United Arab Emirates University

College of Medicine and Health Sciences

Department of Microbiology

PLASMID MEDIATED COLISTIN RESISTANCE IN *ENTEROBACTERIACEAE* ISOLATED FROM FECES OF BROILER CHICKEN IN THE UNITED ARAB EMIRATES

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Microbiology and Immunology)

Under the Supervision of Dr. Agnes Sonnevend

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Declaration of Original Work

I, Sara Abdelkafi Mahmoud Ali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Plasmid Mediated Colistin Resistance in Enterobacteriaceae Isolated from Feces of Broiler Chicken in the United Arab Emirates*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Agnes Sonnevend, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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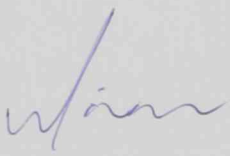
   Signature  Date 29/8/2019

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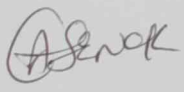
   Signature  Date 29/8/2019

Abstract

Although human infections with colistin resistant Mcr-producing *Enterobacteriaceae* have been reported from the Arabian Peninsula, the presence of *mcr* gene in strains of animal origin has not been studied.

Ten composite fecal samples were collected in each of four geographically distant poultry farms in Abu Dhabi Emirate. Ten colonies from McConkey agar containing 1 mg/L colistin from each sample were PCR-screened for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*. Of the positive ones, one isolate representing each distinct plasmid profiles were selected for susceptibility testing and for whole genome sequencing (WGS). Transfer of the *mcr-1* gene into *Escherichia coli* K12 was attempted. Plasmids were first compared by restriction fragment length polymorphism (RFLP). Representatives of plasmids with distinct RFLP profile were sequenced and their incompatibility type was determined.

mcr-1 positive colonies were identified in 36 of the 40 samples. The 40 isolates selected were resistant to ampicillin and colistin (MIC range 4->256 mg/L) with variable resistance to 3rd generation cephalosporins, tetracyclines, quinolones, co-trimoxazole and aminoglycosides. Thirty-five *Escherichia coli*, two *Escherichia albertii*, two *Klebsiella pneumoniae* and one *Salmonella* Minnesota were identified based on their whole genomes. They represented a remarkable variety of sequence types and in case of *E. coli* core genome multi-locus sequence types. Beyond *mcr-1*, all strains carried at least one beta-lactamase, and aminoglycoside, tetracycline, and co-trimoxazole resistance genes, seven of them ESBL genes and one $bla_{\text{CMY-2}}$. Transfer of the *mcr*-plasmid was successful in case of 6 IncHI2, 26 IncI2 and 4 IncX4 plasmids, respectively. Co-transfer of ampicillin, chloramphenicol and tetracycline resistance with the *mcr-1* gene was observed in case of the IncHI2 plasmids.

From the four chicken farms multiple species of *Enterobacteriaceae* exhibiting a variety of MLST and cgMLST patterns, carrying the *mcr-1* gene on plasmids of three different incompatibility types were isolated. Similar strains and similar plasmids were present in multiple farms. These data show that the farms are heavily infested with *mcr-1* carrying strains with the possibility to transfer it, via the food chain, to humans.

Keywords: Mobile colistin resistance, poultry, United Arab Emirates.

Title and Abstract (in Arabic)

مقاومة عقار الكوليستين عن طريق انتشارالبلازميدات في البكتريا المعوية المعزولة من براز الدجاج من مزارع الدواجن في الامارات العربية المتحدة

الملخص

علي الرغم من وجود تقارير عن حدوث عدوى بشرية بالبكتيريا المعوية المقاومة لعقار الكوليستين المنتجة لجين (MCR) الموجود على البلازميدات في منطقة الخليج العربي إلا انه لا توجد دراسات عن وجود هذا الجين في السلالات ذات المنشأ الحيواني.

تم جمع عشر عينات براز مركبة من كل من أربع مزارع دواجن في إمارة أبوظبي تبعد عن بعضها جغرافيا. من كل عينة تم فحص عشرة مستوطنات مستنبتة على (McConkey agar) المحتوي علي 1ملغ/لتر كوليستين، وقد تم الفحص عن وجود جين ال MCR (1-5) بواسطة تفاعل البلمرة المتسلسل. من العينات الموجبة تم إختيار عزل واحد ممثلا عن كل نمط بلازميدي مختلف لإجراء إختبارات الحساسية للمضادات الحيوية و تسلسل كامل للجينوم (WGS). تمت محاولة نقل جين ال MCR إلى E.coli K12 . تمت مقارنة البلازميدات أولاً عن طريق ال (RFLP)، ثم تم إختيار ممثل عن كل نمط لإجراء فحص التسلسل الجيني و تحديد أنواع ال Incompatibility.

تم تحديد جين ال (MCR-1) في 36 من أصل 40 عينة. ولقد وجدنا أن ال 40 عينة التي تم إختيارها، مقاومة للأمبيسيلين والكوليستين (MIC range 4->256 mg/L) مع مقاومة متغيرة للسيفالوسبورين من الجيل الثالث، التتراسيكلين، الكينولونات، التريميوكسازول والأمينوغليكوزيد. من خلال تسلسل الجينوم الكامل، تم تحديد أنواع البكتيريا، تم تعيين خمسة وثلاثين من الإشريكية القولونية ، وإثنين من الإشريكية البرتي ، وإثنين من كلبسيلا الرئوية والسالمونيلا مينيسوتا. ولقد مثلت هذه المجموعة اختلافاً لافتاً للنظر في أنواع ال ST و في حالة الإشريكية القولونية أنواع مختلفة من ال (cgMLST).

بالإضافة لجين (MCR-1) حملت كل السلالات علي الأقل جين واحداً من بيتا لاكتاماز، أمينوغليكوزيد، تيتراسيكلين، وجينات مقاومة للتريميوكسازول ، سبعة منها جينات ال ESBL وواحد فقط هو جين ال blaCMY-2. كان نقل بلازميد ال MCR ناجحًا في حالة 6 من بلازميدات ال IncHI2 و 26 من ال IncI2 و اربعة من ال IncX4 على التوالي. وقد لوحظ النقل المشترك للجينات المقاومة للأمبيسلين والكلورامفينيكول والتتراسيكلين مع جين ال MCR-1 في حالة بلازميدات ال IncHI2.

تم عزل أنواع متعددة من ال Enterobacteriaceae من مزارع الدواجن الأربعة، وتمثل أنماط متعددة من ال MLST و cgMLST ، حيث تم عزل جين ال MCR-1 على بلازميدات من ثلاثة أنواع مختلفة من ال Incompatibility. توجد سلالات و بلازميدات متشابهة في مزارع متعددة. تظهر هذه البيانات أن المزارع موبوءة بشدة بسلالات تحمل جين ال MCR-1 مع إمكانية نقلها ، عبر السلسلة الغذائية ، إلى البشر.

مفاهيم البحث الرئيسية: نقل مقاومة الكولستين ، الدواجن ، الإمارات العربية المتحدة.

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I'm deeply indebted to my family; their everlasting support and encouragement has been the biggest source of my strength.

Dedication

To my beloved parents and family

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List of Abbreviations

AK	Amikacin
AMC	Amoxicillin/clavulanate
AMP	Ampicillin
ATCC	American type culture collection
AZT	Aztreonam
BMD	Broth microdilution
C	Conjugative
CAZ	Ceftazidime
CCCP	Carbonyl cyanide 3-chlorophennylhydrazone
CF	Cystic fibrosis
cgMLST	Core genome multi-locus sequence typing
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical laboratory standards institute
Cm	conjugation resulted in multiple plasmids containing recipient
CMS	Colistin methane sulfonate
CNS	Central nervous system
CPD	Cefpodoxime
CPX	Conjugate pilus expression
CSF	Cerebrospinal fluid
CTX	Cefotaxime
D	Dextrogyre
Dab	Diaminobutyric acid

DOX	Doxycycline
EDTA	Ethylene diamine tetraacetic acid
ESBL	Extended spectrum beta-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
*f*AUC	Area under the unbound (free) concentration
FDA	Food and drug administration
FOS	Fosfomycin
GCC	Gulf Cooperation Council
GM	Gentamicin
ILe	Isoleucine
Inc	Incompatibility type
IS	Insertion sequence
Kdo	LPS 3 deoxy-D-manno-octulosonic acid
L	Levogyre
L-ara4N	4-amino-4-deoxy-L-arabinase
LB	Luria Bertani Broth
Leu	Leucine
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ ionisation mass spectrometry
MDR	Multi-drug resistent organisms
MHA	Muller-Hinton agar
MHB	Muller-Hinton broth
MIC	Minimum inhibitory concentration
MIU	Million international unit

MLST	Multi-locus sequence typing
NA	Not applicable
NAL	Nalidixic acid
NCTC	National collection of type cultures
ND	Not determined
NDM	New Delhi metallo-beta-lactamase
NST	Not self-transmissible
NT	Non typable
Nva	Norvaline
P-80	Polysorbate 80
PAE	Post antibiotic effect
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PEtN	Phosphoethanolamine
PFGE	Pulsed field gel electrophoresis
Phe	Phenylalanine
PK	Pharmacokinetic
RCS	Regulator of capsule synthesis
RFLP	Restriction fragment length polymorphism
RND	Resistence-nodulation-cell division
rpm	Round per minute
rRNA	Ribosomal RNA
Sap proteins	Sensitive antimicrobial peptides

SDS	Sodium dodecyl sulfate
SLV	Single locus variant
SMR	Small multi-drug resistence protein family
SSC	Saline sodium citrate
ST	Sequence Type
SXT	Co-trimoxazole
TBE Buffer	Tris-Borate-EDTA buffer
TCS	Tow component system
TE Buffer	Tris-EDTA buffer
TET	Tetracycline
TM	Transmembrane
Tn	Transposon
TOB	Tobramycin
Tris	Triaminomethane
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UAE	United Arab Emirates
USA	United States of America
UV	Ultraviolet light
Val	Valine
WGS	Whole genome sequencing

Chapter 1: Introduction

1.1 Overview

The finding of antibiotics and later their commercial availability, ease of administration and efficacy in treating microbial infections has led to dramatic change and to the evolution of modern medicine. Antibiotics have become cornerstone in almost all medical approaches. This great achievement is largely endangered with the surge of antimicrobial resistance mainly in common bacterial pathogens, risking treatment outcomes especially in critically ill patients (World Health Organization, 2014).

In addition to the high mortality rate, infections with multidrug resistant organisms are also a cause of great financial loss (Cosgrove, 2006; DiazGranados et al., 2005; Sydnor and Perl, 2011). This situation is further complicated by the scarcity of new antibiotics in development making emergence of untreatable infections almost inevitable (Munita and Arias, 2016). One of the most important causes of bacterial evolution that usually lead to the development of antimicrobial resistance is horizontal gene transfer (Munita and Arias, 2016). As most of the antimicrobials are natural products, bacteria sharing the same environment usually develop mechanisms to resist these compounds, and hence survive. These bacteria with the intrinsic resistance genes represent the environmental resistome which is the main source of antibiotic resistance genes acquired by clinically important bacteria (Munita and Arias, 2016). Conjugation through plasmids represent a very efficient tool for dissemination of antimicrobial resistance among clinically important bacteria and is usually involved in the emergence of resistance in hospital settings. Gene transfer through conjugation also

occurs with increased frequency in the gastrointestinal tract of humans during antibiotic treatment (Munita and Arias, 2016).

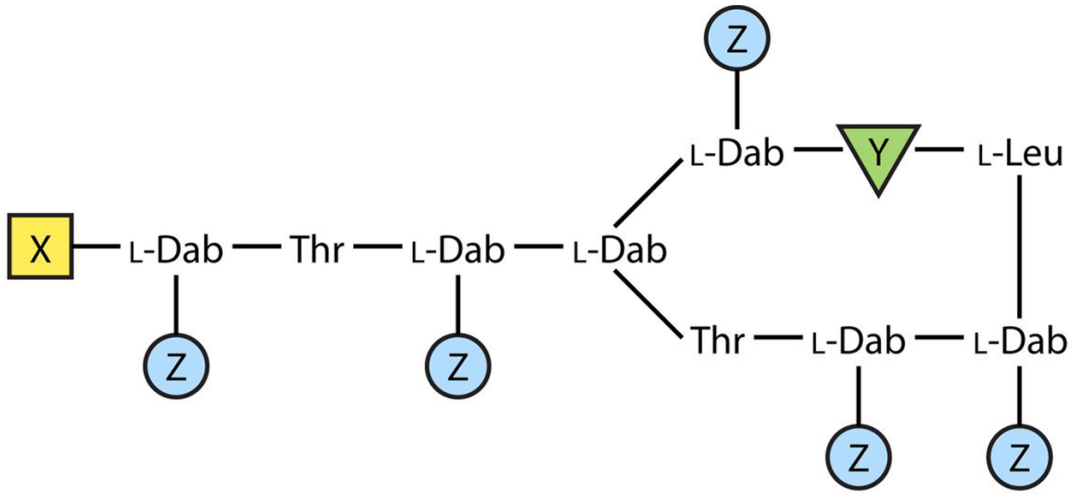
1.2 Polymyxins

Polymyxins are polypeptide antibiotics, discovered in 1947 from various species of *Bacillus polymyxa*. Polymyxins are used to treat serious infections caused by Gram negative bacteria resistant to other, less toxic antibiotics, usually used as a last line treatment against these infections. The recent report (Y. Liu et al., 2016), and the world-wide dissemination of plasmid coded, mobile colistin resistance threatens this last treatment option (Sun et al., 2018). Two polymyxins are used in clinical practice, polymyxin E (Colistin) and polymyxin B, and cross resistance occur between them (J. Li et al., 2006a). Since polymyxins were first discovered in the 1940s, most of the modern drug development procedures were not implemented. Polymyxins have narrow antibacterial spectrum, being active mainly against Gram-negative bacteria (Bergen et al., 2012; J. Li et al., 2006a). Clinical use of polymyxins fell out of favor in 1970s due to reports of side effect, however, their use has been revived as multi-drug resistant bacterial infections have emerged (J. Li et al., 2006a). Worldwide, parenteral colistin is the most common polymyxin in clinical use. In some countries parenteral polymyxin B is available, while in Singapore, USA and Brazil both antibiotics are available (Bergen et al., 2012).

1.2.1 Chemical Structure

Polymyxins are non-ribosomal cyclic lipopeptides, containing ten amino acids. They form an intramolecular cyclic heptapeptide loop. Looping occurs between the amino group of the side chain of diaminobutyric acid (Dab) residue at position 4 and

the carboxyl group at the C-terminal threonine residue at position10. Other structural characteristics of polymyxins include: polycationic nature at physiological pH due to five non proteinogenic diaminobutyric acid, hydrophobic residues at positions 6 and 7, and fatty acyl group at the N terminal. Being amphipathic, *i.e.* having both hydrophilic and lipophilic groups, is necessary for their antibacterial activity (Velkov et al., 2013). Many different groups of polymyxins have been isolated from *Paenibacillus polymyxa*, structural definition of each group is based on the amino acid residues in their sequences. Each group is given a letter as a label. Each group could have different lipopeptide components which differ in their N terminal fatty acyl group. The distinct lipopeptide parts of each group are given a number label (Velkov et al., 2013). Polymyxin B and polymyxin E (colistin) structures have been investigated thoroughly due to their introduction in clinical practice. The available injectable formulations of polymyxin B and polymyxin E for clinical use contain different components, usually polymyxin B1, B2 and polymyxin E1, E2 are the main components (Figure 1). The presence of D-phenylalanine residue at position 6 and a leucine residue at position 7 are the distinct structural characteristic of Polymyxin B group lipopeptide molecule. Apart from the D-phenylalanine at position 6, all other amino acids are in the L-form. The Polymyxin B group comprise seven components; the fatty acyl chains length range between 7-9 carbons and could be branched or non-branched. Polymyxin B1 and B7 (B1-Ile; has an isoleucine residue at position 7 but share the same 6-methyloctanoyl fatty acyl group) have a stereocenter at C6 of the 6-methyloctanoyl fatty acyl group. Polymyxin B6 differs from other polymyxin B lipopeptides in that it has three hydroxy groups at its fatty acyl chain, which contains two stereocenters at C3 and C6 (Velkov et al., 2013).



Dab, Diaminobutyric acid; Thr, Threonine; Phe, Phenylalanine; Leu, Leucine; L, Levogyre; D, Dextrogyre

X — Fatty acid residue differing between the components of the mixtures: 6-methyloctanoic acid for colistin A and polymyxin B1, and 6-methylheptanoic acid for colistin B, and polymyxin B2

Y — Aminoacid differing between colistin and polymyxin B: D-Leu for colistin, and D-Phe for polymyxin B

Z — Groups differing between colistin/polymyxin B and colistimethate: $-NH_2$ for colistin and polymyxin B, and $-NH-CH_2-SO_3H$ for colistimethate

Figure 1: Structures of colistin A and B, colistimethate A and B, and polymyxin B1 and B2 (Poirel et al., 2017)

Polymyxin E on the other hand is defined by having a D-leucine at position 6 and a leucine residue at position 7. Apart from the D-leucine at position 6, all other amino acids are in the L-form. Polymyxin E group comprise six components; polymyxin E1, E2, E3, E4, E7 and E8-Ile being different in their fatty acyl groups. Like polymyxin B, fatty acyl chains length range between 7-9 carbons, and could be branched or non-branched. Polymyxin E1, E1-Val, E1-Ile and E1-Nva have

stereocenter at C6 of the 6-methyloctanoyl fatty acyl group. Many polymyxin E molecules; E1-Val, E1-Ile, E1-Nva, E2-Val, E2-Ile and E8-Ile, share the same N-terminal 6-methyloctanoyl fatty acyl chain, but differ in their branched chain amino acids (valine, norvaline and isoleucine) (Velkov et al., 2013).

1.2.2 Mechanism of Action

The exact mode of action of polymyxins is not fully understood, but the accepted view is that these antibiotics are membrane surface active agents, and their primary target is the lipid A in the outer membrane of Gram-negative bacterial species (Velkov et al., 2010). The outer membrane acts as a barrier that protects bacteria from various detrimental substances including antibiotics (Nikaido, 2003). Structurally the outer membrane is composed of an inner phospholipid layer and an outer layer which contains lipopolysaccharides (LPS), proteins and phospholipids. LPS itself is composed of three parts: The O-antigen, which consists of saccharide unit repeats, a core of oligosaccharides, and lipid A which is the conserved moiety. Lipid A acts as hydrophobic anchor because it is intercalated within the outer leaflet (Raetz and Whitfield, 2002). Lipid A is composed of β-1'-6-linked D-glucosamine disaccharide which is phosphorylated at 1' and 4' positions (Nikaido, 2003). Lipid A hydrocarbons are saturated and are kept packed together within the membrane by van der Waals forces. Calcium (Ca^{+2}) and Magnesium (Mg^{+2}) ions within lipid A phosphoesters help bridging the LPS molecules. The outer membrane has an anionic charge because of lipid A phosphoester moieties and the phosphate and carboxylate groups within the core and O-antigen saccharides (Hancock, 1997; Raetz and Whitfield, 2002). The accepted model for polymyxin interaction with lipid A is called self-promoted uptake pathway, which emphasizes the importance of the amphipathic nature of polymyxins

to its uptake across the outer membrane (Hancock and Chapple, 1999). In this model, the electrostatic attraction between the anionic lipid A and the Dab residues is proposed to be provided by protonation of the free amines on Dab at physiological pH. This results in displacement of Ca^{+2} and Mg^{+2} that help stabilization of the LPS leaflet. This in turn leads to the insertion of the hydrophobic N-terminal fatty acyl chain and the hydrophobic residues at position 6 and 7 (D-Phe-L-Leu in polymyxin B and D-Leu-L-Leu motifs in colistin) into the outer membrane. Insertion of these hydrophobic motifs distorts the packed lipid A fatty acyl chains, which lead to expansion of the outer membrane (Figure 2). The following events are not fully understood; it is supposed that polymyxins mediates fusion of the inner leaflet of the outer membrane and the outer leaflet of the inner membrane, inducing phospholipid exchange, which leads to osmotic imbalance and result in cell death (Hancock and Chapple, 1999). The 3-D nuclear magnetic resonance solution state structure of polymyxin in complex with LPS showed that the polymyxin molecule is folded into two distinct faces; a hydrophobic and a polar face (Pristovsek and Kidric, 1999). The amphipathic structure together with their ability to form pore like structures might lead to outer membrane permeabilization. However, it is suggested that outer membrane permeabilization and bacterial killing are completely uncoupled processes (Soon et al., 2011).

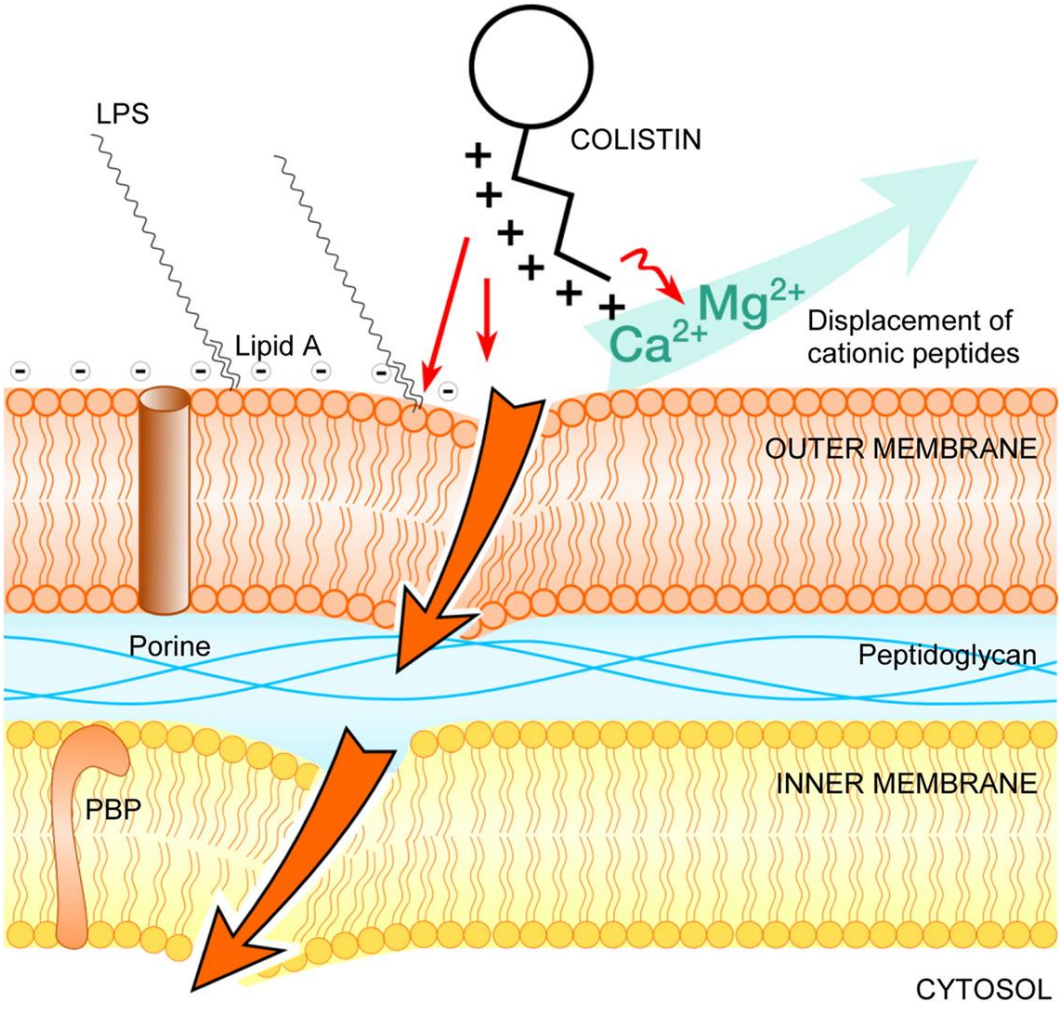


Figure 2: Action of colistin on bacterial membrane (Martis et al., 2014)

1.2.3 Side Effects

Major side effects of polymyxins include nephrotoxicity and neurotoxicity, this has led to their exclusion from routine first line treatment protocols (Falagas and Kasiakou, 2006).

1.2.3.1 Nephrotoxicity

The D-amino acids and the fatty chain in the structure of polymyxins were thought to be implicated in causing nephrotoxicity. The suggested mechanism is that polymyxins increase membrane permeability, which cause increased influx of water,

cations and anions resulting in cell swelling and death (Berg et al., 1996). Another study with mammalian urothelium demonstrated that colistin has increased transepithelial conductance; the magnitude of which seems to be determined by both the length of exposure and concentration of the polymyxin, and the concentration of the divalent cations. Polymyxin B is also suggested to increase transepithelial conductance by the same mechanism as colistin (Lewis and Lewis, 2004). Polymyxin-induced renal toxicity is supposed to be dose dependent**.** Recent studies showed evidence that polymyxins are potentially toxic to renal tubular cells; the proposed mechanisms include; oxidative stress, cell cycle arrest, apoptosis, and autophagy (Azad et al., 2013; Dai et al., 2014; Eadon et al., 2013; Zavascki and Nation, 2017).

