



AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATION OF COLISTIN RESISTANCE AND  
HETERORESISTANCE IN ENTEROBACTERIALES  
ISOLATES FROM POULTRY, WATER AND CLINICAL  
SPECIMENS – ROLE OF *MCR-1* GENES

by  
MARIA JOSEPH EL AZZI

A thesis  
submitted in partial fulfillment of the requirements  
for the degree of Master of Biomedical Science  
to the Department of Experimental Pathology, Immunology and Microbiology  
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# ABSTRACT OF THE THESIS OF

Maria Joseph El Azzi

for Master of Biomedical Science

Major: Experimental Pathology, Immunology,  
and Microbiology

Title: Investigation of Colistin Resistance and Heteroresistance in Enterobacterales Isolates from Poultry, Water and Clinical Specimens – Role of *mcr-1* genes

**Background:** The mobilized colistin resistance, *mcr* genes, have attracted global attention, as they confer resistance to polymyxins, one of the last-resort antimicrobials' family for the treatment of critical infections caused by multidrug-resistant Gram-negative bacteria. This resistance mechanism is plasmid mediated and can be transferred horizontally. Resistance to colistin may go undetected when performing traditional antimicrobial susceptibility testing because of the phenomenon of heteroresistance. This phenotype is denoted by the presence of subpopulations of bacterial cells with higher levels of antimicrobial resistance than those of the rest of the population.

**Aim:** The aim of this study was to determine the prevalence of colistin resistance and to investigate the different mechanisms of colistin resistance and heteroresistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates recovered from poultry feces, water samples and clinical specimens in Lebanon.

**Methods:** Colistin resistance was evaluated in 161 *E. coli* and 92 *K. pneumoniae* isolates recovered from clinical, poultry feces, and water samples using broth micro-dilution (BMD). Moreover, BMD was done on all the colistin resistant isolates using 19 antimicrobials from different antimicrobial families. The prevalence of *mcr* genes (*mcr-1* to *mcr-8*) was assessed by multiplex PCR in all the colistin resistant *E. coli* and *K. pneumoniae* isolates and subjected to whole genome sequencing. To determine the possible presence of colistin resistant subpopulations in colistin susceptible *E. coli* and *K. pneumoniae* isolates, population analysis profiles assay was performed. This was followed by the fitness cost assay to determine the difference in growth rate between the colistin resistant subpopulations and the susceptible ones in the presence and absence of colistin

**Results:** BMD results showed that out of the 253 Enterobacterales isolates, 27 *E. coli* isolates recovered from poultry feces, 10 *E. coli* isolates obtained from water samples and 10 *K. pneumoniae* clinical isolates were colistin resistant. All colistin resistant isolates had also various levels of resistance to a panel of antimicrobial agents. PCR results showed that the *mcr-1* gene was harbored in all the *E. coli* isolates recovered from poultry feces and in 5 out of the 10 *E. coli* isolates obtained from water samples. Moreover, no *mcr* gene was detected in any of the colistin resistant clinical *K. pneumoniae* isolates.

WGS results of 18 out of the 27 colistin resistant *E. coli* isolates recovered from poultry feces showed the presence of 7 different sequence types (ST) with ST1140 being the most prevalent. In addition, the number of antimicrobial resistant determinants detected

in these isolates ranged between 4 and 21 with *mcr-1* being the most prevalent gene. Furthermore, a total of 15 different plasmids were detected in these isolates with IncX4 being predominant.

WGS results of the 5 out of the 10 colistin resistant *E. coli* isolates obtained from water samples showed that they were distributed into 3 different STs, with ST10773 being the most prevalent. Moreover, a total of 23 different antimicrobial resistant determinants were detected in these 5 isolates with *bla*<sub>TEM-1</sub>, *tet(A)-1*, *dfrA-14*, *floR*, and *mcr-1* genes being the most predominant. In addition to that, a total of 10 different plasmids were detected among the isolates.

WGS results of 2 out of the 10 colistin resistant *K. pneumoniae* clinical isolates showed that they belonged to ST383 and ST39. Furthermore, a total of 31 antimicrobial resistant determinants were detected, including the carbapenem resistant gene *bla*<sub>NDM-5</sub> (n=1) and *bla*<sub>OXA-48</sub> (n=2). Besides, 3 different plasmids were harbored in each isolate. Population analysis profiles assay showed that none of the 13 colistin susceptible *E. coli* isolates had a colistin resistant subpopulation. However, 10 out of the 12 colistin susceptible *K. pneumoniae* isolates showed to have a colistin resistant subpopulation. Fitness cost assay showed that in the absence of colistin, both the colistin susceptible *K. pneumoniae* isolates and the colistin resistant subpopulations had the same growth rate. However, in the presence of colistin, a significant decrease in the growth rate of the colistin susceptible isolates occurred when compared to the latter in the absence of colistin. In addition to that, similar growth rates were detected between the colistin resistant subpopulation in the absence and presence of colistin.

**Conclusion:** Plasmid mediated colistin resistance, *mcr-1* gene, in *E. coli* isolates recovered from poultry and water poses a serious threat on public health. This gene could be transferred horizontally into colistin susceptible isolates and they could be introduced into the human community if no strict measures are taken. Moreover, the detection of colistin resistant subpopulations among colistin susceptible *K. pneumoniae* clinical isolates may cause serious complications leading to treatment failure.

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# CHAPTER I

## INTRODUCTION

Antimicrobial resistance (AMR) poses a serious threat on the global public health. It is leading to an increase in morbidity, mortality and healthcare expenses (Smith & Coast, 2002). Bacteria such as *Escherichia coli*, in addition to being an opportunistic pathogen in human, it can cause zoonotic infections. The three compartments: humans, animals and the environment allow the transmission of the bacteria and their mobile genetic elements between them. This interplay between different ecologies induces the dissemination of resistance between diverse bacterial strains (Woolhouse et al., 2015).

Polymyxins are a family of antimicrobials that are positively charged. Their chemical structure consists of a cyclic peptide with a hydrophobic tail. Colistin or polymyxin E, is part of this family and works by disrupting the outer membrane of Gram-negative bacteria through electrostatic interactions with its lipopolysaccharide (LPS) moiety (Sun et al., 2018). Nowadays colistin has been used as a last-line antimicrobial in human medicine for the treatment of severe infections caused by multi-drug resistant Gram-negative bacteria (Kempf et al., 2016). This antimicrobial, like all others is trapped by the phenomenon of resistance (Bialvaei & Samadi Kafil, 2015). Colistin resistance is generally due to modifications in the LPS molecule that induces the decrease of the negative charge on the bacterial membrane lowering the affinity to the cationic antibiotic (Aghapour et al., 2019). Genetic modifications shared only vertically were thought to be the only cause for colistin resistance (R. Wang et al., 2018), until Liu et al, discovered the first mobilized colistin resistance (*mcr-1*) gene in

China. This gene could be transferred horizontally since it is harbored on a plasmid (Liu et al., 2016).

Furthermore, colistin heteroresistance is an underappreciated phenotype among bacterial strains. This phenomenon is explained by the presence of an isolate with both a colistin resistant subpopulation and a population of susceptible ones. Once left undetected, these subpopulations may cause serious complications leading to treatment failure (Band et al., 2021). In this context, we aimed to determine the prevalence of colistin resistance and to investigate the different mechanisms of colistin resistance and heteroresistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates recovered from poultry feces, water samples and clinical specimens in Lebanon.

## CHAPTER II

### LITERATURE REVIEW

#### **A. Antimicrobial Resistance**

Antimicrobial resistance (AMR) poses a serious threat on the global public health (Zhang et al., 2019). AMR is defined by the ability of a bacterium to escape the fatal effects of antimicrobials (W. Wang et al., 2018). This could be achieved through biochemical mechanisms and/or genetic modifications. The four different biochemical mechanisms are the production of an inactivating enzyme, modification of the target of an antibiotic, cell membrane impermeability after modification or quantitative decrease of the porins, and efflux pump. The two genetic modifications are either endogenous through nucleoidic mutations or exogenous through the acquisition of resistant genes on plasmids or transposons (C Reygaert, 2018).

In 2050, 10 million deaths per year will be attributed to AMR that already causes 700,000 deaths/year nowadays. These numbers have overcome the deaths cause by cancer each year (Tagliabue & Rappuoli, 2018). Several factors contribute to the rapid growth of AMR worldwide. This includes the misuse, abuse and overuse of antimicrobials (C Reygaert, 2018). Moreover, the emergence of resistant bacteria is amplified by the usage of antimicrobials to treat sick farm-animals and by utilizing antimicrobials as growth inducers (Prestinaci et al., 2015). The mechanisms of resistance in bacteria recovered from human beings and animals are indistinguishable.

Bacteria and their mobile genetic elements found in animals feces and on their skin can be transferred to other bacteria and to human beings too (Holmes et al., 2016).

Gram-negative bacteria can rapidly develop resistance against clinically utilized antimicrobials. This limits the therapeutic options available to treat infections caused by resistant bacteria. Moreover, they have the ability disseminate resistance between them leading to the emergence of new resistant isolates that may serve as a potential threat to human and animal health (Mukerji et al., 2017).

## **B. Enterobacterales**

Enterobacterales are classified into more than 70 genera with 15 being the most common, such as: *Escherichia*, *Klebsiella*, and *Enterobacter*. Members of this family are Gram-negative, non-spore forming, rod shaped bacteria, oxidase negative, glucose fermenters, and facultative anaerobes. They are found worldwide in soil, water, vegetation, and some are part of the normal flora of animals and humans (Morales-López et al., 2019). Some are one of the most potent pathogens in causing hospital-acquired infections. Plasmids, transposons and other resistance elements are the way by which members of the Enterobacterales family acquire antimicrobial resistance (Iii et al., 2013). In addition to that, nucleoidic mutations are another driver for antimicrobial resistance (C Reygaert, 2018). *Klebsiella pneumoniae* could serve as an example for the emergence of resistance in this family. First *K. pneumoniae* developed resistance against all penicillins, cephalosporins and aztreonam which drifted the spotlight towards carbapenems. After that some strains of *K. pneumoniae* started acquiring carbapenem resistance leaving colistin as an only treatment option. However, the emergence of colistin resistance in these isolates led to extensively (XDR) and pan-drug resistant

(PDR) *K. pneumoniae* isolates. Treating infections caused by these isolates is complicated and, in most cases, requires antimicrobial combination therapy (Karaiskos et al., 2017; Prestinaci et al., 2015).

## **C. Colistin**

### ***1. History of discovery***

Colistin or polymyxin E is one of the last resort antimicrobial agent used for the treatment of infections caused by multi-drug resistant (MDR) and XDR Gram-negative bacteria. *Bacillus polymyxa* subsp. *Colistinus*, a spore forming environmental bacterium that inhabits the soil, was behind the discovery of colistin in Japan by Koyama and coworkers in 1947 (Bialvaei & Samadi Kafil, 2015). The family to which colistin belongs, polymyxins, constituted initially of five compounds with distinct chemical structures (A to E). However, the only two compounds that were introduced into the market are polymyxin B and E (Falagas & Kasiakou, 2005).

### ***2. Chemistry***

The molecule of colistin is composed of 10 D- and L- amino acids (Li et al., 2005), these moieties are D-leucine, L-threonine, and  $\alpha$ - $\gamma$ -diaminobutyric acid (Dab) (Falagas & Kasiakou, 2005). The molecular weight of this polypeptide is 1750 Da. The structure of colistin consists of a heptapeptide ring linked to a tripeptide side chain that is acylated at the amino-terminus by a fatty acid residue (Bialvaei & Samadi Kafil, 2015). Approximately 30 constituents of colistin have been discovered and 13 of them have been studied, these components have distinct amino acids and fatty acids arrangement. Colistin A (polymyxin E<sub>1</sub>) and Colistin B (polymyxin E<sub>2</sub>) are two of the

most important components of colistin (Li et al., 2005). They are composed respectively of 6-methyl-octan-oic acid and 6-methyl-eptanoic acid as the fatty acid chain (Falagas & Kasiakou, 2005). Colistin is an amphiphile because it possesses a positive moiety which is the Dab amino acid and the fatty acid chain. This property facilitates its dissolution in water and blood and its integration in the phospholipid bilayer of eukaryotes and prokaryotes (Evans et al., 1999).

In the market, two forms of colistin are accessible: colistin sulfate and colistimethate sodium (CMS). The latter is used parenterally while colistin sulfate is for topical and oral usage. Both of them can be used in inhalation therapy (Lim et al., 2010). The two forms are not interchangeable because at a pH of 7.4 approximately colistin sulfate is a positively charged whereas CMS is negatively charged (Li et al., 2006). Colistin when reacted with formaldehyde and then with sodium bisulfite gives CMS as a result of a reaction of the  $\gamma$ -amino groups of the Dab amino acids (Li et al., 2005). CMS is less toxic than colistin, however it is less powerful (Falagas & Kasiakou, 2005). Moreover, CMS is not potent enough to kill bacteria so it is labeled as a prodrug of colistin. At room temperature, colistin is stable for up to 12 months (Li et al., 2005) unlike CMS that is directly hydrolyzed in plasma (Bialvaei & Samadi Kafil, 2015).

### ***3. Spectrum of activity***

The polymyxin family of antibiotics inhibit the growth of bacteria (bacteriostatic) when given at minimal concentrations and can kill the bacteria (bactericidal) at high doses (Evans et al., 1999). Colistin have a narrow spectrum of activity (Li et al., 2005) and it is bactericidal on aerobic Gram-negative rods, such as:

*Acinetobacter* spp, *Pseudomonas aeruginosa*, *Haemophilus influenza* and most of the members of the Enterobacterales family. However, some Gram-negative bacilli are naturally resistant to colistin, such as: *Pseudomonas mallei*, *Burkholderia cepacia*, *Proteus* spp, *Providencia* spp, *Serratia* spp, and *Edwardsiella* spp. Moreover, colistin has a significant activity against *Mycobacterium* spp. and *Stenotrophomonas maltophilia*. But, it is not active against Gram-positive bacteria, anaerobes, fungi and parasites (Falagas & Kasiakou, 2005).

#### **4. Toxicity**

The most commonly encountered toxicities related to colistin administration are nephrotoxicity and neurotoxicity (Li et al., 2006). A high dose of colistin in the blood may affect the normal function of the kidneys and cause toxicity because this antimicrobial is mainly excreted through the renal route (Spapen et al., 2011). On the other side, neurotoxicity is manifested by all sorts of neurological disturbances affecting the eyes, the brain and impairs some body functions and causes dizziness, confusion, and ataxia. The most dangerous side effect is neuromuscular blockade where the patient reaches at the end a state of respiratory failure or apnea that can lead to death (Falagas & Kasiakou, 2005). These adverse effects led healthcare workers to stop using colistin in the 1970s. However, colistin was reintroduced into the market as a last resort antimicrobial to treat infection caused by MDR and XDR Gram-negative bacilli (El-Sayed Ahmed et al., 2020). Moreover, several studies showed that nephrotoxicity was very rare upon patients and neurotoxicity started to decrease and then fade away after the cessation of the drug (Bialvaei & Samadi Kafil, 2015).

## ***5. Mode of action***

Colistin mainly targets the outer membrane of Gram-negative bacteria and it interacts with the lipopolysaccharide (LPS) moiety (Falagas & Kasiakou, 2005). The LPS makes the outer membrane selective and prevents the entry of lipophilic and huge antibiotics. Moreover, the LPS is negatively charged, labeling it as a target for positively charged antimicrobials and aids in keeping the outer-membrane coherent and well structured (Bialvaei & Samadi Kafil, 2015). Colistin, as previously discussed is a polycationic molecule that is highly attracted to the negatively charged LPS molecule. First, an electrostatic bond forms between colistin and the LPS molecule (Bialvaei & Samadi Kafil, 2015) leading to the dislocation of calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) (Biswas et al., 2012). These two cations are responsible of the stability of the LPS molecule and their displacement leads to the perturbation of the outer membrane (Falagas & Kasiakou, 2005). The latter leads to the leakage of intracellular contents and eventually bacterial death (Biswas et al., 2012).

Furthermore, colistin has an anti-endotoxin activity which means that it neutralizes the endotoxin of the bacteria (Gupta et al., n.d.). Colistin binds to the lipid A portion of the LPS, neutralize it, and prevent cytokine secretion through a signaling cascade directly associated with CD14 (Bialvaei & Samadi Kafil, 2015).

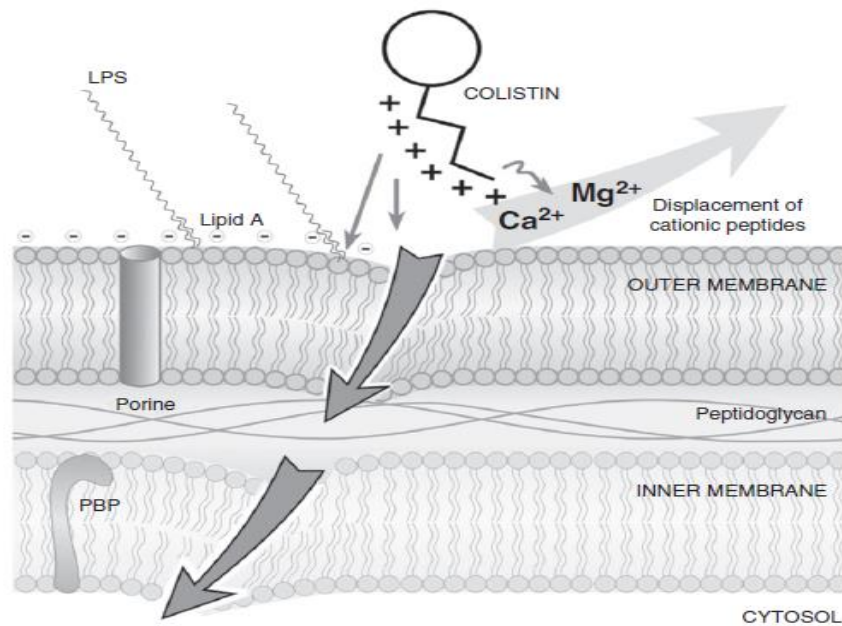


Figure 1: Mode of action of colistin on the bacterial outer membrane (Bialvaei & Samadi Kafil, 2015).

