Resistance to colistin and carbapenem among *Enterobacteriaceae* recovered from human and animal sources is associated with multiple genetic mechanisms

by

Folasade Adekanmbi

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 7, 2021

Keywords: antimicrobial resistance; colistin; carbapenem; ESBL; humans; animals; USA; Nigeria

Approved by

Chengming Wang, Chair, Professor, Pathobiology
Benson Akingbemi, Professor, Anatomy, Physiology and Pharmacology
Kellye Joiner, Associate Professor, Pathobiology
Stuart Price, Associate Professor, Pathobiology
Heather Walz, Assistant Professor, Clinical Sciences

Abstract

Colistin and carbapenem and are important last-resort antimicrobials to treat infections caused by multidrug-resistant Gram-negative bacteria. Understanding the genetic mechanisms conferring resistance to colistin and carbapenem and their prevalence are critical in the development of preventive measures. There is little information on resistance to colistin and carbapenem among *Enterobacteriaceae* in Nigeria and sub-Saharan Africa. Developing nations have poor sanitation practices and policies regulating use of antibiotics, enhancing the spread of antimicrobial resistance. The emergence of resistant bacteria to these last-resort drugs has necessitated a study to understand the genetic mechanisms conferring resistance and their prevalence, which is critical in the development of preventive and therapeutic measures. While there has been quite a number of investigations and good antimicrobial surveillance practices in the United States, very few reports are available about the colistin and carbapenem resistant bacteria in animals.

The research in this thesis aims to investigate the prevalence of colistin and carbapenem resistance from human and animals in Nigeria, and from pigs in the USA, and understand the genetic mechanisms driving the resistance. A total of 1,119 human (stool from outpatients and urine from ICU patients) and animal (rectal swabs from cattle, dogs, pigs, poultry) samples were collected from Benue State, Northcentral, Nigeria. 583 non-duplicate *Enterobacteriaceae* were recovered from these samples using phenotypic methods and whole-genome sequencing (WGS). Of the 583 isolates, 17.0% (99/583) were resistant to colistin, 18.9% (110/583) were resistant to carbapenem, and 9.1% (53/583) had concurrent carbapenem-colistin resistance. PCR (*mcr-1* to

mcr-9) and whole-genome sequencing (WGS) identified *mcr* in 21.2% (21/99) of colistin-resistant isolates: *mcr-1.1* (n = 13), *mcr-8.1* (n = 5), *mcr-1.1* and *mcr-8.1* (n = 2), and *mcr-1.1* and *mcr-5* (n = 1). Of the 21 *mcr*-positive strains, 9 were isolated from human samples, with 8 being *Klebsiella pneumoniae*, and 6 of these human *K. pneumoniae* had a high colistin MIC (>64 μg/mL). In contrast, 9 of the 12 *mcr*-positive animal isolates were *Escherichia coli*, of which only 2 had a colistin MIC of >64 μg/mL.

The carbapenem minimum inhibitory concentrations was between 2 and 32 μl/ml. Interestingly, none of the carbapenem resistant bacteria produced any carbapenemase genes. However, they had a combination of efflux pump mutations, outer membrane protein mutations, and production of extended spectrum beta-lactamases. The population structures of the bacterial isolates carrying concurrent colistin and carbapenem resistance were highly polyclonal, distributed into 37 different sequence types and characterized by the presence of internationally recognized high-risk clones in *Klebsiella pneumoniae* ST11, ST58, ST340-human isolates) and in *E. coli* (ST58, ST744, ST410 -animal isolates). Novel and existing mutations were also observed amongst the resistant isolates. *Escherichia coli* was the most commonly isolated organism from animal samples, while *Klebsiella pneumoniae* was most commonly isolated from human samples.

On the contrary, of the 85 commercial swine fecal samples collected Auburn, Alabama, USA, carbapenem-resistant isolate was not identified. However, molecular analysis by FRET-PCR identified *mcr* genes from 34.5% (38/110) isolates, with *mcr-2* gene as the most prominent 73.7% (28/38). Of the *mcr* positive isolates analyzed for speciation by 16S rRNA, *E. coli* (70.8%) was the most commonly isolated, followed by *K. pneumoniae* (25.0%), and *Salmonella enterica* (4.2%). In two of the *mcr*- positive isolates, WGS identified (*marA, ampC, pmrC,*

pmrE, *pmrF*, and bla_{EC}) known to confer multiple antibiotic resistance, and bla_{AMPH} was identified in only one of the isolates.