1.2.3.2 Neurotoxicity

Neurotoxicity of polymyxins has been attributed to its interaction with neurons, which have a high lipid content. Neurotoxicity is found to be directly related to the active drug concentration (Hoeprich, 1970). Polymyxins act pre-synaptically to inhibit acetylcholine release to the synaptic gap at the neuromuscular junction causing neuromuscular blockade (McQuillen et al., 1967). It is suggested that neurotoxicity occurs in two phases; a short phase in which polymyxins block acetylcholine competitively, and a more prolonged depolarization phase caused by calcium depletion (Falagas and Kasiakou, 2006). Polymyxin-induced neurotoxicity is supposed to be dose dependent. Usual manifestations include dizziness, muscle weakness, paraesthesia, partial deafness, visual disturbances, vertigo, hallucinations, seizures, and confusion. Neuromuscular blockade may cause respiratory failure due to paralysis of the respiratory muscles. Mechanism of paraesthesia appear to be different from neurotransmission blockade (Duncan, 1973, as cited in Falagas and Kasiakou, 2006).

Renal impairment and myasthenia gravis are risk factors for neuromuscular blockade (Decker and Fincham, 1971). Women are more likely to develop neurotoxicity (Koch-Weser et al., 1970).

1.2.3.3 Other Adverse Effects

Mild itching (Olesen and Madsen, 1967, as cited in Falagas and Kasiakou, 2006), rash (Lamb, 1968; Pines et al., 1963) and contact dermatitis associated with topical or ophthalmic use of colistimethate sodium (Inoue and Shoji, 1995, as cited in Falagas and Kasiakou, 2006; Sasaki et al., 1998). These effects might be caused by the histamine releasing effect of the polymyxins, specially polymyxin B (Hoeprich, 1970, as cited in Falagas and Kasiakou, 2006).

Adverse effects of aerosolized polymyxins include chest tightness, bronchoconstriction, cough and sore throat. Several mechanisms are implicated; release of histamine, direct chemical stimulation, and hyperosmolality in the airways. Risk of bronchoconstriction increases in patients with asthma or atopy (Alothman et al., 2005).

1.2.4 Pharmacokinetics / Pharmacodynamics of Polymyxins

1.2.4.1 Pharmacokinetics of Polymyxins

Colistin methanesulfonate/Colistin: Parenteral colistin is administered as prodrug, colistin methanesulfonate (CMS). Pharmacokinetics of CMS differ from that of the positively charged colistin (Nation et al., 2015). While CMS is eliminated mainly by the kidneys, elimination of colistin is thought to be through non renal routes (J. Li et al., 2006a). Formation of colistin after intravenous administration of CMS is a slow process and hence plasma concentration increases slowly. It has been shown

that, with an intravenous dosing regimen of 3 MIU CMS every 8 h, in patients with good renal function, it could take more than 36 hours for the plasma concentration of colistin to reach a steady state of 2 mg/L (Plachouras et al., 2009). This emphasizes that optimization of CMS use in patients could be a great PK/PD challenge. The problem of low initial exposure to colistin could be overcome in part by administration of a loading dose (Plachouras et al., 2009). Two studies found a high likelihood of early bacterial eradication with administration of a loading dose of 6 MIU and 9 MIU, where eight hours after loading dose administration, average colistin plasma concentrations reached 1.34 mg/L and 2.65 mg/L, respectively (Karaiskos et al., 2015; Mohamed et al., 2012). Kidney function and renal replacement therapy were found to have a great impact on the PK/PD of both CMS and formed colistin in critically ill patients (Garonzik et al., 2011; Karvanen et al., 2013). Significant interpatient variation in the average steady state plasma colistin concentration was noticed despite limited variation in the range of daily doses, among patients with comparable creatinine clearance levels and even between those on the same daily doses (Garonzik et al., 2011). Both CMS and colistin were found to be cleared in patients on renal replacement therapy (Garonzik et al., 2011; Karvanen et al., 2013). Renal function greatly influences plasma concentration of formed colistin (Nation et al., 2016). An average colistin steady state concentration of about 2 mg/L need to be maintained as colistin is approximately 50% unbound in human plasma (Cheah et al., 2015). Not much is known about the kinetics of both colistin and CMS at extravascular sites (Tran et al., 2016b). Cerebrospinal fluid (CSF) concentration of formed colistin after intravenous administration of CMS was found to be very low in critically ill patients, with or without central nervous system (CNS) infection but reached >40% of the total colistin serum concentration when combined with intraventricular administration

(Ziaka et al., 2013). Similarly, intravenous administration of colistin in cystic fibrosis patients resulted in low concentration of the drug in the sputum, while inhalational colistin achieved higher colistin concentration in the sputum while keeping plasma concentration of the drug very low (Yapa et al., 2014). This was further emphasized in critically ill patients where administration of CMS via nebulization resulted in higher range of colistin concentration in epithelial fluid lining and lower in plasma compared to intravenous administration of CMS (Boisson et al., 2014).

Polymyxin B: Unlike colistin, polymyxin B is administered as the active drug, eliminating the need for conversion. Elimination of polymyxin B is through non-renal routes, thus its concentration in the urine is low (Zavascki et al., 2008). Renal function does not seem to affect total body clearance. Polymyxin B clearance scaled by total body weight showed low interpatient variability in the plasma concentration (Sandri et al., 2013). Serum half-life of polymyxin B ranges from 3.1 (in neonates) to 13.6 hours (Kwa et al., 2008, 2011; Salvatore et al., 2011; Zavascki et al., 2008). Polymyxin B is about 40% unbound in the plasma, thus a loading dose might greatly improve its pharmacokinetics (Sandri et al., 2013). High concentration of polymyxin B in epithelial lining fluid was obtained using the liposomal form of polymyxin B, which was found to be more effective compared to aqueous form in neutropenic mice models (He et al., 2013).

1.2.4.2 Pharmacodynamics of Polymyxins

Pharmacodynamics of colistin have been studied more than polymyxin B (Bergen et al., 2010; Cheah et al., 2015; Owen et al., 2007; Poudyal et al., 2008). Colistin has been shown to cause rapid concentration-dependent killing against *Acinetobacter baumanii*, *Klebsiella pneumoniae* and *Pseudomonas*

aeruginosa. It was also found to exert minimal post-antibiotic effect (PAE) at concentrations that could be achieved clinically (Bergen et al., 2010; Owen et al., 2007; Poudyal et al., 2008); though the relevance of PAE in patients is questionable due to the long half-life of formed colistin and the little variation in plasma colistin concentration during CMS dosage intervals in critically-ill patients (Bergen et al., 2010). But despite the rapid killing that colistin has showed, regrowth occurred quickly. On the other hand, polymyxin B was found to have comparable pharmacodynamics to colistin, as it has been shown to cause rapid killing of *A. baumanii, K. pneumoniae* and *P. aeruginosa* in vitro, that was also followed by rapid regrowth (Abdul Rahim et al., 2015; Tam et al., 2005; Tran et al., 2016a). Growth of polymyxin-resistant subpopulations within polymyxin hetero-resistant strains were found to be an important contributor to the rapid emergence of resistance (Bergen et al., 2011; J. Li et al., 2006b; Meletis et al., 2011). Both colistin and polymyxin B were shown to have an inoculum effect (Bergen et al., 2011; Tam et al., 2005). The best PK/PD index to better characterize the antimicrobial activity of colistin was found to be the ratio of the area under the unbound (free) concentration–time curve to the MIC (*f*AUC/MIC); as has been shown in a study of *P. aeruginosa* and *A. baumannii* in neutropenic mouse thigh and lung infection models (Cheah et al., 2015). This finding was also obtained from an in vitro study with P. *aeruginosa* (Bergen et al., 2010). On the other hand, the bactericidal activity of polymyxin B has been shown to be concentration dependent and seems to be related to the ratio of the area under the concentration-time curve to the MIC (Tam et al., 2005). However, administration of polymyxin B to patients with good renal function might result in higher *f*AUC/MIC values compared to CMS; because polymyxin B is administered as the active drug and

its distribution is not affected by renal function, but the PD of polymyxin B needs further studies (Tran et al., 2016b).

1.3 Difficulties of In Vitro Polymyxin Susceptibility Testing

1.3.1 Impact of Medium

The two component systems; PhoP/PhoQ and PmrA/PmrB, that basically regulate bacterial response to changes in pH and cation concentration (iron, magnesium and calcium), are involved in causing resistance to polymyxins through LPS modification (Olaitan et al., 2014b). Cation concentrations of Muller-Hinton medium differ greatly from brand to brand and are below the Clinical Laboratory Standards Institute (CLSI) recommended concentrations. This has led the CLSI to recommend the use of cation adjusted Muller-Hinton or cation supplemented culture medium (CLSI, 2019). On the other hand, bactericidal activity of polymyxin antibiotics is found to be inhibited by cations (Chen and Feingold, 1972; Poirel et al., 2017), which could lead to overestimation of colistin antibacterial activity when tested using the recommended cation adjusted Muller-Hinton medium. Another point is that the recommended Ca^{2+} and Mg^{2+} concentration for colistin susceptibility testing is almost half of their concentration in human interstitial space fluid, thus use of the recommended cation adjusted Muller-Hinton media might cause misestimation of colistin MIC at the target site (Matzneller et al., 2015). It is still questionable whether to use cation-adjusted or non-cation-adjusted medium for colistin susceptibility testing (Poirel et al., 2017).

1.3.2 Impact of Powder Composition

Commercial polymyxin B sulfate and colistin sulfate are used to test for MIC. CMS is not used in susceptibility testing as it gives false high MIC results (Landman et al., 2008). As polymyxin B and colistin sulfate are mixtures of chemically related compounds, which differ in their antibacterial activity (Landman et al., 2008); variation in the proportions of these compounds between manufacturers could lead to variations in MIC results. One study showed consistency of the relative proportions of compounds making polymyxin B from four manufacturers, in 10 powder lots (He et al., 2010). Another study found that the MICs with broth microdilution (BMD) using purified major components of polymyxin B were within a $\log_2$ dilution of that using polymyxin powder mixture of the American pharmacopeia (Tam et al., 2011). It could thus be suggested that powder source of polymyxin has no great effect on MICs (Humphries, 2015). On the other hand, the amount of the active drug in a colistin or polymyxin B lot could be significantly different from the standard potency but its consequences on MIC needs to be studied (Humphries, 2015).

1.3.3 Impact of the Composition and Treatment of Plates

Polymyxins being cationic compounds adhere to the negatively charged trays used in BMD; adherence vary depending on the tray material, being higher at low drug concentrations (Karvanen et al., 2017). Different coating of the BMD trays also resulted in significant differences in MIC results (Albur et al., 2014). This can result in significant differences between laboratories as there is no consensus on the type of BMD trays used to test colistin susceptibility (Poirel et al., 2017). Recently the joint CLSI-EUCAST subcommittee on polymyxin susceptibility testing and breakpoints

recommended use of plain polystyrene trays for susceptibility testing of polymyxins (EUCAST, 2016).

1.3.4 Presence or Absence of P-80

Polysorbate 80 is a surfactant that has been used to reduce adherence of polymyxins to BMD trays; with 0.002% final concentration of P-80 in the well resulting in 4-8-fold reduction in polymyxin MICs (Sader et al., 2012). However, P-80 could have synergistic effect with polymyxins resulting in falsely low MICs (Brown and Winsley, 1968, as cited in Poirel et al., 2017). This effect is mostly seen in isolates with MICs of ≤1 μg/ml (Humphries, 2015), possibly supporting the finding that P-80 lyse the inner cell membrane after the permeabilization of the outer membrane by polymyxins (Brown et al., 1979, as cited in Poirel et al., 2017). CLSI recommended BMD testing of polymyxin susceptibility without P-80, but this could make detection of susceptible isolates with MICs of ≤1 μg/ml difficult (Humphries, 2015). Use of glass trays may avoid polymyxin adherence issue, but impractical due to cost and fragility (Humphries, 2015).

1.3.5 Impact of Subcultures

Loss of selective pressure can result in loss of colistin resistance; as it has been shown that 98% of colistin-resistant *A. baumannii* population has lost phenotypic colistin resistance after being passed once in a medium not containing colistin (J. Li et al., 2006b)

1.3.6 Impact of Storage

Storage of isolates at −70°C can lead to loss of colistin resistance; this has been observed in 20% of isolates initially tested resistant to colistin but tested susceptible after 6-8 month of storage at −70°C using the same method (Hindler and Humphries, 2013).

1.3.7 Interpretive Criteria/ Categories and Quality Control Strains

There is no consensus in polymyxin breakpoints between CLSI and EUCAST (CLSI, 2019; EUCAST, 2019a). Zone diameter breakpoints are not available as disc diffusion method of susceptibility testing is not recommended neither by CLSI nor by EUCAST (EUCAST, 2016). CLSI provides colistin and polymyxin B MIC breakpoints for *Pseudomonas* spp. and *Acinetobacter* spp. but not for *Enterobacteriaceae*, while EUCAST provide breakpoints for colistin only and not for polymyxin B (CLSI, 2019; EUCAST, 2019a). On the other hand, EUCAST set up clinical breakpoints for *Enterobacterales*, *Acinetobacter* spp. and *Pseudomonas* spp. with isolates having MIC ≤ 2 mg/L considered as susceptible. CLSI and EUCAST recommend quality control organisms *E. coli* ATCC 25922 with MIC range 0.25-2 mg/L and *P. aeruginosa* ATCC 27853 with MIC range 0.5-4 mg/L for polymyxin susceptibility testing (EUCAST, 2019b). EUCAST has also advised to include an *mcr-1* positive strain *E. coli* NCTC 13846 with MIC of 4 mg/L as a quality control for resistant strains (EUCAST, 2019b).

1.4 Use of Colistin

1.4.1 Clinical Use of Colistin

Clinical use of colistin was revived in in the late 1980s and early 1990s mainly to treat cystic fibrosis (CF) patients infected or colonized with *P. aeruginosa*, where it is administered intravenously or by inhalation. In the late 2000s, colistin use has expanded to cover a range of infections caused by multi-drug resistant Gram-negative bacteria (*P. aeruginosa, K. pneumoniae* and *A. baumannii*), including bacteremia and ventilator associated pneumonia in critically ill, adult patients, mainly administered intravenously; with most of the studies showing satisfactory therapeutic outcomes (Biswas et al., 2012). A large retrospective study in 258 patients treated with colistin for at least 72 hours for multi-drug resistant bacterial infections including 170 (65.9%) *A. baumannii*, 68 (26.4%) *P. aeruginosa*, 18 (7.0%) *K. pneumoniae*, 1 (0.4%) *Stenotrophomonas maltophilia* and 1 (0.4%) *Enterobacter cloacae*; found colistin to be a very effective antibiotic. It has documented cure in 79.1% of patients, with 10% of patients developed nephrotoxicity; moreover, it showed that colistin effectiveness is not dependent on the pathogen type (Falagas et al., 2010). Use of intravenous colistin for the treatment of multi-drug resistant Gram-negative infections in con-CF patients showed a clinical response rate ranging from 47.3% to 5% (Kwa et al., 2007). Colistin was also used in the treatment of ventilator associated pneumonia and bacteremia caused by MDR organisms (Landman et al., 2008; Nation and Li, 2009). Colistin has also been used in combination with other drugs (J. Li et al., 2006a; Petrosillo et al., 2008). Colistin was found to be as effective and safe as carbapenem in a study examining the cure rate of 35 patients with ventilator associated pneumonia caused by *A. baumannii*, treated with intravenous colistin or imipenem (Garnacho- Montero et

al., 2003). These findings were further supported by another study (Kallel et al., 2007), which showed intravenous colistin to be both effective and safe in the treatment of ventilator associated pneumonia caused by pan-drug resistant *A. baumannii* or *P. aeruginosa*; with 75% of patients having favorable clinical outcome and none had developed renal failure. Colistin has been found to be effective in the treatment of infections caused by MDR Gram negative bacteria in burn patients (Cho et al., 2012). Administration of CMS through intraventricular route was found to be effective in the treatment of ventriculitis caused by *A. baumannii* (Dalgic et al., 2009; Lopez-Alvarez et al., 2009). CMS was also found to be effective in the treatment of MDR *P. aeruginosa* infection in cancer patients (Hachem et al., 2007). Administration of CMS through inhalation has been used for the treatment of ventilator associated pneumonia in critically ill patients (Falagas et al., 2009; Michalopoulos et al., 2008). Inhalational CMS has also been reported to enhance eradication of MDR *A. baumannii* from the respiratory tract without significant side effects (Kuo et al., 2012).

1.4.2 Veterinary Use of Polymyxins

Veterinary medicine and animal husbandry sector have largely depended on colistin for different indications. Colistin is used therapeutically in Europe to treat different farm animals with *Enterobacteriaceae* infections. These include sheep, goats, cattle, chickens and pigs. It is also used prophylactically for prevention of diarrhea caused by *E. coli* and *Salmonella spp*. Colistin is also used in aquaculture for the prevention of Gram-negative infections (Catry et al., 2015). Another use of colistin, is its use as a growth promoter in food animals. For this purpose, it is used as an in-feed antibiotic for increasing body weight and feed efficiency. This has been practiced in different countries that include India, China, Vietnam and Japan (Kempf et al., 2016).

In Brazil it is used to promote growth as an in-feed additive in pigs and poultry and its use is FDA approved in the USA (Fernandes et al., 2016b).

Having been used for quite a long time (Kempf et al., 2016), worldwide agricultural consumption of colistin approaches 12.000 tons per year (Y. Liu et al., 2016). The recent report of mobile colistin resistance gene (*mcr-1*) in South China (Y. Liu et al., 2016), and its immense impact on resistance to colistin clearly showed that the sequelae of colistin use were underestimated and demand changes in regulations (Walsh and Wu, 2016).

1.5 Colistin Resistance

Colistin resistance is mediated mainly by modification of lipid A part of lipopolysaccharide. Mechanisms of lipid A modification are different among different genera, but mostly share a common pathway where either phosphoethanolamine (PEtN), or 4-amino-4-deoxy-L-arabinose (L-ara4N) is added to lipid A (Figure 3). (Baron et al., 2016).

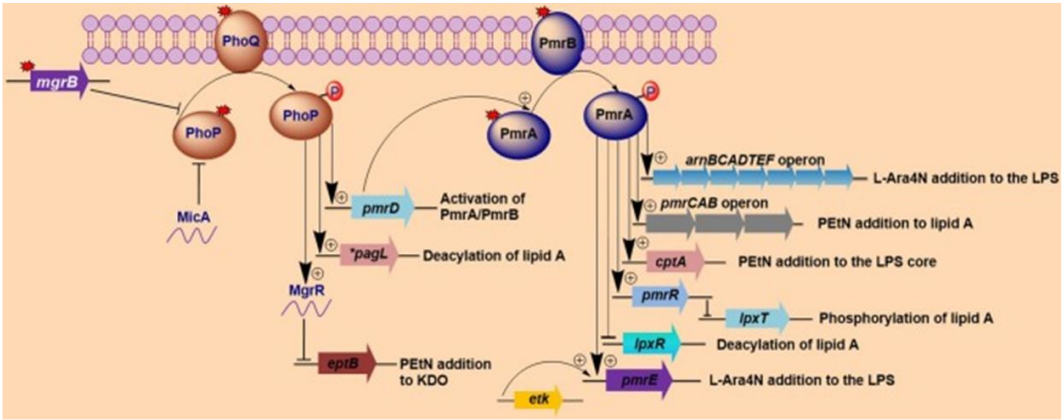


Figure 3: Mechanisms of resistance to colistin (Olaitan et al., 2014b)

Multiple chromosomal mutations have been identified to cause colistin resistance in *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae* (Arduino et al., 2012; Cannatelli et al., 2014a; Johansen et al., 2008). Recently a new mechanism of colistin resistance has been detected; a plasmid mediated mobile colistin resistance gene (*mcr-1*), encoding a phosphoethanolamine transferase. *mcr-1* was detected mainly in *E. coli* strains isolated from pigs and meat in China (Y. Liu et al., 2016).

1.5.1 Modification of lipid A: Addition of 4-amino-4-deoxy-L-arabinose

Modification of the lipopolysaccharide in the outer membrane of Gram-negative bacteria by addition of L-ara4N is a main mechanism of colistin resistance. L-ara4N has two targets in the LPS; the 4'-phosphate group of lipid A is the most common target and the second target being the 1'-phosphate group of the Kdo. Synthesis of L-ara4N and its transfer to lipid A is under the control of *arnBCADTEF* operon (also recognised as *pmrHFIJKLM* operon). L-ara4N is synthesised from uridine diphosphate (Olaitan et al., 2014b). This operon was identified in *K. pneumoniae, E. coli, Salmonella* species and *P. aeruginosa*; but is not present in *A. baumannii* (Olaitan et al., 2014b). Mutations in multiple two-component systems (TCS) lead to constitutive activation of this operon, these include: (a) *PmrAB* mutations in *Salmonella* species (Olaitan et al., 2015; Sun et al., 2009), *P. aeruginosa* (Lee and Ko, 2014), and *A. baumannii* (Bakour et al., 2015), with mutations affecting mainly the PmrB component of the system.

(b) Mutations in PhoP/Q system in *K. pneumoniae* (Cannatelli et al., 2014a; Jayol et al., 2015, 2014), where it is also associated with hetero-resistance to colistin; *P. aeruginosa* (Gutu et al., 2015, 2013; Owusu-Anim and Kwon, 2012; Schurek et al., 2009); and *Salmonella* spp. (Gunn and Miller, 1996). Whereas, *A. baumannii* lacks

PhoP/Q TCS (Baron et al., 2016). *P. aeruginosa* has in addition to PhoPQ, PmrAB other TCS; *parRS, colRS, cprRS* (Gutu et al., 2013; Muller et al., 2011).

The *mgrB* gene exerts negative regulation on PhoP/Q TCS, and its inactivation by various mutations (nonsense, missense, deletions or insertional inactivation) can result in constitutive activation of PhoPQ TCS, which in turn directly activates *arnBCADTEF*, independent of PmrD and PmrAB (Cannatelli et al., 2014b, 2013; Olaitan et al., 2014b; Park et al., 2015). *mgrB* inactivation is suggested to be the main mechanism of colistin resistance in *K. pneumoniae* and *Klebsiella oxytoca* (Bonura et al., 2015; Cannatelli et al., 2014b; Cheng et al., 2015; Gaibani et al., 2014; Giani et al., 2015; Olaitan et al., 2014a; Poirel et al., 2015). Insertion sequence IS*5* family is the most common cause of insertional inactivation of *mgrB,* followed by IS*1*. Insertions can occur in the promoter or in the coding sequence of *mgrB*, with insertion usually being between nucleotide 74 and 75 within *mgrB* (Bonura et al., 2015; Cannatelli et al., 2015, 2014b; Cheng et al., 2015; Olaitan et al., 2014a). Insertional inactivation of *mgrB* was found to be stable during stability assays for IS*5-like*, IS*3-like*, IS*Kpn14* and IS*102* representing different clones of clinical isolates (Cannatelli et al., 2015).

1.5.2 Modification of lipid A: Addition of Phosphoethanolamine

The *ept* genes modify lipid A or Kdo by addition of phosphoethanolamine. Phosphoethanolamine transferase is encoded by different genes, including *eptA (pmrC), eptB (pagC)* and *eptC (cptA)* (Olaitan et al., 2014b; Park et al., 2015; Qureshi et al., 2015). PmrA/B two-component system regulates PmrC. PmrA/B is regulated by PhoP/Q. PmrAB mutations were found to be the major mechanism of colistin resistance in *A. baumannii*, causing activation of PmrC with subsequent addition of

phosphoethanolamine to lipid A (Kim et al., 2015; Park et al., 2015; Qureshi et al., 2015).

1.5.3 Modification of Lipid A: A new TCS: crrAB

The *crrAB* TCS was identified in some *K. pneumoniae* strains and shown to be involved in mediating colistin resistance (Wright et al., 2015). *crr* in the name is for colistin resistance regulation. The system comprises a histidine kinase (*crrB*) and its response regulator (*crrA*). Its absence from some strains of *K. pneumoniae* is proposed to be due to substitution or insertion sequence mediated deletion in the locus. *crrAB* is not present in *E. coli* and *Salmonella enterica*. Wright et al reported missense mutation in *crrB* in two various strains of *K. pneumoniae* that has caused colistin resistance (Wright et al., 2015). Mutations in *crrB* was found to have two consequences: first, activation of two neighbouring genes; H239_3062 and H239_3059 (a proposed glycosyltransferase), and secondly, upregulation of *pmrCAB* but not PhoPQ. Activated *pmrC* can lead to LPS modification by addition of PEtN, while pmrH activation lead to addition of L-ara4N to LPS. The mechanism by which *crrAB* can lead to colistin resistance was further explained by a later study, in which different *K. pneumoniae* strains, with high level colistin resistance were found to have six various missense mutations in the *crrB* (Cheng et al., 2016). Mutations in *crrAB* lead to the activation of the pmrAB through the adjacent gene; H239_3062, which was subsequently named *crrC*. The *crrC* gene connects *crrAB* TCS to *pmrAB* TCS, which when activated lead to upregulation of *arnBCADTEF* and *pmrC* operon. H239_3059, despite being activated by *crrAB* is not implicated in colistin resistance (Cheng et al., 2016).