## 6. Mechanisms of Resistance

### a. Intrinsic Resistance to Colistin in Gram-negative bacteria

Some Gram-negative bacteria are naturally resistant to the family of polymyxins including colistin. The intrinsic resistance to colistin in *Proteus mirabilis* is due to 4-amino-4-deoxy-L-arabinose (L-Ara4N) that are found in the lipid A and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residue of the LPS. In addition to the *eptC* gene which modifies the core LPSs with phosphoethanolamine (PEtN). In *Morganella morganii*, *eptB* gene and *arnBCADTEF* operon mediates the modification of the LPS through PEtN and L-Ara4N respectively. On the other hand, the *arnB* and *arnC* genes in *Serratia marcescens* are the reason behind the intrinsic resistance to colistin. These 2 genes and part of the *arnBCADTEF* operon that is regulated by *phoP*. Moreover, the

natural modification of the LPS moiety in *Burkholderia* spp is the driver for colistin resistance (Biswas et al., 2012; Olaitan et al., 2014)

#### b. Acquired resistance mechanisms

Up to 2015, chromosomal point mutations were thought to be the only known reason for colistin resistance. But many mechanisms can be fundamental for this resistance, especially those related to LPS modification which is the main target of polymyxins (Andrade et al., n.d.).

##### i- LPS Modifications

- a. LPS modifications mediated by the *PmrA/PmrB* and *PhoP/PhoQ* two-component systems in *K. pneumoniae*

Colistin resistant *K. pneumoniae* isolates were found to have 5 times more L-Ara4N attached on the phosphate of the lipid A moiety compared to the susceptible isolates (Helander et al., 1996). This leads to a partial loss of the negative charge of the LPS and weakens the interaction with colistin. The *phoP/phoQ* and *pmrA/pmrB* systems become more active once a *K. pneumoniae* isolate is treated with polymyxins (Kim et al., 2014) . These systems confer resistance by being upregulated due to specific mutations. A mutation in the *pmrA* or *pmrB* genes (missense mutation) will in turn cause the overexpression of the *pmrC* gene and the *arnBCADTEF* operon leading to the addition of PEtN and L-Ara4N to the lipid A portion. On the other hand, mutations or inactivation of the *mgrB* gene, 141 nucleotides in length coding for a 47 amino acids long transmembrane protein, can cause colistin resistance. This resistance is induced by

the inhibition of the synthesis of the protein that is responsible in down-regulating the *phoP/phoQ* regulatory system (Olaitan et al., 2014).

- b. LPS modifications mediated by the *PmrA/PmrB* and *PhoP/PhoQ* two-component systems in *E. coli*

L-Ara4N and 2-aminoethanol are the 2 molecules that characterize the lipid A moiety of a colistin resistant *E. coli* isolates (Nummila et al., 1995). In addition to the *PmrA/PmrB* system, the LPS in *E. coli* is modified under the regulation of a tyrosine kinase, *etk*. (Wösten & Groisman, 1999). This enzyme phosphorylates Ugd protein (a UDP-glucose dehydrogenase) which enhances its activity leading to the massive synthesis of UDP-glucuronic acid. This acid is the base on which the L-Ara4N will start to build up (Lacour et al., 2008). On the other hand, a sRNA known as *mgrR* can affect polymyxin resistance in *E. coli*. Its deletion, inhibits *eptB* gene, induces resistance to colistin. This gene is responsible for the alteration of the LPS by adding PEtN to it, reduces its negative charge, and weakens its interaction with colistin (Raetz et al., 2007; Reynolds et al., 2005)(Moon & Gottesman, 2009).

- c. Underacylation of lipid A in *K. pneumoniae*

An enzyme encoded by the *lpxM* gene is responsible for the attachment of the myristoyl group to the lipid A moiety leading to a hexa-acylated lipid A. In *K. pneumoniae*, a mutation in the *lpxM* gene will result in a penta-acylated lipid A that confers susceptibility to polymyxins including colistin (Clements et al., 2007a). In a study conducted in 2013, the MIC of colistin of a *K. pneumoniae* isolate dropped from 4 mg/L to 0.25 mg/L once the *lpxM* gene got mutated (Velkov et al., 2013). This high susceptibility detected in the underacylated *K. pneumoniae* isolate is attributed to the

better entry of polymyxins into the bacterial membrane (Clements et al., 2007b; Velkov et al., 2013).

a. Underacylation of lipid A in *E. coli*

A hexa-acylated lipid A is an important site for the attachment of L-Ara4N. This modified lipid A is obtained by a reaction called myristoylation induced by the *lpxM* gene. In an *E. coli* isolate that has a mutation in the *lpxM* gene, the lipid A will not be modified and the resistance to colistin will be reduced (Tran et al., 2005).

b. Other LPS alterations

The *lpxR* gene is a hydrolase gene that codes for the 3'-O-deacylase. This enzyme causes the detachment of the 3'-acyloxyacyl moiety from the lipid A molecule leading to the colistin resistance. This mainly occurs in *Salmonella* Typhimurium and other Gram-negative bacteria where the *lpxR* gene is generally dormant (Murray et al., 2007; Reynolds et al., 2006).

Susceptible *K. pneumoniae* isolates were compared at the molecular level to resistant ones and eight mutations were found dispersed between the coding and the non-coding regions.

The colistin MIC in these isolates ranged between 4 and 128 mg/L. These mutated genes were found to code for diverse structural and functional proteins in the bacterium (Snitkin et al., 2012). Moreover, mutations in genes encoding for the machinery of the outer membrane proteins synthesis were detected in colistin resistant *K. pneumoniae* isolates. Some of these genes are the *waaL*, *rfaA*, and *vacJ* genes (Sassera et al., 2014).

Moreover, mutations in the *waaP* gene leads to an increased susceptibility to colistin in some *S. enterica* isolates. This gene encodes a protein that phosphorylates the heptose residue in the LPS (Yethon et al., 2000).

All the above-mentioned genes are associated with the outer-membrane function and integrity and every modification in these genes can lead to colistin resistance in the bacterium (Snitkin et al., 2012).

#### ii- Efflux Pump Induced Resistance

Efflux pumps are proteins involved in the expulsion of toxic substances such as antimicrobials out of the bacterial cell (Webber & Piddock, 2003). The efflux pumps that are responsible for moving colistin out of the bacterial cell are AcrAB and KpnEF. KpnEF is part of a family of MDR efflux pumps. When this pump is mutated in a bacterial isolate, the latter becomes more susceptible to antimicrobials in general and mainly colistin (Srinivasan & Rajamohan, 2013).

#### iii- Role of Capsules in Inducing Colistin Resistance

The bacterial capsule is composed of capsular polysaccharides (CPSs). These CPSs are negatively charged molecule, so they can interact electrostatically with the positively charged colistin molecule. This interaction will trap the antibiotic, decrease the concentration of colistin reaching the outer membrane, leading to colistin resistance (Llobet et al., 2008).

Moreover, the Ugd protein, encoded by the *ugd* gene, is involved in the CPSs and L-Ara4N biosynthesis (Lacour et al., 2006). The addition of a phosphate group to this gene is an important step that links capsular synthesis to colistin resistance (Lacour et al., 2008).

#### iv- Mobilized-Colistin Resistance (*mcr*) genes

Until 2015, genetic modifications were thought to be the only reason behind colistin resistance. These mutations can only be shared vertically from one bacterial progeny to the other (R. Wang et al., 2018). Liu et al. isolated a colistin resistant *E. coli* from a pig in china and it was designated as *E. coli* SHP45. This isolate harbored the first ever detected plasmid-borne colistin-resistance gene, named *mcr-1* gene. This gene showed the ability to be horizontally transferred between different bacterial isolates, causing a serious threat due to ability of its spread to humans (Liu et al., 2016).

The *mcr* genes are mainly detected in isolates recovered from animals because of the biased use of colistin in the veterinary sector (Rhouma et al., 2016). Twelve veterinary drugs were found to contain colistin. First, these drugs were used to treat poultry and then they were used as a regimen for several animal diseases (Kassem et al., 2019). Nowadays, *mcr-1* gene is disseminated in a large number of countries from the five different continents (R. Wang et al., 2018). From 2015 and onwards, researchers discovered several variants of the *mcr* gene. Until 2019, 9 different *mcr* genes (*mcr-1* to *mcr-9*) were discovered by scientists. The *mcr-1*, *mcr-3*, *mcr-7.1* and *mcr-8* genes were first reported in China. *mcr-1* and *mcr-3* genes were detected in *E. coli* while the *mcr-7.1* and *mcr-8* genes were first reported in *K. pneumoniae*. *mcr-6* gene was detected in a *Moraxella pluranimalium* isolate in the United Kingdom. The *mcr-4*, *mcr-5*, and *mcr-9* genes were all first encountered in *Salmonella* spp isolates in Italy, Germany and the United States respectively (Gharaibeh & Shatnawi, 2019). In 2020, the *mcr-10* gene was added to the family. It was first detected in an *Enterobacter roggenkampii* clinical isolate in China. It showed a high percentage of similarity with the *mcr-9* gene (C. Wang et al., 2020).

The *mcr-1* gene encodes an enzyme known as the PEA-lipid A transferase. This enzyme links a PEA group to the 1(4')-phosphate of the glucosamine moiety of the lipid A in the LPS on the bacterial outer membrane. This will reduce its negative charge and consequently results in a lower affinity to colistin (Hinchliffe et al., 2017; Zhang et al., 2019). This gene is mainly harbored in the IncI2, IncHI2, and IncX4 plasmids (R. Wang et al., 2018). The *mcr-1* gene have 11 genetic variants ranging from *mcr-1.2* to *mcr-1.12*. This gene is usually associated with MDR bacteria and is accompanied with other resistance genes such as the Extended-spectrum  $\beta$  lactamases (ESBLs) or *bla<sub>NDM</sub>* genes. This may lead to the dissemination of PDR Gram-negative bacteria (Feng, 2018).

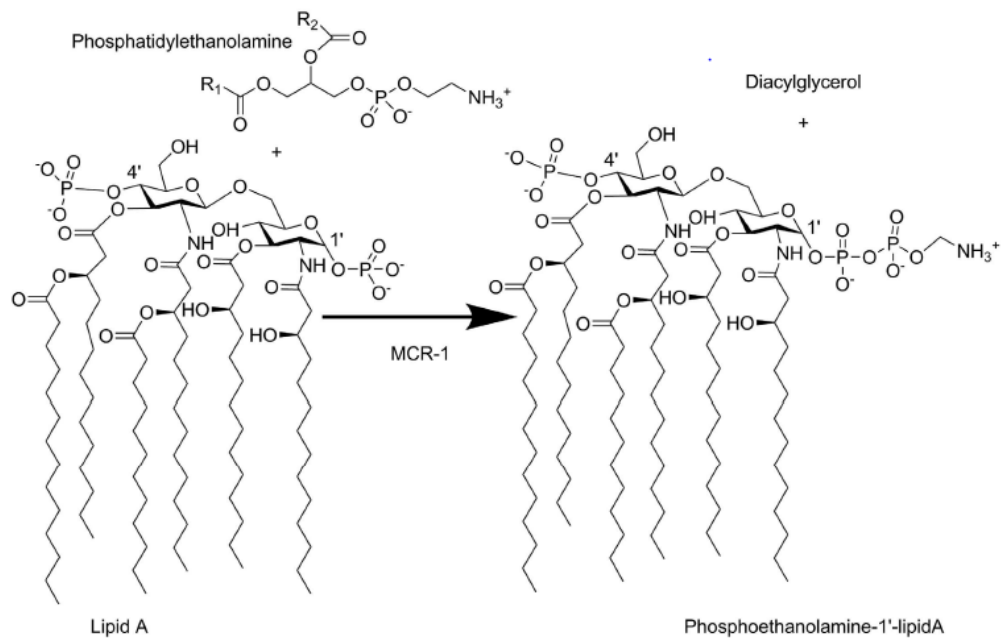


Figure 2: Modification of lipid A by the *mcr-1* gene (Hinchliffe et al., 2017).

#### **D. Colistin Heteroresistance**

Heteroresistance to an antimicrobial agent is manifested by the existence of resistant subpopulations in a susceptible population of the same isolate (El-Halfawy & Valvano, 2015). It was first identified in *Haemophilus influenza* in 1947 (Alexander & Leidy, 1947). Heteroresistance was detected in several microorganisms, such as: *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* (Nicoloff et al., 2019). This phenotype was mostly recorded against bactericidal antibiotics, such as:  $\beta$ -lactams, fluoroquinolones, aminoglycosides, metronidazole, glycopeptides, and antimicrobial peptides (El-Halfawy & Valvano, 2015). The Population Analysis Profiles (PAPs) is the optimal technique to detect heteroresistance (Charretier et al., 2018). The key difference between heteroresistance and persistence is that persister cells do not grow in the presence of the antimicrobial (Figure 3) (Dewachter et al., 2019).

Colistin heteroresistance occurs when a colistin susceptible bacterial isolate ( $\text{MIC} \leq 4 \mu\text{g/ml}$ ) has a resistant subpopulations that are able to propagate in the presence of colistin ( $\text{MIC} \geq 4 \mu\text{g/ml}$ ) (Liao et al., 2020). Colistin heteroresistance has been attributed to many factors. Cheong et al. reported that amino acid mutations in the PmrAB or PhoPQ systems in *K. pneumoniae* induced colistin resistance in the resistant subpopulations (Cheong et al., 2019). This is also the main reason behind colistin heteroresistance in *E. coli* (Kuang et al., 2020). Heteroresistance should not be ignored, since it is reported as the main reason of recurrent of chronic and fatal infections (El-Halfawy & Valvano, 2015).

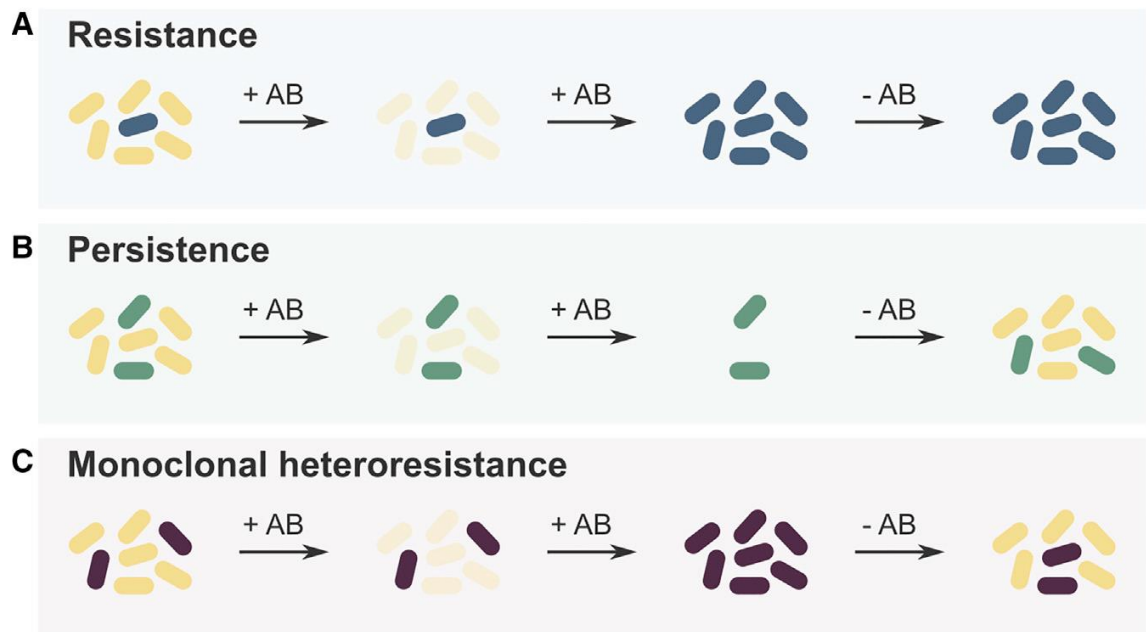


Figure 3: Key differences between resistance, persistence, and heteroresistance (Dewachter et al., 2019).

### E. Colistin Resistance in Enterobacterales

Several studies tackling colistin resistance were previously done at the country level. In 2015, Dandachi et al. reported the first detection of a colistin resistant *E. coli* harboring *mcr-1* gene from poultry farms in Lebanon (Dandachi et al., 2018). In 2017, Dandachi et al. studied 23 colistin resistant *E. coli* isolates recovered from fecal samples collected from swine farms in Lebanon. PCR results showed that all the 23 *E. coli* isolates harbored the *mcr-1* gene (Dandachi et al., 2019). Moreover, Al Mir et al. reported the first encounter of *E. coli* clinical isolates harboring the *mcr-1* gene in Lebanon (Al-Mir et al., 2019). In 2018, Salloum et al. reported a *K. pneumoniae* isolate recovered from a patient admitted to the American University of Beirut Medical Centre harboring the *mcr-8.1* gene (Salloum et al., 2020). At the American University of Beirut, Sourenian et al. detected the presence of the *mcr-1* gene in *E. coli* isolates

obtained from water samples collected from the Mediterranean Sea, suggesting environmental contamination (Sourenian et al., 2020). Recently, Jouman et. al reported that the emergence of the *mcr-1* gene in *E. coli* isolates has reached reputable community daycares and started appearing in the fecal matters of some toddlers (Jouman et al., 2021).