This study is the first to report *mcr-1* in *Alcaligenes faecalis* and the emergence of *mcr-5* and *mcr-8* in Nigeria. WGS determined that *mcr-1* was localized on an IncX4 plasmid and that 95.2% of *mcr-1* harboring isolates (20/21) transferred colistin resistance successfully by conjugation. This study adds valuable information regarding resistance to colistin and carbapenem with implications for both human and animal health. These findings highlight the global spread of colistin resistance and emphasize the urgent need for coordinated global action to combat resistant bacteria. The presence of high-risk clones in these isolates provides worrisome evidence that humans and animals may serve as reservoirs and vectors for global microbial spread and should therefore be continuously monitored.

Acknowledgment

I would be unable to complete my thesis without my graduate mentor, Dr. Chengming Wang, accepting me into his lab, training and mentoring me, and entrusting me with this project. Thank you for being an incredible model and leader in academia.

I would also like to appreciate my graduate committee members, Drs. Akingbemi, Joiner, Price, and Walz, who were supportive during the journey to completing my thesis while being generous with their time and patience.

My family members are my best support system, and I thank them all for supporting and cheering me on all through. My parents, Ebenezer and Bolajoko Adekanmbi; my siblings, Mrs. Catherine Fadoju, Tosin, and Mayokun Adekanmbi; and my favorite Uncle, Babatunde Adekanmbi. I do not take your love and immense support for granted.

Several hours of laboratory work were involved in getting this work done. I must thank my lab colleagues- Dr. Anil Poudel, Dr. Anwar Kalalah, Dr. Monirul Hoque, and Ms. Subarna Barua for their help. Worthy of mention is also Dr. Emmanuel Ngbede, whom I collaborated with for generating data for samples from Nigeria. It was a great experience working with you.

Table of Contents

List of Tables

List of Figures

List of abbreviations

AMR antimicrobial resistance

MGE mobile genetic element

HGT horizontal gene transfer

ESBL extended-spectrum beta lactamase

MDR multidrug resistant

CRE carbapenem-resistant *Enterobacteriaceae*

LPS lipid polysaccharide

PBP penicillin binding protein

CP-CRE carbapenemase-producing- carbapenem-resistant *Enterobacteriaceae*

OMP outer membrane protein

CARD comprehensive antibiotic resistance database

EDTA ethylenediaminetetraacetic acid

IDSA infectious disease society of America

LTAC long-term acute care

AMU antimicrobial use

ICU intensive care unit

CLSI clinical & laboratory standards institute

WGS whole genome sequencing

BLAST basic local alignment search tool

MLST multi locus sequence typing

CIM carbapenem inactivation method

Chapter 1

Literature review

1.1 Antimicrobial resistance

Antibiotics are natural or chemically synthesized agents capable of inhibiting the growth of microorganisms (bacteriostatic) or ultimately killing the microorganisms (bactericidal) (Fymat, 2017). Penicillin, a β-lactam antibiotic, was the first discovered antimicrobial compound (Van Hoek et al., 2011). Antibiotics function to improve health through several mechanisms of actions which serve as the basis of classification into groups, including i) protein synthesis inhibition by macrolide, chloramphenicol, aminoglycoside, and tetracycline, ii) bacterial cell wall inhibition as seen in β-lactam and glycopeptide, iii) interruption of DNA and RNA synthesis in quinolone and rifampin and modification of cell energy metabolism (Van Hoek et al., 2011).

While antibiotics have saved numerous lives since their inception and effective use, the continued dependence and possibly over-dependence for both human and animal health globally also has its attendant detriments, in the form of antimicrobial resistance (AMR) (O ’Neill, 2015). AMR is the ability of microorganisms to counteract the effect of antibiotics used to treat infections by developing mechanisms that render the microorganisms resistant (Umeokonkwo et al., 2019). Antibiotic resistance crisis has been associated with antibiotics overuse, misuse, unregulated use, extensive agricultural use, and lack of new drugs in the pharmaceutical industry (Lee Ventola, 2015). Antibiotic-resistant bacteria cause infections resulting in reduced life quality, increased mortality, and recurring infection rates (Umeokonkwo et al., 2019). Microorganisms have undergone evolutionary changes overtime for adaptive living to circumvent the effects of antibiotics and to survive in their hosts.