1.5.4 Other Modifications of Lipid A

In *Vibrio cholerae*, a newly identified TCS, VprAB, that mediates fixation of glycine in lipid A is implicated in the development of resistance to polymyxins. This TCS act through stimulation of a three-gene operon; *almEFG* expression, which result in transport of glycine across the cell membrane to lipid A (Herrera et al., 2014). *carRS*, a TCS that is recognised as a negative regulator of biofilm formation; is found to increase the expression of *almEFG* by binding directly to *almEFG* operon (Bilecen et al., 2015). Recent studies in *E. coli* showed that PmrD is activated in low Mg^{+2} levels and can result in the addition of L-ara4N and PEtN to lipid A. PmrD activation is shown to be essential for polymyxin resistance in *E. coli* and other bacteria. PmrD appear to act independent of PhoPQ system, suggesting the presence of an unknown system capable of separately activating PmrD under low Mg^{+2} levels and hence causing lipid A modifications (Rubin et al., 2015). $LpxL_{Ab}$ and $LpxM_{Ab}$ are putative acyltransferases identified in *A. baumannii*, which are implicated in lipid A acylation independent of PagP pathway (Boll et al., 2015). The observation of hepta-acylated lipid A in *A. baumanii*, lacking PagP homologue, has led to the identification of $LpxL_{Ab}$ and $LpxM_{Ab}$. $LpxL_{Ab}$ adds one lauroyl acyl chain to lipid A, while $LpxM_{Ab}$ adds two. High susceptibility to polymyxins was observed in $LpxM_{Ab}$ mutant *A. baumanii*, which stresses its role in polymyxin resistance (Boll et al., 2015). Alterations in another two-component system; *mrgRS* causing its constitutive activation were linked to high level colistin resistance in *Burkholderia pseudomallei* (Kumar et al., 2016), but whether to attribute colistin resistance to the presence of *mrgRS* TCS or to mutations in *mrgS* is not yet clear.

1.5.5 Colistin Resistance Conferred by Beta Lactam Resistance

Insertion of a carbapenem gene carrying insertion sequence in the *mgrB* gene was identified in pan-drug resistant *K. pneumoniae* ST147 strains. IS*Ecp1* carrying $bla_{\text{OXA-181}}$ insertion into *mgrB* caused both carbapenem and colistin resistance (Sonnevend et al., 2017; Zowawi et al., 2015). In a second report, IS*Ecp1* carrying $bla_{\text{CTX-M-15}}$ insertion in *mgrB* resulted in both cephalosporin and colistin resistance (Jayol et al., 2016).

1.5.6 Other LPS Modifications

Various genes in LPS synthesis pathway has been found to contribute to the development of polymyxin resistance. Mutations in an O-antigen acetyltransferase encoding gene, decreases colistin resistance due to impairment of arabinose fixation. Other mutations in O-antigen synthesis genes that can result in complete loss of O-antigen like *rfaH* gene in *Yersinia entercolitica* (Leskinen et al., 2015), *naxD* in *Francisella spp*. (Llewellyn et al., 2012), reduces resistance to polymyxin B. Also, mutants of *K. pneumoniae* that have lost O-antigen showed decreased resistance to polymyxins (Campos et al., 2004). Regulators of lipid A biosynthesis can also affect resistance to polymyxins. *ramA* is a newly identified, universal lipid A biosynthesis regulator, that regulate expression of more than 68 genes involved in lipid A synthesis, including *lpxC, lpxL*, and *lpxO*. Several *Enterobacteriaceae* were found to have this regulator such as *K. pneumoniae, Salmonella spp, Citrobacter spp, and Enterobacter spp*. *ramA* locus contains three genes; *ramA, romA* and *ramR*; *ramR* is found to be the repressor of *ramA* and *romA*. *ramA* could increase resistance to polymyxins through unknown pathways, as survival assays showed that *K. pneumoniae* with a mutated

ramA are less susceptible to colistin (De Majumdar et al., 2015). Glycosylation of the phosphate group of lipid A with galactosamine is another LPS modification associated with colistin resistance that has been identified in *A. baumannii* (Leskinen et al., 2015).

1.5.7 Loss of Lipopolysaccharide

This mechanism of polymyxin resistance has been identified in *A. baumannii* (Selasi et al., 2015). Complete loss of lipopolysaccharide occurs because of mutations in the first three genes implicated in lipid A biosynthesis; *lpxA, lpxB,* and *lpxC*. Mutation in any of these genes can lead to termination of lipid A synthesis and stops LPS formation (Moffatt et al., 2010). Complete loss of lipopolysaccharide can result also from mutations in LptD, which is an outer membrane protein involved in the translocation of LPS across the periplasm and the outer membrane (Bojkovic et al., 2016; Moffatt et al., 2010). Comparing metabolites of colistin susceptible *A. baumannii* and colistin resistant *A. baumannii* lacking LPS showed other amino acids and carbohydrate metabolites disturbances and depletion of metabolites of peptidoglycan synthesis (Maifiah et al., 2016; Wand et al., 2015). LPS-deficient *A. baumannii* is found to be more susceptible to azithromycin, vancomycin and rifampicin (Garcia-Quintanilla et al., 2015). Loss of the LPS-barrier function could lead to increased passive diffusion to the hydrophobic antibiotics, azithromycin and rifampicin and hence increased susceptibility to them. Colistin-resistant *A. baumannii* showed reduced virulence. LPS-deficient *A. baumannii* showed high level of expression of genes implicated in lipoprotein biosynthesis and transport, carbapenem resistance, and membrane biosynthesis and transport. Modifications in peptidoglycan biosynthesis could explain the increased susceptibility to vancomycin (Vila-Farrés et al., 2015).

1.5.8 Efflux Pump Mechanisms

Efflux pumps might also contribute to colistin resistance. Many efflux pumps or efflux pump regulators have been associated with colistin resistance but in general, activation of these pumps results in increased resistance to several antibiotics. Multiple efflux pumps were identified in several species, including Sap proteins (sensitive antimicrobial peptides), BrIR, KpnEF, and AcrAB-TolC (Chambers and Sauer, 2013; Srinivasan and Rajamohan, 2013; Warner and Levy, 2010). Sap proteins are encoded by SapABCDF operon, these proteins are found to be composed of five proteins (Parra-Lopez et al., 1993). Sap proteins were found to be necessary for resistance to some antimicrobial peptides like; melittin and protamine, but for others such as defensins it is not efficient. But in *Proteus mirabilis,* Sap operon seems to play a role in colistin resistance, as its mutation has resulted in increased susceptibility to polymyxin B (McCoy et al., 2001). At physiological levels of NaCl, *A. baumannii* can upregulate expression of eighteen presumptive efflux pumps with resultant reduced susceptibility to polymyxins, including CarO porin (Hood et al., 2010). MexXY-OprM efflux pump in *P. aeruginosa* is implicated in resistance to fluroquinolones, cefepime and aminoglycosides (Muller et al., 2011). MexXY expression is regulated by mexZ, but MexXY expression can be regulated independently by parRS two component system. Mutations and subinhibitory concentrations of antimicrobial peptides can induce activation of parRS TCS. This activation has two sequelae; first, upregulation of mexXY and downregulation of oprD expression resulting in carbapenem resistance and secondly, upregulation of pmrAB TCS and overexpression of arnBCADTEF-ugd operon. BrIR, a member of MerR regulator family, is an activator of a multi-drug efflux pump. BrIR binding to the oprH promoter of the oprH-PhoPQ operon can

downregulate the PhoPQ TCS, which results in increased susceptibility to colistin as a result of reduced transcription of the PmrAB and arnT systems (Chambers and Sauer, 2013). KpnEF efflux pump is a member of the Cpx regulon that is involved in capsule synthesis and antibiotic resistance to colistin, rifampicin, erythromycin and ceftriaxone in *K. pneumoniae* (Srinivasan and Rajamohan, 2013). KpnEF, EbrAB in *Bacillus subtilis* and AbeS in *A. baumannii* are members of the small multi-drug resistance protein family (SMR). Lastly, AcrAB-TolC transporter, which belongs to the family of RND (resistance -nodulation-cell division), that is known to effectively pump drugs out of the bacterial cytoplasm (Warner and Levy, 2010). AcrAB mutant *E. coli* showed 8-fold increase in polymyxin susceptibility, while TolC mutants showed an increase in polymyxin susceptibility of 10,000-fold, which could be explained by colistin being a substrate of this efflux pump. Moreover, AcrAB-TolC expression is PhoPQ dependent (X.Z Li et al., 2015). Inhibition of the efflux pump with CCCP (carbonyl cyanide 3-chlorophennylhydrazone) in *K. pneumoniae*, *P. aeruginosa*, *A. baumannii* and *Stenotrophomonas maltophilia*, showed the involvement of the pump in colistin resistance (Ni et al., 2016).

1.5.9 Capsule Formation

Capsule contribution to polymyxin resistance has been characterized in several naturally polymyxin resistant bacteria such as *Neisseria meningitidis* and *Campylobacter jejuni*. A 10-fold increase in polymyxin B susceptibility is noticed in *N. meningitidis* upon loss of capsule only or loss of capsule and lipopolysaccharide (Spinosa et al., 2007). In *C. jejuni*, an increased susceptibility to polymyxin B is noticed after truncation of sialic acid; a sugar found in LPS (Keo et al., 2011). Additionally, *K. pneumoniae* isolates with several capsule layers showed more colistin

resistance in comparison to monolayer isolates (Formosa et al., 2015). Furthermore, purified capsular polysaccharides from *K. pneumoniae*, *Streptococcus pneumoniae* and *P. aeruginosa* increased polymyxin B resistance in unencapsulated strains of *K. pneumoniae*, *E. coli* and *P. aeruginosa* (Llobet et al., 2008). Also, capsule formation regulators seem to be implicated in colistin resistance through activation of KpnEF efflux pump in case of Cpx (conjugative pilus expression) and by regulation of PhoPQ by RCS (regulator of capsule synthesis) regulon (Olaitan et al., 2014b).

1.6 Mobile Colistin Resistance Genes

Carrying mobile colistin resistance gene *mcr-1*, provides in addition to resistance the tool for its transfer, in contrary to chromosomal mediated colistin resistance (Y. Liu et al., 2016). To complicate things further *mcr-1* is found to be a developing gene as 11 more versions were reported from different countries (*mcr-1.2*, *mcr-1.3*, up to *mcr-1.12*) (Sun et al., 2018). *mcr-1* being such a dynamic gene might indicate the presence of a continuous selective pressure in a wide host range (Sun et al., 2018). Ten species of *Enterobacteriaceae* have been found to carry the *mcr-1* gene, these include *E. coli* (Arcilla et al., 2016; Y. Liu et al., 2016; Olaitan et al., 2016a), *K. pneumoniae* (Y. Liu et al., 2016; Stoesser et al., 2016), *Salmonella* (Campos et al., 2016; Figueiredo et al., 2016; Webb et al., 2016), *K. aerogenes* (Zeng et al., 2016), *Enterobacter cloacae* (Zeng et al., 2016), *Cronobacter sakazakii* (B. Liu et al., 2017), *Shigella sonnei* (Pham Thanh et al., 2016), *Kluyvera species* (Zhao and Zong, 2016), *Citrobacter species* (X.P. Li et al., 2017; Sennati et al., 2017) and *Raoultella ornithinolytica* (Luo et al., 2017). This wide distribution of *mcr-1* carrying species among *Enterobacteriaceae* is also associated with different host and environmental sources that include humans (Y. Wang et al., 2017; Ye et al., 2016; X. Zhang et al.,

2016), animals (Chiou et al., 2017; Duggett et al., 2017; Q. Wang et al., 2017), food (Doumith et al., 2016; Grami et al., 2016; Luo et al., 2017; Monte et al., 2017) and natural environments (Fernandes et al., 2017; Guenther et al., 2017; Trung et al., 2017).

Following the detection of *mcr-1*, multiple new variants of *mcr-1* have been identified in different countries. *mcr-2* was identified in Belgium by Xavier *et al.* in *E. coli* that has only 80% identity to *mcr-1* (Xavier et al., 2016b). *mcr-2* was found to be carried on an IncX4 plasmid; it has been recently detected also in China in pigs and poultry (J. Zhang et al., 2018). There is no biochemical proof of the progenitors of *mcr-1* and *mcr-2* (Sun et al., 2018), but use of genetic and bioinformatic tools shows that *mcr-1/2* genes have possibly originated from *Moraxella* species (Kieffer et al., 2017; Poirel et al., 2017a).

Six more *mcr* genes were subsequently identified in *E.coli* and/or *Salmonella;* these are: *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli et al., 2017), *mcr-5* (Borowiak et al., 2017), *mcr-6.1* (AbuOun et al., 2018), *mcr-7.1* (Yang et al., 2018), and *mcr-8* (X. Wang et al., 2018). They are phylogenetically different from *mcr-1/2* (Sun et al., 2018). A new *mcr-9* gene has also been identified (preprint manuscript) (Carroll et al., 2019).

mcr-3 has been identified in *E. coli* isolated from animal and human sources. *mcr-3* is genetically diverse as multiple variants with point substitutions have been identified (Litrup et al., 2017; L. Liu et al., 2017; Roer et al., 2017). *mcr-4* is also found to have a variant, *mcr-4.2* that might co-occur with *mcr-3.7*. However, mere co-existence of *mcr-4.2* and *mcr-3.7* does not entail increased resistance to polymyxins (Teo et al., 2018). *mcr-5* has been first detected in a collection of *Salmonella enterica* subsp. *enterica* serovar Paratyphi B, where it was found to be part of a transposon of

the Tn3 family in ColE-type plasmids; with chromosomal location of the transposon in one isolate (Borowiak et al., 2017). *mcr-5* has recently been identified in *Aeromonas hydrophila* isolated from pigs in China, which imply the ability of *mcr-5* to disseminate between different bacterial species and genera (Ma et al., 2018).

1.6.1 Mobile Colistin Resistance Genes: Different Plasmids and Genetic Contexts

The first reported *mcr-1* gene was found to be carried on IncI2 type plasmid pHNSHP45 (Y. Liu et al., 2016), subsequently more than ten plasmid types were found to harbor *mcr-1* gene; IncI2, IncX4 and IncHI2 being the main plasmid carriers of *mcr-1* (Sun et al., 2018). Other plasmid types include IncP (F. Zhao et al., 2017), IncIH1 (Zurfluh et al., 2016), IncFII (Xavier et al., 2016a), IncFI (Zeng et al., 2016), IncFIB (Nordmann et al., 2016), F18:A-:B+ (McGann et al., 2016), IncY (C. Zhang et al., 2017), IncK (Dona et al., 2017) and a phage-like plasmid (R. Li et al., 2017a). Genomes of all these plasmids were found to be highly comparable despite being found in different species, isolated from wide host range and being collected from various locations. This could indicate that these plasmids have been extensively exchanged between various species of *Enterobacteriaceae* (Sun et al., 2018). IncX4 plasmid type is one of the main types carrying *mcr-1* (Sun et al., 2018). *Enterobacteriaceae* are the main host of IncX plasmids; IncX plasmids were detected in *Enterobacteriaceae* collected before the use of medical antibiotics (Datta and Hughes, 1983). *mcr-1* harboring IncX4 plasmids were found to have closely related backbones by comparative genomics (Sun et al., 2017a). IncX4 type plasmids are the main contributors to the global spread of mcr-1 gene being highly self-transferable (Fernandes et al., 2016a). *mcr-1* was also found to be carried on hybrid plasmids such as IncX3-IncX4 hybrid (Sun et al., 2016), and IncI2-IncFIB (Q. Wang et al., 2017).

Genetic recombination between *mcr-1* harboring plasmids could explain the presence of *mcr-1* on hybrid plasmids (Sun et al., 2018). One *E. coli* strain isolated from a pig in China was found to harbor two *mcr-1* carrying plasmids of IncI2 and IncX4 replicon type (Q. Wang et al., 2017). Such complex spread of *mcr-1* carrying plasmids could be a result of increased use of colistin in animal production causing high selective pressure (Sun et al., 2018). *mcr-1* could also be transposed into the chromosome from plasmids (Zhou et al., 2017). Other *mcr* genes were also found to be carried by varied plasmid replicon types ; unlike *mcr-1, mcr-2* is harbored only by IncX4 plasmid type (Xavier et al., 2016b), where it is found to be located between two insertion sequences of IS*Ec69* (Partridge, 2017; Sun et al., 2017b). On the other hand, *mcr-3* is carried by IncHI2 plasmid type, but its genetic variant mcr3.7 which has three-point substitutions is harbored by a self-transferable IncP type plasmid where it is located within an TnAs3-like transposon (L. Liu et al., 2017). *mcr-1* was found to coexist with *mcr-3* in *Salmonella* (Litrup et al., 2017) and *E. coli* (Hernandez et al., 2017) and with *mcr-5* in *E. coli* (Fukuda et al., 2018). Though uncommon, *mcr-1* also co-occurred with ESBL (Haenni et al., 2016; H. Zhang et al., 2016; Sonnevend et al., 2016), NDM-1 (Delgado-Blas et al., 2016; Sonnevend et al., 2016), NDM-5 (Du et al., 2016), and NDM-9 (Yao et al., 2016). In one study, an *E. coli* clinical isolate was found to carry *mcr-3, mcr-1* and $bla_{\text{NDM-5}}$ on three different plasmids (L. Liu et al., 2017). The newly detected mcr-*4* (Carattoli et al., 2017) and *mcr-5* (Borowiak et al., 2017) were found to be carried by ColE type plasmids. Being harbored by several plasmid replicon types, *mcr-1* is also found to have complex genetic contexts; mostly *mcr-1* gene is located within mcr-1 cassette, a 2600 base pair fragment, where it is followed downstream by a hypothetical protein proposed to have a phosphoesterase activity (Poirel et al., 2016). In the first reported *mcr-1* harboring plasmid, an insertion sequence, IS*Apl1* was found

to be located directly upstream of *mcr-1* gene, which is believed to be the mobilizing tool of *mcr-1*. Being part of IS30 type, IS*Apl1* can form circular intermediates which help translocate antibiotic resistance (Tegetmeyer et al., 2008) and consequently two to three base pairs will be duplicated at the new insertion site (Mahillon and Chandler, 1998). It is also possible that IS*Apl1* flank *mcr-1* cassette at both sides forming a transposon, Tn*6330*, thought to be an important translocation mechanism that has helped transposition of *mcr-1* into various plasmid types (R. Li et al., 2017b). It was shown in vitro that this transposon could locate in A-T rich DNA region and cause duplication of two base pairs (Poirel et al., 2017b). *mcr-2* is flanked by two insertion sequences IS*Ec69*, forming a transposon which presumably plays an important role in mcr-2 mobilization through circular intermediate formation (Partridge, 2017). In *mcr-3* carrying plasmids, *mcr-3* gene is found to be downstream of a truncated transposon Tn*As2* (L. Liu et al., 2017; Yin et al., 2017). In ColE10 plasmid, *mcr-4* gene is found downstream of the insertion sequence IS*5* (Carattoli et al., 2017). *mcr-5* is found within a Tn*3* transposon in ColE type plasmid (Borowiak et al., 2017). Presence of mcr genes within mobile genetic elements enhances the spread of *mcr* genes by horizontal gene transfer, which could be further escalated by recombination events between mobile genetic elements (Sun et al., 2018).

1.6.2 MCR-1 and MCR-2 Mechanism of Action

Colistin attaches electrostatically to the negatively charged lipopolysaccharide layer of the bacterial cell membrane. Changes in LPS structure can reduce the negative charge resulting in colistin resistance (Dixon and Chopra, 1986). As discussed earlier, attachment of L-aminoarabinose and phosphoethanolamine to lipid A are the two main modifications that usually cause intrinsic resistance to polymyxins (Petrou et al., 2016;

Tamayo et al., 2002). EptA, an alkaline phosphatase of *Neisseria* is the most investigated lipid A phosphoethanolamine transferase (Cox et al., 2003). Using phosphatidylethanolamine (PE) as its substrate, the enzyme catalyses attachment of phosphoethanolamine to lipid A (Kandler et al., 2014; Wanty et al., 2013). MCR-1 and MCR-2 are annotated as lipid A-4'-PEA transferases (Sun et al., 2018). MCR-1 and MCR2 structures were modeled from neisserial EptA X-ray structure (Anandan et al., 2017). It has been predicted that, MCR-1 and MCR-2 are integral membrane proteins (Gao et al., 2016; Sun et al., 2017b). MCR-1 and/or MCR-2 has two domains; N terminus which is the TM domain with five helices and a C terminus which forms the catalytic domain facing the periplasmic space (Coates et al., 2017; Stojanoski et al., 2016). It is speculated that like neisserial EptA, MCR-1 and MCR-2 catalyze phosphoethanolamine transfer to lipid A through a two-step reaction: first; hydrolysis of PE into diacyl glycerol and phosphoethanolamine which remain bound to MCR-1/2. Second; phosphoethanolamine transfer to Kdo2 of lipid A (Wanty et al., 2013). In vivo activity of MCR-1/2 in catalyzing the transfer of phosphoethanolamine from its substrate PE to LPS lipid A has been demonstrated by MALDI-TOF mass spectrometry (Sun et al., 2018).

1.6.3 Structure and Function of MCR-1, MCR-2

MCR-1, and MCR-2 have low identity of 34.4% (Y. Liu et al., 2016) and 34.5% (Xavier et al., 2016b) respectively to the neisserial EptA; with identity of the TM region of MCR-1 and MCR-2 being as low as 28.6% and 26.1% respectively in comparison to neisserial EptA. The low identity is further supported by phylogenic studies showing that MCR-1 and MCR-2 are evolutionarily distinct from neisserial EptA. On the other hand, EptA (Anandan et al., 2017; Wanty et al., 2013), MCR-1

(Gao et al., 2016), MCR-2 (Sun et al., 2017b) were found to share a conservative zinc binding pocket which could serve as the enzyme catalytic site and is necessary for phenotypic polymyxin resistance.

A nucleophilic threonine 285 residue is proposed to coordinate with the zinc ion in the MCR-1 active site, this has been inferred from studying the structural features of the soluble domain of MCR-1 enzyme (Gao et al., 2016; Hinchliffe et al., 2017; Hu et al., 2016; Ma et al., 2016; Stojanoski et al., 2016). Later the crystal structure of MCR-1 in complex with an analogue to its natural substrate was presented (P. Wei et al., 2018), raising the possibility that MCR-1 has developed two substrate binding sites. Not much is known about the TM domain of MCR-1 or MCR-2. Study of the crystal structure of *Neisseria meningitidis* EptA characterized the TM domain to have charged residues that might be involved in substrate binding (Anandan et al., 2017). Neisserial EptA structure was used to model a full-length structure of MCR-1, which might help identify the phosphoethanolamine binding site (Xu et al., 2018a). To examine and compare the catalytic domains of EptA, MCR-1 and MCR-2, site-specific mutagenesis was used to generate point mutants of these enzymes (Sun et al., 2017b). MCR-1 was found to confer more resistance to colistin than EptA (Gao et al., 2016). On the other hand, MCR-2, provided increased growth advantage in the presence of colistin compared to MCR-1 (Sun et al., 2017b). Recipients of the wild-type of *mcr-1* or *mcr-2* were resistant to colistin concentration up to 16 µg/ml, whereas recipients of the mutants with inactivated catalytic domain showed no significant growth with over 0.5 µg/ml colistin concentration. Minimum inhibitory concentration measurement showed comparable results, with the wild-type EptA expressing *E. coli* having colistin MIC of 2 µg/ml, while wild-type MCR-1, MCR-2 expressing *E. coli* having colistin

MIC of 4 µg/ml (Sun et al., 2017b). On the other hand, the *E. coli* strains with the catalytic point mutations in MCR-1 or MCR-2 showed a colistin MIC of 0.25 µg/ml. Moreover, MALDI-TOF MS clearly showed that all of the *E.coli* strains with point mutations in MCR-1, MCR-2 catalytic domains were unable to accomplish addition of phosphoethanolamine to 4'lipid A, which emphasizes that all the residues in the catalytic domain (E246, T285, H395, D465, and H466) are essential for its enzymatic action and subsequently colistin resistance (Sun et al., 2017b). The TM domain ensure the right positioning of the periplasmic part of MCR-1 and MCR-2 and is integral to the complete function of MCR-1 and MCR-2, as genetic deletion of the TM domain lead to complete loss of MCR-1 and MCR-2 function (Gao et al., 2016; Sun et al., 2017b). Replacing the TM region of MCR-1 with that of MCR-2 or vice versa did not change the enzymatic activity of the new chimeric enzyme compared to the original enzymes; this functional interchangeability might indicate functional convergence and convergent evolution (Sun et al., 2017b). However functional exchangeability is not possible between the TM domains of MCR-1, MCR-2 and the corresponding domain of EptA, suggesting two different origins of MCR-1/2 and EptA (Sun et al., 2017b), further backing the proposal that *mcr-1* (AbuOun et al., 2017; Kieffer et al., 2017) and *mcr-2* (Kieffer et al., 2017; Poirel et al., 2017a) might have originated from *Moraxella* species.