# CHAPTER III

## MATERIALS AND METHODS

### **A. Source of Bacterial Isolates**

A total of 161 *E. coli* and 92 *K. pneumoniae* isolates from clinical and environmental origins were assessed for colistin resistance. This study included 47 colistin resistant isolates distributed as: 27 *E. coli* isolates recovered from poultry feces, 10 *E. coli* isolates obtained from water samples and 10 *K. pneumoniae* clinical isolates.

### **B. Broth Microdilution**

#### ***1. Materials***

- 96-well plates (Corning).
- Mueller Hinton broth Cation adjusted (Sigma- Aldrich).
- MacConkey agar (LAB M).
- 5 mL polystyrene tubes (Falcon).
- 15 mL conicals (Falcon).
- Petri plates (JRZ Plastilab).
- Dimethyl sulfoxide (Sigma- Aldrich).
- Densitometer (Vitek).
- Colistin sulfate (Sigma-Aldrich).
- Amikacin (Anfarm).
- Tetracycline (Sigma-Aldrich).
- Azithromycin (Sigma-Aldrich).
- Trimethoprim/sulfamethoxazole (Sigma- Aldrich).

- Piperacillin/tazobactam (Wyeth Lederle S.P.A).
- Meropenem (AstraZeneca).
- Imipenem (MSD).
- Ertapenem (MSD).
- Cefuroxime (Hikma Pharmaceuticals)
- Ceftazidime (Sigma- Aldrich)
- Cefepime (Gulf Pharmaceutical Industries)
- Gentamicin (Sigma-Aldrich)
- Ciprofloxacin (Hikma Pharmaceuticals)
- Levofloxacin (Sanofi).
- Tigecycline (Wyeth Lederle)
- Ceftolozane/tazobactam (Merke Sharp & Dohme)
- Fosfomicin (Laboratorios ERN)
- Aztreonam
- 1.5 mL Eppendorf tubes (Corning).
- Nuclease-free water.

## ***2. Method***

To determine the minimal inhibitory concentration (MIC) of the colistin resistant isolates against antimicrobials from different antimicrobial families, broth-microdilution (BMD) was performed. This assay was conducted on 19 different antimicrobials: meropenem, imipenem, ertapenem, cefuroxime ceftazidime, cefepime, aztreonam, ciprofloxacin, levofloxacin, colistin, amikacin, gentamicin, azithromycin, fosfomicin, tetracycline, tigecycline, piperacillin/tazobactam, ceftolozane/tazobactam, and trimethoprim/sulfamethoxazole.

#### a. Preparation of Antimicrobial Suspensions

The antimicrobial suspensions were prepared as follow: a stock solution with a concentration of 5 mg/mL for azithromycin, colistin, tetracycline, and levofloxacin (available as a liquid solution) and 10 mg/mL for all the rest except for ciprofloxacin that was available as a liquid solution with a concentration of 2 mg/mL. Azithromycin, tigecycline, and trimethoprim/sulfamethoxazole were dissolved in Dimethyl sulfoxide (DMSO) while the rest were dissolved in nuclease free water.

#### b. Broth Microdilution assay

Bacterial isolates selected for broth microdilution were subcultured on MacConkey agar and placed in the incubator at 37 °C for 18-24 hours. In the next day, 90 µL of Cation-adjusted Mueller Hinton Broth (MHCAB) were pipetted in the wells of columns 2 to 12 of a 96-well plate. However, in the first column, 180 µL of MHCAB minus the volume of the designated antimicrobial were added. Subsequently, the corresponding antimicrobial will be added to the first column in order to obtain a final concentration of 1024 µg/mL in the wells. The only 3 exceptions were azithromycin with 128 µg/mL being the initial concentration, and trimethoprim/sulfamethoxazole and tigecycline with 256 µg/mL being that starting concentration. The MHCAB/antimicrobial mixture in column 1 was mixed 3 times then 90 µL were transferred to column 2 and so on till column 11 was reached. This was done in order to achieve a 2-fold decrease in the concentration of the antimicrobial agents. Column 12 served as a positive control (MHCAB without antimicrobial and inoculated with the bacterial suspension) and a negative control (MHCAB only).

After that, bacterial suspensions were prepared by adding 2-3 colonies of each bacterium into MHCAB till it reaches a turbidity equivalent to 0.5 McFarland (using a densitometer). Then 750  $\mu$ L of the latter were added to 14.25 mL MHCAB to reach a bacterial suspension with a concentration of  $5 \times 10^6$  CFU/mL. After that, 10  $\mu$ L of the latter were added to all the wells except the negative control to have a bacterial suspension with a final concentration of  $5 \times 10^5$  CFU/mL. The experiment was run in duplicates for each antimicrobial and the plates were then placed on the shaker (160 rpm) in the incubator at 37 °C for 18-24 hours. In the next day, the well preceding the first well with bacterial growth was considered as the well containing the MIC. The results were interpreted as susceptible, intermediate, or resistant according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

## **C. Population Analysis Profiles (PAPs)**

### ***1. Materials***

- MacConkey agar (LAB M).
- Petri plates (JRZ plastilab).
- Luria – Bertani Broth (LAB M).
- 5 mL polystyrene tubes (Falcon).
- Luria-Bertani agar (LAB M).
- Colistin sulfate (Sigma).

### ***2. Method***

Bacterial isolates were subcultured on MacConkey agar and placed in the incubator at 37 °C for 18-24 hours. After that, bacterial suspensions were prepared by adding 2-3 colonies of each bacterium into Luria-Bertani (LB) broth till it reaches a

turbidity equivalent to 0.5 McFarland (detected on the densitometer). From each bacterial suspension, 30  $\mu$ L were inoculated separately on LB agar plates supplemented with increasing concentrations of colistin (0, 0.25, 0.5, 1, 2, 4, 8 and 16  $\mu$ g/mL). Then the plates were placed in the incubator at 37 °C for 48 hours. Once done, the colony forming units of each plate were counted. For each isolate, one colony was selected from the plates supplemented with the highest concentration of colistin for storage and BMD. To assess if these isolates were maintaining their heteroresistant phenotype, BMD against colistin was reevaluated after serial passages in colistin-free LB broth (Liao et al., 2020).

#### **D. Fitness Cost Assay**

##### ***1. Materials***

- MacConkey agar (LAB M).
- 15 mL falcon tubes (Falcon).
- Mueller Hinton broth Cation adjusted (Sigma).
- 96-well plates (Corning).
- Plate reader (Berthold).

##### ***2. Method***

Bacterial isolates were subcultured on MacConkey agar and placed in the incubator at 37 °C for 18-24 hours. Then, 3-5 colonies were transferred from the agar plates into 10 mL of sterile MHCAB placed in the incubator at 37 °C for 18-24 hours. In the next day, the bacterial suspension of each isolate was diluted at a 1:1000 ratio. The latter was then transferred into 4 distinct wells (200  $\mu$ L each) of a 96 well-plate. Some conditions required the addition of colistin with a concentration of 4  $\mu$ g/mL in the

assigned wells. The division rate of each tested isolate was measured using a plate reader (OD 600 nm) for 16 hours with reads being recorded every 30 minutes. The obtained results were then normalized, averaged and plotted as graphs using PRISM software (Sleiman et al., 2021).

## **E. Polymerase Chain Reaction**

### ***1. DNA extraction***

#### **a. Materials**

- Luria-Bertani agar (LAB M).
- Petridishes (JRZ Plastilab).
- 1.5 mL Eppendorf tubes (Corning).
- Nuclease-free water (Quiagen).
- Heat block (Thermo-Fisher).
- Centrifuge (Thermo Scientific).
- Nanodrop (Denovix).

#### **b. Method**

Each bacterial isolate was subcultured on LB agar plates and placed in the incubator at 37 °C for 18-24 hours. In the next day, for each bacterial isolate, 2-3 colonies were transferred to an Eppendorf tube containing 100 µL of nuclease-free water. Once homogenized by vortexing, each Eppendorf tube was placed on a heat block at 100 °C for 10 minutes. After that centrifugation at 13,000 rpm for 5 minutes was done to collect the supernatant that contains the extracted DNA. The quality and purity of the DNA were evaluated using a Nanodrop and all the extracted DNA were stored at -20 °C (Dimitrakopoulou et al., 2020).

## ***2. Multiplex PCR***

### **a. Materials**

- Primers (BioRad).
- 0.2 mL Eppendorf tubes (Corning).
- Nuclease free water (Quiagen).
- Mini-spin (Eppendorf).
- Vortex (Benmark).
- Master-mix (Solis biodyne).
- PCR machine (Bio-Rad).
- 96-well plates (Bio-Rad).

### **b. Methods**

First all the primers required for the run were diluted. This was achieved by adding 20  $\mu\text{L}$  of each primer stock to 80  $\mu\text{L}$  of nuclease-free water. This was followed by the preparation of the PCR mix. The volume of the PCR mix allocated for each sample was 27  $\mu\text{L}$ , distributed as the following: 6  $\mu\text{L}$  of the Master Mix, 0.5  $\mu\text{L}$  of each primer (forward and reverse) (Table 1), and 13  $\mu\text{L}$  of nuclease free water. The PCR mix was prepared for all the samples at once by multiplying the indicated volumes by the number of samples included in the run. In each allocated well of the 96-well plate, 27  $\mu\text{L}$  of the PCR mix and 3  $\mu\text{L}$  of the DNA of the designated sample were added. Once all the samples were loaded, the 96-well plate was sealed and placed in the PCR machine. The following condition were followed: 94  $^{\circ}\text{C}$  for 15 min, followed by 25 cycles of (94  $^{\circ}\text{C}$  for 30 sec, 58  $^{\circ}\text{C}$  for 90 sec, and 72  $^{\circ}\text{C}$  for 60 sec), followed by 72 $^{\circ}\text{C}$  for 10 min (Rebelo et al., 2018).

Table 1: Description of the primers of the 8 mcr genes

Primer	F/R	Sequence	Tm °C	GC%
mcr-1-	F	5'-AGTCCGTTTGTCTTGTGGC-3'	58.4	50
mcr-1-	R	5'-AGATCCTTGGTCTCGGCTTG-3'	60.5	55
mcr-2-	F	5'-CAAGTGTGTTGGTCGCAGTT-3'	58.4	50
mcr-2-	R	5'-TCTAGCCCGACAAGCATAACC-3'	60.5	55
mcr-3-	F	5'-AAATAAAAATTGTTCCGCTTATG-3'	54	26.1
mcr-3-	R	5'-AATGGAGATCCCCGTTTTT-3'	53	42.1
mcr-4-	F	5'-TCACTTTCATCACTGCGTTG-3'	56.4	45
mcr-4-	R	5'-TTGGTCCATGACTACCAATG-3'	56.4	45
mcr-5-	F	5'-ATGCGGTTGTCTGCATTTATC-3'	57.4	42.9
mcr-5-	R	5'-TCATTGTGGTTGTCCTTTTCTG-3'	58.4	40.9
mcr-6-	F	5'-GTCCGGTCAATCCCTATCTGT-3'	61.3	52.4
mcr-6-	R	5'-ATCACGGGATTGACATAGCTAC-3'	60.3	45.5
mcr-7-	F	5'-TGCTCAAGCCCTTCTTTTCGT-3'	59.4	47.6
mcr-7-	R	5'-TTCATCTGCGCCACCTCGT-3'	59.5	57.9
mcr-8-	F	5'-AACCGCCAGAGCACAGAATT-3'	58.4	50
mcr-8-	R	5'-TTCCCCAGCGATTCTCCAT-3'	60.5	55

### 3. Gel Electrophoresis

#### a. Materials

- Tris-base (NZY tech).
- Acetic acid
- 0.5M EDTA (VWR Life science).
- PH meter (Thermo scientific).
- Agarose powder (Bio-Rad)
- Microwave
- Ethidium bromide (Bio-Rad).
- Gel chamber (Bio-Rad).
- Electrophoresis unit (Bio-Rad).

- 100 bp ladder (Solis biodyne).
- Loading dye (Invitrogen).
- Parafilm (Bemis).
- Gel Doc (Bio-Rad).

#### b. Method

The first step was the preparation of 200 mL of 50X TAE buffer. First, 48.4 g of tris-base (MW=121.14g/mol) were dissolved in 140 mL of distilled water. Then, 11.5 mL of acetic acid, 20 mL of 0.5M EDTA (PH 8.0), and 29 mL of distilled water were added to the 140 mL of tris-base solution. The PH of the 50X TAE was adjusted to 8.5 followed by autoclaving. Once sterile and at room temperature, the 50X TAE buffer was diluted to 1X by adding 20 mL of 50X TAE to 980ml of sterile Milli-Q water. The gel was prepared by adding 100 mL of 1X TAE to 2 g of agarose powder in a 500 mL Erlenmeyer flask. Then the flask was placed in the microwave until the powder was dissolved and the mixture was clear. Then, under the hood, 10  $\mu$ L of ethidium bromide were added and mixed well by shaking. The final mixture was then poured in the gel chamber (with the comb already settled) and left for 30 min to solidify. Once solidified the comb was removed and the gel was moved to the electrophoresis unit which should already filled with 1X TAE buffer. In the first well of the gel, 10  $\mu$ L of the 100 bp ladder was added. Then on a parafilm, 2  $\mu$ L of the loading dye were mixed with 10  $\mu$ L of each PCR product and then they were loaded into the wells consecutively. Once all the wells were loaded, the lid was closed, and the run was started at 100 V. After 40-50 minutes (once proper diffusion was detected on the gel), the run was stopped and the gel was taken to be visualized using a Gel Doc (Rebelo et al., 2018).

## **F. Whole-Genome Sequencing**

### ***1. Materials***

- QIAamp DNA mini kit (QIAGEN).
- Nanodrop
- Qubit
- Nextera XT DNA library preparation kit (Illumina)
- MinION sequencer
- Nanopore ligation sequencing kit

### ***2. Method***

Whole Genome Sequencing (WGS) was performed by our collaborators at King Abdullah International Medical Research Center, Saudi Arabia. Genomic DNA was extracted from overnight cultures using the QIAamp DNA mini kit (QIAGEN, USA) according to manufacturer's instructions. The quality and yield of the extracted DNA were checked on the nanodrop and qubit and then prepared for sequencing using the Nextera XT DNA library preparation kit (Illumina, UK). Sequencing was performed on the MiSeq instrument using the 2x300 paired-end protocol.

### ***3. Bioinformatics***

Generated reads were assembled into contigs using shovill 1.0.9 (<https://github.com/tseemann>) with the defaults options. MLSTs were inferred from assembled genomes with the mlst-v2.18.1 software while antimicrobial resistance genes were identified using Abricate 0.9.8 (<https://github.com/tseemann>). Chromosomal mutations associated with antimicrobial resistance were detected using gene\_finder ([https://github.com/phe-bioinformatics/gene\\_finder](https://github.com/phe-bioinformatics/gene_finder)). DNA from selected isolates were

subjected to low coverage sequencing on the MinION sequencer using the nanopore ligation sequencing kit according to the manufacturer's instructions. Hybrid assembly of short and long reads were performed using Unicycler 0.4.8 (Wick et al., 2017). Circularized plasmid from assembled genomes were compared using Blastn and BRIG (Alikhan et al., 2011).

## CHAPTER IV

### RESULTS

#### A. Antimicrobial Susceptibility Testing

In order to determine the prevalence of colistin resistance among Enterobacterales, BMD was done on 161 *E. coli* and 92 *K. pneumoniae* isolates. The results showed that 37 out of the 161 *E. coli* isolates were colistin resistant with MIC values ranging between 4 µg/mL and 8 µg/mL (Table 2). Moreover, 10 out of the 92 *K. pneumoniae* isolates were colistin resistant with MIC values ranging from 4 µg/mL to 256 µg/mL (Table 3).