Resistance genes in bacteria can be inherited through generations (intrinsic resistance) or through acquired resistance. Intrinsic resistance, defined as resistance due to the bacteria's inherent properties, includes the presence of chromosomal genes. This resistance type is through chromosomal genes present in the bacteria genome, and is seen in bacteria that have never been susceptible to the antibiotics (J. H. Lee, 2019). This resistance ability is innate and intrinsically related to the general physiology or anatomy of the microorganism, conferring resistance through several mechanisms and is not affected by the use (or misuse) of antibiotics (Capita & Alonso-Calleja, 2013). Acquired resistance, defined as resistance exhibited when a previously sensitive bacterium becomes non-susceptible through the acquisition of genetic materials exogenously (Christaki et al., 2020) on mobile genetic elements (MGEs), such as plasmids, through a phenomenon known as horizontal gene transfer (HGT). It can also occur through impermeability of the outer membrane as seen in Gram-negative bacteria, and removal of antibiotics out of the cells using the efflux pump through mutations Through HGT, antibiotic resistance can be transferred among interspecies and intraspecies of bacteria (Read & Woods, 2014). This transfer occurs through the spread of resistance genes from one bacterium to another by virus (bacteriophages) through transduction, and plasmid (extra-chromosomal DNA with a self-replicating ability) transfer conjugation. Plasmids, extrachromosomal genetic material capable of autonomous replication, are the most effective means of acquired resistance mechanism and can be transmitted vertically and horizontally (Alanis, 2005). Conjugative plasmids are known to be readily transmitted horizontally, intra-species and inter-species and are crucial in pathogenic bacteria evolution (Johnson & Nolan, 2009).

1.2 Mechanisms of resistance in Gram-negative *Enterobacteriaceae*

Enterobacteriaceae are significant causes of serious infections, and many of the most important members of this family are becoming increasingly resistant to currently available antibiotics (Paterson, 2006)**.** Bacteria have developed several antibiotic resistance mechanisms, including: i) alteration of antimicrobial targets in bacteria cells (e.g., antimicrobial intracellular receptor modification), ii) efflux pumps (by pumping out antimicrobials which have successfully penetrated the cell), iii) modifications to membrane permeability to antibiotics (e.g., outer membrane changes in some Gram-negative bacteria conferring impermeability to hydrophobic antibiotics such as beta-lactams or macrolides) (Ferri et al., 2017) iv) antibiotic enzymatic degradation, v) overproduction of target enzyme, and vi) acquisition of other metabolic pathways to those inhibited by the drug (Van Hoek et al., 2011).

1.2.1 Target site alteration

One of the most common bacterial antibiotic resistance mechanisms is target site modification, applicable to almost all families of antibiotics (Munita & Arias, 2016). Through this resistance mechanism, the antimicrobial target site is altered, resulting in the prevention of the antimicrobial from binding to the bacterial cell, thereby disabling antimicrobial activity (Verraes et al., 2013). Such changes include enzymatic modifications to the binding site, point mutations in genes encoding the target site, and replacement of the original target. The goal of these changes is a reduction in the affinity of the antibiotic for the target site (Munita & Arias, 2016). An example of both target site modification and the structural gene mutation is seen in fluoroquinolone resistance through modification to the gene encoding DNA gyrase and DNA topoisomerase IV, respectively (Mukerji et al., 2017).

1.2.2 Enzymatic degradation

Through enzymatic degradation, antibiotics are inactivated using specific enzymes produced by the bacteria. Examples of enzymatic degradation include, secretion of β-lactamases such as AmpC β-lactamases, extended-spectrum-β-lactamases (ESBLs), and carbapenemases, which hydrolyze the β-lactam ring, conferring resistance to carbapenems, penicillins, and cephalosporins (Livermore & Woodford, 2006).