1.6.4 Mechanism of Action of MCR-3

mcr-3 gene is evolving rapidly, with more *mcr-3* variants being detected including *mcr3.2* (Bi et al., 2017), *mcr3.3* (Bi et al., 2017), *mcr3.4* (Roer et al., 2017), *mcr3.5* (Haenni et al., 2018), and *mcr3.7* (L. Liu et al., 2017). Phylogenetic analysis showed MCR-3 to be distinct in evolution from MCR-1, but closely cluster with MCR-

like proteins that are chromosomally encoded in some species of *Aeromonas* (Ling et al., 2017; Xiaoming Wang et al., 2017). MCR-3 being almost identical to MCR-1 (Gao et al., 2016), MCR-2 (Sun et al., 2017b), and EptA (Cox et al., 2003) is an integral membrane protein with five N terminal helices (Xu et al., 2018b). Its mass is 61.3 kDa. The structure of MCR-3 is composed of two domains; N-terminal TM domain and a periplasmic C-terminal catalytic domain (Xu et al., 2018b). The catalytic domain hydrolase fold is composed of ten alpha helices and seven beta sheets, while the TM domain consists of six alpha helices. The catalytic domain connects to the TM domain by four short periplasmic loops, a bridge helix and a long-coiled loop (Xu et al., 2018b). Sequence alignment showed conserved residues between MCR-1, MCR-2, EptA, and MCR-3, which include five potential zinc binding residues (E238, T277, H375, D450 and H451) and seven residues (N103, T107, E111, G322, K325, H380, and H463) possibly involved in substrate binding, in consistence with evidence from EptA (Anandan et al., 2017), and MCR-1 (Xu et al., 2018a). More biochemical analysis is needed to determine the role of these residues in the catalytic activity of MCR-3. Experimental evidence shows that MCR-3 enzymatically catalyses the removal of phosphoethanolamine from the lipid substrate and the modification of lipid A by the phosphoethanolamine (PEA) (Xu et al., 2018b). From this evidence and the similarity of the Thr280-PEA adduct seen in EptA (Anandan et al., 2017; Wanty et al., 2013) and its counterparts involved in alkaline phosphatase-like phosphate transferases (Stec et al., 2000), it can be hypothesized that MCR-3 first bind PE releasing diacyl glycerol, which results in MCR-3 bound PEA adduct. Transfer of PEA from this adduct to the 1(4′)-phosphate position of lipid A in the second part of the reaction then generates PPEA-4′- lipid A. Domain swapping analysis, making hybrid proteins where either the catalytic or the TM domain of MCR-3 is swapped for that of

MCR-1 or EptA, showed that hybrid proteins confers no colistin resistance. The incompatibility of the MCR-3 domain with those of MCR-1/2 and EptA might indicate that MCR-3 is evolutionary distant from them. Multiple sequence alignment and molecular docking showed the substrate binding cavity of MCR-3 to be composed of 12 residues, five of which (E238, T277, H375, D450 and H451) are likely involved in zinc binding and seven (N103, T107, E111, G322, K325, H380 and H463) involved in substrate recognition. Apart from glycine (G322) in MCR-3 in place of the conserved serine in EptA, MCR-1 and MCR-2 (S325, S330 and S328, respectively), all other eleven residues are highly conserved between them. Generating alanine mutants in these 12 residues showed that all the five residues involved in zinc binding are important in enzymatic activity and none of their mutant's support growth in the presence of colistin. While mutations in N103A and T107A in the substrate binding domain results in proteins retaining partial activity; mutation in G322A results in a completely functional protein. Mutations in the remaining four residues lead to completely nonfunctional proteins (Xu et al., 2018b). Colistin is known to activate a downstream hydroxyl radical mediated cellular death pathway (Dong et al., 2015; Sampson et al., 2012). Presence of MCR-1/2 and its progenitor ICR-Mo is found to prevent the formation of reactive oxygen species (W. Wei et al., 2018). MCR-3 expression is suggested to prevent reactive oxygen species production *in vivo* by preventing colistin entry into the cells. Expression of MCR-3 is found to negatively affect bacterial growth and viability (Xu et al., 2018b).

1.6.5 Mechanism of Action of MCR-4

The MCR-4 protein showed 82–99% identity to phosphoethanolamine transferases found in *Shewanella* species suggesting that *mcr-4* might have originated

in *Shewanella spp*. On the other hand, MCR-4 protein showed only 34.0%, 35.0% and 49.0% amino acid sequence identity to MCR-1, MCR-2 and MCR-3 respectively (Carattoli et al., 2017). The plasmid borne *mcr-4.3* variant, which does not confer phenotypic colistin resistance was found to be 100% identical to a chromosomal DNA fragment (~1.7 kb) of *S. frigidimarina* NCIMB 400 (Accession entry: CP000447) that covers a putative phosphoethanolamine transferase-encoding gene and its predicted promoter of 59 bp. *mcr-4.3* is supposed to be the inactive form of MCR-4 predominantly existing in *S. frigidimarina*; as its two revertant mutants were able to cause phenotypic colistin resistance. It is believed that functional MCR-4 enzyme has progressed from the silenced MCR-4.3 present in *S. frigidimarina* (H. Zhang et al., 2019). MCR-4 is supposed to have two distinct domains linked by a flexible linker. MCR-4 is anchored to the inner membrane at the N terminus by five α-helix TM domain that is connected to periplasmic domain with the catalytic activity that accomplishes addition of phosphoethanolamine to lipid A in a two-step reaction: first, MCR-4 is supposed to cleave phosphoethanolamine group from its lipid precursor, and covalently attaches it to the enzyme; second, the phosphoethanolamine group is transferred to lipid A generating PPEA-4'-lipid A (H. Zhang et al., 2019). Molecular docking studies showed the head group of the PE substrate has docked well into MCR-4; a zinc atom was found above the substrate binding pocket, with five metal-interacting residues being predicted including E240, T278, H377, D452, and H453. Mutations in the zinc binding residues resulted in completely nonfunctional enzyme as were mutants of the substrate binding residues. Domain swabbing showed MCR-4 to be incompatible with MCR-1 and MCR-3.

This shows that MCR enzymes function through similar mechanism despite being evolutionary different (H. Zhang et al., 2019).

1.7 The Epidemiology of the *mcr-1* Gene

1.7.1 Worldwide Distribution of *mcr-1* in Animals

The *mcr-1* has been detected in five continents in more than 40 regions (XiuNa Wang et al., 2017) just few years since its first identification in 2015 in China (Y. Liu et al., 2016). This clearly shows that *mcr-1* is an efficient transfer tool for polymyxin resistance (XiuNa Wang et al., 2017). Carriage rate of *mcr-1* in *Enterobacteriaceae* varies according to where and when samples are collected and the source of the sample. *mcr-1* has been detected in chicken isolates from 1980s in China; retrospective studies have shown six times increase in the carriage rate of *mcr-1* in 2014 compared to the rate in 2009 (Z. Shen et al., 2016). This clearly corresponds with the start of using colistin in 2000s and China being one of the top consumers of colistin in 2015 (Y. Liu et al., 2016). Likewise, increase in the carriage rate is reported from Vietnam (Trung et al., 2017). On the other hand, in countries or regions where colistin is not used to promote growth, *mcr-1* carriage rate in food animals is very low; in the USA, with prevalence in food animals at slaughter is 0.1% (Meinersmann et al., 2017) and Europe, with a prevalence of 3.8% in Germany (Irrgang et al., 2016).

However, in two European countries, Portugal (Nicolas et al., 2017) and Italy (Curcio et al., 2017), where colistin is used to treat postweaning diarrhea or used as metaphylaxis, *mcr-1* carriage rate is found to be very high (72.5-98%).

Detection of *mcr-1* in migratory birds in Asia (Mohsin et al., 2016), Europe (Ruzauskas and Vaskeviciute, 2016) and South America (Liakopoulos et al., 2016)

might have helped in the worldwide spread of *mcr-1* (Fierer et al., 2012; Sun et al., 2018). In Brazil, *mcr-1* was identified in Magellanic penguins (Sellera et al., 2017). *mcr-1* was also detected from pet animals, in one study, an *E. coli* strain isolated from a pet cat was found to harbor both *mcr-1* and bla_{NDM-5} (Sun et al., 2016). Moreover, transfer of *mcr-1* carrying *E. coli* between pet animals and humans has been documented (Lei et al., 2017), which could facilitate the spread of *mcr-1*. It has been also detected from boot swabs, dog faeces, stable flies and manure in the environment surrounding farms with *mcr-1* -positive swine herds in Germany (Guenther et al., 2017).

1.7.2 Worldwide Distribution of *mcr-1* Positive *Enterobacteriaceae* in Humans

In more than 40 countries *Enterobacteriaceae* is found to be asymptomatically carried by healthy, as well as, ill people, either in sporadic or in endemic cases (Sun et al., 2018). The first report of human carriage rate of *mcr-1* harboring *Enterobacteriaceae* came from screening of inpatients in China and was found to be 1%. (Y. Liu et al., 2016). Subsequently, *mcr-1* was detected in sporadic cases in other countries like India (Bernasconi et al., 2016), Singapore (Teo et al., 2016), Italy (Corbella et al., 2017), Algeria (Berrazeg et al., 2016; Yanat et al., 2016) and USA (Mediavilla et al., 2016). However, in Spain (Ortiz de la Tabla et al., 2017) and in Taiwan (Kuo et al., 2016) *mcr-1* was found to be carried by members of the epidemic *E. coli* ST131 clone. Generally, worldwide *mcr-1* carriage rate in humans, both in healthy carriers and in patients, is 1%, which is much lower than in animals (Sun et al., 2018). But using enriched culture in screening children intestinal flora increased detection rate to 9.8% (Y. Hu et al., 2017). Two big studies examined the prevalence of *mcr-1* positive *Enterobacteriaceae* in inpatients with blood stream infections,

concluded that *E. coli* is the main *mcr-1* carrying species (1%), with only 0.2% of *K. pneumoniae* being positive for *mcr-1* (Quan et al., 2017; Y. Wang et al., 2017). Studying the contexts of acquiring an *mcr-1* related infection, as well as, the complications of such infection is worthy, as human isolation of *mcr-1* carrying isolates is still low, and isolates are usually susceptible to other antibiotics (Sun et al., 2018). Three factors were found to be associated with *mcr-1* positive *Enterobacteriaceae* infections; immunosuppression, male gender and previous antibiotic exposure, specifically carbapenems and quinolones. On the other hand, colon colonization of inpatients correlates with use of antibiotics before being hospitalized (Y. Wang et al., 2017). Intestinal flora carriage rate is found to be 3.5% in provincial regions in China; all *mcr-1* positive *E. coli* isolates in this study showed phenotypic polymyxin resistance, and all were extended spectrum beta lactamase producers (Bi et al., 2017). An epidemic of *mcr-1* positive *Enterobacteriaceae* infections occurred in a hospital in China and resulted in two deaths out of six patients affected. Species identification showed that five of the strains were *K. pneumoniae* of the same sequence type (ST11). The sixth strain was found to be *E. coli*. In all strains *mcr-1* was carried on IncX4 type plasmids (Tian et al., 2017), suggesting the possibility that colistin use is not a prerequisite for a very fast dissemination of *mcr-1* in hospitals. Sporadic cases of blood stream infections caused by *mcr-1* positive *Enterobacteriaceae* were detected in Denmark (Hasman et al., 2015), Italy (Corbella et al., 2017), Brazil (Rocha et al., 2017) and the USA (Mediavilla et al., 2016), where *mcr-1* was found to coexist with NDM-5 in an *E. coli* isolated from a patient with urinary tract infection. Also, an *E. coli* strain carrying *mcr-1* on IncX4 plasmid isolated from human blood stream infection was found to be closely related to an avian pathogenic *E. coli* (Macesic et al., 2017).

1.7.3 Epidemiology of *mcr-1* in Countries of the Gulf Cooperation Council

1.7.3.1 Humans

The first reported *mcr-1* gene carrying isolates from the GCC countries were described in 2016 by Sonnevend *et al.* (Sonnevend et al., 2016). In a collection of seventy-five colistin resistant *E. coli* and *K. pneumoniae* isolated from patients in 2012-2015 in the UAE, Oman, Bahrain, Kuwait and the Kingdom of Saudi Arabia (KSA), four *E. coli* strains were found to carry *mcr-1* gene. Two from Bahrain (BA76, BA77), one from the KSA (SA26) and one from the UAE (ABC149). All four strains were found to be resistant to 3rd generation cephalosporins, tetracycline, trimethoprim/sulfamethoxazole and gentamicin, with variable resistance to amikacin, carbapenems, chloramphenicol and ciprofloxacin. No clonal relation was found between the isolates, and they belonged to widely distributed sequence types. one strain belonged to the *E. coli* ST131 (ABC149) associated with multi-drug resistance phenotype. All isolates were found to carry ESBL or carbapenemase genes. One strain (SA26) showing high level resistance to carbapenem was found to carry $bla_{\text{NDM-1}}$ on a non typable plasmid of 95 Kb size. Another strain BA76 with intermediate susceptibility to some carbapenems, was found not to harbor any of the tested carbapenemase genes. *E. coli* BA76 from Bahrain carried *rmtB*, while SA26 from Saudi carried *rmtC* 16S RNA methylase genes. All strains carried disinfectant resistance genes but showed no increase in MIC to the tested disinfectants compared to the laboratory strain *E. coli* K12. *mcr-1* carrying plasmids were all transferrable by conjugation into *E. coli* J53RAZ. In case of ABC149, *mcr-1* harboring plasmid was suggested to be co-mobilized as conjugation experiments resulted in transfer of multiple plasmids. Transfer of pABC149 was subsequently achieved by

transformation into *E. coli* DH5α. BA76, BA77 and ABC149 carried *mcr-1* in 60 Kb IncI2 plasmids. An *ISEcp1*-driven $bla_{CTX\text{-}M\text{-}64}$ gene was found to coexist with *mcr-1* in pBA76-MCR. All three IncI2 plasmids showed high backbone similarity to the first described *mcr-1* plasmid pHNSHP45 (Y. Liu et al., 2016). On the other hand, in SA26, mcr-1 was carried on a 240 Kb plasmid of IncHI2 type. Backbone of this plasmid showed high similarity to two *mcr-1* negative multi-resistant plasmids; pHK0653 and pHXY0908 harbored by *Salmonella typhimurium* isolated from human and poultry samples and to the second *mcr-1* positive plasmid, pHNSHP45-2 in SHP45 strain (Zhi et al., 2016). pSA26-mcr-1 is found to have a different resistance island compared to those three plasmids which contained 13 resistance genes; however, *mcr-1* gene was located outside the resistance island flanked by two complete IS*Apl1*.

The second report on *mcr-1* from the region came from France (Leangapichart et al., 2016), where they investigated the acquisition of *mcr-1* gene during Hajj. They performed two cohort studies in 2013 and 2014, where rectal swabs were collected before and after pilgrimage. The prevalence of *mcr-1* positive isolates has increased significantly after pilgrimage; from 1.55% to 8.53% in 2013 and from 1.02% to 9.18% in 2014. *mcr-1* gene was carried by both *E. coli* and *K. pneumoniae* and its sequence showed 100% identity to the first described gene (Y. Liu et al., 2016). In two occasions, the same ST type of *E. coli* was carried by two unrelated pilgrims, while a couple carried strains with two different ST types. Overcrowding during Hajj probably facilitated the spread of antibiotic resistant bacteria (Leangapichart et al., 2016). While use of antibiotics during travel might help selecting for antibiotic resistant bacteria (Kantele et al., 2015), spread of *mcr-1* positive, colistin resistant strains can occur without use of colistin showing a low transmission fitness cost, as has been shown in

some of the pilgrims being colonized by *mcr-1* strain without use of any antibiotics in the Hajj period. Despite not identifying infection sources and transmission modes, identification of different sequence types in pilgrims suggested that various sources and modes of transmission were involved in the acquisition of *mcr-1*. Furthermore, spread of *mcr-1* carrying plasmids may occur upon return of pilgrims to their countries (Leangapichart et al., 2016).

Another report of mcr came from Oman, where they identified a single *mcr-1* positive non-MDR *E. coli* by screening a collection of colistin resistant isolates between 2014 and 2016. Screening for *mcr-2* was negative. The *mcr-1* harboring strain (OM97) is of ST10, a clone known of spreading ESBL and quinolone resistance genes globally. The *mcr-1* gene was found to be carried by a 63 Kb transferrable, IncI2 type plasmid not carrying other resistance genes. IS*Apl1* element was not found in the *mcr-1* surroundings (Mohsin et al., 2016).

In another screening for *mcr-1* of a collection of different pathogenic gram-negative bacteria from GCC countries, one *E. coli* strain from Qatar was found to harbor *mcr-1* gene (Forde et al., 2018). The strain (MS8345), of ST95 sequence type, was resistant to polymyxins, as well as, to many beta lactam and non-beta lactam antibiotics. MS8345 carried two plasmids; a multi-drug resistant plasmid of 241Kb, which harbored the *mcr-1* gene (pMS8345A), and a second 133Kb virulence plasmid (pMS8345B). The *mcr-1* harboring plasmid was of IncHI2 type, which carried15 resistance genes in a multidrug resistance island. *mcr-1* gene was found to be separate from the island with IS*Apal*1 being upstream of the *mcr-1* gene. The plasmid had high backbone similarity to the *mcr-1* positive pSA26-MCR1 (GenBank accession number KU743384) (Sonnevend et al., 2016) being different in the structure and

content of the MDR island, with pMS8345A having an extra eight resistance genes not found in the MDR island of pSA26-MCR1.

1.7.3.2 Animals

The first study to investigate antibiotic resistance in veterinary sector came from Qatar (Eltai et al., 2018), aiming to have a baseline knowledge on the prevalence of antibiotic resistance in food animals. Ninety commensal *E. coli* isolates from fecal samples collected from one live bird market and two broiler chicken farms were subjected to susceptibility testing to many antibiotics of clinical relevance. Using E-test method, 15.5% of the isolates were resistant to colistin. *mcr-1* was detected in these isolates with PCR.

1.7.4 Antimicrobial Resistance Surveillance/Statistics in GCC

Searching official web sites (listed in Table 1) of the relevant ministries in the GCC with the key words: Antibiotic use in animal fodders, colistin, colistin resistant bacteria, colistin resistance gene showed no results from Bahrain, Kuwait, Oman, Qatar, Saudi Arabia and the UAE. From Oman a national action plan emphasizing the prudent use of antibiotics and prohibiting the use of critical antibiotics in animal husbandry was retrieved, but no factual data on use of antibiotics or more specifically, colistin, was available (Al-Saidi and Al Sajwani, 2016).

Table 1: Official web sites of the relevant ministries in countries of the GCC

Country	Ministry	Official Web Site
Bahrain	Ministry of Works Municipalities Affairs and Urban Planning	https://www.mun.gov.bh/
Kuwait	Public Authority of Agriculture Affairs and Fish Resources	http://www.paaf.gov.kw/
Oman	Ministry of Agriculture and Fisheries Ministry of Health & Ministry of Agriculture & Fisheries	http://www.maf.gov.om/ http://extwprlegs1.fao.org/docs/pdf/oma171516.pdf
Qatar	Ministry of Municipality and Environment	http://www.mme.gov.qa/
Saudi Arabia	Ministry of Environment Water and Agriculture	https://www.mewa.gov.sa/
UAE	Ministry of Climate Change and Environment	https://www.moccae.gov.ae/

1.8 Hypothesis, Aims and Objectives

Since MCR-producing human bacterial isolates were found earlier in the UAE and other GCC countries, our hypothesis was that such resistant bacteria are present in farmed animals in the UAE, as well. Therefore, the aim of our study was to identify and characterize *Enterobacteriaceae* carrying mobile colistin resistance genes by screening composite stool samples of broiler chicken and by whole genome sequencing of the isolates harboring the *mcr* gene to establish their similarity to those isolates identified in the region from humans, as well as, to compare them to *mcr* positive bacteria identified worldwide.

Chapter 2: Methods

2.1 Sample Collection

Four randomly selected broiler poultry farms in Abu Dhabi Emirate (UAE) were visited over a 2-month period for sample collection. All farms were privately owned by different independent owners and were located within a minimum of 30 km from each other. Each farm was composed of 2-4 broiler houses. The number of broilers in each house ranged between 5,000 and 7,000 birds, and all were healthy, *i.e.* no outbreaks of infectious diseases occurred in the broiler flocked during the sample collection. During each sampling visit to a farm, one broiler house, where birds were 3-weeks of age, was randomly selected and sampled. Ten composite fecal samples (~100 g each) were collected and placed in sterile plastic containers. Each composite sample was composed of three individual fresh fecal droppings. The sampling was carried out using a zig-zag pattern through the entire broiler house as described earlier (Alali et al., 2010). The fecal samples were transferred to our laboratory and processed immediately on the day of collection.

2.2 Isolation of *mcr* Gene Positive *Enterobacterales*

A 10 µl loopful of composite fecal samples (approx. 0.1 g) was cultured overnight in 4 ml Tryptic Soy Broth (TSB) (MAST, Merseyside, UK) supplemented with 1µg/ml colistin sulfate (Sigma-Aldrich, St Louise, USA) and 8 µg/ml vancomycin (Sigma-Aldrich, St Louise, USA). Next day, 10 µl and 100 µl of the TSB culture was sub-cultured on McConkey agar plates (Oxoid, Basingstoke, UK) containing 1 µg/ml colistin sulfate (Sigma-Aldrich, St Louise, USA), and incubated overnight at 37°C. Colonies present after the incubation were subcultured as

macrocolonies on 3% agar containing Tryptic Soy Agar (MAST, Merseyside, UK) supplemented with 1µg/ml colistin sulfate (Sigma-Aldrich, St Louise, USA) . If less than 10 isolated colonies were present, all were sub-cultured, otherwise maximum 10 colonies per sample, if present, with different colony morphology, were selected for subculture.

2.2.1 Genomic DNA Extraction by Heat Treatment

An approximately 1 µl loopful part of macrocolonies was suspended in 200 µl sterile distilled water. Thermal lysis was used to extract the DNA, where the tubes containing the suspension were incubated for 10 minutes at 99°C in thermo-block (Eppendorf, Germany). This is followed by centrifuging the tubes at 14800 rpm for 10 minutes, after which the supernatants were collected from each tube and used as template in PCR.

2.2.2 Multiplex PCR for Detecting Mobile Colistin Resistance (*mcr*) Genes

The above DNA samples were tested for the presence of mobile colistin resistance determinants by multiplex PCR detecting *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* (Rebelo et al., 2018); Primers and PCR reaction conditions are listed in Table 2.

Isolates positive by the above PCR were stored in TSB (MAST, Merseyside, UK) containing 20% glycerol at -80˚C in our laboratory until further investigation.

Table 2: Primers and PCR conditions for multiplex PCR for the detection of the *mcr* genes

Gene	Primer	Sequences (5'-3')	Initial denaturation	Cycle	Final extension	Product (bp)	Reference
mcr-1	mcr1_320bp_fw	AGTCCGTTTGTTCTTGTGGC	5' at 94°C	30X 30" at 94°C, 90" at 58°C 60" at 72°C	10' at 72°C	320	(Rebelo et al., 2018)
	mcr1_320bp_rev	AGATCCTTGGTCTCGGCTTG					
mcr-2	mcr2_700bp_fw	CAAGTGTGTTGGTCGCAGTT				715	
	mcr2_700bp_rev	TCTAGCCCGACAAGCATACC					
mcr-3	mcr3_900bp_fw	AAATAAAAATTGTTCCGCTTATG				929	
	mcr3_900bp_rev	AATGGAGATCCCCGTTTTT					
mcr-4	mcr4_1100bp_fw	TCACTTTCATCACTGCGTTG				1,116	
	mcr4_1100bp_rev	TTGGTCCATGACTACCAATG					
mcr-5	MCR5_fw	ATGCGGTTGTCTGCATTTATC				1,644	(Borowiak et al., 2017)
	MCR5_rev	TCATTGTGGTTGTCCTTTTCTG					

2.3 Molecular Comparison of *mcr* Positive Isolates

From each of the 40 fecal samples a maximum of 3 isolates positive by PCR for an *mcr* gene were selected for further analysis. If positive isolates exhibited different colony morphology, one of each type was selected.

