Dimitrakopoulou, M.-E., Stavrou, V., Kotsalou, C. and Vantarakis, A., 2020. Boiling Extraction Method VS Commercial Kits for Bacterial DNA Isolation from Food Samples. *Journal of Food Science and Nutrition Research*, 03(04), pp.311–319.

Dominguez, J.E., Redondo, L.M., Figueroa Espinosa, R.A., Cejas, D., Gutkind, G.O., Chacana, P.A., Di Conza, J.A. and Fernández Miyakawa, M.E., 2018. Simultaneous Carriage of mcr-1 and Other Antimicrobial Resistance Determinants in Escherichia coli From Poultry. *Frontiers in Microbiology*, 9, p.1679.

Duggett, N.A., Randall, L.P., Horton, R.A., Lemma, F., Kirchner, M., Nunez-Garcia, J., Brena, C., Williamson, S.M., Teale, C. and Anjum, M.F., 2018. Molecular epidemiology of isolates with multiple mcr plasmids from a pig farm in Great Britain: the effects of colistin withdrawal in the short and long term. *Journal of Antimicrobial Chemotherapy*, 73(11), pp.3025–3033.

El-Sayed Ahmed, M.A.E.-G., Zhong, L.-L., Shen, C., Yang, Y., Doi, Y. and Tian, G.-B., 2020. Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerging Microbes & Infections*, 9(1), pp.868–885.

Elbediwi, Li, Paudyal, Pan, Li, Xie, Rajkovic, Feng, Fang, Rankin and Yue, 2019. Global Burden of Colistin-Resistant Bacteria: Mobilized Colistin Resistance Genes Study (1980–2018). *Microorganisms*, 7(10), p.461.

FDA, 2022. *Combating Antibiotic Resistance*. [online] Available at: <https://www.fda.gov/consumers/consumer-updates/combating-antibiotic-resistance#:~:text=The agency has issued drug,of taking them as directed.> [Accessed 21 August 2022].

Formosa, C., Herold, M., Vidaillac, C., Duval, R.E. and Dague, E., 2015. Unravelling of a mechanism of resistance to colistin in Klebsiella pneumoniae using atomic force microscopy. *Journal of Antimicrobial Chemotherapy*, 70(8), pp.2261–2270.

Fukuda, A., Sato, T., Shinagawa, M., Takahashi, S., Asai, T., Yokota, S., Usui, M. and Tamura, Y., 2018. High prevalence of mcr-1 , mcr-3 and mcr-5 in Escherichia coli derived from diseased pigs in Japan. *International Journal of Antimicrobial Agents*, 51(1), pp.163–164.

Gogry, F.A., Siddiqui, M.T., Sultan, I. and Haq, Q.M.R., 2021. Current Update on Intrinsic and Acquired Colistin Resistance Mechanisms in Bacteria. *Frontiers in Medicine*, 8, p.677720.

Gomez, M., Gomez, M., Karbowski, J.P. and Yates, I., 2007. Assay: Accuracy and Precision with Serial Dilution. *Genetic Engineering & Biotechnology News*, [online] 27(6). Available at: <https://www.genengnews.com/magazine/67/assay-accuracy-and-precision-with-serial-dilution/> [Accessed 5 August 2022].

Gough, M., Hancock, R.E.W. and Kelly, N.M., 1996. Antiendotoxin activity of cationic peptide antimicrobial agents. *Infection and Immunity*, 64(12), pp.4922–4927.

Gupta, N., 2019. DNA extraction and polymerase chain reaction. *Journal of Cytology*, 36(2), p.116.

Hancock, R.E., 1997. Peptide antibiotics. *The Lancet*, 349(9049), pp.418–422.

Hudzicki, J., 2012. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology*, (December 2009), pp.1–13.

Humphries, R.M., 2021. Re-Exploring the Intermediate Interpretive Category. *AST News Update*, 6(1), pp.7–11.