1.2.3 Decreased permeability of the bacterial outer membrane

In Gram-negative bacteria, the outer membrane serves as a permeability barrier for many substances, including antibiotics. The outer membrane structure of Gram-negative bacteria is unique due to the presence of outer membrane proteins or porins, which are absent in Gram-positive bacteria (Mukerji et al., 2017). The innate low permeability of the bacterial outer membrane to antibiotics is the reason for intrinsic resistance in some Gram-negative bacteria. However, alterations in the outer membrane can result in acquired resistance. Considering the selective passage of substances into the cell through the outer membrane, a reduction in porin expression or porin loss changes the cell membrane permeability. Therefore, it restricts the entry of antimicrobials into the bacterial cell, inhibiting antibiotic activity. The number, type, and structure of porins affect substance entry, including antibiotics, into the cell and the susceptibility of the bacteria to them. Generally, changes in porin expression alone lead to a low-level antibiotic resistance, but the resistance is enhanced in addition to other resistance mechanisms (Fernández & Hancock, 2012).

Porins are the primary route of entry of hydrophilic antibiotics. Hence, β-lactams, tetracyclines, and some fluoroquinolones are particularly affected by changes in the permeability of the outer membrane because they are hydrophilic (Pagès et al., 2008).

1.2.4 Efflux pumps

Efflux pumps are energy-dependent complex bacterial systems present on the cytoplasmic membrane capable of pumping toxic molecules out of the cell. By extruding substances, including antibiotics, out of the cells, antibiotics cannot reach the required concentration for antibacterial activity. The action of antibiotics is thus hindered, leading to resistance (Verraes et al., 2013). Efflux pumps have been identified in Gram-positive and Gram-negative bacteria and affect a wide range of antibiotics, including protein synthesis inhibitors, fluoroquinolones, β-lactams, carbapenems, and polymyxins. The efflux pump system could exhibit a broad-spectrum specificity by transporting multiple unrelated substances, as seen in MDR bacteria, or they may be substrate-specific, as seen in *tet* determinants for tetracycline and *mef* genes for macrolides in pneumococci (Poole, 2005). Genes encoding efflux pumps can be chromosomally encoded, contributing to intrinsic resistance in some bacteria e.g. *E. faecalis* inherent resistance to streptogramin A or located on MGEs in some others (Singh et al., 2002).

1.3 Resistance to antimicrobials of last resort

While the emergence of bacterial resistance to first-line antibiotics is an issue of concern, more disturbing is the resistance to drugs of last resort, also called last-line drugs. According to World Health Organization, WHO, drugs of last resort or in the reserve group are antibiotics to be used in the most severe circumstances when all other alternatives have failed. These extreme

circumstances are those infections caused by multidrug-resistant organisms (WHO, 2019). Resistance to these last-line drugs has led to the fear of pan-drug resistance. The major critically important last-resort antimicrobials used to treat MDR Gram-negative bacteria include fluoroquinolones, extended-spectrum cephalosporins, carbapenems, and colistin (WHO, 2018).

1.4 Colistin resistance mechanisms

Colistin is a cationic polypeptide antibiotic, a member of the polymyxin family of molecules, comprising five different chemical compounds (polymyxins A, B, C, D, and E) (Stefaniuk & Tyski, 2019a). One of the few remaining options for treating life-threatening infections mediated by MDR bacteria is colistin (polymyxin E). Colistin was first isolated in Japan from the spore-forming soil bacterium *Bacillus polymyxa subsp. colistinus* in 1947, and first used as an intravenous formulation in the 1950s (Bialvaei & Samadi Kafil, 2015b). Colistin was approved by the US FDA and has been in use since 1959 for treating infections caused by Gram-negative bacteria, including infectious diarrhea and urinary tract infections, as well as in bowel decontamination and for topical formulations to treat eye and ear infections (Landman et al., 2008).

As a drug of last resort, colistin is of great importance in treating conditions caused by carbapenem-resistant *Enterobacteriaceae* (CRE) that belong to multi-resistant isolates and has been classified as one of the critically important for human medicine (WHO, 2017). Despite its usefulness in treating MDR infections, the high rate of nephrotoxicity associated with colistin in humans prompted its disuse and subsequent replacement with safer antibiotics. However, the recent resistance to other drugs of last resort has necessitated the use of colistin again in humans. Colistin has a narrow spectrum activity and acts based on the electrostatic interaction between

colistin amino groups and lipid A subunits of lipopolysaccharide (LPS). In the LPS, colistin displaces Mg2+ and Ca2+ ions, disrupting the cell outer membrane, resulting in increased cell membrane permeability and eventual cell death.