2.3.1 Plasmid Profile Analysis

Plasmids were isolated by the alkaline lysis method of Kado and Liu (Kado and Liu, 1981) with minor modifications. Bacterial strains were cultured on TSA plates, incubated overnight at 37°C to obtain a confluent growth. Cells were collected from the plates and suspended in 250µl of lysing solution (3% SDS, 50 µM Tris, pH 12.56-12.58), mixed by gentle agitation till they became homogenous and viscous. The suspensions were incubated in a thermoblock at 60°C for 45 minutes, being mixed gently every 15 minutes. Then 250 µl of phenol-chloroform (1:1) was added, mixed gently followed by centrifugation at 13000 rpm for 15 minutes. About 100 µl of the top aqueous layer was transferred to another tube containing 5 µl loading dye. Samples were loaded in 0.8% agarose gel and subjected to electrophoresis at 130 V for 4 hours. Gels were stained with ethidium bromide for 20 minutes, de-stained for 10 minutes and photographed using a gel documentation system (Biometra, Germany). For plasmid size control, *E. coli* V517 (Macrina et al., 1978) and *E. coli* 39R861 (Threlfall et al., 1986) were used. *E. coli* $J53_{RAZ}$ lacking any plasmid was used to identify the chromosomal band. Plasmid patterns were analyzed and compared by the Dice similarity index using GelComparII software (Bionumerics, Sin Martens, Belgium).

2.3.2 Species Identification

Species identification of bacterial strains exhibiting different plasmid profiles was performed by PCR amplifying the conserved part of the 16S rRNA coding gene and direct sequencing the PCR products (Watanabe et al., 2001). The primers and conditions used are listed in Table 3.

Table 3: Primers and PCR conditions for the partial amplification of the 16*SrRNA* gene

Primer	Sequences (5'-3')	Initial denaturation	Cycles	Final extension	Produc t size
E8F	AGAGTTTG ATCCTGGC TCAG	94 °C- 3 min	35X 94°C- 15 sec 55.5°C-30 sec 72°C- 1 min	72°C- 10 min	525 bp
E533R	TIACCGIIIC TICTGGCAC				

PCR products were loaded on 1% agarose gel for electrophoresis, followed by gel purification using Wizard PCR and Gel Purification Kit (Promega, USA), as per the manufacturer's instructions. The sequencing was done using the Big Dye Cycle Terminator V.3.1 (Applied Biosystems) kit on the 3130X Genetic Analyzer (Applied Biosystems). Samples were sequenced in both directions, and analyzed using the MEGA7 software (MEGA7, 2016). Species was identified by BLASTn (BLAST ®) analysis of the sequences obtained.

2.3.3 Pulsed Field Gel Electrophoresis (PFGE)

Macrorestriction pattern of bacterial strains exhibiting different plasmid profiles were compared by PFGE, as described in (Gautom, 1997). Briefly, after overnight culture on TSA, bacterial strains were collected and suspended in 2ml cell suspension buffer (100

mM Tris:100 mM EDTA, pH 8.0), to a density of 2-3 McFarland unit. Plug agarose was prepared by melting 1% plug agarose (Sigma-Aldrich, St. Louise, USA) and 1% SDS in TE buffer (10 mM Tris:1mM EDTA); The molten agarose was kept at 54°C. 500 µl of the cell suspension were transferred to microcentrifuge tubes, to which a 25 µl of 20 mg/ml proteinase K (Invitrogen), and 525 µl of 1% plug agarose were added, mixed gently, transferred to 1ml syringes and allowed to solidify. 1mm thick slices of the plugs were cut into 50 ml tubes containing 5ml cell lysis buffer (50mM Tris:50mM EDTA, 1% Sarkosyl, pH 8.0) and 25µl proteinase K (20 mg/ml). The tubes were then incubated for 2 hours at 50°C in water bath with shaking. The plugs were then washed twice with 10 ml of sterile preheated distilled water for 20 minutes, then washed four times with 10 ml preheated TE buffer for 20 minutes. All the washing steps were done at 50°C, shaking; plugs were then stored at 4°C in 5 ml TE buffer. One plug of each strain was digested by overnight incubation in 100 µl restriction enzyme mixture (10 µl of Cutsmart buffer, 1.5 µl *Xba*I enzyme (20 U/µl) (NE Biolabs, USA) and 87.5 µl of distilled water) at 37°C. Next day, restriction mixture was removed, and plugs were incubated in 250 µl of 0.5X TBE buffer, at room temperature for 30 minutes. Plugs were inserted into 1.4% PFGE running agarose (Sigma-Aldrich, St. Louise, USA) gel that was prepared in 0.5X TBE buffer. Plugs of *Salmonella* Braenderup H9812 digested with *Xba*I the same way as described above was used as a control. Electrophoresis of the gels was run in 0.5X TBE, in electrophoresis chamber of CHEF DNA Mapper, at 14°C temperature. The electrophoresis run was for 24 hours at 6 V/cm with 120° angle and an initial switch time of 2.2 seconds and a final switch time of 54.2 seconds with linear ramp. GelComparII software (Bionumerics, Sin Martens, Belgium) was used to analyze the PFGE fingerprints.

2.3.4 Genomic DNA Preparation by Wizard® Genomic DNA Purification Kit

Genomic DNA of bacterial strains exhibiting different plasmid profiles was also extracted by Wizard® Genomic DNA Purification Kit (PROMEGA, USA) as per the manufacturer's protocol for further investigation. Briefly, strains were cultured in 10 ml TSB, at 37°C, overnight. 1.5 ml of an overnight culture was centrifuged at 13000 rpm for 3 minutes, supernatant was removed carefully by vacuum. Pellets were resuspended in 600 µl of nuclei lysis solution, incubated at 80°C in the thermoblock for 5 minutes. Suspensions were allowed to cool to room temperature, after that 3 µl of RNase solution was added, mixed and the mixture incubated for 45 minutes at 37°C. Then 200 µl of protein precipitation was added, the mixture was vortexed vigorously for 20 seconds, then incubated in ice for 5 minutes. The mixture was then centrifuged at 14800 rpm for 10 minutes. 600 µl of the supernatant was transferred to a clean tube containing 600 µl isopropanol at room temperature, and mixed. Then centrifuged at 13000 rpm for 3 minutes, supernatant was decanted, and 600 µl of 70% ethanol was added, mixed, and centrifuged again at 13000 rpm for 3 minutes, the supernatant was decanted by flipping the tube. Pellets were airdried, then rehydrated in 100 µl nuclease free water, stored at 4°C. Optical density of the preps was measured using ND-1000 spectrophotometer (Nano Drop Technologies, USA). DNA prepared by this method was sent for Illumina HiSeq 150 bp paired-end whole genome sequencing (Illumina, 2019), performed in Novogene Company Limited, Hong Kong (Novogene (HK), 2019).

2.3.5 Analysis of the Whole Genome Sequences

The paired-end reads received from Novogene Company Limited, Hong Kong were assembled into contigs using CLC Genomic Workbench v10.0 (QIAGEN Aarhus, Denmark). These contigs were uploaded to Pathogenwatch (Pathogenwatch, 2019) to confirm species identification; and to the ResFinder and PlasmidFinder at Center for Genomic Epidemiology webpage (Center for Genomic Epidemiology, 2011) for identification of acquired antibiotic resistance genes, as well as to identify plasmids with various incompatibility types. The assembled contigs were also used to define the strains' multi-locus sequence types (MLST) and core genome MLST types (cgMLST) using the SeqSphere+ software (Ridom© GmbH, Münster, Germany).

2.4 Analysis of the *mcr* Gene Carrying Plasmids

2.4.1 Southern Blot and Hybridization

Southern blotting was done to localize the *mcr* gene on plasmids of selected wild-type strains; plasmid gels were prepared using the described method (Kado and Liu, 1981). Rulers were placed around the gel, then photographed to help locate the bands. Gels were then depurinated in 0.25 M HCl, then denatured in 0.5 M NaOH, 1 M NaCl, and lastly neutralized in 1 M Tris, 0.6 M NaCl. Each of these steps were done twice for 15 minutes, with gentle agitation, at room temperature; with the gels being rinsed with distilled water between the steps.

Using capillary action, gels were then transferred to Hybond N+ membranes; being soaked overnight in 20X SSC (saline-sodium citrate). The following day,

membranes were cross-linked using UV at 70 000 microjoules. Hybridization was done using the DIG DNA labeling and detection kit (Roche, Germany).

Probe preparation: The PCR product of the *mcr-1* control strain (ABC149) (Sonnevend et al., 2016) was quantified using NanoDrop 1000 spectrophotometer. DIG DNA Labelling Kit was used to label the probe; where 200 µg of the purified DNA was denatured by heating in boiling water bath for 10 minutes and quickly chilled on ice, after which labelling proceeded according to manufactures instructions. The blot membrane was incubated in hybridization buffer, at hybridization temperatures for 30 minutes as a prehybridization step, after which the hybridization buffer containing the probe was added, incubated overnight at the hybridization temperature. Hybridization temperature was calculated based on the size and the GC ratio of the probe using the formula; T_{opt}= T_m-20°C with T_m= 49.82 + 0.4 1 (%G+C) - 600/L (L is the length of the probe in base pairs), with the GC of the probe being 48%, size being 309 bp, optimum temperature for hybridization was calculated to be 48°C. Next day, membranes were washed twice with 2X SSC / 1% SDS for 5 minutes at room temperature, followed by washing twice in 0.1X SSC/ 1% SDS for 15 minutes at 68°C. Following this, they were rinsed in washing buffer at room temperature, and incubated in blocking solution for 30 minutes, then incubated in antibody solution for 30 minutes. After that, membranes were washed twice in washing buffer, and equilibrated in detection buffer. Membranes were then incubated in color substrate solution, kept in the dark without shaking till the appearance of the labelled band.

2.4.2 Plasmid Transfer

2.4.2.1 Conjugation

In order to assess whether the *mcr*-carrying plasmids were self-transmissible, conjugation experiments were carried out using in-house generated rifampicin and Na-azide resistant *E. coli* $J53_{RAZ}$ as recipient. After an overnight culture on TSA plates, both donor and recipient were inoculated into 5 ml of pre-warmed TSB, kept at 37°C, shaking for 4 hours. Then donor and recipient were combined in the ratio of 1 (donor): 4 (recipient) in a volume of 4 ml in 50 ml falcon tubes and incubated at 37°C for 4 hours without shaking. The tubes were then centrifuged at 3500 rpm for 15 minutes, supernatant was removed, the pellet was suspended in 200 µl TSB and vortexed. A 100 µl of the suspension was plated into two plates (without antibiotics) as a drop in the center of the plates; plates were incubated overnight upside-down at 37°C. Next day the bacterial growth from the two plates was collected and suspended into 5 ml 1x PBS, centrifuged at 3500 rpm for 15 minutes and the supernatant was removed. This step was repeated once more after which the pellet was suspended in 3 ml of 1x PBS and serially diluted. 200 µl from each dilution was plated on plates containing 2 µg/ml colistin and 100 µg/ml Na-azide and incubated at 37°C for 24-48 hours. Colonies were then selected, tested by PCR for the *mcr* gene to confirm the transfer. Their plasmid profile was also established, after plasmid purification by the alkaline lysis method (Kado and Liu, 1981), to prove that the derivative contained a single plasmid.

2.4.2.2 Transformation

If the conjugation did not result in a single plasmid containing derivative, attempts were made to transfer the *mcr*-plasmid by heat-shock transformation. Plasmid purified from the wild-type strain using the Qiagen Plasmid Maxi Prep (Qiagen, Germany) were transformed into competent cells of *E. coli* DH5α.

For plasmid isolation using the Qiagen Plasmid Maxi Prep (Qiagen, Germany) strains were inoculated in 100 ml of Luria Bertani broth (Oxoid, UK), incubated overnight at 37°C, with vigorous shaking. Bacterial culture was harvested by centrifugation at 6000 x g for 15 min at 4°C. This was followed by resuspending the pellet in 10 ml buffer P1; 10 ml of buffer P2 was added, mixed thoroughly and incubated at room temperature for 15-25 minutes. After that 10 ml of pre-chilled buffer P3 was added, mixed thoroughly and incubated on ice for 20 minutes. The mixture was centrifuged at ≥ 20,000 x g for 30 min at 4°C. Qiagen columns were equilibrated by 10 ml buffer QBT and allowed to drain by gravity. Supernatant of the last centrifugation was applied to the Qiagen tip and allowed to enter the resin by gravity flow. The Qiagen column was washed twice with 30ml of buffer QC. DNA was then eluted with 15 ml buffer QF, allowed to flow by gravity. DNA was precipitated by adding 10.5 ml room temperature isopropanol to the eluted DNA, the mixture was centrifuged at 4000 x g for 75 minutes at 4°C, supernatant was decanted. The pellet was washed with 5ml 70% ethanol, centrifuged at 4000 x g at 4°C for 30 minutes, supernatant was decanted. The pellet was air dried and dissolved in nuclease free water. ND-1000 spectrophotometer (Nano Drop Technologies, USA) was used to measure the DNA concentration.

For the transformation 1 µg of the purified plasmid DNA was added to the thawed competent cells (recipient) and incubated for 20 minutes on ice. After that they were heat shocked at 42°C for 5 minutes and incubated again on ice for 2 minutes. Then mixtures were added to 1 ml of TSB and incubated for 1 hour at 37°C shaking. Of these cultures, 200 µl and 800 µl were plated onto plates containing 2 µg colistin and incubated at 37°C overnight. DNA prepared from colonies was tested for *mcr* gene by PCR, also tested for the presence of a single plasmid as described above.

2.4.3 Plasmid Restriction Fragment Length Polymorphism (RFLP)

Half µg of plasmid DNA extracted from the single *m*cr-plasmid containing derivatives by the Qiagen Plasmid Maxi Prep kit (Qiagen, Germany) was digested with restriction enzymes: 60 units of *Hinc*II, *Nsi*I, and 120 units of *Nde*I were used for the approx. 60 Kb plasmids, other plasmids were digested using *BamH*I, *Hind*III, and *EcoR*I restriction enzymes, 120 units each. Digested plasmids were subjected to electrophoresis using 1% agarose gel, at 130 volts for 2 hours, and the patterns were manually compared.

2.4.4 Sequencing of the *mcr*-Plasmids

Plasmid DNA purified of selected single plasmid containing transconjugants or transformants were sent for commercial next generation sequencing on the Illumina MiSeq platform (Illumina, 2019), (performed at the CCIB DNA Core Facility in Massachusetts General Hospital (Cambridge, MA, USA). The reads were assembled into contigs using CLC Genomic Workbench v9.0 (QIAGEN Aarhus, Denmark). These contigs were uploaded o the ResFinder and PlasmidFinder at Center for Genomic

Epidemiology webpage (Center for Genomic Epidemiology, 2011) for identification of acquired antibiotic resistance genes co-located on the mcr-plasmids, as well as to identify these plasmids' incompatibility types.

2.5 Antibiotic Susceptibility Testing

Susceptibility of the selected wild-type isolates and their respective transconjugants or transformants to twenty antibiotics was tested by disc diffusion (Kirby-Bauer test) using antibiotic discs produced by MAST (Merseyside, UK) and Mueller Hinton agar (Oxoid, Basingstoke, UK). The following antibiotics were tested: amoxicillin/clavulanate, ampicillin, cefoxitin, cefpodoxime, ceftazidime, cefotaxime, aztreonam, piperacillin/tazobactam, imipenem, ertapenem, meropenem, gentamicin, amikacin, tobramycin, chloramphenicol, doxycycline, and tetracycline, ciprofloxacin, nalidixic acid and co-trimoxazole. Fosfomycin susceptibility was tested by agar dilution using Muller-Hinton Agar (MAST Merseyside, UK) supplemented with 25 mg/L glucose-6-phosphate. The minimum inhibitory concentration of colistin was determined by broth microdilution (BMD), using colistin sulfate powder produced by Sigma-Aldrich (St. Louise, USA) and cation adjusted Muller-Hinton Broth (Oxoid, Basingstoke, UK). In all tests the Clinical Laboratory Standards Institute Guidelines were followed (CLSI, 2019). *E. coli* ATCC25922 was used for quality control. For colistin BMD an *mcr-1* positive *E. coli* isolate (ABC149) (Sonnevend et al., 2016) with colistin MIC of 4 mg/L was also included.

Chapter 3: Results

3.1 Screening for *mcr* Gene Positive Isolates

Of the 40 composite chicken stool samples collected from four broiler poultry farms in Abu Dhabi Emirate (UAE) 36 samples yielded colonies positive by the screening PCR. All of them were positive for the *mcr-1* gene allele. The distribution of positive colonies detected is shown in Table 4. After enrichment in colistin and vancomycin supplemented TSB and subculture on colistin containing McConkey agar altogether 218 *mcr-1* positive colonies were identified. To investigate the variety of species, and potentially the different genetic support of the *mcr-1* gene, from each sample a maximum of 3 colonies exhibiting different morphology were selected. The selected 97 colonies are color marked in Table 4.

Table 4: Distribution of positive colonies in the stool samples tested

Samples		No of mcr-1 positive colonies	Positive colonies										No of isolates further tested
FARM A	Sample 1	6	FA1-1	-	-	FA1-4	FA1-5	-	-	FA1-8	FA1-9	FA1-10	3
	Sample 2	5	FA2-1	-	FA2-3	FA2-4	FA2-5	-	FA2-7	-	-	FA2-10	3
	Sample 3	5	FA3-1	-	-	FA3-4	-	FA3-6	FA3-7	-	-	FA3-10	3
	Sample 4	7	FA4-1	-	-	FA4-4	FA4-5	-	FA4-7	-	FA4-9	FA4-10	3
	Sample 5	7	FA5-1	-	FA5-3	-	FA5-5	FA5-6	FA5-7	-	FA5-9	FA5-10	3
	Sample 6	4	FA6-1	FA6-2	-	-	-	FA6-6	-	-	-	-	3
	Sample 7	4	FA7-1	-	-	-	FA7-5	FA7-6	-	-	FA7-9	-	3
	Sample 8	10	FA8-1	FA8-2	FA8-3	FA8-4	FA8-5	FA8-6	FA8-7	FA8-8	FA8-9	FA8-10	3
	Sample 9	4	FA9-1	FA9-2	-	-	FA9-5	-	FA9-7	-	-	-	3
	Sample 10	3	FA10-1	FA10-2	FA10-3	-	-	-	-	-	-	-	3
FARM B	Sample 1	10	FB1-1	FB1-2	FB1-3	FB1-4	FB1-5	FB1-6	FB1-7	FB1-8	FB1-9	FB1-10	3
	Sample 2	10	FB2-1	FB2-2	FB2-3	FB2-4	FB2-5	FB2-6	FB2-7	FB2-8	FB2-9	FB2-10	3
	Sample 3	8	FB3-1	FB3-2	FB3-3	FB3-4	FB3-5	FB3-6	FB3-7	FB3-8	x	x	3
	Sample 4	0	x	x	x	x	x	x	x	x	x	x	0
	Sample 5	0	x	x	x	x	x	x	x	x	x	x	0
	Sample 6	3	FB6-1	FB6-2	FB6-3	x	x	x	x	x	x	x	3
	Sample 7	10	FB7-1	FB7-2	FB7-3	FB7-4	FB7-5	FB7-6	FB7-7	FB7-8	FB7-9	FB7-10	3
	Sample 8	2	-	-	-	-	FB8-5	-	-	-	FB8-9	-	2
	Sample 9	0	x	x	x	x	x	x	x	x	x	x	0
	Sample 10	1	FB10-1	x	x	x	x	x	x	x	x	x	1
FARM C	Sample 1	9	FC1/1	FC1-2-1	FC1-3	FC1-4	FC1-5-1	-	FC1-7	FC1-8	FC1-9	FC1-10	3
	Sample 2	4	FC2-1-1	-	FC2-3-1	-	-	-	-	-	FC2-9-1	FC2-9-2	3
	Sample 3	10	FC3-1	FC3-2	FC3-3	FC3-4	FC3-5	FC3-6	FC3-7	FC3-8	FC3-9	FC3-10	3
	Sample 4	9	FC4-1	FC4-2	-	FC4-4-1	FC4-5	FC4-6	FC4-7	FC4-8	FC4-9-1	FC4-10-1	3
	Sample 5	10	FC5-1	FC5-2	FC5-3	FC5-4	FC5-5	FC5-6	FC5-7-1	FC5-8	FC5-9	FC5-10	3
	Sample 6	9	FC6-1	-	FC6-3	FC6-4-1	FC6-5	FC6-6	FC6-7	FC6-8	FC6-9	FC6-10-1	3
	Sample 7	0	x	x	x	x	x	x	x	x	x	x	0
	Sample 8	2	-	-	FC8-3-2	FC8-4-2	-	-	-	-	-	x	2
	Sample 9	10	FC9-1	FC9-2	FC9-3	FC9-4	FC9-5-1	FC9-6	FC9-7	FC9-8	FC9-9	FC9-10	3
	Sample 10	1	FC10-1	x	x	x	x	x	x	x	x	x	1
FARM D	Sample 1	5	FD1-1	-	-	FD1-4	FD1-5	FD1-6	-	-	-	FD1-10	3
	Sample 2	1	-	-	-	-	-	-	FD2-7	-	-	-	1
	Sample 3	3	FD3-1	-	-	-	-	FD3-6	-	FD3-8	-	-	3
	Sample 4	10	FD4-1	FD4-2	FD4-3	FD4-4	FD4-5	FD4-6	FD4-7	FD4-8	FD4-9	FD4-10	3
	Sample 5	8	FD5-1	FD5-2	FD5-3	-	FD5-5	FD5-6	FD5-7	-	FD5-9	FD5-10	3
	Sample 6	9	FD6-1	FD6-2	FD6-3	FD6-4	FD6-5-1	FD6-6	-	FD6-8	FD6-9	FD6-10	3
	Sample 7	10	FD7-1-1	FD7-2	FD7-3	FD7-4	FD7-5-2	FD7-6	FD7-7-1	FD7-8	FD7-9-2	FD7-10	3
	Sample 8	1	-	-	x	-	-	-	-	FD8-8	-	-	1
	Sample 9	6	FD9-1	-	FD9-3-1	-	FD9-5-1	FD9-6	FD9-7-1	-	-	-	3
	Sample 10	2	-	FD10-2	-	-	-	-	-	FD10-8	-	-	2
ALL		218											97

The selected 97 isolates exhibited 40 different plasmid profiles as shown in Figure 4.

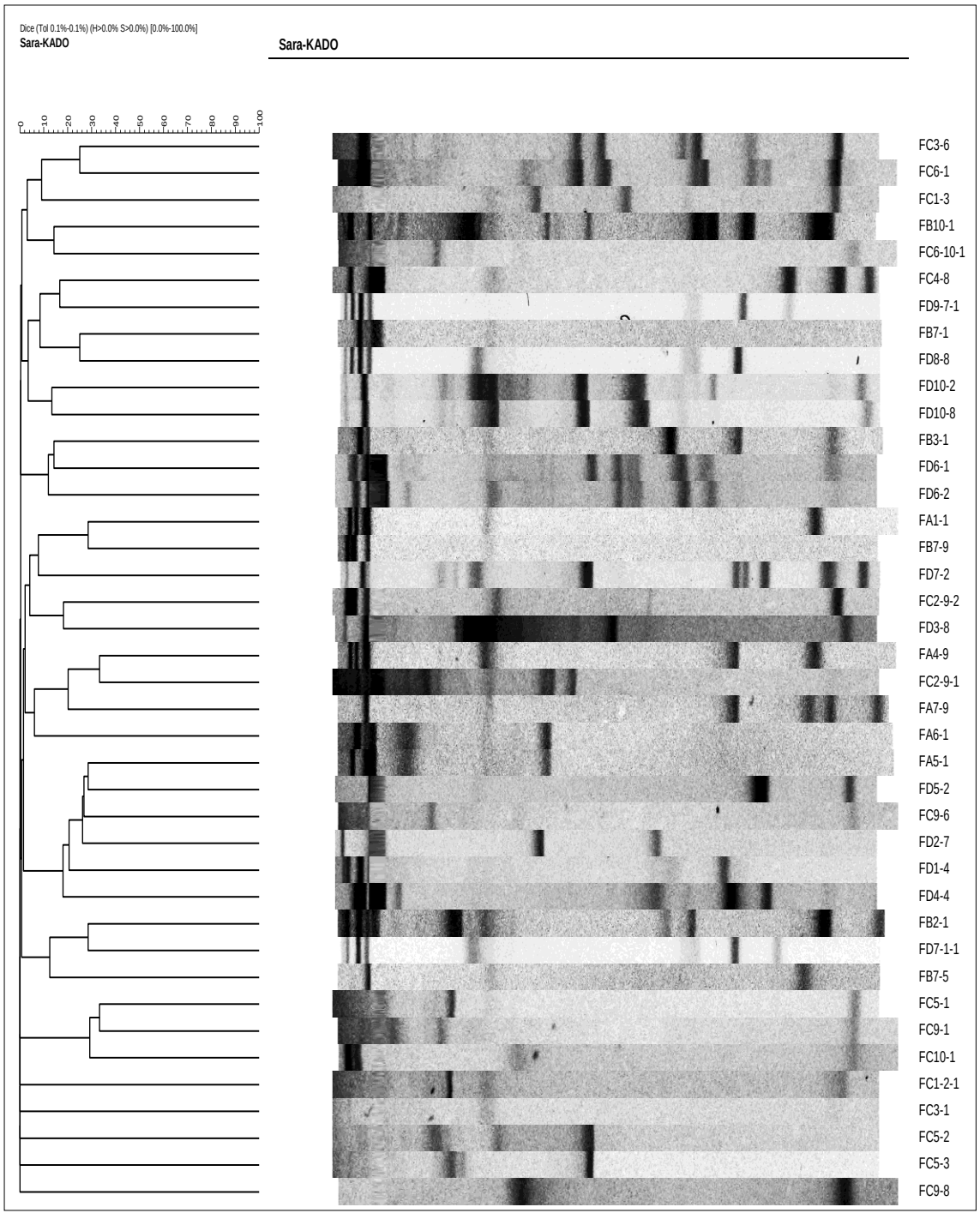


Figure 4: Plasmid profile of the 40 isolates exhibiting non-identical patterns

3.2 Species Identification

Sequencing of the 16S rRNA coding gene revealed that the 40 isolates included 35 *Escherichia coli*, two *Klebsiella pneumoniae/variicola* and one *Salmonella enterica*. The species of two isolates could not be established without ambiguity as the sequenced part of the 16S rRNA gene was identical to the corresponding *Escherichia albertii*, *Shigella boydii* and *E. coli* sequences, as well (Table 5).