Hutchings, M.I., Truman, A.W. and Wilkinson, B., 2019. Antibiotics: past, present and future. *Current Opinion in Microbiology*, 51, pp.72–80.

Imlay, J.A., 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nature Reviews Microbiology*, 11(7), pp.443–454.

Javed, H., Saleem, S., Zafar, A., Ghafoor, A., Shahzad, A. Bin, Ejaz, H., Junaid, K. and Jahan, S., 2020. Emergence of plasmid-mediated mcr genes from Gram-negative bacteria at the human-animal interface. *Gut Pathogens*, 12(1), p.54.

Koyama, Y., 1950. A new antibiotic 'colistin' produced by spore-forming soil bacteria. *J. Antibiot.*, 3, pp.457–458.

Kumazawa, J. and Yagisawa, M., 2002. The history of antibiotics: The Japanese story. *Journal of Infection and Chemotherapy*, 8(2), pp.125–133.

Langlois, B.R., Cromwell, G.L., Stahly, T.S., Dawson, K.A. and Hays, V.W., 1983. Antibiotic resistance of fecal coliforms after long-term withdrawal of therapeutic and subtherapeutic antibiotic use in a swine herd. *Applied and Environmental Microbiology*, 46(6), pp.1433–1434.

Lentz, S.A.M., Dalmolin, T.V., Barth, A.L. and Martins, A.F., 2021. mcr-1 Gene in Latin America: How Is It Disseminated Among Humans, Animals, and the Environment? *Frontiers in Public Health*, 9, p.648940.

Lim, L.M., Ly, N., Anderson, D., Yang, J.C., Macander, L., Jarkowski, A., Forrest, A., Bulitta, J.B. and Tsuji, B.T., 2010. Resurgence of Colistin: A Review of Resistance, Toxicity, Pharmacodynamics, and Dosing. *Pharmacotherapy*, 30(12), pp.1279–1291.

Liu, Y.-Y., Chandler, C.E., Leung, L.M., McElheny, C.L., Mettus, R.T., Shanks, R.M.Q., Liu, J.-H., Goodlett, D.R., Ernst, R.K. and Doi, Y., 2017. Structural Modification of Lipopolysaccharide Conferred by mcr-1 in Gram-Negative ESKAPE Pathogens. *Antimicrobial Agents and Chemotherapy*, 61(6), pp.e00580-17.

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H. and Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases*, 16(2), pp.161–168.

Luo, Q., Wang, Y. and Xiao, Y., 2020. Prevalence and transmission of mobilized colistin resistance (mcr) gene in bacteria common to animals and humans. *Biosafety and Health*, 2(2), pp.71–78.

Luo, Q., Yu, W., Zhou, K., Guo, L., Shen, P., Lu, H., Huang, C., Xu, H., Xu, S., Xiao, Y. and Li, L., 2017. Molecular Epidemiology and Colistin Resistant Mechanism of mcr-Positive and mcr-Negative Clinical Isolated Escherichia coli. *Frontiers in Microbiology*, 8, p.2262.

McPhee, J.B., Lewenza, S. and Hancock, R.E.W., 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in Pseudomonas aeruginosa. *Molecular Microbiology*, 50(1), pp.205–217.

Mentasti, M., David, S., Sands, K., Khan, S., Davies, L., Turner, L. and Wootton, M., 2021. Rapid detection and differentiation of mobile colistin resistance (mcr-1 to mcr-10) genes by real-time PCR and melt-curve analysis. *Journal of Hospital Infection*, 110, pp.148–155.

Mitton, B., Kingsburgh, C., Kock, M.M., Mbelle, N.M. and Strydom, K., 2019. Evaluation of an In-House Colistin NP Test for Use in Resource-Limited Settings. *Journal of Clinical Microbiology*, 57(10), pp.e00501-19.

Olaitan, A.O., Morand, S. and Rolain, J.-M., 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*, 5, p.643.

Poirel, L., Jayol, A. and Nordmann, P., 2017. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. *Clinical Microbiology Reviews*, 30(2), pp.557–596.