Colistin has been used in human medicine and has also been used extensively in veterinary medicine over the years (Bos et al., 2013) in treating intestinal infections in pigs, poultry, and cattle, which were caused by *Enterobacterales* strains, mainly *E.coli* and *Salmonella* spp. (Liu et al. 2016). Bacterial colistin resistance can be mediated by plasmid-mediated resistance genes, known as the mobile colistin resistance gene (*mcr*) and chromosomal mutations.

1.4.1 Plasmid-mediated *mcr* genes

So far, ten variants of the *mcr* genes have been reported (*mcr*-1 to 10) (Stefaniuk & Tyski, 2019b; Wang et al., 2020). In 2015, the first plasmid-mediated colistin resistance was detected in an *E. coli* strain isolated from food animals in China (Liu et al., 2016). There has been a surge of several reports of *mcr*- variants reported globally since then (Izdebski et al., 2016). The *mcr-2* gene was first identified in Belgium in 2016 from *E. coli* strains isolated from calves and pigs; this MCR-2 protein showed 80.65% identity to the *mcr-1* (Xavier et al., 2016). Meanwhile, in 2017, *mcr-3* was also isolated from *E. coli* in China, and the amino acid sequence of MCR-3 showed 32.5 and 31.7% amino acid identity to MCR-1 and MCR-2, respectively (Yin et al., 2017). The *mcr-4* gene was detected on *Salmonella* in 2017 (Carattoli et al., 2017) while *mcr-5* was identified in fermenting *Salmonella enterica subsp. enterica* serovar Paratyphi B (Borowiak et al., 2017). In 2018, three other *mcr* genes, *mcr-6 ,-7* and *-8* were identified (AbuOun et al., 2017, 2018; Wang et al., 2018; Yang et al., 2018). Furthermore, *mcr-9* was isolated in 2019 from a strain of *Salmonella* in Washington State, USA (Carroll et al., 2019).

These *mcr* variants have varying degrees of identity to the *mcr*-1 gene: *mcr*-2, -3, -4, -5, -6, -7, and -8, which share 81%, 32%, 34%, 36%, 83%, 35%, and 31% amino acid sequence identity, respectively, with mcr-1 (Nang et al., 2019). The *mcr-9* is most closely related to *mcr-3*, with a 4.5% amino acid identity, and is more commonly found in *Salmonella* than other *mcr* gene variants that are identified in *E. coli* and *Klebsiella* (Carroll et al., 2019). The *mcr*- genes are mostly localized on the IncI2, IncX4, and IncHI2 plasmids.

1.4.2 Chromosomal mutations

Other important colistin-resistant mechanisms are overexpression of efflux pumps, outer membrane porin modifications, and reductions in the overall negative charge of the LPS (Bialvaei & Samadi Kafil, 2015a). In Gram-negative bacteria, colistin resistance is usually due to the reduced binding of colistin to the bacterial outer membrane, resulting from changes in the LPS due to changes in the two-component regulatory systems, PhoPQ and PmrAB (Bialvaei & Samadi Kafil, 2015a). In *K. pneumoniae*, the most critical chromosomal colistin resistance mechanism is modifying the *mgrB* gene, encoding the negative regulator of phoP/phoQ system (Jayol et al., 2015). The *mcr-1* has also been found to be associated with ESBL-producing isolates bearing $bla_{\text{CTX-M-1}}$ and a human isolate with a $bla_{\text{KPC-2}}$ carbapenemase gene (Al-Tawfiq et al., 2017).