Table 5: Species identification based on the sequence of the 16S rRNA coding gene of the 40 isolates with different plasmid profiles, confirmation by WGS is shown in Table 7

Strain	Species
FB10-1	*Escherichia coli*
FD6-1	*K. pneumoniae/variicola*
FD6-2	*K. pneumoniae/variicola*
FC3-6	*Escherichia albertii/E.coli/S.boydii*
FC6-1	*Escherichia albertii/E.coli/S.boydii*
FA1-1	*Escherichia coli*
FA4-9	*Escherichia coli*
FB7-9	*Escherichia coli*
FC2-9-2	*Escherichia coli*
FD1-4	*Escherichia coli*
FD8-8	*Escherichia coli*
FD9-7-1	*Escherichia coli*
FD7-1-1	*Escherichia coli*
FC4-8	*Escherichia coli*
FD4-4	*Escherichia coli*
FB2-1	*Escherichia coli*
FA5-1	*Escherichia coli*
FA6-1	*Escherichia coli*
FB1-1	*Escherichia coli*
FC2-9-1	*Escherichia coli*
FA7-9	*Escherichia coli*
FB3-1	*Escherichia coli*
FB7-1	*Escherichia coli*
FC10-1	*Escherichia coli*
FD3-8	*Escherichia coli*
FC3-1	*Escherichia coli*
FD7-2	*Salmonella enterica*
FD10-2	*Escherichia coli*
FD10-8	*Escherichia coli*
FB7-5	*Escherichia coli*
FD2-7	*Escherichia coli*
FC6-10-1	*Escherichia coli*
FD5-2	*Escherichia coli*
FC9-1	*Escherichia coli*
FC5-1	*Escherichia coli*
FC1-3	*Escherichia coli*
FC1-2-1	*Escherichia coli*
FC9-8	*Escherichia coli*
FC5-2	*Escherichia coli*
FC5-3	*Escherichia coli*

3.3 Pulsed-Field Gel Electrophoresis Comparison

Macrorestriction pattern analysis of the *K. pneumoniae* and the *Escherichia* spp. isolates are shown in Figure 5 and Figure 6. The two *K. pneumoniae* were indistinguishable (Figure 5).

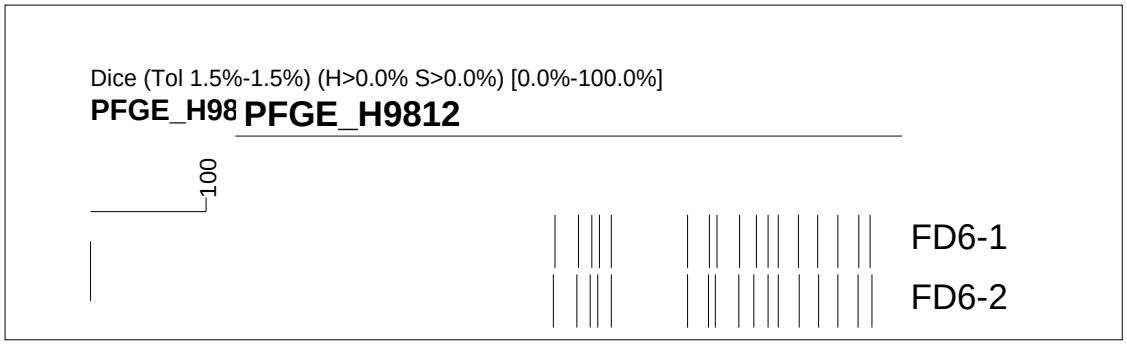


Figure 5: PFGE comparison of the two *mcr-1* positive K. pneumoniae isolates

Of the 37 *Escherichia* spp. 28 were typable by PFGE (Figure 6), DNA of the remaining 9 isolates autolyzed during electrophoresis. Using the > 80% similarity threshold, the typable isolates were diverse, they exhibited 12 PF patterns.

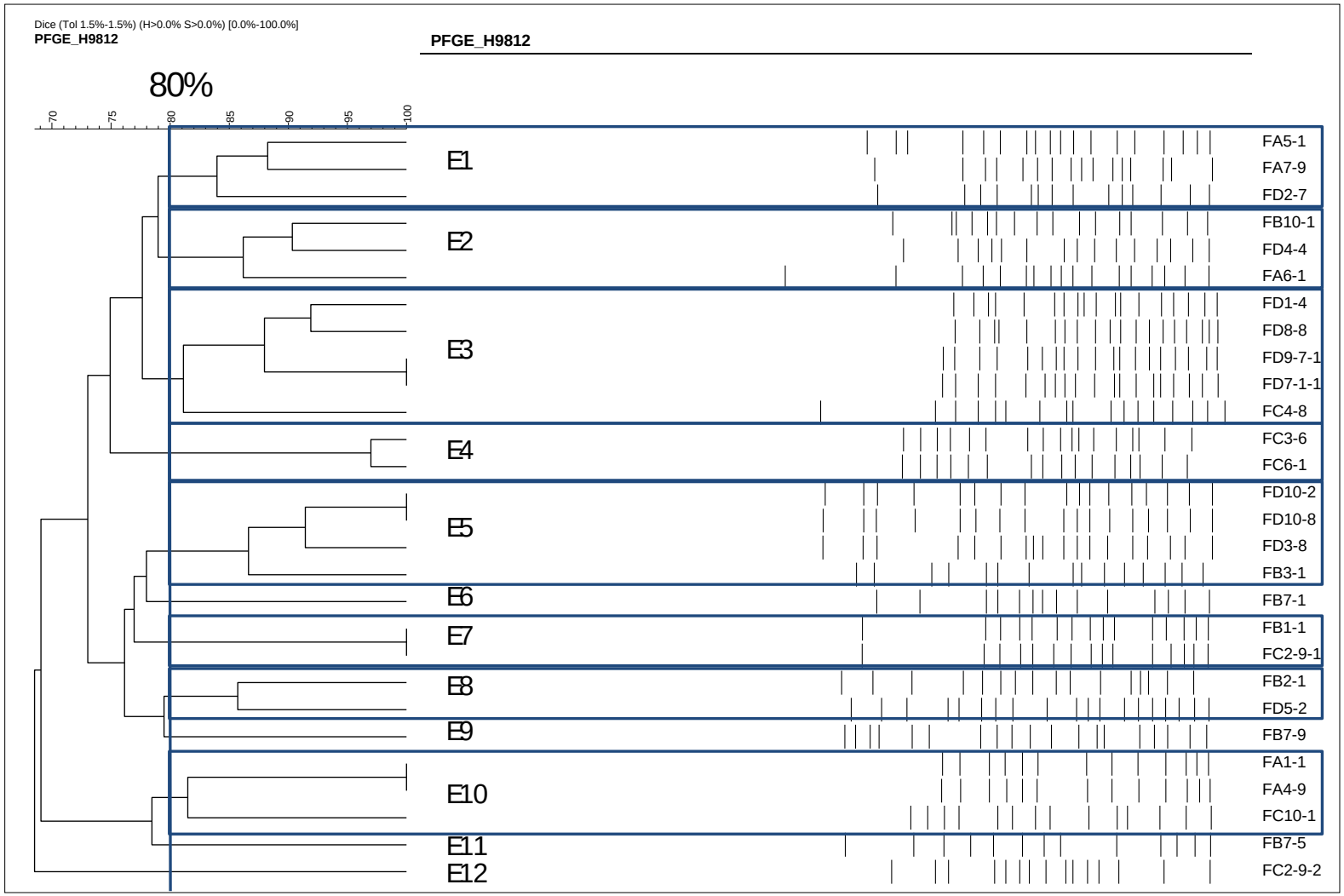


Figure 6: PFGE comparison of the 28 typable *Escherichia* spp. isolates

3.4 Analysis of the Whole Genome Sequences

The selected 40 isolates exhibiting different plasmid patterns were also subjected to whole genome sequencing. The result of the CLC Genomic Workbench v10.0 assembly of the Illumina reads, as well as the percentage of good cgMLST targets found by SeqSphere+ is summarized in Table 6. Analysis of the assembled contigs revealed that the identification by 16S rRNA gene sequences were correct and the two isolates with ambiguous results indeed were *E. albertii*. Based on the WGS results the serotype of *S. enterica* FD7-2 was *S.* Minnesota.

Table 6: WGS statistics of the *mcr-1* positive isolates

Strain	Genome Length	No. Contigs	Smallest Conti	Largest Contig	Average Contig Length	N50	GC Content	% Good cgMLST Targets#
FA1-1	5075470	195	550	257769	26028	92949	50.5	99.5
FA4-9	5026512	158	501	300384	31813	115026	50.5	99.5
FA5-1	5163762	137	506	391090	37691	121621	50.4	99.5
FA6-1	5188210	133	501	388627	39009	103817	50.3	99.4
FA7-9	4815426	204	501	351030	23605	136395	50.5	99.7
FB10-1	5047363	159	502	260516	31744	100943	50.3	99.3
FB1-1	5293874	135	509	640641	39213	189214	50.5	99.7
FB2-1	5960285	256	482	351821	23282	95211	50.2	99.2
FB3-1	5135638	186	501	283432	27610	76037	50.6	99.2
FB7-1	4972003	90	481	484275	55244	223278	50.7	99.6
FB7-5	4849735	122	501	297161	39751	132214	50.6	99
FB7-9	6958746	2531	500	71059	2749	4630	50.3	67.8
FC10-1	5014450	107	508	456029	46864	184832	50.5	99.6
FC1-2-1	5105144	195	511	263006	26180	119063	50.6	99.6
FC1-3	5633324	170	501	353225	33137	145060	50.4	99
FC2-9-1	5426045	193	525	369391	28114	189214	50.6	99.7
FC2-9-2	5220520	143	527	365330	36507	130894	50.6	99.5
FC3-1	5463042	112	534	410266	48777	243392	50.2	99.3
FC3-6	5174377	231	523	305003	22399	136421	49.4	NA
FC4-8	5199467	227	541	238560	22905	90065	50.4	98.9
FC5-1	5118159	184	519	282368	27816	119164	50.6	99.6
FC5-2	5495415	141	529	760534	38974	243335	50.2	99.3
FC5-3	5491123	139	479	550066	39504	210372	50.2	99.3
FC6-1	5303963	307	502	315676	17276	150155	49.4	NA
FC6-10-1	5098252	169	502	282393	30167	125719	50.6	99.5
FC9-1	5061790	164	502	282431	30864	125816	50.6	99.6
FC9-8	5482670	134	563	667667	40915	216652	50.2	99.3
FD10-2	5600318	168	503	358163	33335	141588	50.2	99
FD10-8	5603101	179	565	358353	31302	141659	50.2	99
FD1-4	5418810	205	513	198806	26433	85808	50.2	99.2
FD2-7	4899692	152	510	224401	32234	102396	50.5	99.4
FD3-8	5593930	173	503	343858	32334	141602	50.2	99
FD4-4	4942732	411	183	166993	12026	66227	50.7	98.8
FD5-2	5040246	124	482	579690	40647	223696	50.6	99.3
FD6-1	6138266	804	501	426273	7634	167943	56.5	NA
FD6-2	5771971	330	505	426169	17490	147508	56.9	NA
FD7-1-1	5462231	240	531	220776	22759	102355	50.2	99.3
FD7-2	4955158	94	504	647023	52714	225301	52.1	NA
FD8-8	5379295	189	530	220781	28461	107828	50.2	99.3
FD9-7-1	5412841	204	500	220774	26533	107828	50.2	99.3

only for *E. coli* isolates, NA not applicable

The result of the multi-locus sequence typing (MLST) established from the WGS and the core genome MLST (cgMLST) clustering of the *E. coli* isolates is

exhibited in Figure 7 and Table 7. Repeated sequencing of one of the 35 *E. coli* isolates did not yield enough target to perform the MLST or cgMLST. The 34 cgMLSTed *E. coli* formed 7 clusters with the threshold of ≤ 10 non-identical targets out of 2513. In Table 7, clusters of cgMLST are marked with C, and isolates not clustering, termed as singletons, are marked with S.

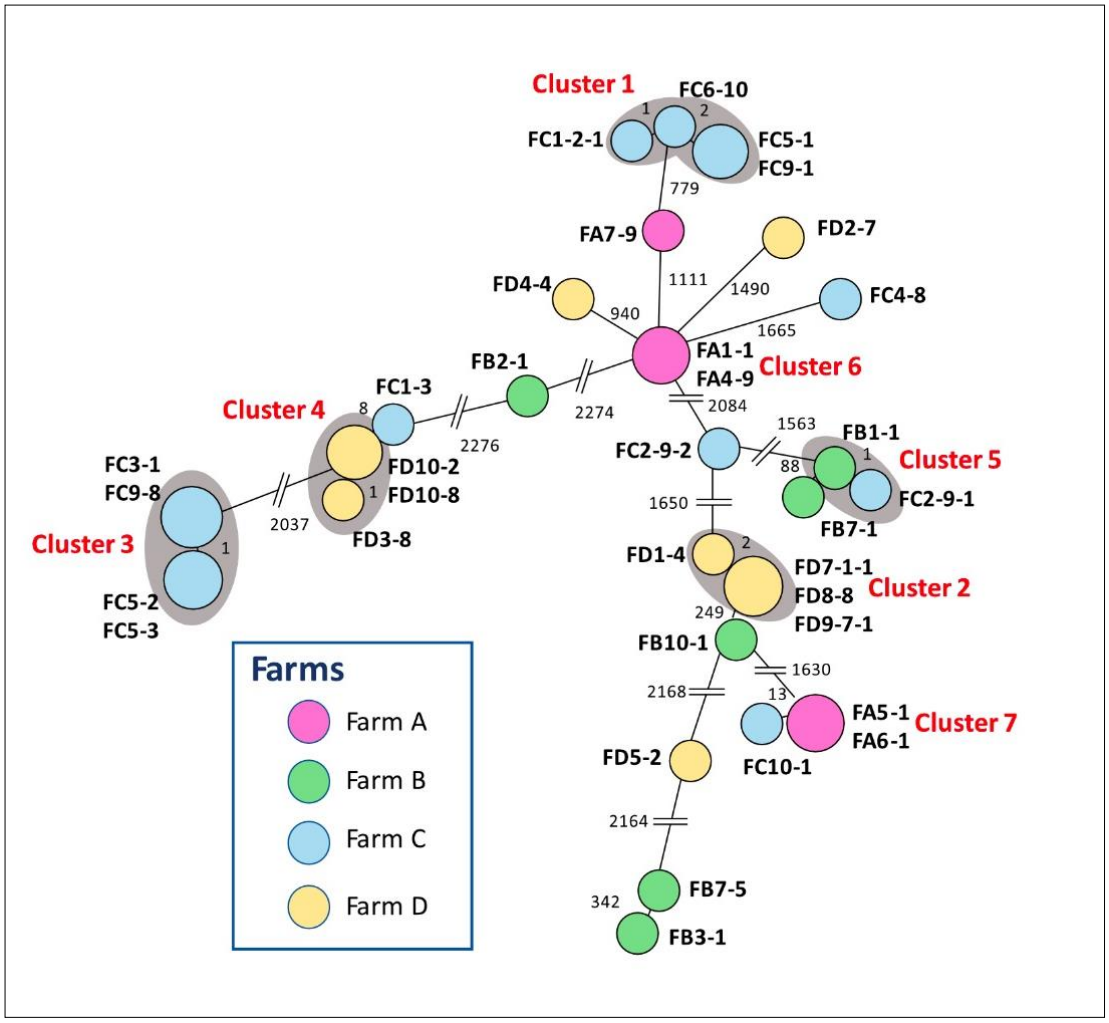


Figure 7: Minimum spanning tree based on cgMLST of *mcr-1* producing *E. coli* isolates (color coded based on the farm they originated from)

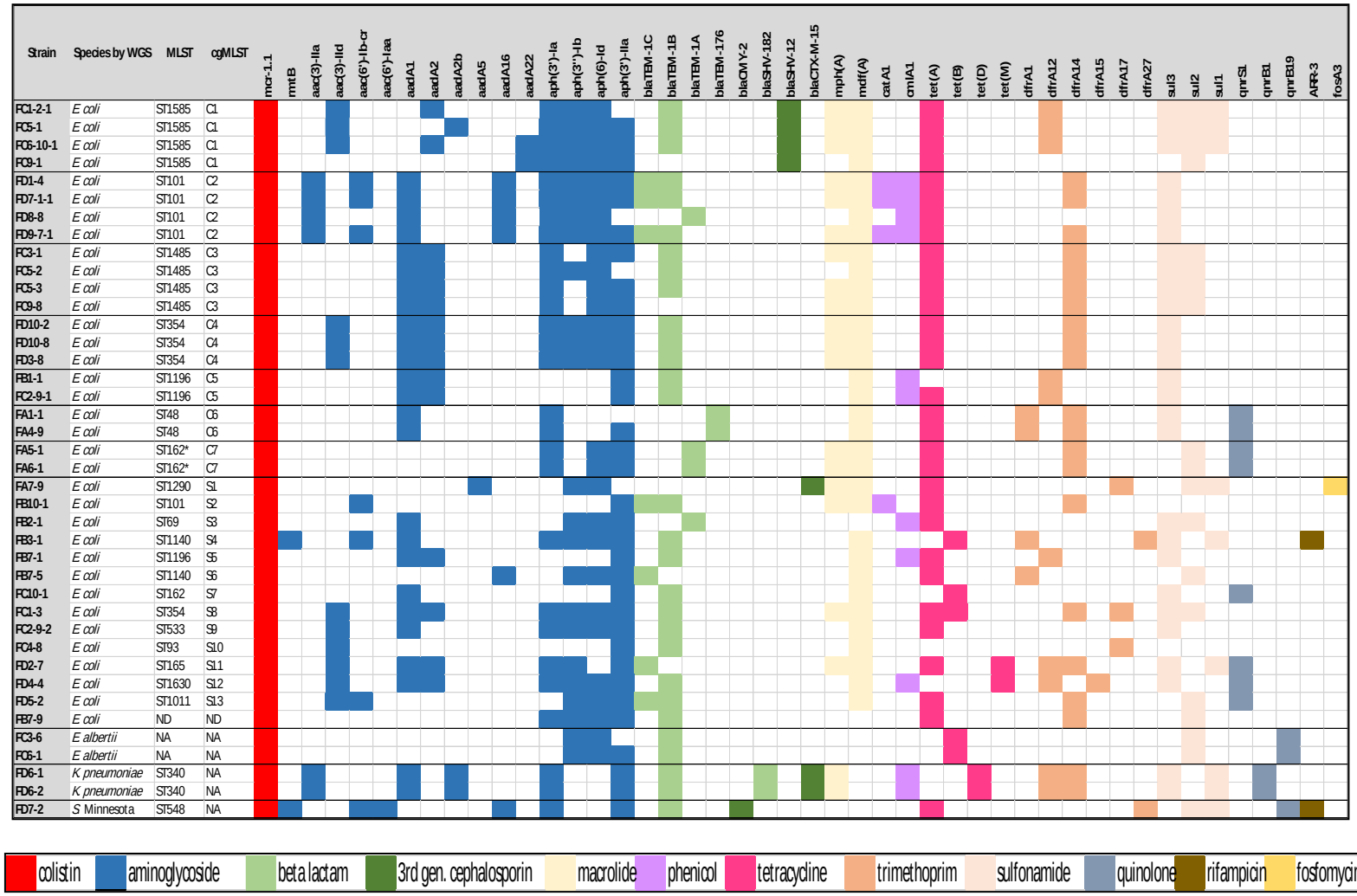
Table 7: Species identification, MLST, and cgMLST of the *mcr-1* positive isolates

Strain	Species by WGS	PFGE Pattern	MLST	cgMLST
FC1-2-1	E. coli	NT	ST1585	C1
FC5-1	E. coli	NT	ST1585	C1
FC6-10-1	E. coli	NT	ST1585	C1
FC9-1	E. coli	NT	ST1585	C1
FD1-4	E. coli	EC3	ST101	C2
FD7-1-1	E. coli	EC3	ST101	C2
FD8-8	E. coli	EC3	ST101	C2
FD9-7-1	E. coli	EC3	ST101	C2
FC3-1	E. coli	NT	ST1485	C3
FC5-2	E. coli	NT	ST1485	C3
FC5-3	E. coli	NT	ST1485	C3
FC9-8	E. coli	NT	ST1485	C3
FD10-2	E. coli	EC5	ST354	C4
FD10-8	E. coli	EC5	ST354	C4
FD3-8	E. coli	EC5	ST354	C4
FB1-1	E. coli	EC7	ST1196	C5
FC2-9-1	E. coli	EC7	ST1196	C5
FA1-1	E. coli	EC10	ST48	C6
FA4-9	E. coli	EC10	ST48	C6
FA5-1	E. coli	EC1	ST162-SLV	C7
FA6-1	E. coli	EC2	ST162-SLV	C7
FB7-9	E. coli	EC9	ND	ND
FA7-9	E. coli	EC1	ST1290	S1
FB10-1	E. coli	EC2	ST101	S2
FB2-1	E. coli	EC8	ST69	S3
FB3-1	E. coli	EC5	ST1140	S4
FB7-1	E. coli	EC6	ST1196	S5
FB7-5	E. coli	EC11	ST1140	S6
FC10-1	E. coli	EC10	ST162	S7
FC1-3	E. coli	NT	ST354	S8
FC2-9-2	E. coli	EC12	ST533	S9
FC4-8	E. coli	EC3	ST93	S10
FD2-7	E. coli	EC1	ST165	S11
FD4-4	E. coli	EC2	ST1630	S12
FD5-2	E. coli	EC8	ST1011	S13
FC3-6	E. albertii	EC4	NA	NA
FC6-1	E. albertii	EC4	NA	NA
FD6-1	K. pneumoniae	KP1	ST340	NA
FD6-2	K. pneumoniae	KP1	ST340	NA
FD7-2	S. enterica serovar Minnesota	S1	ST548	NA

NT non-typable, SLV single locus variant, ND not determined, NA not applicable

As it is shown in Table 7 and in Figure 7, cgMLST mostly clustered isolates from the same farm, except for Cluster 5 (C5) with an isolate from Farm B and another one from Farm C. The acquired resistance gene content of isolates was also determined based on the WGS data. As shown in Table 8, all strains tested harbored the *mcr-1.1* gene, and further to that an array of aminoglycoside, beta-lactam (including extended spectrum beta-lactamase), macrolide, tetracycline, phenicol, trimethoprim, sulfonamide, quinolone or even fosfomycin resistance genes. Isolates belonging to the same cgMLST cluster were not always possessing the same array of resistance genes.

Table 8: Acquired antibiotic resistance genes of the mcr-1 positive isolates



3.5 Antibiotic Susceptibility of the *mcr-1* Positive Isolates

The results of the antibiotic susceptibility testing confirmed phenotypically the results of the WGS. As shown in Table 9, the majority of isolates were not only resistant to colistin, but all of them were multi-drug resistant, *i.e*. non-susceptible to 3 or more classes of antibiotics.