Prescott, J.F., 2014. The resistance tsunami, antimicrobial stewardship, and the golden age of microbiology. *Veterinary Microbiology*, 171(3–4), pp.273–278.

Quinlan, S., 2018. *Types of Agar Plates*. [online] Sciencing.com. Available at: <https://sciencing.com/types-agar-plates-8131230.html> [Accessed 21 August 2022].

Schembri, M.A., Blom, J., Krogfelt, K.A. and Klemm, P., 2005. Capsule and Fimbria Interaction in Klebsiella pneumoniae. *Infection and Immunity*, 73(8), pp.4626–4633.

Senturk, S., 2005. Evaluation of the anti-endotoxic effects of polymyxin-E (colistin) in dogs with naturally occurred endotoxic shock. *Journal of Veterinary Pharmacology and Therapeutics*, 28(1), pp.57–63.

Shahrezaei, V. and Marguerat, S., 2015. Connecting growth with gene expression: of noise and numbers. *Current Opinion in Microbiology*, 25, pp.127–135.

Shrestha, A., Soriano, S., Song, M. and Chihara, S., 2014. Intravenous colistin-induced acute respiratory failure: A case report and a review of literature. *International Journal of Critical Illness and Injury Science*, 4(3), p.266.

Srinivasan, V.B. and Rajamohan, G., 2013. KpnEF, a New Member of the Klebsiella pneumoniae Cell Envelope Stress Response Regulon, Is an SMR-Type Efflux Pump Involved in Broad-Spectrum Antimicrobial Resistance. *Antimicrobial Agents and Chemotherapy*, 57(9), pp.4449–4462.

Tadesse, D.A., Zhao, S., Tong, E., Ayers, S., Singh, A., Bartholomew, M.J. and McDermott, P.F., 2012. Antimicrobial Drug Resistance in Escherichia coli from Humans and Food Animals, United States, 1950–2002. *Emerging Infectious Diseases*, 18(5), pp.741–749.

Tamma, P.D., Turnbull, A.E., Milstone, A.M., Hsu, A.J., Carroll, K.C. and Cosgrove, S.E., 2012. Does the Piperacillin Minimum Inhibitory Concentration for Pseudomonas aeruginosa Influence Clinical Outcomes of Children With Pseudomonal Bacteremia? *Clinical Infectious Diseases*, 55(6), pp.799–806.

Tan, T.Y., 2006. Comparison of three standardized disc susceptibility testing methods for colistin. *Journal of Antimicrobial Chemotherapy*, 58(4), pp.864–867.

Tankeshwar, A., 2022. *Broth Dilution Method for MIC Determination*. [online] Available at: <https://microbeonline.com/minimum-inhibitory-concentration-mic-broth-dilution-method-procedure-interpretation/> [Accessed 21 August 2022].

Uwizeyimana, J.D., Kim, D., Lee, H., Byun, J.-H. and Yong, D., 2020. Determination of Colistin Resistance by Simple Disk Diffusion Test Using Modified Mueller-Hinton Agar. *Annals of Laboratory Medicine*, 40(4), pp.306–311.

Vidaillac, C., Benichou, L. and Duval, R.E., 2012. In Vitro Synergy of Colistin Combinations against Colistin-Resistant Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae Isolates. *Antimicrobial Agents and Chemotherapy*, 56(9), pp.4856–4861.

Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M. and Zong, Z., 2020. Identification of novel mobile colistin resistance gene mcr-10. *Emerging Microbes & Infections*, 9(1), pp.508–516.

Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., Zhang, S., Shen, J., Shen, Z. and Wang, Y., 2018. Emergence of a novel mobile colistin resistance gene, mcr-8 , in NDM-producing Klebsiella pneumoniae. *Emerging Microbes & Infections*, 7(1), pp.1–9.

Wellcome, 2022. *Why is it so hard to develop new antibiotics?* [online] Available at: <https://wellcome.org/news/why-is-it-so-hard-develop-new-antibiotics> [Accessed 21 August 2022].