1.5 Carbapenem resistance mechanisms

Carbapenems, including meropenem, imipenem, ertapenem, and doripenem, are drugs of last resort, used to treat infections caused by multidrug-resistant Gram-negative bacteria. Carbapenems have broad-spectrum activity against bacterial activity and a unique structure,

having a β-lactam ring, which offers protection to the carbapenems against most carbapenem degrading enzymes such as β-lactamases, including Metallo-β-lactamases and extended-spectrum β-lactamases (ESBLs) (Hwang & Gums, 2016). Bacterial cell walls are composed of a peptidoglycan polymer, whose synthesis is enabled by transpeptidase enzymes called the penicillin-binding proteins (PBPs). Carbapenems act by binding to the active sites of these PBPs irreversibly, resulting in the inhibition of transpeptidation of the peptidoglycan layer through crosslinking, thereby disrupting the cell wall synthesis (Fisher et al., 2005; Kapoor et al., 2017) Continued autolytic action of autolysins, a group of bacterial surface enzymes, results in final cell death. While the exact mechanism is uncertain, it is speculated that the autolysins function to create nicks in the cell wall, which act as attachment points for new peptidoglycan units. Hence, cell wall biosynthesis inhibition by β-lactams, together with continued cell wall autolysis, creates weak spots in the cell wall. The cell membrane, too weak to keep the hypertonic cell from rupturing by osmotic shock, finally ruptures, and the cell dies (Van Heijenoort, 2001).

Carbapenems, have been used extensively over the years due to failure of treatment with penicillin and cephalosporin, resulting in the emergence of carbapenem resistance (Patrice Nordmann et al., 2009). Carbapenem resistance in *Enterobacteriaceae* is mediated majorly by mutations in a target site, efflux pumps, porin, and enzymatic inactivation. Of these, enzyme production is the most common mcahnism. Generally, carbapenem resistant *Enterobacteriaceae* are classified as carbapenemase-producing CRE (CP-CRE) and non-carbapenemase-producing CRE (Suay-García & Teresa Pérez-Gracia, 2019). In CP-CRE, the main mechanism of carbapenem relies on the destruction of these compounds by the action of carbapenemases and other β- lactamases. These enzymes destroy the amide bond of the β-lactam ring, rendering the antimicrobial ineffective (Munita & Arias, 2016). The non-carbapenemase-producing CRE

produce ESBLs and AmpC enzymes and lose outer membrane porin (OMP) proteins (Satlin et al., 2017).

1.5.1 Carbapenemases

Amongst members of the family *Enterobacteriaceae*, plasmid-encoded carbapenemases were first seen in *K. pneumoniae*, however, carbapenem resistance has been reported in *E. coli* and other members of the *Enterobacteriaceae* (Cuzon et al., 2010; Perez et al., 2016). *Enterobacteriaceae*, such as *Escherichia coli, Klebsiella pneumoniae, Citrobacter freundii*, and *Enterobacter cloacae*, are the most common pathogenic bacteria for nosocomial infections. However, *E. coli* and *K. pneumoniae* are of the greatest interest because they are also the most clinically important, causing infections in the clinical setting (Hsu et al., 2017). While carbapenem resistance is more frequently observed in humans, there have been reports of carbapenem-resistant bacteria in food animals (poultry, cattle, pigs) and companion animals (dogs, cats, and horses) and in the environment (Mataseje et al., 2012; P. Nordmann et al., 2011). This is particularly alarming since carbapenems are not registered for use in animals, although off-label use occurs in cats and dogs. Carbapenemases that have been identified in *Enterobacteriaceae* are classified into 3 classes: the Ambler classes A, B, and D β-lactamases. Members of classes A and D, are beta-lactamases, having serine at their active site, while those belonging to class B are metalloenzymes, with zince at their active sites (Queenan & Bush, 2007).

Class A carbapenemases comprise some chromosomally encoded and plasmid-mediated enzymes. These beta-lactamases have been detected in *Enterobacter cloacae, Serratia marcescens*, and *Klebsiella spp*. (Queenan & Bush, 2007) and they all actively hydrolyze

carbapenems and are partially inhibited by clavulanic acid. NmcA (not metalloenzyme carbapenemase A), SME (Serratia marcescens enzyme), IMI-1 (Imipenem-hydrolysing β-lactamase), and SFC-1 (Serratia fonticola carbapenemase-1 are all chromosomally-encoded, while KPC (KPC-2 to KPC-13), IMI (IMI-1 to IMI-3), derivatives (GES-1 to GES-20) of GES (Guiana extended-spectrum), are plasmid-mediated (Bedenić et al., 2014; P. Nordmann et al., 2011). KPCs are the most widespread and of clinical importance amongst members of this class and are most commonly reported in *Klebsiella*. Their global dissemination has been reported in Asian, North American, European, and African countries (Codjoe & Donkor, 2017).