Table 9: Colistin MIC and non-susceptibility to other antibiotics

Strain	Species by WGS	cgMLST	Colistin MIC (mg/L)	Non-susceptibility to other antibiotics
FC1-2-1	*E. coli*	C1	4	AMP, CPD, NAL, CIP, SXT, TET, DOX, GM
FC5-1	*E. coli*	C1	4	AMP, CPD, NAL, CIP, SXT, TET, DOX, GM
FC6-10-1	*E. coli*	C1	4	AMP, CPD, NAL, CIP, SXT, TET, DOX, GM
FC9-1	*E. coli*	C1	4	AMP, CPD, NAL, CIP, TET, DOX
FD1-4	*E. coli*	C2	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FD7-1-1	*E. coli*	C2	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FD8-8	*E. coli*	C2	4	AMP, NAL, CIP, TET, DOX, CHL, GM
FD9-7-1	*E. coli*	C2	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FC3-1	*E. coli*	C3	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FC5-2	*E. coli*	C3	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FC5-3	*E. coli*	C3	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FC9-8	*E. coli*	C3	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FD10-2	*E. coli*	C4	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FD10-8	*E. coli*	C4	8	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FD3-8	*E. coli*	C4	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FB1-1	*E. coli*	C5	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FC2-9-1	*E. coli*	C5	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FA1-1	*E. coli*	C6	4	AMP, NAL, CIP, SXT, TET, DOX
FA4-9	*E. coli*	C6	4	AMP, NAL, CIP, SXT, TET, DOX
FA5-1	*E. coli*	C7	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FA6-1	*E. coli*	C7	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FA7-9	*E. coli*	S1	4	AMP, CPD, CTX, NAL, CIP, SXT, TET, DOX, CHL, FOS
FB10-1	*E. coli*	S2	4	AMP, NAL, CIP, TET, DOX, CHL
FB2-1	*E. coli*	S3	4	AMP, NAL, CIP, TET, GM, TOB
FB3-1	*E. coli*	S4	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, AK, GM, TOB
FB7-1	*E. coli*	S5	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FB7-5	*E. coli*	S6	4	AMP, NAL, CIP, SXT, TET, DOX, CHL
FC10-1	*E. coli*	S7	4	AMP, NAL, CIP, TET, DOX
FC1-3	*E. coli*	S8	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM
FC2-9-2	*E. coli*	S9	4	AMP, NAL, CIP, TET, DOX, CHL, GM, TOB
FC4-8	*E. coli*	S10	8	AMP, NAL, CIP, CHL, GM
FD2-7	*E. coli*	S11	4	AMP, NAL, CIP, SXT, TET, DOX, GM, TOB
FD4-4	*E. coli*	S12	4	AMP, NAL, SXT, TET, DOX, CHL, GM
FD5-2	*E. coli*	S13	4	AMP, NAL, CIP, SXT, TET, DOX, CHL, GM, TOB
FB7-9	*E. coli*	ND	4	AMP, NAL, CIP
FC3-6	*E. albertii*	NA	8	AMP, NAL, TET, DOX
FC6-1	*E. albertii*	NA	8	AMP, NAL, CIP, TET, DOX
FD6-1	*K. pneumoniae*	NA	>256	AMP, CPD, CAZ, CTX, AZT, NAL, CIP, SXT, TET, DOX, CHL, GM
FD6-2	*K. pneumoniae*	NA	>256	AMP, CPD, CAZ, CTX, AZT, NAL, CIP, SXT, TET, DOX, CHL, GM
FD7-2	*S.* Minnesota	NA	8	AMP, AMC, FOX, CPD, CAZ, CTX, CIP, SXT, TET, DOX, AK, GM, TOB

AMP ampicillin, CPD, cefpodoxime, NAL nalidixic acid, CIP ciprofloxacin, SXT co-trimoxazole, TET tetracycline, DOX doxycycline, GM gentamicin, CHL chloramphenicol, CTX cefotaxime, FOS fosfomycin, TOB tobramycin, AK amikacin, AZT aztreonam, CPD cefpodoxime, CAZ ceftazidime, AMC amoxicillin/clavulanate

3.6 Analysis of the *mcr* Gene Carrying Plasmids

Southern blot of the plasmid electrophoresis gels and hybridization with the *mcr-1* probe confirmed the plasmid localization of the mcr-1 gene in 36 of the 40 selected isolates. Based on the alkaline lysis extracted *E. coli* 39R861 plasmid size controls, in 26 strains the size of the *mcr*-plasmid was approximately 60kb, in six strains it was >154 kb, and in four the *mcr*-probe hybridized with an approximately 80 kb plasmid band (Table 10).

The majority of the *mcr*-plasmids (n=27) were self-transmissible, but in six of them the conjugation resulted in the transfer of multiple plasmids (Table 10). In these latter cases, and in case of a non-self-transmissible plasmid single plasmid containing derivative was achieved by transformation of the *mcr*-plasmid into *E. coli* DH5α. In *E. coli* FC1-3 and FC9-8 the hybridization of the plasmid profile of the wild-type strain has not revealed a plasmid-borne *mcr* gene, nevertheless conjugation resulted in transfer of *mcr-1* gene carrying plasmids with a size of >154 kb (Table 10).

Table 10: Characteristics of the mcr-1 carrying plasmids

Strain				*mcr*-plasmid				
Name	Species by WGS	MLST	cgML ST	Detected by hybridization	Approximate size (kb)	Self-transmissibility	Plasmid RFLP pattern	Inc type based on plasmid sequencing
FC1-2-1	*E. coli*	ST1585	C1	Yes	60	C	S1	NT
FC5-1	*E. coli*	ST1585	C1	Yes	60	C	S1b	NT
FC6-10-1	*E. coli*	ST1585	C1	Yes	60	C	S1	NT
FC9-1	*E. coli*	ST1585	C1	Yes	60	C	S1b	IncI2
FD1-4	*E. coli*	ST101	C2	Yes	60	C	S2b	IncI2
FD7-1-1	*E. coli*	ST101	C2	Yes	60	C	S2	NT
FD8-8	*E. coli*	ST101	C2	Yes	60	Cm	S2a	IncI2
FD9-7-1	*E. coli*	ST101	C2	Yes	60	Cm	S2a	NT
FC3-1	*E. coli*	ST1485	C3	Yes	>154	C	B3	IncHI2
FC5-2	*E. coli*	ST1485	C3	No	NA	NA	NA	NT
FC5-3	*E. coli*	ST1485	C3	No	NA	NA	NA	NT
FC9-8	*E. coli*	ST1485	C3	No	>154	C	B2	IncHI2-IncFIB
FD10-2	*E. coli*	ST354	C4	Yes	>154	NST	B1	NT
FD10-8	*E. coli*	ST354	C4	Yes	>154	NST	B1	NT
FD3-8	*E. coli*	ST354	C4	Yes	>154	NST	B1	IncHI2
FB1-1	*E. coli*	ST1196	C5	Yes	60	NST	S1	NT
FC2-9-1	*E. coli*	ST1196	C5	Yes	60	NST	S1d	IncI2
FA1-1	*E. coli*	ST48	C6	Yes	60	C	S1a	IncI2
FA4-9	*E. coli*	ST48	C6	Yes	60	Cm	S1	NT
FA5-1	*E. coli*	ST162*	C7	Yes	60	C	S1	IncI2
FA6-1	*E. coli*	ST162*	C7	Yes	60	NST	S1	NT
FA7-9	*E. coli*	ST1290	S1	Yes	60	C	S1	NT
FB10-1	*E. coli*	ST101	S2	Yes	60	Cm	S1c	IncI2
FB2-1	*E. coli*	ST69	S3	Yes	60	C	S2	NT
FB3-1	*E. coli*	ST1140	S4	Yes	60	C	S1	NT
FB7-1	*E. coli*	ST1196	S5	Yes	80	NST	NT	IncX4
FB7-5	*E. coli*	ST1140	S6	Yes	60	C	S1a	NT
FC10-1	*E. coli*	ST162	S7	Yes	60	C	S1	NT
FC1-3	*E. coli*	ST354	S8	No	>154	C	B1a	IncHI2
FC2-9-2	*E. coli*	ST533	S9	Yes	60	NST	S1	NT
FC4-8	*E. coli*	ST93	S10	Yes	60	C	S2	NT
FD2-7	*E. coli*	ST165	S11	Yes	>154	NA	NA	NT
FD4-4	*E. coli*	ST1630	S12	Yes	60	C	S2	IncI2
FD5-2	*E. coli*	ST1011	S13	No	NA	NA	NA	NT
FB7-9	*E. coli*	ND	ND	Yes	60	C	S1	NT
FC3-6	*E. albertii*	NA	NA	Yes	60	C	S1	IncI2
FC6-1	*E. albertii*	NA	NA	Yes	60	C	S1	NT
FD6-1	*K. pneumoniae*	ST340	NA	Yes	80	Cm	M1	IncX4
FD6-2	*K. pneumoniae*	ST340	NA	Yes	80	Cm	M1	NT
FD7-2	*S.* Minnesota	ST548	NA	Yes	80	NST	NT	IncX4

C conjugative, Cm conjugation resulted in multiple plasmids containing recipient, NST not self-transmissible, NA not applicable, NT not tested

The antibiotic susceptibility of the single *mcr*-plasmid containing derivatives is shown in Table 11. All derivatives had elevated colistin MIC compared to the recipients. Only six derivatives exhibited non-susceptibility to other antibiotics tested, these were the derivatives having the >154kb mcr-plasmid.

Table 11: Antibiotics resistance of transconjugants/transformants carrying a single *mcr*-plasmids

Transconjugant/ transformant	Colistin MIC (mg/L)	Resistance co-transferring with the *mcr*-plasmid
J53RAZ(pFC1-2-1)	2	none
J53RAZ(pFC5-1)	2	none
J53RAZ(pFC6-10-1)	2	none
J53RAZ(pFC9-1)	2	none
J53RAZ(pFD1-4)	2	none
J53RAZ(pFD7-1-1)	4	none
DH5α(pFD8-8)	4	none
DH5α(pFD9-7-1)	4	none
J53RAZ(pFC3-1)	2	AMP, TET, DOX, CHL
J53RAZ(pFC9-8)	2	AMP, TET, DOX, CHL
DH5α(pFD10-2)	4	AMP, TET, CHL
DH5α(pFD10-8)	4	AMP, TET, CHL
DH5α(pFD3-8)	2	AMP, TET, CHL
DH5α(pFB1-1)	4	none
DH5α(pFC2-9-1)	8	none
J53RAZ(pFA1-1)	4	none
J53RAZ(pFA4-9)	4	none
J53RAZ(pFA5-1)	2	none
J53RAZ(pFA6-1)	4	none
J53RAZ(pFA7-9)	2	none
DH5α(pFB10-1)	2	none
J53RAZ(pFB2-1)	2	none
J53RAZ(pFB3-1)	2	none
DH5α(pFB7-1)	2	none
J53RAZ(pFB7-5)	2	none
J53RAZ(pFC10-1)	2	none
J53RAZ(pFC1-3)	2	AMP, TET, DOX, CHL
J53RAZ(pFC2-9-1)	2	none
J53RAZ(pFC4-8)	4	none
J53RAZ(pFD4-4)	2	none
J53RAZ(pFB7-9)	2	none
J53RAZ(pFC3-6)	2	none
J53RAZ(pFC6-1)	2	none
DH5α(pFD6-1)	4	none
DH5α(pFD6-2)	4	none
DH5α(pFD7-2)	2	none
J53RAZ	≤0.5	none
DH5α	≤0.5	none

AMP ampicillin, TET tetracycline, DOX doxycycline, CHL chloramphenicol

All *mcr*-plasmids were purified from the single plasmid containing derivatives and were subjected to restriction fragment length pattern (RFLP) analysis. (The actual RFLP patterns are shown in the Appendix). The RFLP results are listed in Table 10. Altogether 16 plasmids were selected for sequencing based on varying plasmid sizes

and RFLP patterns and/or hosted by a different species. From the plasmid sequences the incompatibility type of the plasmid (Table 10), the immediate genetic surrounding of the mcr-1.1 gene (Figure 8) and the presence of other antibiotic resistance genes (Figure 9) was established. However, as we used short Illumina sequencing reads, determining the complete plasmid sequences was beyond the scope of this study.

As it is listed in Table 10, plasmids having an approximate size of 60kb were incompatibility type (Inc) I2 plasmids, irrespective whether they exhibited RFLP pattern S1, S1a, S1b, S1c, S1d, S2, S2a or S2b. The plasmids with estimated size of 80 kb based on the alkaline lysis plasmid preparation were IncX4 plasmids with a size of approximately 35 kb, based on the next generation sequencing results. The large plasmids (>154kb) were all IncHI2 types, with RFLP pattern B2 plasmid being a likely fusion plasmid with IncHI2 and IncFIb replicases.

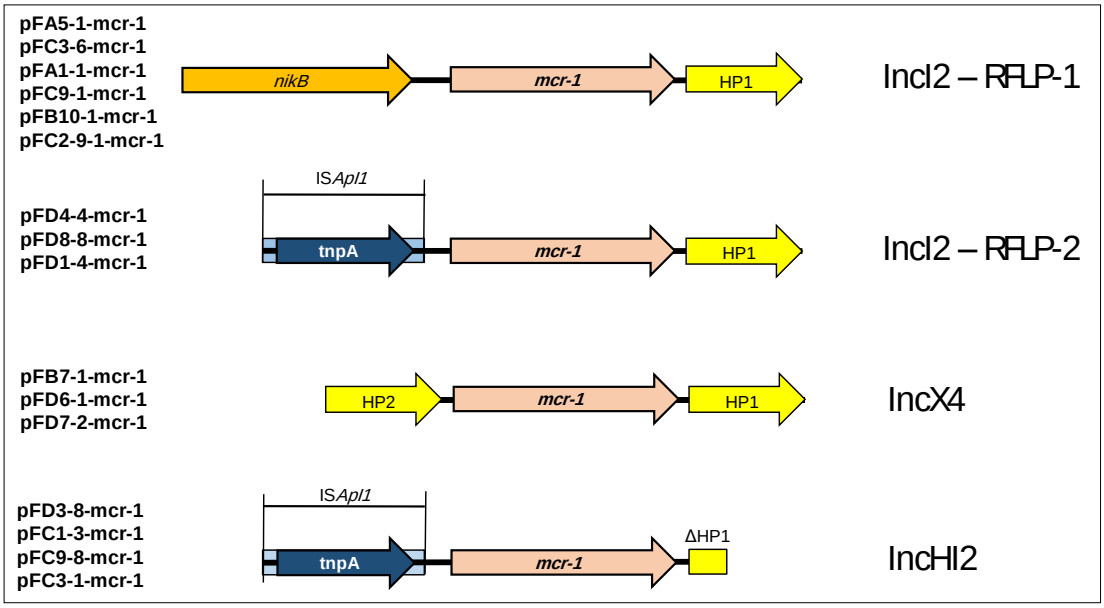


Figure 8: Genetic surrounding of the mcr-1 gene in the mcr-plasmids sequenced

The immediate genetic surrounding of the *mcr-1* gene (shown in Figure 8) was correlating with the plasmid incompatibility types, with the notable difference in IncI2

plasmids, in which RFLP type S1, S1a, S1b, S1c, S1d plasmids did not possess IS*Apl1*. This insertion element, commonly attributed to mobilize the *mcr-1* gene, was present in IncI2 plasmids with RFLP pattern S2, S2a and S2b and in all IncHI2 plasmids.

Contigs generated from the plasmid sequence were also uploaded to the ResFinder website to assess whether they carry antibiotic resistance genes beyond the *mcr-1.1*. Further antibiotic resistance genes were not present in any of the IncI2 and IncX4 type plasmids. Nevertheless, all IncHI2 plasmids, beyond the *mcr-1.1*, possessed *bla*$_{\text{TEM-1B}}$, *dfrA14*, sul3, *tet(A)*, *mph(A)*, *floR*, *aadA1*, *aadA2*, *sat1*, *aphA*, *stra* and *strB* genes coding for beta-lactam, co-trimoxazole, tetracycline, macrolide, phenicol and aminoglycoside resistance. This array of resistance genes was the same as the resistance island gene array of an IncHI2 plasmid previously described from Saudi Arabia (pSA26-mcr-1, Genbank Accession No. KU743384) (Sonnevend et al., 2016). Therefore, we mapped the reads of pFD3-8-mcr-1, pFC1-3-mcr-1, pFC9-8-mcr-1 and pFC3-1-mcr-1 to KU743384 and in all these plasmids the reads mapped to the resistance island of pSA26-mcr-1 (Figure 9).

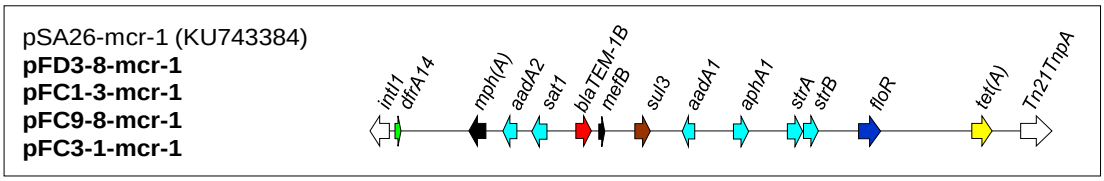


Figure 9: Resistance island of the four IncHI2 type *mcr*-plasmid in this study and KU43384 (Sonnevend et al., 2016)

Chapter 4: Discussion

The severe global problem of the rising antibiotic resistance cannot be solved by isolated steps taken in human medicine only. Therefore, the concept of One Health became the approach to tackle the issue (WHO, 2017), and more and more studies examine the effect of antibiotic use and the presence of drug-resistant bacteria, not only in the human population, but in the animal husbandry and generally in the environment. Such studies are frequently published from Europe, North and South-America and part of Asia, *e.g.* South Korea and China (Pormohammad et al., 2019; Qiao et al., 2018), but very few studies were conducted in the Middle East (Dandachi et al., 2018), and data are almost completely missing from the Arabian Peninsula, where a report on antibiotic resistant marine isolates in Kuwait (Al-Sarawi et al., 2018) and two others from Saudi Arabia (Altalhi et al., 2010) and Qatar (Eltai et al., 2018) on poultry isolates, and a further one on animal *Salmonella* isolates from the UAE (Münch et al., 2012) are the only recent data on non-human isolates of antibiotic resistant *Enterobacteriaceae*.

The recently discovered plasmid mediated colistin resistance caused a great concern, as colistin is one of the last-resort drugs to treat multi-drug resistant organisms, which are even resistant to carbapenems. The fact that colistin was widely used in animal husbandry, and also epidemiological data, suggested that mobile colistin resistance (*mcr*) genes have emerged in animals and possibly transferred to humans (Apostolakos and Piccirillo, 2018). Poultry, and other food producing animals, were described worldwide as carriers of MCR-producing bacteria, and they were found especially frequently in animals in East and South-East Asia (Kempf et al., 2016). In the Arabian Peninsula, sporadic cases of MCR-producing *Enterobacteriaceae* have

been reported from humans (Alghoribi et al., 2019; Mohsin et al., 2017; Sonnevend et al., 2016). However, data on animal carriage of this gene is almost completely lacking, with a single study reporting 15.5% of commensal broiler chicken *E. coli* isolates investigated possessing the *mcr-1* gene in Qatar (Eltai et al., 2018). In our study, 90% of the samples tested positive for *mcr-1* carrying isolates; and testing several colonies from the positive samples identified 40 isolates with different plasmid profiles. These rates, however, cannot be directly compared, as we used composite stool samples and pre-enriched the specimens in colistin containing medium, hence enhancing the chance to isolate MCR-producing organisms.

The 40 isolates with different plasmid profiles belonged to four species of *Enterobacteriaceae*: *E. coli, K. pneumoniae*, *E. albertii* and *Salmonella* Minnesota. The latter two may cause foodborne gastrointestinal infections in humans (FDA, 2019; Q. Li et al., 2018). Although the species, *Salmonella enterica* is frequently associated with *mcr* genes, to the best of our knowledge, our study is the first identifying *S. enterica* serovar Minnesota carrying an *mcr-1* gene (Lima et al., 2019). Worryingly, the *S.* Minnesota isolate was not only resistant to colistin, but to seven other classes of antibiotics including extended spectrum cephalosporins (ESC), due to the presence of 14 resistance genes beyond *mcr-1*. Such MDR *Salmonella* strains usually belong to *S.* Typhimurium serotype, however in Brazil they recorded *S.* Minnesota isolated from poultry meat, which was resistant to ESC due to the production of CMY-2, a plasmid coded AmpC type cephalosporinase (Moura et al., 2017). Furthermore, a study from Portugal also described CMY-2 producing, multi-drug resistant *S.* Minnesota isolated from poultry meat imported from Brazil (Campos et al., 2018). Interestingly, the MLST of the isolate recovered from Portugal was the same as the one isolated in our study (ST548), but worryingly beyond the ESC, tetracycline, ciprofloxacin and co-

trimoxazole resistance, FD7-2 *S.* Minnesota isolate from the UAE was also resistant to all aminoglycosides due to the presence of *rmtB* ribosomal methylase, and to colistin due to the presence of *mcr-1*.

E. albertii, a human and bird pathogen species of *Enterobacteriaceae*, is frequently colonizing birds. This species was isolated in several countries from processed chicken, suggesting that poultry is the origin of human diarrheal diseases (Bhatt et al., 2019). Furthermore, it has been described from China to be multi-drug resistant, producing ESBL and to carry the *mcr-1* gene (Q. Li et al., 2018). The isolates in this study exhibited resistance to colistin, tetracycline, nalidixic acid and ampicillin, but were not producing an ESBL.

On the other hand, the two clonally related *K. pneumoniae* identified were not only MCR-1, but also ESBL producers harboring the $bla_{\text{CTX-M-15}}$ gene. Although, our isolates did not possess a carbapenemase gene, their sequence type, ST340 belongs to the worldwide spreading clonal complex 258 (CC258) usually carrying bla_{KPC}. Polymyxin resistant *K. pneumoniae* ST340 has been described from Brazil, but the mechanism of resistance was a chromosomal mutation in that isolate (Braun et al., 2018).

The majority of our isolates found to possess *mcr-1* gene were *E. coli* reflecting the worldwide trend of *E. coli* being the most frequent carrier of the *mcr* genes (Sun et al., 2018). The 35 *E. coli* isolates exhibited 16 sequence types (STs) with one being untypable based on the WGS data. In all farms, multiple STs were present, and in certain STs the cgMLST further differentiated strains (Table 7 and Figure 7). This variety of *E. coli* is not surprising as the *mcr* genes mostly spread via horizontal gene transfer, and rarely show clonality (Matamoros et al., 2017; Sun et al., 2018). However,

although the zoonotic origin of human MCR-1 producing isolates were suggested (Poirel and Nordmann, 2016), none of the STs identified from poultry feces overlapped with human clinical MCR-1 producing isolates described from the Arabian Peninsula (Alghoribi et al., 2019; Leangapichart et al., 2016; Mohsin et al., 2017; Sonnevend et al., 2016).

E. coli ST101 was represented with the largest number of isolates (n=5) (Table 6). This sequence type of *E. coli* was reported as one of the most frequent carbapenem resistant, New Delhi metallo-beta-lactamase producing *E. coli* ST found among human isolates worldwide (Dadashi et al., 2019). Moreover, ESBL-/AmpC-producing *E. coli* belonging to the same ST was recovered from meat products imported into the European Union from third countries (Müller et al., 2018). Furthermore, it was also recognized in environmental samples collected on Chinese public transport vehicles harboring *mcr-1* (C. Shen et al., 2018) and from poultry farms in Vietnam (Trung et al., 2017).

In the collection of isolates with different plasmid profiles *E. coli* ST354, ST1485 and ST1585 were represented by four isolates each. ST1585 has not been reported to carry *mcr* genes or other resistance genes, yet. Further to *mcr-1*, all four isolates in our collection also carried and ESBL gene, $bla_{\text{SHV-12}}$.

E. coli ST1485 was described from Japan to be ESBL, colonizing humans in the community (Nakamura et al., 2016), and recently a human non-MDR, MCR-1 producing *E. coli* ST1485 was also reported from the USA from a urine sample of a patient returning from Portugal (Gilrane et al., 2017).

Multi-drug resistant, ESBL or carbapenemase producer *E. coli* ST354 clinical and animal isolates are reported from various continents (Bedenić et al., 2018; Dagher et al., 2018; Dandachi et al., 2018; Mora et al., 2011; Sallem et al., 2015; Vangchhia et al., 2016). MCR-1 producer ST354 was also isolated from human, as well as from animal samples in several countries (Matamoros et al., 2017). Thus, this clone may be considered a high-risk MDR clone with a conceivable ability to spread, and colonize or infect various species (Woodford et al., 2011).

E. coli ST1196 represented by three isolates in our collection, and like ST354, this lineage was also reportedly isolated from humans and animals. This clone has been identified to carry *mcr-1* gene in Spanish leukemia patients, in Chinese hospital sewage water, and in Taiwanese retail chicken meat (Kuo et al., 2016; Lalaoui et al., 2019; Zhao et al., 2017). Furthermore, it was also reported to harbor $bla_{\text{NMD-5}}$ in clinical isolates of Myanmar, and to possess $bla_{\text{OXA-48}}$ in companion animals in Germany (Aung et al., 2018; Pulss et al., 2018).

E. coli ST162 and its single-locus variant was also found in our study to carry *mcr-1*. This clone has been identified in animals, mostly chicken to carry *mcr-1* in South-East Asia and in Europe (Matamoros et al., 2017). The same clone also carried $bla_{\text{CTX-M}}$ in an isolate from the Choqueyapu River in La Paz, Bolivia and in retail raw chicken meat portions in Japan (Guzman-Otazo et al., 2019; Hayashi et al., 2018). However, to the best of our knowledge, human clinical isolate of this clone has not been described yet.

Two *E. coli* ST48 and ST1140 were among our isolates, respectively. The former is a frequently reported avian pathogen, *i.e.* a causative agent of colibacillosis in chicken (Cordoni et al., 2016; Zhuge et al., 2019). It has also been recognized as

enterotoxigenic *E. coli* from human diarrheal samples (Y. Li et al., 2017). *E. coli* ST48 was also described to carry *mcr-1* gene from chicken isolates of China (Zhuge et al., 2019). Furthermore, a novel NDM-variant, NDM-17 was also found in a chicken isolate of this clone in China (Z. Liu et al., 2017).