WHO, 2018. The detection and reporting of colistin resistance. *World Health Organisation*, WHO/WSI/AM, pp.1–17.

Xavier, B.B., Lammens, C., Ruhal, R., Kumar-Singh, S., Butaye, P., Goossens, H. and Malhotra-Kumar, S., 2016. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. *Eurosurveillance*, 21(27), p.30280.

Yang, Y.-Q., Li, Y.-X., Lei, C.-W., Zhang, A.-Y. and Wang, H.-N., 2018. Novel plasmid-mediated colistin resistance gene mcr-7.1 in Klebsiella pneumoniae. *Journal of Antimicrobial Chemotherapy*, 73(7), pp.1791–1795.

Yin, W., Li, H., Shen, Y., Liu, Z., Wang, S., Shen, Z., Zhang, R., Walsh, T.R., Shen, J. and Wang, Y., 2017. Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in Escherichia coli. *mBio*, 8(3), pp.e00543-17.

Yu, Z., Qin, W., Lin, J., Fang, S. and Qiu, J., 2015. Antibacterial Mechanisms of Polymyxin and Bacterial Resistance. *BioMed Research International*, 2015, pp.1–11.

Zhang, J., Chen, L., Wang, J., Butaye, P., Huang, K., Qiu, H., Zhang, X., Gong, W. and Wang, C., 2018a. Molecular detection of colistin resistance genes (mcr-1 to mcr-5) in human vaginal swabs. *BMC Research Notes*, 11(1), p.143.

Zhang, J., Chen, L., Wang, J., Yassin, A.K., Butaye, P., Kelly, P., Gong, J., Guo, W., Li, J., Li, M., Yang, F., Feng, Z., Jiang, P., Song, C., Wang, Y., You, J., Yang, Y., Price, S., Qi, K., Kang, Y. and Wang, C., 2018b. Molecular detection of colistin resistance genes (mcr-1, mcr-2 and mcr-3) in nasal/oropharyngeal and anal/cloacal swabs from pigs and poultry. *Scientific Reports*, 8(1), p.3705.

APPENDIX

Table A: Absorbance readings of the broth cultures with different concentrations of colistin.