Table 2: Broth microdilution results of the 161 *E. coli* isolates against colistin

Isolate code	MIC (µg/mL)	Interpretation
<i>E. coli</i> 1	<0.25	S
<i>E. coli</i> 2	<0.25	S
<i>E. coli</i> 3	<0.25	S
<i>E. coli</i> 4	<0.25	S
<i>E. coli</i> 5	<0.25	S
<i>E. coli</i> 6	<0.25	S
<i>E. coli</i> 7	<0.25	S
<i>E. coli</i> 8	<0.25	S
<i>E. coli</i> 9	<0.25	S
<i>E. coli</i> 10	<0.25	S
<i>E. coli</i> 11	<0.25	S
<i>E. coli</i> 12	<0.25	S
<i>E. coli</i> 13	<0.25	S
<i>E. coli</i> 14	<0.25	S
<i>E. coli</i> 15	<0.25	S
<i>E. coli</i> 16	<0.25	S
<i>E. coli</i> 17	<0.25	S
<i>E. coli</i> 18	<0.25	S
<i>E. coli</i> 19	<0.25	S
<i>E. coli</i> 20	<0.25	S

<i>E. coli</i> 21	<0.25	S
<i>E. coli</i> 22	<0.25	S
<i>E. coli</i> 23	<0.25	S
<i>E. coli</i> 24	<0.25	S
<i>E. coli</i> 25	<0.25	S
<i>E. coli</i> 26	<0.25	S
<i>E. coli</i> 27	<0.25	S
<i>E. coli</i> 28	<0.25	S
<i>E. coli</i> 29	<0.25	S
<i>E. coli</i> 30	<0.25	S
<i>E. coli</i> 31	<0.25	S
<i>E. coli</i> 32	<0.25	S
<i>E. coli</i> 33	<0.25	S
<i>E. coli</i> 34	<0.25	S
<i>E. coli</i> 35	<0.25	S
<i>E. coli</i> 36	<0.25	S
<i>E. coli</i> 37	<0.25	S
<i>E. coli</i> 38	<0.25	S
<i>E. coli</i> 39	<0.25	S
<i>E. coli</i> 40	<0.25	S
<i>E. coli</i> 41	<0.25	S
<i>E. coli</i> 42	<0.25	S
<i>E. coli</i> 43	<0.25	S
<i>E. coli</i> 44	<0.25	S
<i>E. coli</i> 45	<0.25	S
<i>E. coli</i> 46	<0.25	S
<i>E. coli</i> 47	<0.25	S
<i>E. coli</i> 48	<0.25	S
<i>E. coli</i> 49	<0.25	S
<i>E. coli</i> 50	<0.25	S
<i>E. coli</i> 51	<0.25	S
<i>E. coli</i> 52	<0.25	S
<i>E. coli</i> 53	<0.25	S
<i>E. coli</i> 54	<0.25	S
<i>E. coli</i> 55	<0.25	S
<i>E. coli</i> 56	<0.25	S
<i>E. coli</i> 57	<0.25	S
<i>E. coli</i> 58	<0.25	S
<i>E. coli</i> 59	<0.25	S
<i>E. coli</i> 60	<0.25	S
<i>E. coli</i> 61	<0.25	S

<i>E. coli</i> 62	<0.25	S
<i>E. coli</i> 63	<0.25	S
<i>E. coli</i> 64	<0.25	S
<i>E. coli</i> 65	<0.25	S
<i>E. coli</i> 66	<0.25	S
<i>E. coli</i> 67	<0.25	S
<i>E. coli</i> 68	<0.25	S
<i>E. coli</i> 69	<0.25	S
<i>E. coli</i> 70	<0.25	S
<i>E. coli</i> 71	<0.25	S
<i>E. coli</i> 72	<0.25	S
<i>E. coli</i> 73	<0.25	S
<i>E. coli</i> 74	0.25	S
<i>E. coli</i> 75	0.25	S
<i>E. coli</i> 76	0.25	S
<i>E. coli</i> 77	0.5	S
<i>E. coli</i> 78	0.5	S
<i>E. coli</i> 79	0.5	S
<i>E. coli</i> 80	0.5	S
<i>E. coli</i> 81	0.5	S
<i>E. coli</i> 82	0.5	S
<i>E. coli</i> 83	0.5	S
<i>E. coli</i> 84	0.5	S
<i>E. coli</i> 85	0.5	S
<i>E. coli</i> 86	0.5	S
<i>E. coli</i> 87	0.5	S
<i>E. coli</i> 88	0.5	S
<i>E. coli</i> 89	0.5	S
<i>E. coli</i> 90	0.5	S
<i>E. coli</i> 91	0.5	S
<i>E. coli</i> 92	0.5	S
<i>E. coli</i> 93	0.5	S
<i>E. coli</i> 94	0.5	S
<i>E. coli</i> 95	0.5	S
<i>E. coli</i> 96	0.5	S
<i>E. coli</i> 97	0.5	S
<i>E. coli</i> 98	0.5	S
<i>E. coli</i> 99	0.5	S
<i>E. coli</i> 100	0.5	S
<i>E. coli</i> 101	1	S
<i>E. coli</i> 102	1	S

<i>E. coli</i> 103	1	S
<i>E. coli</i> 104	1	S
<i>E. coli</i> 105	1	S
<i>E. coli</i> 106	1	S
<i>E. coli</i> 107	1	S
<i>E. coli</i> 108	1	S
<i>E. coli</i> 109	1	S
<i>E. coli</i> 110	1	S
<i>E. coli</i> 111	1	S
<i>E. coli</i> 112	1	S
<i>E. coli</i> 113	1	S
<i>E. coli</i> 114	1	S
<i>E. coli</i> 115	1	S
<i>E. coli</i> 116	1	S
<i>E. coli</i> 117	1	S
<i>E. coli</i> 118	1	S
<i>E. coli</i> 119	1	S
<i>E. coli</i> 120	1	S
<i>E. coli</i> 121	1	S
<i>E. coli</i> 122	1	S
<i>E. coli</i> 123	1	S
<i>E. coli</i> 124	1	S
<i>E. coli</i> 125	4	R
<i>E. coli</i> 126	4	R
<i>E. coli</i> 127	4	R
<i>E. coli</i> 128	4	R
<i>E. coli</i> 129	4	R
<i>E. coli</i> 130	4	R
<i>E. coli</i> 131	4	R
<i>E. coli</i> 132	4	R
<i>E. coli</i> 133	4	R
<i>E. coli</i> 134	4	R
<i>E. coli</i> 135	4	R
<i>E. coli</i> 136	4	R
<i>E. coli</i> 137	4	R
<i>E. coli</i> 138	4	R
<i>E. coli</i> 139	4	R
<i>E. coli</i> 140	4	R
<i>E. coli</i> 141	4	R
<i>E. coli</i> 142	4	R
<i>E. coli</i> 143	4	R

<i>E. coli</i> 144	4	R
<i>E. coli</i> 145	4	R
<i>E. coli</i> 146	4	R
<i>E. coli</i> 147	4	R
<i>E. coli</i> 148	4	R
<i>E. coli</i> 149	4	R
<i>E. coli</i> 150	4	R
<i>E. coli</i> 151	4	R
<i>E. coli</i> 152	4	R
<i>E. coli</i> 153	4	R
<i>E. coli</i> 154	4	R
<i>E. coli</i> 155	8	R
<i>E. coli</i> 156	8	R
<i>E. coli</i> 157	8	R
<i>E. coli</i> 158	8	R
<i>E. coli</i> 159	8	R
<i>E. coli</i> 160	8	R
<i>E. coli</i> 161	8	R

MIC: Minimum inhibitory concentration; S: Susceptible; R: Resistant

Table 3: Broth microdilution results of the 92 *K. pneumoniae* isolates against colistin

<b>Isolate code</b>	<b>MIC (µg/mL)</b>	<b>Interpretation</b>
<i>K. pneumoniae</i> 1	<0.25	S
<i>K. pneumoniae</i> 2	<0.25	S
<i>K. pneumoniae</i> 3	<0.25	S
<i>K. pneumoniae</i> 4	<0.25	S
<i>K. pneumoniae</i> 5	<0.25	S
<i>K. pneumoniae</i> 6	<0.25	S
<i>K. pneumoniae</i> 7	<0.25	S
<i>K. pneumoniae</i> 8	<0.25	S
<i>K. pneumoniae</i> 9	<0.25	S
<i>K. pneumoniae</i> 10	<0.25	S
<i>K. pneumoniae</i> 11	<0.25	S
<i>K. pneumoniae</i> 12	<0.25	S
<i>K. pneumoniae</i> 13	<0.25	S
<i>K. pneumoniae</i> 14	<0.25	S
<i>K. pneumoniae</i> 15	<0.25	S
<i>K. pneumoniae</i> 16	<0.25	S
<i>K. pneumoniae</i> 17	<0.25	S

<i>K. pneumoniae</i> 18	<0.25	S
<i>K. pneumoniae</i> 19	<0.25	S
<i>K. pneumoniae</i> 20	<0.25	S
<i>K. pneumoniae</i> 21	<0.25	S
<i>K. pneumoniae</i> 22	<0.25	S
<i>K. pneumoniae</i> 23	<0.25	S
<i>K. pneumoniae</i> 24	<0.25	S
<i>K. pneumoniae</i> 25	<0.25	S
<i>K. pneumoniae</i> 26	<0.25	S
<i>K. pneumoniae</i> 27	<0.25	S
<i>K. pneumoniae</i> 28	<0.25	S
<i>K. pneumoniae</i> 29	<0.25	S
<i>K. pneumoniae</i> 30	<0.25	S
<i>K. pneumoniae</i> 31	<0.25	S
<i>K. pneumoniae</i> 32	<0.25	S
<i>K. pneumoniae</i> 33	<0.25	S
<i>K. pneumoniae</i> 34	<0.25	S
<i>K. pneumoniae</i> 35	<0.25	S
<i>K. pneumoniae</i> 36	<0.25	S
<i>K. pneumoniae</i> 37	<0.25	S
<i>K. pneumoniae</i> 38	<0.25	S
<i>K. pneumoniae</i> 39	<0.25	S
<i>K. pneumoniae</i> 40	<0.25	S
<i>K. pneumoniae</i> 41	<0.25	S
<i>K. pneumoniae</i> 42	<0.25	S
<i>K. pneumoniae</i> 43	<0.25	S
<i>K. pneumoniae</i> 44	<0.25	S
<i>K. pneumoniae</i> 45	<0.25	S
<i>K. pneumoniae</i> 46	<0.25	S
<i>K. pneumoniae</i> 47	<0.25	S
<i>K. pneumoniae</i> 48	<0.25	S
<i>K. pneumoniae</i> 49	0.5	S
<i>K. pneumoniae</i> 50	0.5	S
<i>K. pneumoniae</i> 51	0.5	S
<i>K. pneumoniae</i> 52	0.5	S
<i>K. pneumoniae</i> 53	0.5	S
<i>K. pneumoniae</i> 54	0.5	S
<i>K. pneumoniae</i> 55	0.5	S
<i>K. pneumoniae</i> 56	0.5	S
<i>K. pneumoniae</i> 57	0.5	S
<i>K. pneumoniae</i> 58	0.5	S

<i>K. pneumoniae</i> 59	0.5	S
<i>K. pneumoniae</i> 60	<1	S
<i>K. pneumoniae</i> 61	<1	S
<i>K. pneumoniae</i> 62	<1	S
<i>K. pneumoniae</i> 63	<1	S
<i>K. pneumoniae</i> 64	<1	S
<i>K. pneumoniae</i> 65	<1	S
<i>K. pneumoniae</i> 66	1	S
<i>K. pneumoniae</i> 67	1	S
<i>K. pneumoniae</i> 68	1	S
<i>K. pneumoniae</i> 69	1	S
<i>K. pneumoniae</i> 70	1	S
<i>K. pneumoniae</i> 71	1	S
<i>K. pneumoniae</i> 72	1	S
<i>K. pneumoniae</i> 73	1	S
<i>K. pneumoniae</i> 74	1	S
<i>K. pneumoniae</i> 75	1	S
<i>K. pneumoniae</i> 76	<2	S
<i>K. pneumoniae</i> 77	<2	S
<i>K. pneumoniae</i> 78	<2	S
<i>K. pneumoniae</i> 79	<2	S
<i>K. pneumoniae</i> 80	<2	S
<i>K. pneumoniae</i> 81	<2	S
<i>K. pneumoniae</i> 82	<2	S
<i>K. pneumoniae</i> 83	4	R
<i>K. pneumoniae</i> 84	4	R
<i>K. pneumoniae</i> 85	8	R
<i>K. pneumoniae</i> 86	16	R
<i>K. pneumoniae</i> 87	16	R
<i>K. pneumoniae</i> 88	32	R
<i>K. pneumoniae</i> 89	32	R
<i>K. pneumoniae</i> 90	64	R
<i>K. pneumoniae</i> 91	64	R
<i>K. pneumoniae</i> 92	256	R

MIC: Minimum inhibitory concentration; S: Susceptible; R: Resistant

## B. Epidemiology of colistin resistant isolates

The 37 colistin resistant *E. coli* isolates were divided into 27 isolates retrieved from poultry feces from farms in Lebanon and 10 isolates obtained from water samples collected from different regions in Lebanon. The isolates recovered from poultry feces were distributed as 17 from farms in the North of Lebanon and 10 from farms located in the South of Lebanon.

Moreover, the 10 other *E. coli* isolates were obtained from water samples collected from 6 different regions in Lebanon. They were distributed as the following: 3 isolates from Ouzaai, 3 isolates from Tabarja, 1 isolate from Manara, 1 isolate from Batroun, 1 isolate from Damour and 1 isolate from Saida (Table 4). Furthermore, all the 10 colistin resistant *K. pneumoniae* isolates were recovered from patients admitted to the American University of Beirut Medical Center.

Table 4: The geographic distribution of the colistin resistant *E. coli* isolates recovered from water samples

Region	No. of isolates (%)
Ouzaai	3 (30%)
Tabarja	3 (30%)
Manara	1 (10%)
Batroun	1 (10%)
Damour	1 (10%)
Saida	1 (10%)

## C. Antimicrobial Susceptibility Testing

In order to determine the profiles of resistance of all the colistin resistant isolates, BMD was done against 19 antimicrobials from different antimicrobial families.

### 1. *E. coli* Isolates Recovered from Poultry Feces

BMD results of the 27 *E. coli* isolates recovered from poultry feces showed that all the isolates were resistant to colistin, 22 (81%) to tetracycline, 21 (78%) to trimethoprim/sulfamethoxazole, 20 (74%) to tigecycline, 19 (70%) to ciprofloxacin and levofloxacin, 17 (63%) to cefuroxime and fosfomycin, 15 (56%) to piperacillin/tazobactam, 14 (52%) to azithromycin, 13 (48%) to ceftazidime and gentamicin, 9 (33%) to aztreonam, and 4 (15%) to cefepime. Moreover, all the isolates were susceptible to ertapenem, meropenem, imipenem, amikacin, and ceftolozane/tazobactam (Tables 5 & 6).

Table 5: Broth microdilution results of the 27 *E. coli* isolates recovered from poultry feces

Antibiotics	No. Resistant (%)	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>
Cefuroxime	17 (63%)	2- >1024	32	1024
Ceftazidime	13 (48%)	<1 - 64	2	32
Cefepime	4 (15%)	<1 - 64	<1	16
Meropenem	0 (0%)	<1	<1	<1
Imipenem	0 (0%)	<1-2	<1	<1
Ertapenem	0 (0%)	<1	<1	<1
Aztreonam	9 (33%)	<1 - 64	8	32
Ciprofloxacin	19 (70%)	<1 - 32	16	32
Levofloxacin	19 (70%)	<1 - 32	8	32
Colistin	27 (100%)	4 - 8	4	8
Amikacin	0 (0%)	<1 - 4	2	4
Gentamicin	13 (48%)	<1 - 512	2	512
Azithromycin	14 (52%)	<0.125 - >128	32	>128
Fosfomycin	17 (63%)	2 - >1024	>1024	>1024
Tetracycline	22 (81%)	<1 - 256	128	256
Tigecycline	20 (74%)	<0.25 - 2	0.5	1
Ceftolozane/Tazobactam	0 (0%)	<1 - 8	2	8
Piperacillin/Tazobactam	15 (56%)	<1 - 16	8	16
Trimethoprim/Sulfamethoxazole	21 (78%)	<0.25 - >256	>256	>256

MIC: Minimum inhibitory concentration, MIC<sub>50</sub>: MIC value at which 50% of the 27 *E. coli* isolates were inhibited; MIC<sub>90</sub>: MIC value at which 90% of the 27 *E. coli* isolates were inhibited.

Antibiotics	Cefuroxime	Ceftazidime	Cefepime	Meropenem	Imipenem	Ertapenem	Aztreonam	Ciprofloxacin	Levofloxacin	Colistin	Amikacin	Gentamicin	Azithromycin	Fosfomycin	Tetracycline	Tigecycline	Ceftolozane/Tazobactam	Piperacillin/Tazobactam	Trimethoprim/Sulfamethoxazole
Isolates																			
<i>E. coli</i> 125	R	R	S	S	S	S	S	R	R	R	S	R	R	R	R	R	S	R	R
<i>E. coli</i> 126	R	R	S	S	S	S	S	R	R	R	S	R	R	R	R	R	S	R	R
<i>E. coli</i> 158	R	S	R	S	S	S	R	S	S	R	S	S	S	R	R	R	S	R	S
<i>E. coli</i> 127	R	R	S	S	S	S	S	S	S	R	S	S	R	R	R	R	S	R	S
<i>E. coli</i> 128	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	S	S	S	R
<i>E. coli</i> 129	R	R	S	S	S	S	R	R	R	R	S	R	R	R	R	R	S	R	R
<i>E. coli</i> 159	R	R	R	S	S	S	S	S	S	R	S	S	R	R	R	R	S	R	S
<i>E. coli</i> 130	R	S	S	S	S	S	S	S	S	R	S	R	S	R	R	R	S	R	R
<i>E. coli</i> 131	R	R	S	S	S	S	R	R	R	R	S	R	R	R	R	R	S	R	S
<i>E. coli</i> 132	R	R	S	S	S	S	S	R	R	R	S	S	S	R	S	S	S	R	R
<i>E. coli</i> 133	R	R	R	S	S	S	S	R	R	R	S	S	S	R	R	R	S	R	S
<i>E. coli</i> 134	R	R	S	S	S	S	R	R	R	R	S	S	S	R	S	S	S	R	R
<i>E. coli</i> 160	R	R	S	S	S	S	R	S	S	R	S	S	S	R	S	R	S	R	S
<i>E. coli</i> 135	R	S	S	S	S	S	R	S	S	R	S	R	S	R	R	S	S	S	R
<i>E. coli</i> 136	R	R	S	S	S	S	R	R	R	R	S	S	S	R	S	R	S	R	R
<i>E. coli</i> 137	R	R	S	S	S	S	R	R	R	R	S	S	S	R	S	R	S	R	R
<i>E. coli</i>	R	S	S	S	S	S	S	S	S	R	S	R	S	R	R	S	S	S	R

<b>161</b>																			
<b><i>E. coli</i> 138</b>	R	R	R	S	S	S	R	R	R	R	S	R	S	S	R	R	S	R	R
<b><i>E. coli</i> 139</b>	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	R	S	S	R
<b><i>E. coli</i> 140</b>	S	S	S	S	S	S	S	R	R	R	S	S	R	S	R	R	S	S	R
<b><i>E. coli</i> 141</b>	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	S	S	S	R
<b><i>E. coli</i> 142</b>	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	R	S	S	R
<b><i>E. coli</i> 143</b>	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	R	S	S	R
<b><i>E. coli</i> 144</b>	S	S	S	S	S	S	S	R	R	R	S	S	R	S	R	R	S	S	R
<b><i>E. coli</i> 145</b>	S	S	S	S	S	S	S	R	R	R	S	S	S	S	R	S	S	S	R
<b><i>E. coli</i> 146</b>	S	S	S	S	S	S	S	R	R	R	S	R	R	S	R	R	S	S	R
<b><i>E. coli</i> 147</b>	S	S	S	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S	R

Table 6: Detailed representation of the broth microdilution results of the 27 *E. coli* isolates recovered from poultry feces.