The only publication on *E. coli* ST1140 was a report on an *mcr-1* carrying isolate from a German swine farm (Guenther et al., 2017).

Further single isolates of *E. coli* ST69, ST93, ST165, ST533, ST1011, ST1290, ST1630 were identified to carry *mcr-1*. Of these sequence types, ST69 is a well-recognized human ExPEC lineage, which has been found in animal and food products as well (Riley, 2014). *mcr-9*, an inducible gene encoding an acquired phosphoethanolamine transferase, was described recently from this *E. coli* sequence type (Kieffer et al., 2019).

E. coli ST93 carrying *mcr-1* has been described from Chinese companion animals, and also from a human stool sample in Finland (Gröndahl-Yli-Hannuksela et al., 2018; J. Wang et al., 2018).

E. coli ST1011 was described from a German swine farm to possess the *mcr-1* gene (Guenther et al., 2017).

E. coli ST165, ST533, ST1290 and ST1630 has not been described carrying *mcr* genes, yet (Matamoros et al., 2017).

The *E. coli* ST1290 isolate, FA7-9 from Farm A, also had a unique resistance gene profile, harboring $bla_{\text{CTX-M-15}}$ ESBL and *fosA3* fosfomycin resistance genes. This latter gene has rarely been encountered in the Arabian Peninsula (Algowaihi et al., 2016; Dantas Palmeira et al., 2018), and as fosfomycin is a frequently used oral

treatment for urinary tract infection caused by an ESBL *E. coli,* co-occurrence of these two genes in an *E. coli* isolate is worrying.

Nonetheless, it is of great concern that all *mcr-1* possessing isolates examined in our sturdy were not only resistant to colistin, but to a broad range of antibiotics, qualifying as multi-drug resistant using the definition laid out by (Magiorakos et al., 2012).

The mcr-1 gene was located on plasmid in 36 of the 40 isolates investigated. Seventy-five percent of these plasmids were transmissible. These 36 plasmids belonged to three plasmid incompatibility types: IncHI2, IncI2 and IncX4. The plasmid Inc types were mostly related to clonality except for the *E. coli* ST1196 isolates, which either carried IncI2 or IncX4 plasmids. The three *mcr-1*-plasmids' Inc types in our study are the ones encountered most frequently worldwide in association with this gene (Matamoros et al., 2017). The IncI2 and IncX4 mcr-plasmids may confer fitness advantage to those isolates bearing them, which could be a possible explanation for their extensive spread (Wu et al., 2018).

Only IncHI2 plasmids carried multiple resistance determinants, suggesting that those isolates having the IncI2 or IncX4 plasmids were MDR due to possession of other additional mobile genetic elements and possible chromosomal mutations, but investigating these traits was beyond the scope of our current study.

The immediate genetic surrounding of the *mcr-1* gene (Figure 8) was similar to those described earlier from the Arabian Peninsula (Sonnevend et al., 2016) or in case of IncX4 plasmid, which has not been described from the region earlier, elsewhere (Sun et al., 2018). A notable difference between the IncHI2 plasmids of our study and

the same incompatibility type mcr-plasmids of human isolates from the Arabian Peninsula was that the IS*Apl1* did not bracket the resistance gene, it was only found upstream of the *mcr-1* (Alghoribi et al., 2019; Sonnevend et al., 2016). Nevertheless, the IncHI2 plasmids of chicken origin harbored the same resistance island of 13 resistance genes, as a human isolate of Saudi Arabia (Figure 9), suggesting possible transfer between animals and humans (Sonnevend et al., 2016).

The limitation of our study was its small scale and the lack of data on several factors that could potentially impact the spread of Mcr-producing strains. These include the use of antibiotics in the chicken flocks, the nationalities and personal hygiene of the workers, the infection control practices implemented, the waste disposal systems, the presence or absence of manure recycling locally or after being sold to other animal farms, its use as feed in fish farms. All this information, once collected in future, more extensive studies would give a more comprehensive picture on the environmental effect and possible infection transmission routes. Nonetheless, we think that the paucity of data on antibiotic resistance of isolates of animal origin from the UAE makes our investigation valuable. Moreover, even from this small sample size, a considerable variety of MCR-producing multi-drug resistant *Enterobacteriaceae* were isolated and characterized, suggesting the magnitude of the problem in the country. It is even more important to highlight these issues, as the use of antibiotics in animal husbandry in the UAE is not always transparent and well documented. The epidemiological situation suggested by this study is highly complex and necessitates a surveillance program for the full assessment of antibiotic resistance problem in animal husbandry across the whole UAE, and to investigate the possible routes of transmission of MCR-producing *Enterobacteriaceae* from animals to humans in the UAE. Interdisciplinary coordinating body between the relevant ministries could ensure

implementation of WHO recommendations, national regulations and best practices in animal husbandry as well as ensure documentation, accessibility, and transparency regarding information on antibiotic use in animal husbandry. Public health surveillance as well as inclusion of colistin in routine susceptibility testing of clinical isolates could reveal the magnitude of mcr-1 carriage in humans.

Chapter 5: Conclusion

Our study identified a large number of MCR-producing *Enterobacteriaceae* in composite stool samples of broiler chicken. These isolates exhibited a broad variety, belonged to four different species, and in case of the *E. coli* isolates, to 16 different sequence types and 20 core genome MLST types.

All MCR-producing chicken stool isolates were multi-drug resistant, harbored a broad range of resistance genes beyond the *mcr-1*, and the majority of them were of globally encountered lineages of *Enterobacteriaceae* causing infections in humans too.

In most of the isolates the *mcr-1* gene was located on plasmids of IncHI2, IncI2 and IncX4 types. The IncHI2 and IncI2 plasmids were similar to the respective Inc type mcr-plasmids previously identified in the Arabian Peninsula from clinical isolates.

Based on these findings, assessment of the antibiotic resistance problem in animal husbandry across the whole UAE is warranted, as in our investigation the sample collection was geographically limited.

Also, further studies should investigate the possible route of transmission of MCR-producing *Enterobacteriaceae* from animals to humans in the UAE.

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Appendix

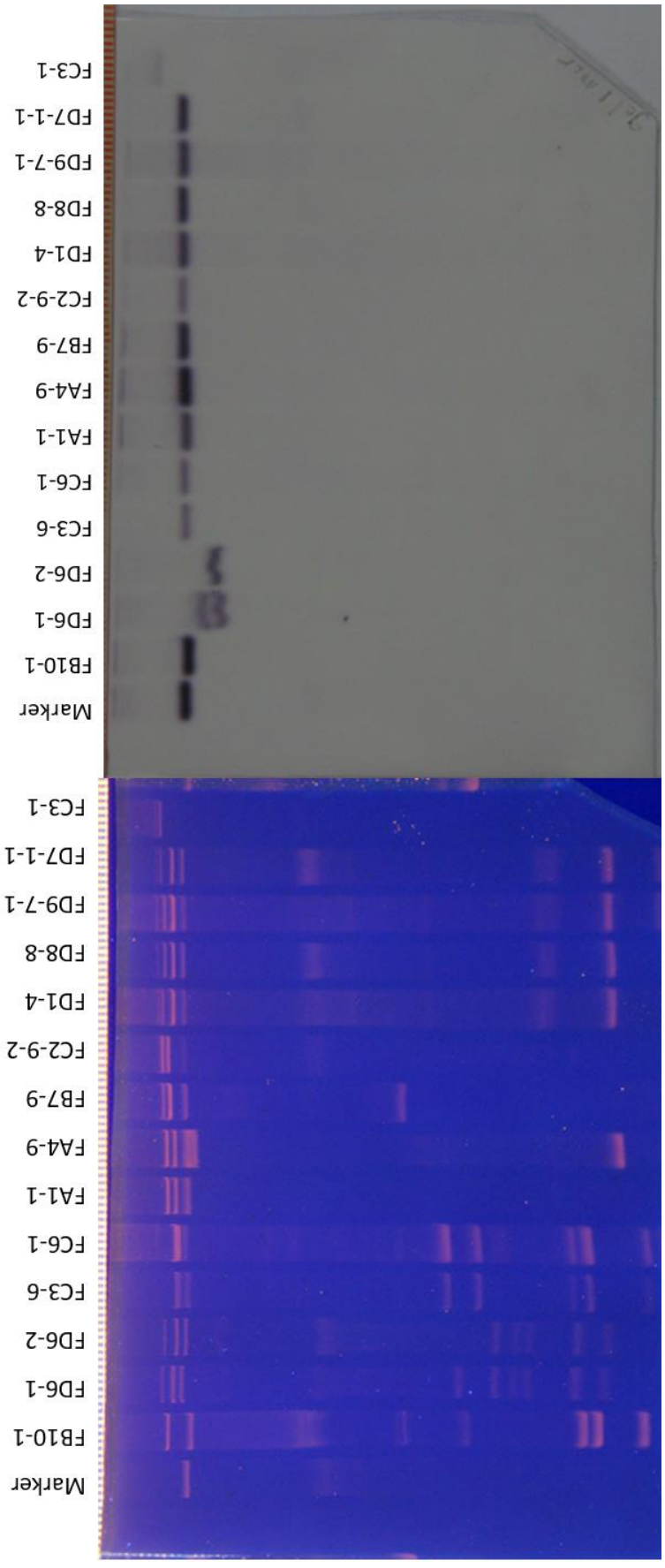

Figure A.1: Hybridization and mcr-1 developed membranes of the representative plasmids (First batch)

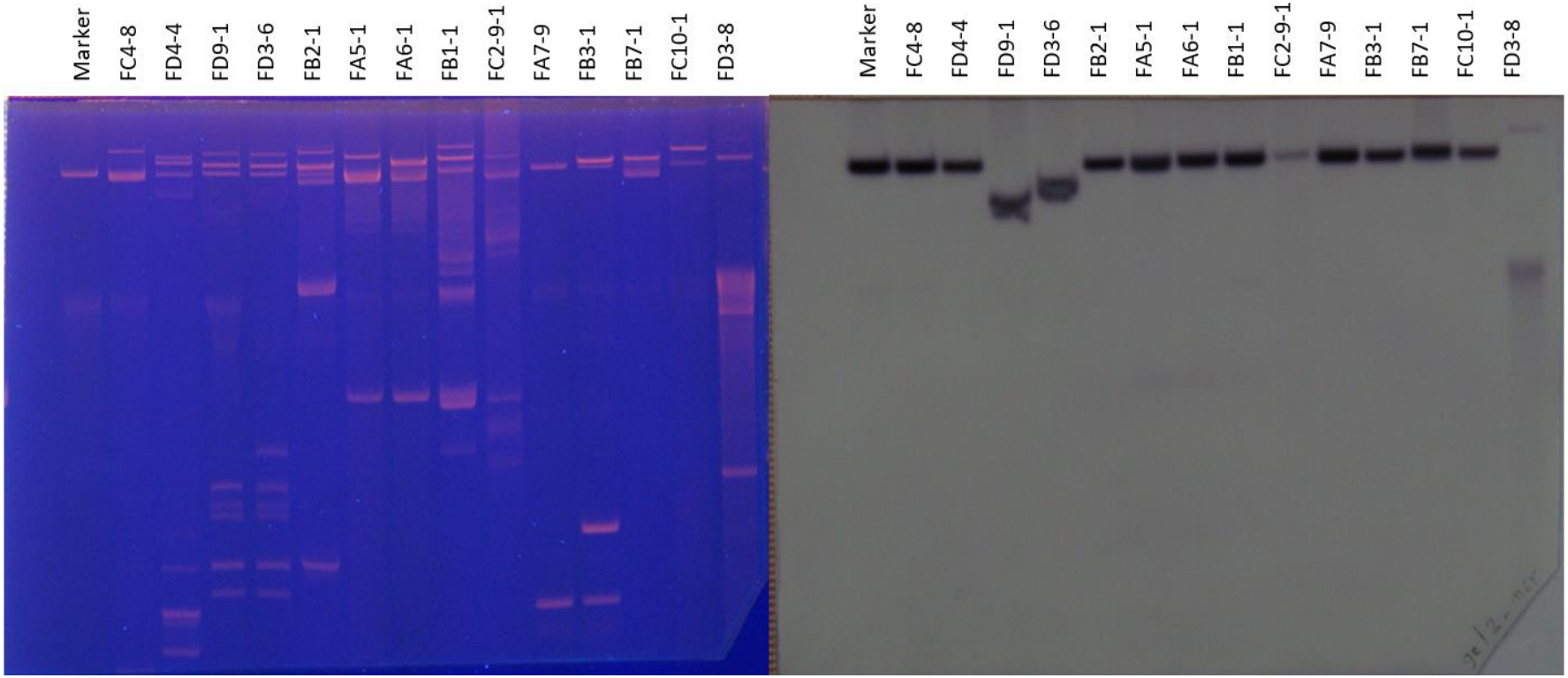

Figure A2: Hybridization and mcr-1 developed membranes of the representative plasmids (Second batch)

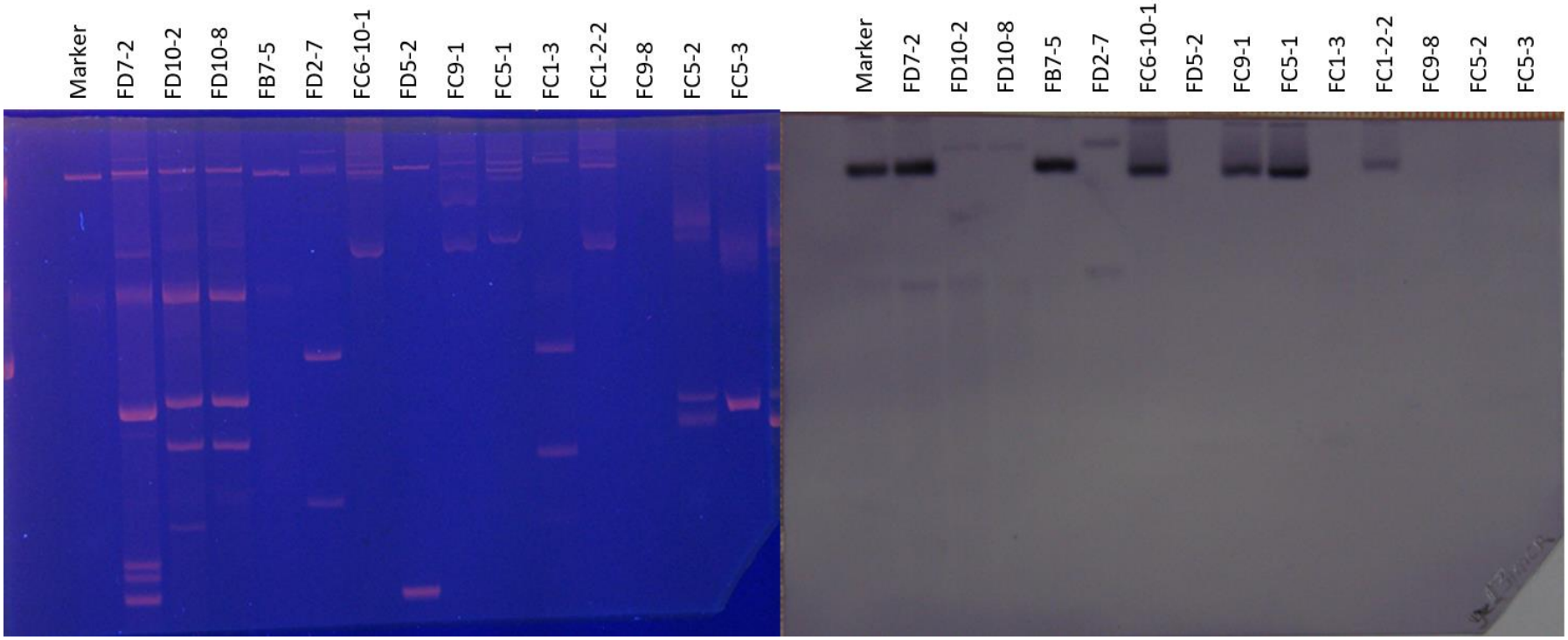

Figure A3: Hybridization and mcr-1 developed membranes of the representative plasmids (Third batch)

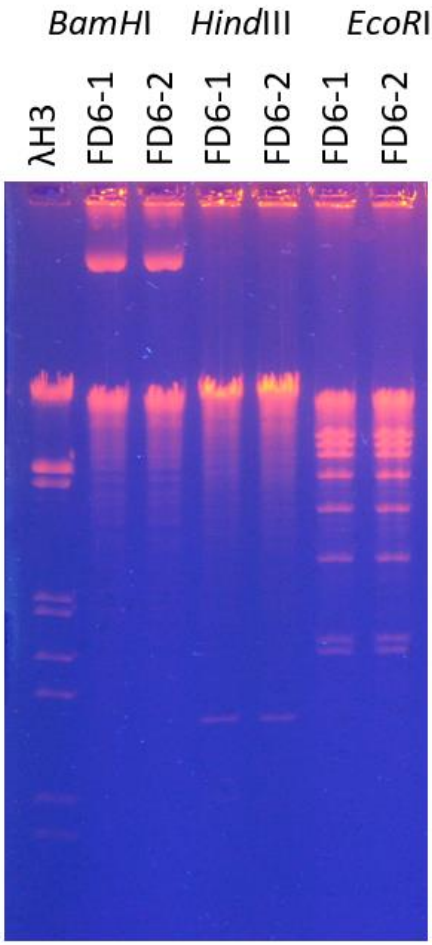

Figure B: RFLP pattern of the plasmids of the two Klebsiella pneumoniae strains digested with *BamH*I, *Hind*III, and *Eco*RI

United

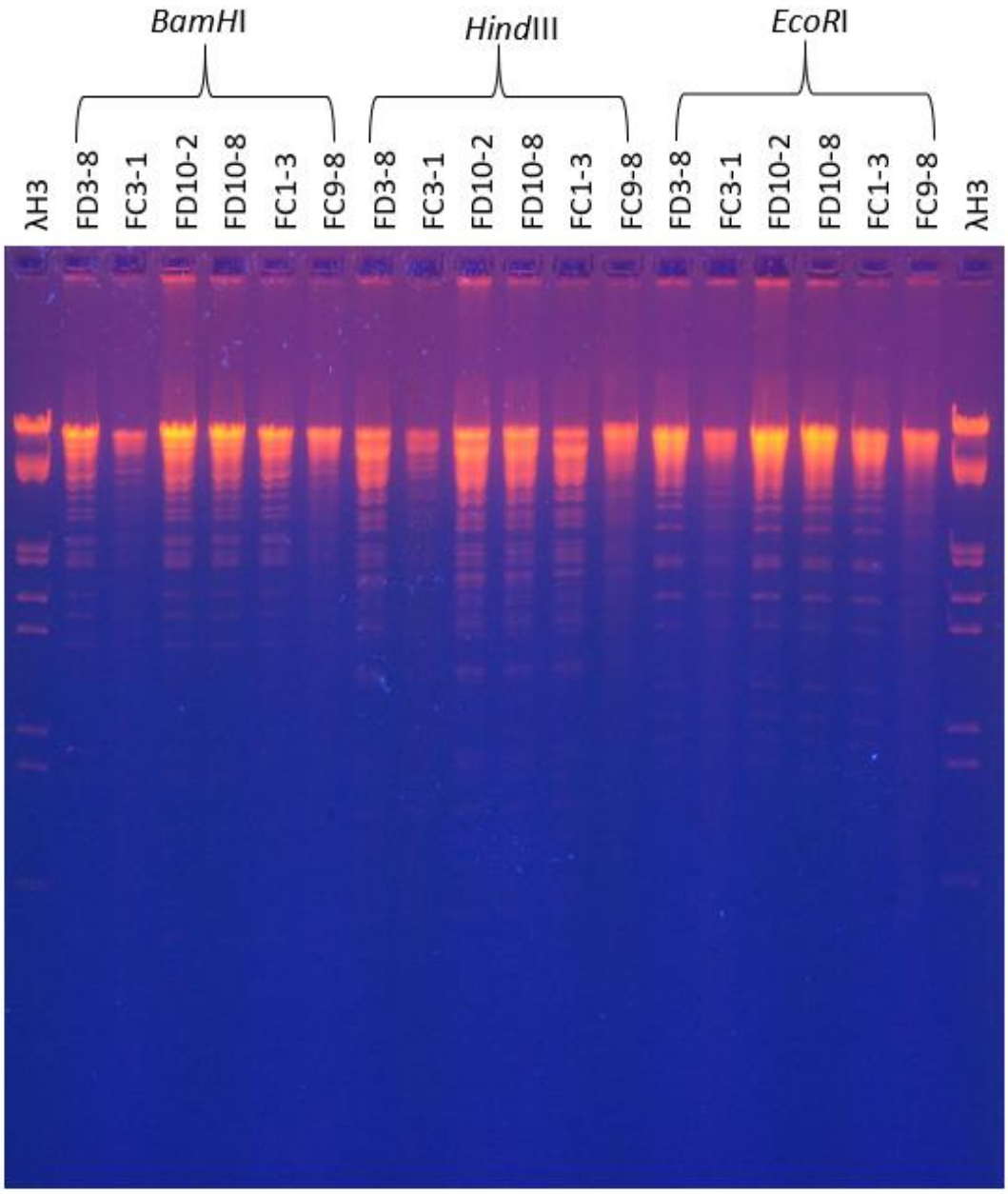

Figure C: Digestion of the 154Kb plasmids with *BamH*I, *Hind*III, and *Eco*RI

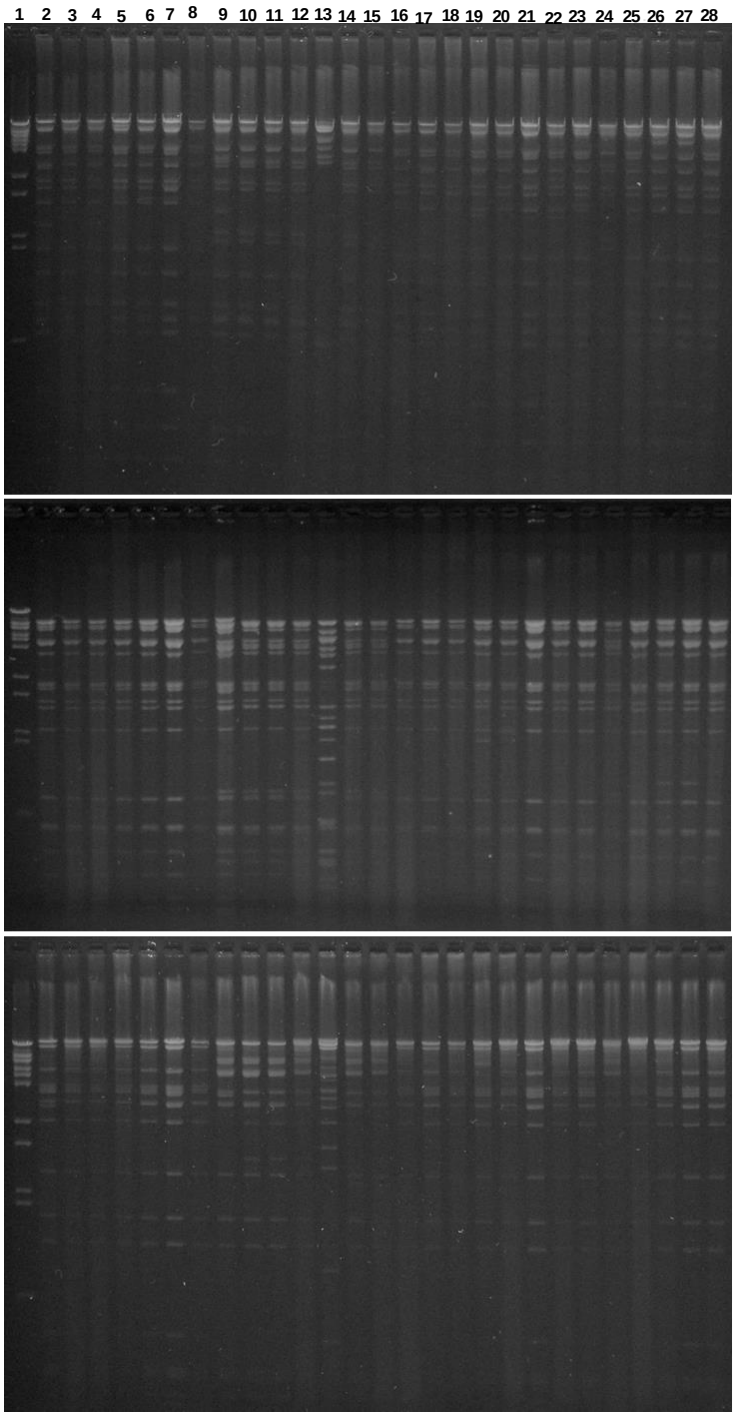

Figure D: RFLP pattern of the 60Kb plasmids digested with *Nsi*I, *Hinc*II, and *Nde*I, restriction enzymes