Isolate	Replicate	Absorbance reading at 600 nm											
		Medium control	Concentration of colistin (µg/mL)										Growth control
			128	64	32	16	8	4	2	1	0.5	0.25	
FS1	1	0.053	0.054	0.054	0.057	0.056	0.056	0.057	0.056	0.487	0.472	0.480	0.589
	2	0.054	0.052	0.055	0.054	0.054	0.054	0.055	0.055	0.385	0.411	0.445	0.510
	3	0.052	0.053	0.055	0.055	0.056	0.055	0.083	0.060	0.396	0.480	0.468	0.491
FS2	1	0.053	0.050	0.052	0.052	0.052	0.060	0.053	0.055	0.232	0.412	0.470	0.573
	2	0.053	0.050	0.053	0.052	0.056	0.058	0.053	0.055	0.184	0.435	0.417	0.570
	3	0.052	0.054	0.052	0.052	0.053	0.055	0.054	0.055	0.230	0.487	0.553	0.591
FS3	1	0.0515	0.0520	0.0571	0.0525	0.0532	0.1690	0.0544	0.4790	0.6440	0.7430	0.6870	0.6930
	2	0.0523	0.0504	0.0516	0.0536	0.0566	0.0575	0.0509	0.4520	0.3840	0.6890	0.7430	0.6780
	3	0.0530	0.0500	0.0519	0.0538	0.0533	0.0546	0.0400	0.4940	0.4630	0.6420	0.6960	0.6830
FS5	1	0.0522	0.0502	0.0517	0.0523	0.0534	0.0567	0.0532	0.5730	0.5750	0.6220	0.4910	0.6100
	2	0.0521	0.0509	0.0510	0.0520	0.0535	0.0520	0.0547	0.4700	0.4370	0.6050	0.5200	0.5830
	3	0.0524	0.0525	0.0513	0.0615	0.0504	0.0510	0.0551	0.4460	0.5820	0.5100	0.5870	0.5910
FS6	1	0.0505	0.0487	0.0502	0.0525	0.0503	0.0512	0.0637	0.0467	0.5800	0.5840	0.5870	0.6070
	2	0.0489	0.0480	0.0475	0.0481	0.0502	0.0488	0.0485	0.0499	0.5990	0.5580	0.5880	0.6100
	3	0.0488	0.0498	0.0476	0.0487	0.0485	0.0504	0.0415	0.0521	0.5430	0.6110	0.6800	0.6290
FS11	1	0.0496	0.0513	0.0508	0.0463	0.0510	0.0487	0.0531	0.0486	0.0496	0.3760	0.5910	0.6510
	2	0.0492	0.0490	0.0475	0.0495	0.0491	0.0643	0.0494	0.0500	0.0478	0.3370	0.4850	0.4970
	3	0.0619	0.0482	0.0469	0.1280	0.0496	0.0488	0.0493	0.0516	0.0486	0.3840	0.4760	0.5160
FS12	1	0.0527	0.0484	0.0485	0.0475	0.0492	0.0516	0.0489	0.4390	0.4960	0.4170	0.4940	0.5520
	2	0.0492	0.0484	0.0498	0.0510	0.0537	0.0506	0.0499	0.3860	0.4200	0.4440	0.4700	0.5010
	3	0.0539	0.0492	0.0500	0.0505	0.0523	0.0527	0.0537	0.4530	0.3960	0.3890	0.5490	0.5730
PS47	1	0.0536	0.0515	0.0528	0.0524	0.0516	0.0544	0.0597	0.3270	0.4060	0.3990	0.4240	0.4590
	2	0.0571	0.0504	0.0515	0.0505	0.0508	0.0522	0.0564	0.1770	0.2580	0.3580	0.3370	0.4000
	3	0.0535	0.0502	0.0512	0.0519	0.0528	0.0538	0.0609	0.3190	0.2580	0.2410	0.5210	0.4110
W7	1	0.050	0.051	0.051	0.051	0.050	0.051	0.050	0.258	0.396	0.357	0.409	0.438
	2	0.051	0.050	0.052	0.056	0.053	0.052	0.054	0.407	0.363	0.405	0.377	0.465
	3	0.050	0.050	0.053	0.054	0.051	0.052	0.057	0.441	0.446	0.463	0.502	0.354

Shading indicates positive growth of bacteria in the broth culture

Table B: Diameter of clear zone.

Antibiotics	Zone diameter (mm)								
	FS1	**FS2**	**FS3**	**FS5**	**FS6**	**FS11**	**FS12**	**PS47**	**W7**
AMP	NI	NI	NI	NI	NI	NI	NI	NI	NI
CPM	22	21	22	19	20	19	19	25	26
CTX	21	23	18	16	19	20	21	27	25
CTR	21	23	18	19	20	18	21	29	19
CM	NI	NI	NI	NI	7	9	NI	27	NI
CIP	15	16	15	14	15	15	12	24	24
CT	10	9	9	9	10	10	10	11	10
IPM	24	24	20	21	21	7	24	17	23
PRL	15	11	11	14	10	10	NI	30	14
SXT	NI	NI	NI	NI	NI	NI	NI	20	NI
TE	NI	NI	NI	NI	NI	NI	NI	12	8
TOB	19	12	16	18	18	17	17	19	17

AMP: Ampicillin; CPM: Cefepime; CTX: Cefotaxime; CTR: Ceftriaxone; CM: Chloramphenicol; CIP: Ciprofloxacin; CT: colistin; IPM: Imipenem; PRL: Piperacillin; SXT: Sulfamethoxazole-trimethoprim; TE: Tetracycline; TOB: Tobramycin; NI: No inhibition.