## 2. *E. coli* Isolates Recovered from Water Samples

BMD results of the 10 *E. coli* isolates recovered from water samples showed that all the isolates were resistant to colistin, 9 (90%) to tetracycline and trimethoprim/sulfamethoxazole, 7 (70%) to azithromycin, 5 (50%) to tigecycline and fosfomicin, 4 (40%) to levofloxacin, 3 (30%) to ciprofloxacin, and 2 (20%) to gentamicin. Moreover, all the isolates were susceptible to cefuroxime, ceftazidime, cefepime, ertapenem, meropenem, imipenem, aztreonam, amikacin, ceftolozane/tazobactam, and piperacillin/tazobactam (Tables 7 & 8).

Table 7: Broth microdilution results of the 10 *E. coli* isolates recovered from water samples

Antibiotics	No. Resistant (%)	MIC range	MIC <sub>50</sub>	MIC <sub>90</sub>
Cefuroxime	0 (0%)	2 - 16	4	8
Ceftazidime	0 (0%)	<1	<1	<1
Cefepime	0 (0%)	<1	<1	<1
Meropenem	0 (0%)	<1	<1	<1
Imipenem	0 (0%)	<1	<1	<1
Ertapenem	0 (0%)	<1	<1	<1
Aztreonam	0 (0%)	<1	<1	<1
Ciprofloxacin	3 (30%)	<1 - 8	<1	4
Levofloxacin	4 (40%)	<1 - 8	2	4
Colistin	10 (100%)	4 - 8	4	4
Amikacin	0 (0%)	<1 - 4	<1	4
Gentamicin	2 (20%)	<1 - 256	<1	128
Azithromycin	7 (70%)	2 - >128	32	>128
Fosfomicin	5 (50%)	<1 - >1024	128	>1024
Tetracycline	9 (90%)	<1 - 256	256	256
Tigecycline	5 (50%)	<0.25 - 2	0.5	1
Ceftolozane/Tazobactam	0 (0%)	<1	<1	<1
Piperacillin/Tazobactam	0 (0%)	<1 - 16	4	16
Trimethoprim/Sulfamethoxazole	9 (90%)	<0.25 - >256	>256	>256

MIC: Minimum inhibitory concentration, MIC<sub>50</sub>: MIC value at which 50% of the 10 *E. coli* isolates were inhibited; MIC<sub>90</sub>: MIC value at which 90% of the 10 *E. coli* isolates were inhibited.

Table 8: Detailed representation of the broth microdilution results of the 10 *E. coli* isolates recovered from water samples.

Antibiotics	Cefuroxime	Ceftazidime	Cefepime	Meropenem	Imipenem	Ertapenem	Aztreonam	Ciprofloxacin	Levofloxacin	Colistin	Amikacin	Gentamicin	Azithromycin	Fosfomicin	Tetracycline	Tigecycline	Ceftolozane/Tazobactam	Piperacillin/Tazobactam	Trimethoprim/Sulfamethoxazole
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Isolate																				
<i>E. coli</i> 148	S	S	S	S	S	S	S	S	R	R	R	S	S	R	R	R	S	S	S	R
<i>E. coli</i> 155	S	S	S	S	S	S	S	S	S	R	R	S	R	R	R	R	R	S	S	R
<i>E. coli</i> 149	S	S	S	S	S	S	S	S	S	R	S	R	R	S	R	R	S	S	R	
<i>E. coli</i> 150	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	S	S	S	R	
<i>E. coli</i> 151	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	
<i>E. coli</i> 152	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S	S	S	R	
<i>E. coli</i> 156	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S	S	S	R	
<i>E. coli</i> 153	S	S	S	S	S	S	S	R	R	R	S	S	R	S	R	R	S	S	R	
<i>E. coli</i> 154	S	S	S	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	R	
<i>E. coli</i> 157	S	S	S	S	S	S	S	R	R	R	S	S	R	R	R	R	S	S	R	

S: Susceptible; R: Resistant

### 3. *K. pneumoniae* Clinical Isolates

BMD results of the 10 *K. pneumoniae* clinical isolates showed that all the isolates were resistant to colistin, fosfomycin, and piperacillin/tazobactam, 9 (90%) to ceftazidime, cefepime, azithromycin, and ceftolozane/tazobactam, 8 (80%) to aztreonam, tigecycline, and trimethoprim/sulfamethoxazole, 7 (70%) to cefuroxime, ertapenem, levofloxacin, gentamicin, and tetracycline, 6 (60%) to meropenem, imipenem, and ciprofloxacin, and 5 (50%) to amikacin (Tables 9 & 10). Moreover, 5 out of the 10 *K. pneumoniae* isolates were PDR since they showed resistance to all the tested antibiotics (Table 10).

Table 9: Broth microdilution results of the 10 *K. pneumoniae* clinical isolates

Antibiotics	No. Resistant (%)	MIC range	MIC50	MIC90
Cefuroxime	7 (70%)	2 - >2048	>1024	>2048
Ceftazidime	9 (90%)	<1 - >2048	512	>2048
Cefepime	9 (90%)	<1 - 2048	512	2048
Meropenem	6 (60%)	<1 - 512	4	512
Imipenem	6 (60%)	<1 - 1024	4	1024
Ertapenem	7 (70%)	<1 - 1024	8	1024
Aztreonam	8 (80%)	<1 - >1024	256	512

Ciprofloxacin	6 (60%)	<1 - 256	16	128
Levofloxacin	7 (70%)	<1 - 128	8	64
Colistin	10 (100%)	4 - 256	16	64
Amikacin	5 (50%)	<1 - >2048	32	>2048
Gentamicin	7 (70%)	<1 - >2048	128	2048
Azithromycin	9 (90%)	8 - >256	>128	>128
Fosfomycin	10 (100%)	256 - >2048	>1024	2048
Tetracycline	7 (70%)	<1 - 512	128	512
Tigecycline	8 (80%)	<0.25 - 8	4	8
Ceftolozane/Tazobactam	9 (90%)	<1 - >2048	64	>2048
Piperacillin/Tazobactam	10 (100%)	64 - 2048	256	1024
Trimethoprim/Sulfamethoxazole	8 (80%)	0.5 - >256	>64	>256

MIC: Minimum inhibitory concentration, MIC<sub>50</sub>: MIC value at which 50% of the 10 *K. pneumoniae* isolates were inhibited; MIC<sub>90</sub>: MIC value at which 90% of the 10 *K. pneumoniae* isolates were inhibited.

Table 10: Detailed representation of the broth microdilution results of the 10 *K. pneumoniae* clinical isolates

Antibiotics	Cefuroxime	Ceftazidime	Cefepime	Meropenem	Imipenem	Ertapenem	Aztreonam	Ciprofloxacin	Levofloxacin	Colistin	Amikacin	Gentamicin	Azithromycin	Fosfomycin	Tetracycline	Tigecycline	Ceftolozane/Tazobactam	Piperacillin/Tazobactam	Trimethoprim/Sulfamethoxazole
	Isolate																		
<i>K. pneumoniae</i> 83	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R	S
<i>K. pneumoniae</i> 84	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
<i>K. pneumoniae</i> 85	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
<i>K. pneumoniae</i> 86	S	R	R	S	S	S	R	S	S	R	S	S	R	R	S	S	R	R	S
<i>K. pneumoniae</i>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

<i>niae 87</i>																			
<i>K. pneumoniae 88</i>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
<i>K. pneumoniae 89</i>	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
<i>K. pneumoniae 90</i>	S	R	R	R	S	S	R	S	R	R	S	S	R	R	S	R	R	R	R
<i>K. pneumoniae 91</i>	R	R	R	S	R	R	S	R	R	R	S	R	R	R	R	R	R	R	R
<i>K. pneumoniae 92</i>	R	R	R	S	S	R	R	S	S	R	S	R	R	R	R	R	R	R	R

S: Susceptible; R: Resistant

#### D. Polymerase Chain Reaction

PCR results showed a consistent band at 320 bp which is equivalent to the *mcr-1* gene. Moreover, the results showed that all the 27 colistin resistant *E. coli* isolates recovered from poultry feces harbored the *mcr-1* gene. Furthermore, 5 out of the 10 colistin resistant *E. coli* isolates recovered from water samples harbored the *mcr-1* gene (Figure 4). In addition to that, none of the colistin resistant *K. pneumoniae* clinical isolates harbored any of the *mcr* genes (*mcr-1* to *mcr-8*).

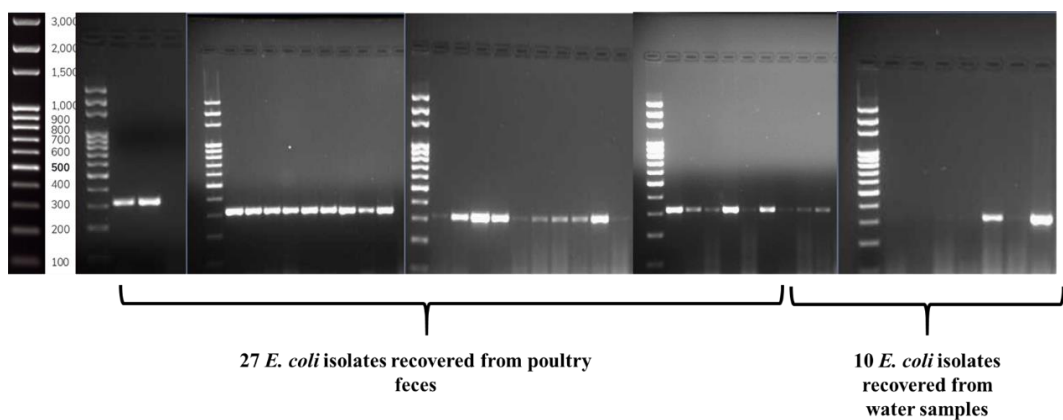


Figure 4: PCR results of the 37 colistin resistant *E. coli* isolates

## **E. Whole Genome Sequencing**

WGS was done by our collaborators at King Abdullah International Medical Research Center in Saudi Arabia on 25 out of the 47 colistin resistant Enterobacterales isolates. They were distributed as the following: 18 *E. coli* isolates recovered from poultry feces, 5 *E. coli* isolates obtained from water samples, and 2 colistin resistant *K. pneumoniae* isolates.

### ***1. E. coli Isolates Recovered from Poultry Feces***

WGS results showed that the 18 *E. coli* isolates belonged to 7 different sequence types (ST), distributed as: ST1140 (n=7), ST226 (n=3), ST2705 (n=3), ST162 (n=2), ST2936 (n=1), ST3288 (n=1), and ST6448 (n=1). The most prevalent sequence type was ST1140 where 7 out of the 18 *E. coli* isolates belonged to it (Table 11). They were also the most prevalent among isolates recovered from poultry feces from farms in the South and it was detected in 2 *E. coli* isolates obtained from poultry feces from farms in the North. Moreover, *E. coli* isolates belonging to STs 162 and 6448 were only found in isolates recovered from poultry feces from farms in the South. Whereas, *E. coli* isolates belonging to STs 226, 2705, 2936, and 3288 were only obtained from poultry feces from farms in the North (Table 12).

Table 11: Distribution of the 18 *E. coli* isolates among the 7 detected sequence types

<b>Sequence type</b>	<b>No. of isolates (%)</b>
1140	7 (39%)
2705	3 (17%)

226	3 (17%)
162	2 (11%)
2936	1 (6%)
3288	1 (6%)
6448	1 (6%)

Table 12: Distribution of the 18 *E. coli* isolates based on their sequence types and region of isolation.

Isolate code	Sequence type	Region
<i>E. coli</i> 125	226	North
<i>E. coli</i> 126	226	North
<i>E. coli</i> 129	2936	North
<i>E. coli</i> 130	3288	North
<i>E. coli</i> 131	226	North
<i>E. coli</i> 134	1140	North
<i>E. coli</i> 135	2705	North
<i>E. coli</i> 137	1140	North
<i>E. coli</i> 138	6448	South
<i>E. coli</i> 141	1140	South
<i>E. coli</i> 142	1140	South
<i>E. coli</i> 143	1140	South
<i>E. coli</i> 144	162	South
<i>E. coli</i> 145	1140	South
<i>E. coli</i> 146	1140	South
<i>E. coli</i> 147	162	South
<i>E. coli</i> 160	2705	North
<i>E. coli</i> 161	2705	North

Table 12: Distribution of the 18 *E. coli* isolates based on their sequence types and region of isolation.

The number of antimicrobial resistant determinants ranged from 4 to 21 among the 18 *E. coli* isolates. Other than the *mcr-1* gene (n=18), *tetA* and *floR* genes were the most frequent and they were detected in 15 out of the 18 isolates. Moreover, several  $\beta$ -lactamase genes were detected, including: *bla*<sub>TEM-1</sub> (n=12), *bla*<sub>CMY-2</sub> (n=7), *bla*<sub>TEM-122</sub>

(n=5), *bla*<sub>CTX-M-3</sub> (n=4) and *bla*<sub>CTX-M-55</sub> (n=1). Furthermore, resistance to fluoroquinolones was either due to 2 mutations in the *gyrA* gene (S83L and D87N) and one in the *parC* gene (S80I) (n=13) or one mutation in the *gyrA* gene (S83L) (n=1). In addition to that, 12 out of the 18 *E. coli* isolates harbored the macrolides resistance genes *lnu(F)* and *mph(A)*. Finally, 8 different aminoglycosides resistance genes were detected (Table 13).

Table 13: Antimicrobial resistance genes harbored in the 18 *E. coli* isolates

Antimicrobial family	AMR genes	No. of isolates (%)
<b>β-lactams</b>	<i>CMY-2</i>	7 (39%)
	<i>CTXM-3</i>	4 (22%)
	<i>CTXM-55</i>	1 (6%)
	<i>CTXM-9</i>	1 (6%)
	<i>SHV-53</i>	1 (6%)
	<i>TEM-1</i>	12 (67%)
	<i>TEM-122</i>	5 (28%)
<b>Aminoglycosides</b>	<i>aac(3)-IId</i>	13 (72%)
	<i>aadA1</i>	3 (17%)
	<i>aadA2</i>	12 (67%)
	<i>aadA5</i>	2 (11%)
	<i>strAB</i>	9 (50%)
	<i>aadA</i>	2 (11%)
	<i>aac(6')-Ib-cr</i>	1 (6%)
	<i>aph(3')-Vib</i>	1 (6%)
<b>Fluoroquinolones</b>	<i>gyrA</i>	14 (78%)
	<i>parC</i>	13 (72%)
	<i>qnrS-1</i>	7 (39%)
	<i>qnrB-1</i>	1 (6%)
<b>Macrolides</b>	<i>lnu-(F)</i>	12 (67%)
	<i>mph-(A)</i>	12 (67%)
	<i>sat2A</i>	8 (44%)
	<i>mdf(A)</i>	1 (6%)
<b>Sulfonamides</b>	<i>dfrA-17</i>	2 (11%)
	<i>dfrA-14</i>	4 (22%)

	<i>dfrA-1</i>	8 (44%)
	<i>dfrA-12</i>	2 (11%)
	<i>sul-3</i>	9 (50%)
	<i>sul-2</i>	9 (50%)
<b>Tetracyclines</b>	<i>tet(A)-1</i>	15 (83%)
	<i>tet(M)</i>	8 (44%)
<b>Chloramphenicol</b>	<i>cml-1</i>	8 (44%)
	<i>floR</i>	15 (83%)
<b>Fosfomycin</b>	<i>fosA-3</i>	10 (56%)
<b>Tellurium resistance proteins</b>	<i>terF</i>	6 (33%)
	<i>terE</i>	7 (39%)
	<i>terD</i>	6 (33%)
	<i>terC</i>	6 (33%)
	<i>terB</i>	7 (39%)
<b>Polymyxins</b>	<i>mcr-1</i>	18 (100%)

AMR: Antimicrobial resistance

The *mcr-1.1* gene, detected in 16 out of the 18 *E. coli* isolates, was located on a 33.3 kb circular contig sharing high similarity with IncX4-type plasmids harboring *mcr* variants (coverage 97-100%, identity 99-100 %) which were previously reported in various Enterobacterales species, including *Salmonella enterica*, *E. coli* and *Klebsiella pneumoniae* (Figure 5B). The draft genome of isolate 80 located *mcr-1* on a 64.7 kb circular contig that was most similar to the IncI2-type self-conjugative plasmid bearing *mcr-1.8* (92% coverage with 99.46% identity, accession number: KY792081.2). Sequence comparison confirmed that isolate 83 had the same IncI2-type *mcr-1*-harboring plasmid (Figure 5A).

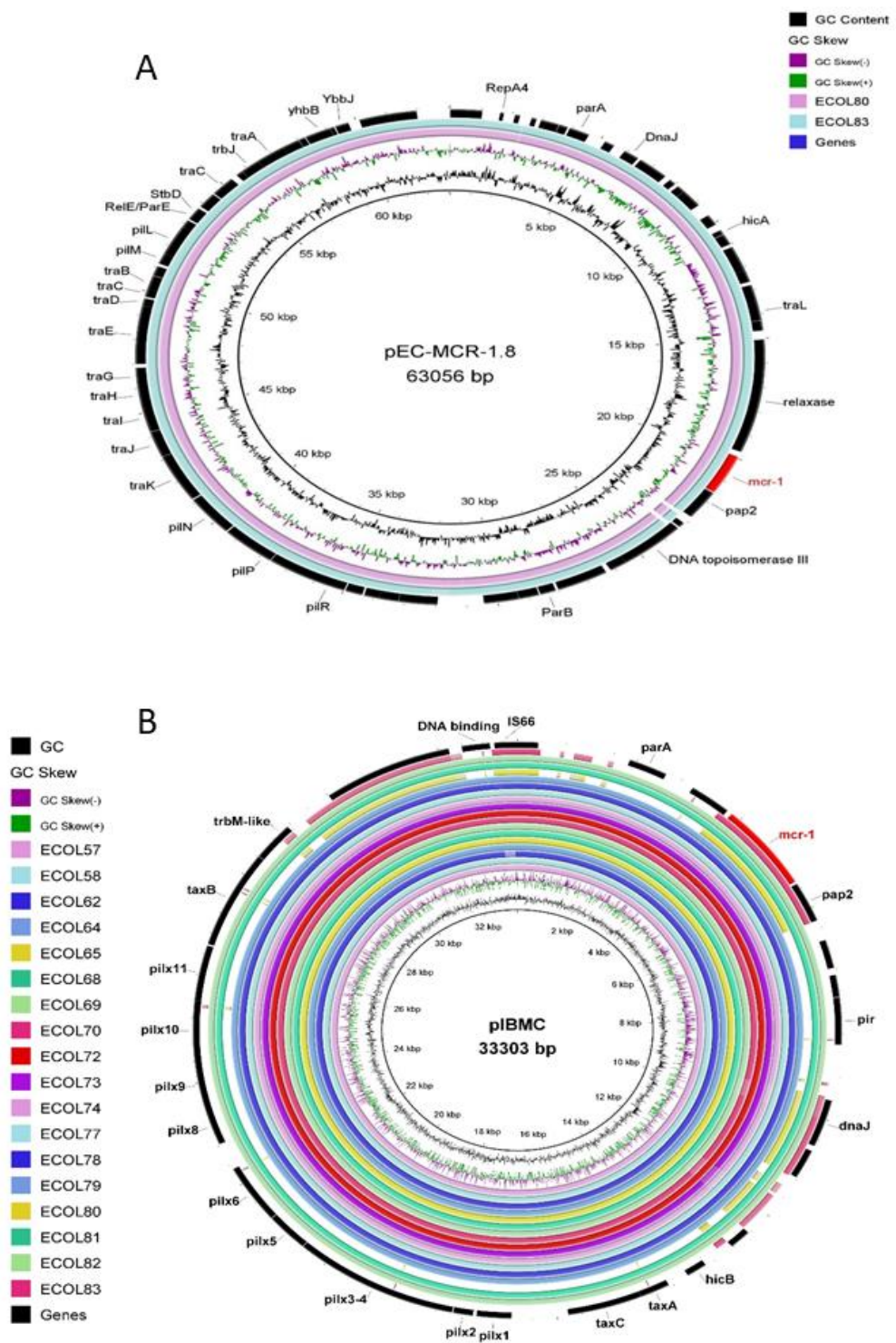


Figure 5: Circular graphical map showing BLAST comparisons of the *mcr-1* carrying plasmids identified in

A) Isolates 80 and 83 against IncI2-type plasmid pEC-*mcr*-1.8 (accession number: KY792081)

B) The remaining isolates against IncX4-type plasmid pIBMC-*mcr*1 (accession number: MF449287.1). The *mcr*-1 gene location is shown in the figure with all the other genes.

A total of 15 different plasmids were detected in the 18 *E. coli* isolates, distributed as follows: IncX4 (n=16), IncFII(pCoo) (n=10), IncI2(Delta) (n=7), IncHI2 (n=6), IncHI2A (n=6), IncFIB(K) (n=6), IncFIB(AP001918) (n=5), IncFIA (n=4), IncI1-I(Alpha) (n=4), IncFII (n=4), IncFIC(FII) (n=3), p0111 (n=3), IncFII(29) (n=2), IncI2 (n=1) and IncFIB(pLF82-PhagePlasmid) (n=1) (Table 14).

Table 14: Plasmids harbored in the 18 *E. coli* isolates

<b>Plasmid</b>	<b>No. of isolates (%)</b>
IncX4	16 (89%)
IncFII(pCoo)	10 (56%)
IncI2(Delta)	7 (39%)
IncHI2	6 (33%)
IncHI2A	6 (33%)
IncFIB(K)	6 (33%)
IncFIB(AP001918)	5 (28%)
IncFIA	4 (22%)
IncI1-I(Alpha)	4 (22%)
IncFII	4 (22%)
p0111	3 (17%)
IncFIC(FII)	3 (17%)
IncFII(29)	2 (11%)
IncFIB(pLF82-PhagePlasmid)	1 (6%)
IncI2	1 (6%)

## 2. *E. coli* Isolates Recovered from Water Samples

WGS results showed that the 5 *E. coli* isolates belonged to 3 different STs, distributed as: ST10773 (n=3), ST93 (n=1), and ST 949 (n=1) (Table 15). The 3 *E. coli* isolates belonging to ST10773 were recovered from water samples collected from Ouzaai and Saida. Moreover, the *E. coli* isolate belonging to ST93 was obtained from water sample collected from Damour. Finally, the *E. coli* isolate belonging to ST949 was retrieved from water sample collected from Tabarja (Table 16).

Table 15: Distribution of the 5 *E. coli* isolates among the 3 detected sequence type

Sequence type	No. of isolates (%)
10773	3 (60%)
949	1 (20%)
93	1 (20%)

Table 16: Distribution of the 5 *E. coli* isolates based on their sequence types and region of isolation

Isolate code	Sequence type	Region
<i>E. coli</i> 148	10773	Ouzaai
<i>E. coli</i> 150	949	Tabarja
<i>E. coli</i> 153	10773	Saida
<i>E. coli</i> 156	93	Damour
<i>E. coli</i> 157	10773	Ouzaai

The number of antimicrobial resistant determinants ranged from 7 to 20 among the 5 *E. coli* isolates. The *mcr-1*, *bla*<sub>TEM-1</sub>, *floR*, *tet(A)-1*, *dfrA-14* genes were harbored in all the isolates. The *bla*<sub>TEM-1</sub> (n=5) was the only one  $\beta$ -lactamase gene detected among the isolates. Moreover, two aminoglycosides resistance genes, *aadA2* and *strAB*,

were detected in 4 out of the 5 isolates. Furthermore, resistance to fluoroquinolones was either due to 2 mutations in the *gyrA* gene (S83L and D87N) and one in the *parC* gene (S80I) (n=1) or one mutation in the *gyrA* gene (S83L) (n=4). The *qnrS-1* gene (n=4) also encoded for resistance against fluoroquinolones. Resistance to macrolides was encoded by the *mph-(A)* gene (n=4). In addition to that, *tet(M)* (n=3) and *tet(A)-1* (n=5) genes encoded for resistance against tetracyclines (Table 17).

Table 17: Antimicrobial resistance genes harbored in the 5 *E. coli* isolates

Antimicrobial family	AMR genes	No. of isolates (%)
<b>β-lactams</b>	<i>TEM-1</i>	5 (100%)
<b>Aminoglycosides</b>	<i>aadA2</i>	4 (80%)
	<i>strAB</i>	4 (80%)
<b>Fluoroquinolones</b>	<i>gyrA</i> [S83L]	4 (80%)
	<i>gyrA</i> [S83L; D87N]	1 (20%)
	<i>qnrS-1</i>	4 (80%)
	<i>parC</i> [S80I]	1 (20%)
<b>Macrolides</b>	<i>mph-(A)</i>	4 (80%)
<b>Sulfonamides</b>	<i>dfrA-1</i>	3 (60%)
	<i>dfrA-14</i>	5 (100%)
	<i>sul-2</i>	3 (60%)
	<i>sul-3</i>	4 (80%)
<b>Tetracyclines</b>	<i>tet(M)</i>	3 (60%)
	<i>tet(A)-1</i>	5 (100%)
<b>Chloramphenicol</b>	<i>cml-1</i>	4 (80%)
	<i>floR</i>	5 (100%)
<b>Fosfomicin</b>	<i>fosA-3</i>	3 (60%)
<b>Tellurium resistance proteins</b>	<i>terF</i>	3 (60%)
	<i>terE</i>	3 (60%)
	<i>terD</i>	3 (60%)
	<i>terC</i>	3 (60%)
	<i>terB</i>	3 (60%)
<b>Polymyxins</b>	<i>mcr-1</i>	5 (100%)

AMR: Antimicrobial resistance

A total of 10 different plasmids were detected in the 5 *E. coli* isolates, distributed as follows: Col440I (n=3), IncFII(pCoo) (n=3), IncHI2 (n=3), IncHI2A (n=3), p0111 (n=3), IncFIB(AP001918) (n=2), IncFII (n=2), IncX4 (n=2), IncII-I(Alpha) (n=1) and IncX1 (n=1) (Table 18).

Table 18: Plasmids harbored in the 5 *E. coli* isolates

Plasmid	No. of isolates (%)
Col440I	3 (60%)
IncFII(pCoo)	3 (60%)
IncHI2	3 (60%)
IncHI2A	3 (60%)
p0111	3 (60%)
IncFIB(AP001918)	2 (40%)
IncFII	2 (40%)
IncX4	2 (40%)
IncII-I(Alpha)	1 (20%)
IncX1	1 (20%)

### 3. *K. pneumoniae* Clinical Isolates

WGS was done on 2 out of the 10 colistin resistant *K. pneumoniae* isolates. The results showed that *K. pneumoniae* 88 and *K. pneumoniae* 91 belonged to ST383 and ST39 respectively.

A total of 16 and 25 antimicrobial resistance determinants were detected in *K. pneumoniae* 91 and *K. pneumoniae* 88 respectively. Resistance to carbapenems was encoded by *bla*<sub>OXA-48</sub> (n=2) and *bla*<sub>NDM-5</sub> (n=1) genes. Moreover, seven genes encoding resistance for  $\beta$ -lactams were detected, such as: *bla*<sub>CTX-M-15</sub> (n=2), *bla*<sub>CTX-M-14</sub> (n=2), *bla*<sub>SHV-1</sub> (n=1), and *bla*<sub>TEM-1</sub> (n=2). Aminoglycoside resistance was encoded by 7 different genes, such as: *aph(3')-VIb* (n=2) and *strAB* (n=2) genes. Fluoroquinolone

resistance was either due to 2 mutation in the *gyrA* gene (S83F; D87N) and 1 in the *parC* gene (S80I) (n=1), and/or encoded by *qnrS-1* (n=2) and *qnrB-1* (n=1) genes. Furthermore, *mph-(A)* (n=1), *mph-(E)* (n=1), and *msr-(E)* (n=1) genes encoded for macrolide resistance. In addition to that, *tet(A)-1* (n=2) and *fosA-v3* (n=1) genes encoded for tetracycline and Fosfomycin resistance respectively (Table 19).

Each *K. pneumoniae* isolate harbored 3 different plasmids. *K. pneumoniae* 88 harbored the following plasmids: IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR) and IncL. On the other hand, IncFIB(K), IncFII(K) and IncM1 plasmids were found in *K. pneumoniae* 91.

Table 19: Information on the 2 *K. pneumoniae* isolates from clinical samples including antimicrobial resistance genes

Antimicrobial family	AMR Genes	No. of isolates (%)
<b>Carbapenems</b>	<i>NDM-5</i>	1 (50%)
	<i>OXA-48</i>	2 (100%)
<b>β-lactams</b>	<i>CTX-M-15</i>	2 (100%)
	<i>CTX-M-14</i>	2 (100%)
	<i>OXA-9</i>	1 (50%)
	<i>SHV-1</i>	1 (50%)
	<i>TEM-1</i>	2 (100%)
	<i>OXA-1</i>	1 (50%)
	<i>SHV-11</i>	1 (50%)
<b>Aminoglycosides</b>	<i>aph(3')-VIb</i>	2 (100%)
	<i>aadA2</i>	1 (50%)
	<i>armA</i>	1 (50%)
	<i>aac(6')-Ib</i>	1 (50%)
	<i>strAB</i>	2 (100%)
	<i>aac(3)-Iia</i>	1 (50%)
	<i>aac(6')-Ib-cr</i>	1 (50%)
<b>Fluoroquinolones</b>	<i>gyrA [S83F; D87N]</i>	1 (50%)
	<i>parC [S80I]</i>	1 (50%)
	<i>qnrS-1</i>	2 (100%)
	<i>qnrB-1</i>	1 (50%)
<b>Macrolides</b>	<i>mph-(A)</i>	1 (50%)

	<i>msr-(E)</i>	1 (50%)
	<i>mph-(E)</i>	1 (50%)
<b>Sulfonamides</b>	<i>dfrA-5</i>	1 (50%)
	<i>dfrA-14</i>	1 (50%)
	<i>sul-2</i>	2 (100%)
	<i>sul-1</i>	1 (50%)
	<b>Tetracyclines</b>	<i>tet(A)-1</i>
<b>Chloramphenicol</b>	<i>catA-1</i>	1 (50%)
<b>Fosfomicin</b>	<i>fosA-v3</i>	1 (50%)
<b>Bleomycin</b>	<i>ble</i>	1 (50%)

AMR: Antimicrobial resistance

## F. Colistin Heteroresistance

### 1. Colistin Susceptible *E. coli* Isolates

The PAPs assay was performed on 13 colistin susceptible *E. coli* isolates. In all the isolates, no growth was detected on agar plates supplemented with 4 µg/mL colistin and above after 48 hours of incubation at 37 °C (Table 20). This shows that none of these isolates have colistin resistant subpopulations.

Table 20: Population Analysis Profiles results of the 13 *E. coli* isolates

Isolates	Concentration of colistin in the plate (µg/mL)							
	0	0.25	0.5	1	2	4	8	16
<i>E. coli</i> 58	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 104	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 7	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 18	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 41	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 10	Growth	Growth	No growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 19	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 16	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 8	Growth	Growth	Growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 28	Growth	Growth	No growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 35	Growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
<i>E. coli</i> 30	Growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth

<i>E. coli</i> 44	Growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
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## 2. Colistin Susceptible *K. pneumoniae* Isolates

The PAPs assay was performed on 12 colistin susceptible *K. pneumoniae* isolates. After 48 hours of incubation at 37 °C, the results showed that 10 out of the 12 isolates have colistin resistant subpopulations that grew on agar plates supplemented with colistin (4 µg/ml and above) (Table 21).

Table 21: Population Analysis Profiles results of the 12 *K. pneumoniae* isolates

Isolates	Concentration of colistin in the plate (µg/mL)							
	0	0.25	0.5	1	2	4	8	16
<i>K. pneumoniae</i> 76	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 64	Growth	Growth	Growth	Growth	Growth	Growth	No growth	<b>Growth</b>
<i>K. pneumoniae</i> 60	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 5	Growth	Growth	Growth	Growth	No growth	<b>Growth</b>	No growth	No growth
<i>K. pneumoniae</i> 2	Growth	Growth	Growth	Growth	No growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 16	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 15	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 19	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 23	Growth	Growth	Growth	Growth	No growth	No growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 26	Growth	Growth	Growth	Growth	Growth	Growth	Growth	<b>Growth</b>
<i>K. pneumoniae</i> 67	Growth	Growth	Growth	Growth	Growth	No growth	No growth	No growth
<i>K. pneumoniae</i> 63	Growth	Growth	Growth	Growth	Growth	No growth	No growth	No growth

Moreover, BMD was done to validate that the colistin resistant subpopulations were colistin resistant. The results confirmed that they were all colistin resistant with MIC values ranging between 8 µg/mL and 32 µg/mL. Moreover, to confirm that these colistin resistant subpopulation would not lose this phenotype, BMD was repeated after

1 week of serial passages in antimicrobial-free broth. The results confirmed that they all remained colistin resistant (Table 22).

Table 22: Broth microdilution results of the 10 colistin susceptible *K. pneumoniae* isolates and their colistin resistant subpopulation

Isolate	Initial MIC (µg/mL)	Highest concentration of growth in PAPs (µg/mL)	Colistin resistant subpopulation's MICs before serial passages (µg/mL)	Colistin resistant subpopulation's MICs after 1 week of serial passages (µg/mL)
<i>K. pneumoniae</i> 5	<0.25	4	32	32
<i>K. pneumoniae</i> 2	<0.25	16	64	32
<i>K. pneumoniae</i> 15	<0.25	16	8	8
<i>K. pneumoniae</i> 16	<0.25	16	16	8
<i>K. pneumoniae</i> 19	<0.25	16	32	16
<i>K. pneumoniae</i> 76	<2	16	8	8
<i>K. pneumoniae</i> 64	<1	16	16	16
<i>K. pneumoniae</i> 60	<1	16	16	16
<i>K. pneumoniae</i> 23	<0.25	16	32	16
<i>K. pneumoniae</i> 26	<0.25	16	16	16

### 3. Fitness Cost

Fitness cost assay was done to determine the effect of the heteroresistant phenotype on the growth rate. This was achieved by comparing the colistin susceptible *K. pneumoniae* isolates with their corresponding colistin resistant subpopulation in the

presence and absence of colistin. Fitness cost results showed a significant (P-value <0.0001) decrease in the growth rate of the colistin susceptible *K. pneumoniae* 60 clinical isolate in the presence of colistin when compared to the one in the absence of colistin (Figure 6A). Moreover, the results showed similar growth rates between the colistin resistant subpopulation of *K. pneumoniae* 60 in the absence and presence of colistin (Figure 6B). Furthermore, in the absence of colistin, the colistin susceptible *K. pneumoniae* 60 clinical isolates had similar growth rates compared to the colistin resistant subpopulation (Figure 6C). All the remaining colistin susceptible *K. pneumoniae* 2 (Figure 7), *K. pneumoniae* 5 (Figure 8), *K. pneumoniae* 15 (Figure 9), *K. pneumoniae* 16 (Figure 10), *K. pneumoniae* 19 (Figure 11), *K. pneumoniae* 23 (Figure 12), *K. pneumoniae* 26 (Figure 13), *K. pneumoniae* 64 (Figure 14), and *K. pneumoniae* 76 (Figure 15) clinical isolates and their corresponding colistin resistant subpopulation showed the same results.

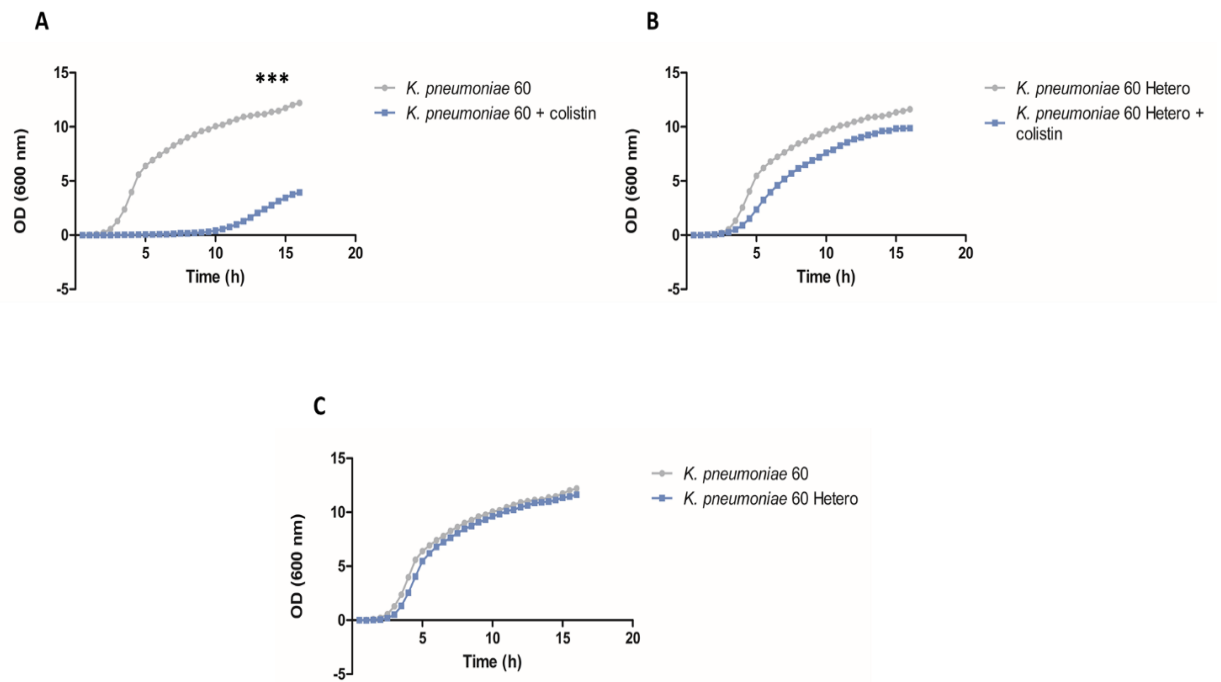
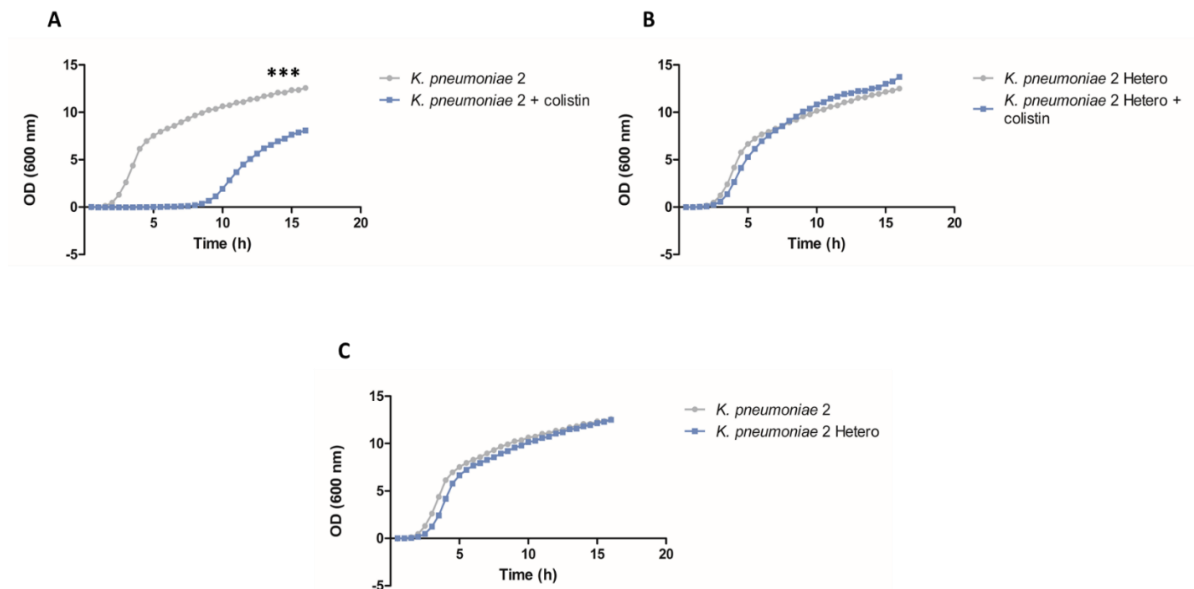


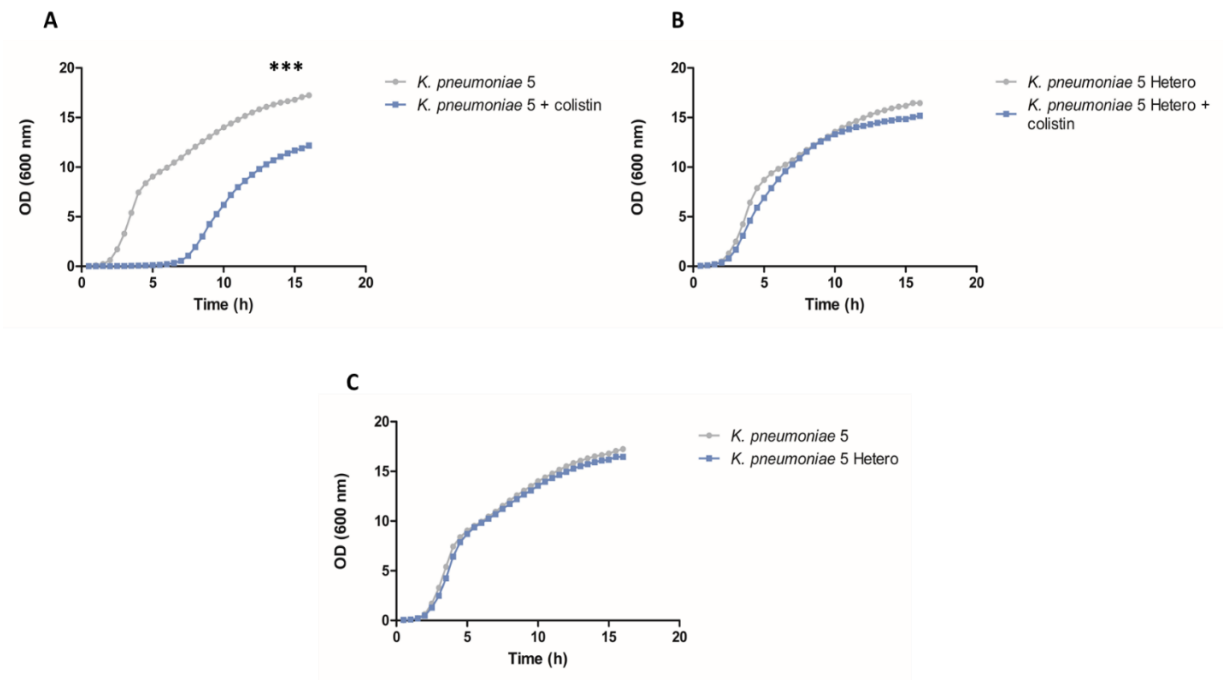
Figure 6: Fitness cost results of *K. pneumoniae* 60 and its colistin resistant subpopulation.

\*\*\*: Significant (P-value <0.0001)



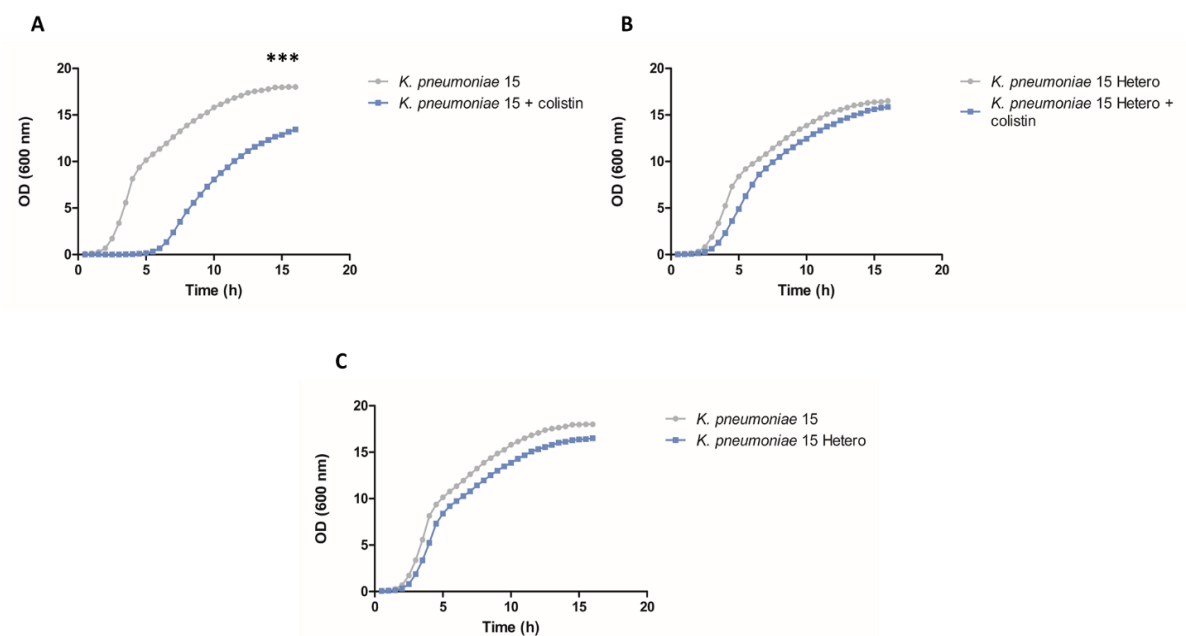
\*\*\*: Significant (P-value <0.0001)

Figure 7: Fitness cost results of *K. pneumoniae* 2 and its colistin resistant subpopulation.



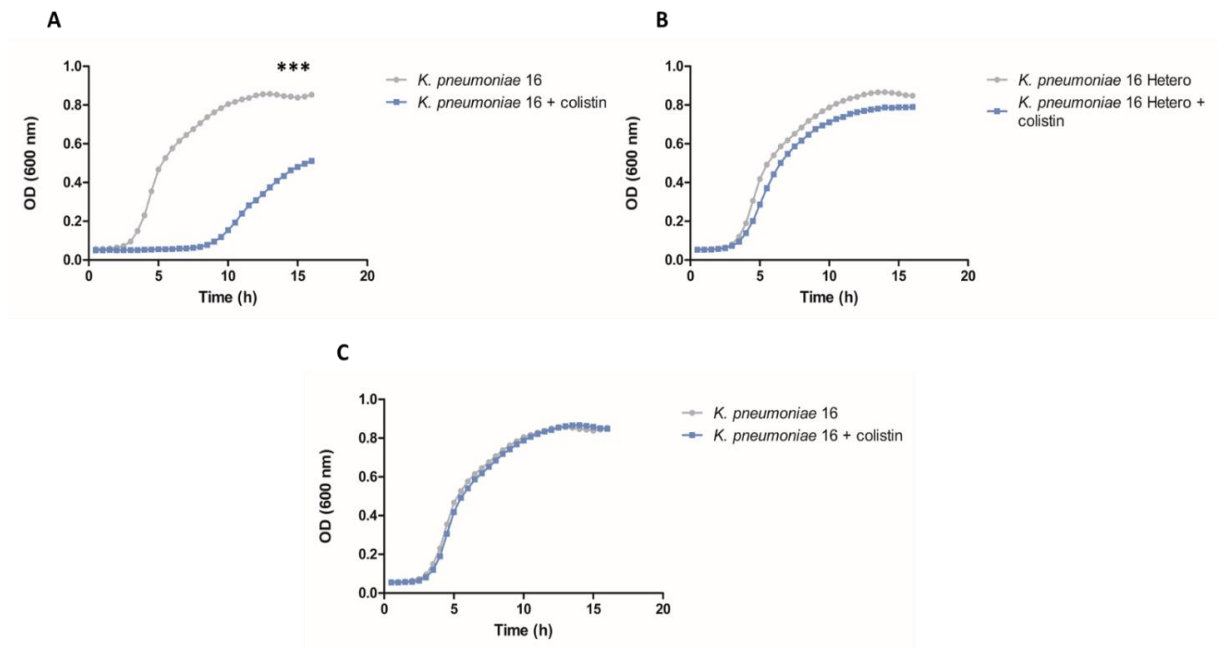
\*\*\*: Significant (P-value <0.0001)

Figure 8: Fitness cost results of *K. pneumoniae* 5 and its colistin resistant subpopulation.



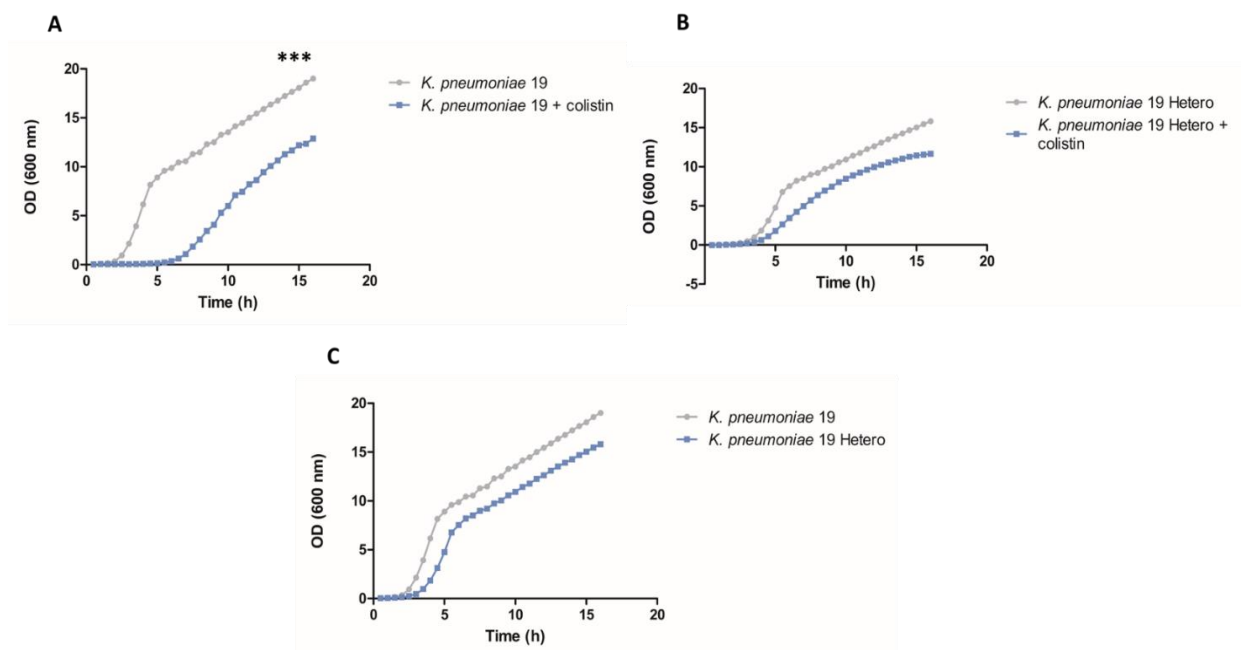
\*\*\*: Significant (P-value <0.0001)

Figure 9: Fitness cost results of *K. pneumoniae* 15 and its colistin resistant subpopulation



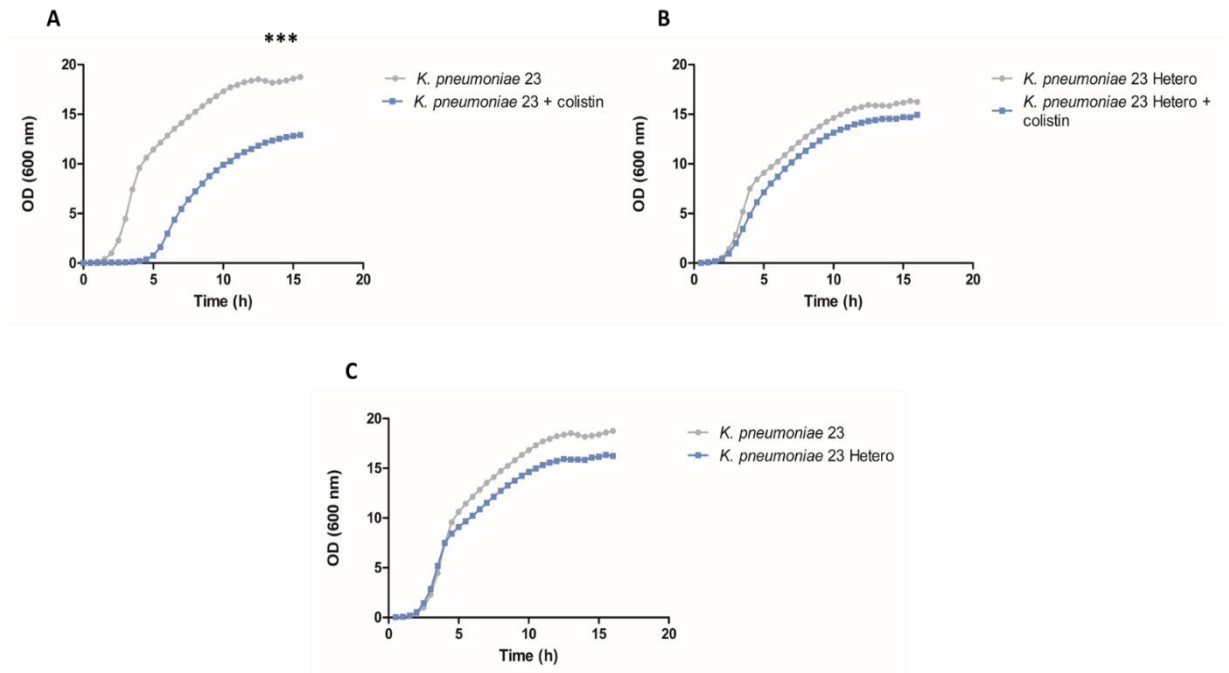
\*\*\*: Significant (P-value <0.0001)

Figure 10: Fitness cost results of *K. pneumoniae* 16 and its colistin resistant subpopulation



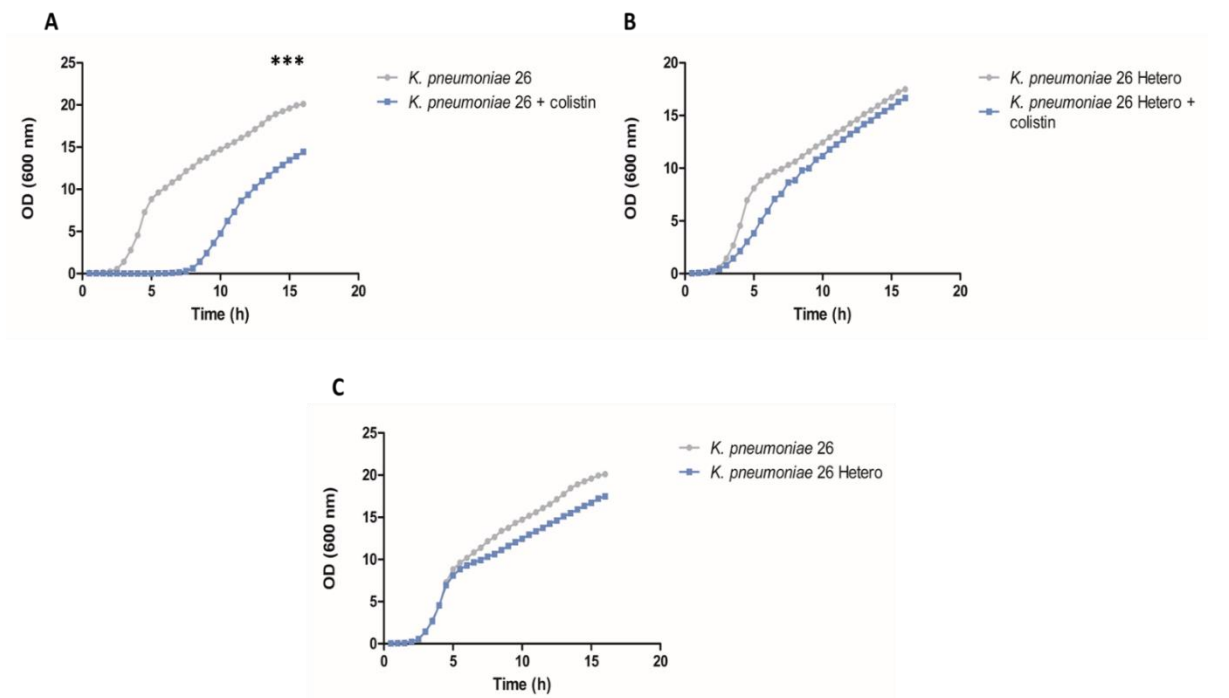
\*\*\*: Significant (P-value <0.0001)

Figure 11: Fitness cost results of *K. pneumoniae* 19 and its colistin resistant subpopulation



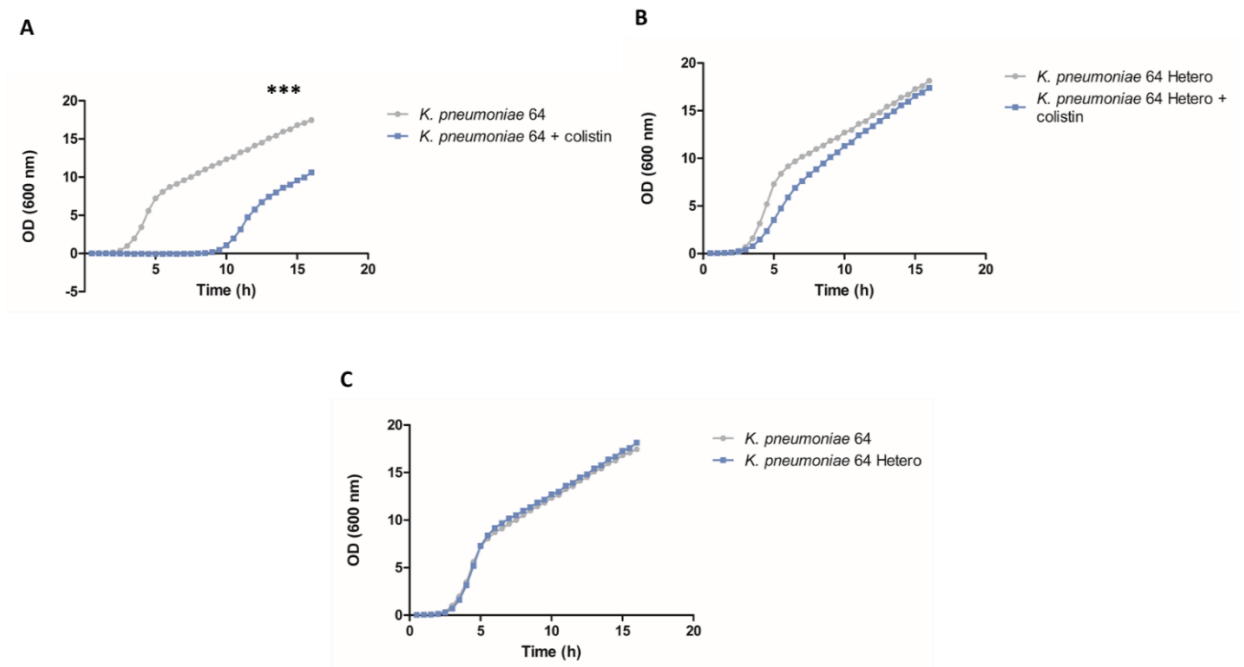
\*\*\*: Significant (P-value <0.0001)

Figure 12: Fitness cost results of *K. pneumoniae* 23 and its colistin resistant subpopulation



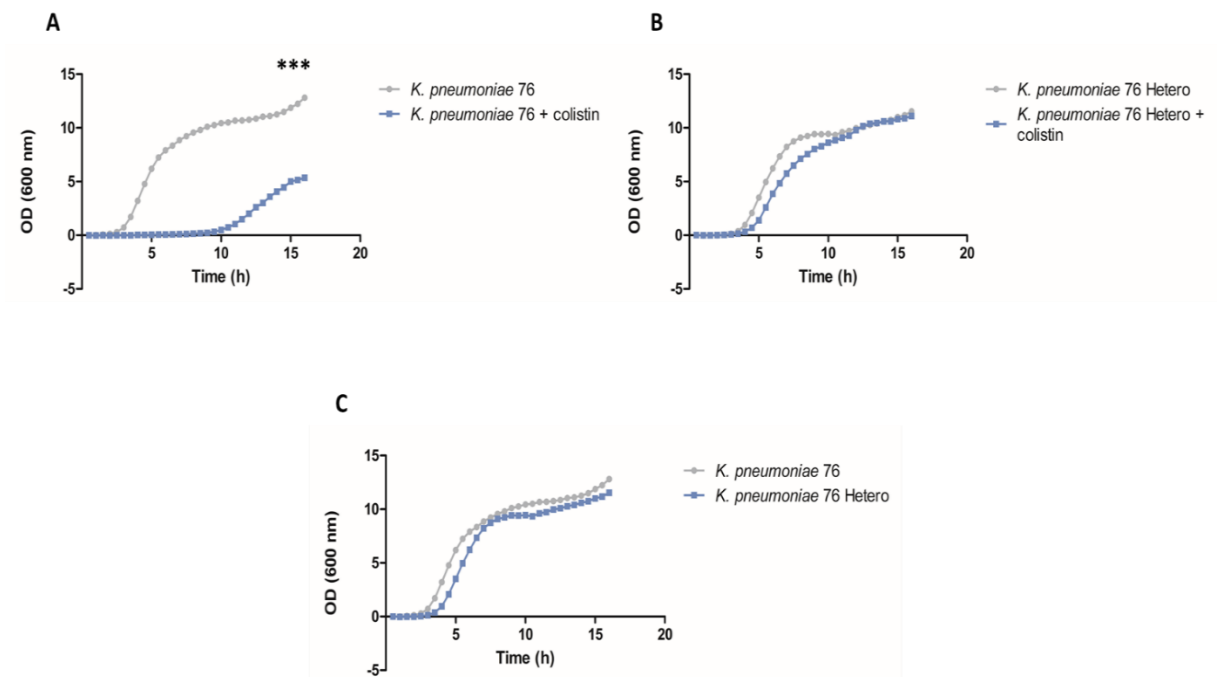
\*\*\*: Significant (P-value <0.0001)

Figure 13: Fitness cost results of *K. pneumoniae* 26 and its colistin resistant subpopulation



\*\*\*: Significant (P-value <0.0001)

Figure 14: Fitness cost results of *K. pneumoniae* 64 and its colistin resistant subpopulation



\*\*\*: Significant (P-value <0.0001)

Figure 15: Fitness cost results of *K. pneumoniae* 76 and its colistin resistant subpopulation

## CHAPTER V

### DISCUSSION

Colistin, one of the last-line antimicrobials, was reintroduced in the market as a treatment option due to the emergence of MDR and XDR Gram-negative bacteria (Aghapour et al., 2019). However, this does not change the fact that the more this antimicrobial is used the higher the chances are for the development of colistin resistance (Bialvaei & Samadi Kafil, 2015). One of the main focuses of this study was the plasmid mediated colistin resistance due to the *mcr* genes. These genes are found in animals especially in poultry, were in 2015, the first *mcr-1* gene harbored in a colistin resistant *E. coli* isolate recovered from poultry in Lebanon was reported (Dandachi et al., 2018). This gene was also reported in *E. coli* isolates recovered from irrigation water samples and the Mediterranean sea (Hmede & Kassem, 2018; Sourenian et al., 2020). The *mcr* genes are carried on plasmids. Different types of plasmids are well known to harbor these genes, such as: IncI2, IncX4, IncF, IncHI1, IncHI2, IncP and IncY plasmids (Zurfluh et al., 2017). IncI2 and IncX4, were the two plasmids that harbored the *mcr-1* gene detected in colistin resistant *E. coli* isolates recovered from poultry feces and water samples in this study.

A total of 37 colistin resistant *E. coli* isolates were included in this study. They were divided into 27 isolates retrieved from poultry feces from farms in Lebanon and 10 isolates obtained from water samples from different regions in Lebanon. Seventeen out of the 27 *E. coli* isolates obtained from poultry feces were from farms in the North of Lebanon and the remaining 10 were from farms located in the South of Lebanon. Whole genome sequencing was done on 18 out of the 27 *E. coli* isolates. Eight out of the 18 *E.*

*coli* isolates were from those recovered from poultry feces from farms located in the South of Lebanon. Their WGS results showed that 5 out of these 8 isolates belonged to ST1140. Moreover, the remaining 10 *E. coli* isolates were from those obtained from poultry feces from farms in the North of Lebanon. Their WGS results showed that 2 out of the 10 isolates belonged to ST1140, which was the most prevalent sequence type among the isolates recovered from farms in the south. This indicates the possible dissemination of colistin resistant *E. coli* isolates belonging to ST1140 among farms at the country level. Moreover, *E. coli* isolates belonging to sequence type 1140 can cause infections in humans. In a study done by Mavroidi et al., an *E. coli* isolate attributed to the sequence type 1140 was recovered from a patient in the University Hospital of Larissa, Central Greece (Mavroidi et al., 2012). Furthermore, an *E. coli* isolate belonging to ST2705 was recovered from a stool sample of a patient admitted to Hôtel-Dieu de France hospital in Beirut (Al-Mir et al., 2019). This isolate matches with the sequence types of three of the *E. coli* isolates retrieved from poultry feces in this study. This emphasizes on the possibility of these colistin resistant isolates in causing infection in humans and the potential risk of their transmissibility from poultry to humans.

A total of 10 *E. coli* isolates were recovered from water samples collected from 6 different regions across Lebanon. WGS was done on 5 out of the 10 isolates and its results showed that the most prevalent sequence type was the ST10773. This sequence type was attributed to 2 isolates recovered from the Ouzaai region and 1 isolate from Saida which may be explained due to the relatively short distance separating these 2 regions.

The *mcr-1* gene was harbored in 32 out of the 37 colistin resistant *E. coli* isolates. In addition to the *mcr-1* genes, many other antimicrobial resistance genes were

detected in these isolates, such as *bla*<sub>CMY-2</sub>, *aadA*, *gyrA*, *mph*-(A), *tet*(A)-1, and *fosA*-3 genes encoding resistance to  $\beta$ -lactams, aminoglycosides, fluoroquinolones, macrolides, tetracyclines, and fosfomycin respectively. The *E. coli* isolates recovered from poultry feces harbored a wider variety of resistance genes compared to those recovered from water samples.

A total of 10 colistin resistant *K. pneumoniae* clinical isolates were included in this study and none of these isolates harbored any *mcr* gene (*mcr*-1 to *mcr*-8). In such cases, mutations in the LPS molecule or the presence of efflux pumps may be the main driver behind colistin resistance in these isolates (Webber & Piddock, 2003). WGS was done on 2 out of the 10 colistin resistant *K. pneumoniae* clinical isolates (*K. pneumoniae* 88 and *K. pneumoniae* 91). WGS results confirmed that colistin resistance was neither caused by an acquired resistance gene nor due to efflux pumps. However, *K. pneumoniae* 91 had a mutation in the *mgrB* gene that is directly related to colistin resistance. Both isolates harbored the *bla*<sub>OXA-48</sub> gene in addition to several antimicrobial resistance genes, such as *bla*<sub>CTX-M-15</sub>, *aadA*2, *gyrA*, *tet*(A)-1, *fosA*-v3, and *mph*-(A) genes encoding resistance to  $\beta$ -lactams, aminoglycosides, fluoroquinolones, tetracyclines, fosfomycin and macrolides respectively. These 2 isolates are a serious threat to the public health since in addition to their resistance to several antimicrobials from different antimicrobial families, they are simultaneously resistant to colistin and carbapenems. Additionally, *K. pneumoniae* 88 belongs to ST383 which has been linked to nosocomial outbreaks. Guo et al., reported an outbreak of *bla*<sub>OXA-48</sub> producing *K. pneumoniae* belonging to ST383 in a Chinese hospital (Guo et al., 2016).

Out of the 25 colistin susceptible clinical isolates belonging to the Enterobacterales family (13 *E. coli* and 12 *K. pneumoniae*), only 10 *K. pneumoniae*

isolates had a colistin resistant subpopulations. This phenotype deeply alters the antimicrobial treatment efficacy (Band & Weiss, 2019). According to the FDA susceptibility testing guidelines, classifying colistin heteroresistant isolates as colistin susceptible is a “very major discrepancy” (FDA et al., 2009). Fitness cost results showed that, in the absence of colistin, the colistin susceptible *K. pneumoniae* clinical isolates had similar growth rates compared to the colistin resistant subpopulation. This shows that in the absence of colistin, no pressure was exerted on either of the 2, so similar growth rates were recorded. Moreover, fitness cost results showed similar growth rates between the colistin resistant subpopulation in the absence and presence of colistin. This assures the idea of heteroresistance that indicates the ability of the antimicrobial resistant subpopulations to propagate in the presence of the antimicrobial. Furthermore, fitness cost results showed a significant decrease in the growth rate of the colistin susceptible *K. pneumoniae* clinical isolates in the presence of colistin when compared to the ones in the absence of colistin. This indicates that the colistin susceptible population, that are the majority, died in the presence of colistin. Plasmid mediated colistin resistance, *mcr-1* gene, in *E. coli* isolates recovered from poultry and water poses a serious threat on public health. This gene could be transferred horizontally into colistin susceptible isolates and they could be introduced into the human community if no strict measures were taken. Moreover, the detection of colistin resistant subpopulations among colistin susceptible *K. pneumoniae* clinical isolates may cause serious complications leading to treatment failure.

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