Class B carbapenemases all possess the ability to hydrolyze but are unable to hydrolyze aztreonam and are susceptible to inhibition by ethylenediaminetetraacetic acid (EDTA), EDTA chelates Zn2+ and other divalent cations, resulting in the unique characteristic of this class of enzymes. The interaction of beta-lactams with zinc ions in the active site of the enzyme is what causes them to be inhibited by EDTA, a chelator of Zn2+ and other divalent cations. The most prominent members include New Delhi metallo-β-lactamase 1 (NDM-1), Imipenem-resistant Pseudomonas (IMP)-type carbapenemases, VIM (Verona integron-encoded metallo-β-lactamase), GIM (German imipenemase) and SIM (Seoul imipenemase). These enzyme-encoding genes are usually found inside a variety of integron structures and inserted into gene cassettes (Giakkoupi et al., 2003; Queenan & Bush, 2007). The first NDM case was reported in Klebsiella in India in 2009 (Yong et al., 2009) and has since been seen in most *Enterobacteriaceae* (Cui et al., 2019), while the first *Enterobacteriaceae* carrying IMP was seen in Japan (Watanabe et al., 1991) and has been reported in other Asian countries, with the highest report in China (P. Nordmann et al., 2011), and has since spread to the United States and Australia (Queenan & Bush, 2007).

Class D Carbapenemases are serine-lactamases, which are only moderately inhibited by EDTA or clavulanic acid. These carbapenemases belong to the OXA enzyme family and have a low carbapenem activity. In most countries including the United States and the United Kingdom, the enzymes are present mainly in non-fermenter species such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and occasionally in isolates of the *Enterobacteriaceae* family (Moquet et al., 2011; Laurent Poirel et al., 2011). The ability of OXA carbapenemases to rapidly mutate and increase their range of activity is a major source of concern (Codjoe & Donkor, 2017). Over the years, 102 unique OXA sequences were identified of which 9 are ESBLs and at least 37 are considered to be carbapenemases (Walther-Rasmussen & Høiby, 2006). In Turkey, the Middle East, North Africa, and Europe, OXA-48 is the most commonly seen in *K. pneumoniae.* Environmental *Acinetobacter* species have been found to carry the non-nosocomial OXA-24 strain, while the OXA-23 type, which is found worldwide, is more prevalent in the United States and Europe, and the OXA-58 group was identified in several outbreaks around the world (Evans & Amyes, 2014).

Carbapenemases have been known to commonly confer resistance amongst CP-CRE. However, other ESBLs have been identified to confer carbapenem resistance, often in association with porin and or efflux pump alterations (Codjoe & Donkor, 2017). Carbapenems are also used in treating infections caused by multidrug-resistant bacteria, producing extended-spectrum β-lactamases (ESBLs) and AmpC enzyme, as carbapenems are stable against these enzymes. However, in recent times, ESBLs have been known to confer carbapenem resistance, often in association with porin or efflux pump mutation (Ye et al., 2018).

1.5.2 Extended Spectrum Beta-lactamases (ESBL)

ESBLs are β-lactamases, which have the ability to offer bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems), and which are inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson & Bonomo, 2005a). This is the differing characteristics between ESBL and AmpC, as AmpC – producing organisms e.g., *Enterobacter cloacae* are not inhibited by clavulanic acid. The total number of ESBLs now characterized exceeds 200 (Paterson & Bonomo, 2005b).

Amongst the ESBLs, the most frequently encountered and clinically important are found in the Temoniera (TEM), sulfhydryl variable (SHV), and cefotaximase-Munich (CTX-M) families (Nwafia et al., 2019). ESBL producing isolates are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* (Yusuf et al., 2014), and these resistant strains are associated with a high rate of mortality rate, a higher financial burden on infected patients due to extended hospital stays and health costs (Nwafia et al., 2019).

TEM- is a common plasmid-mediated ESBL and was first isolated from *E. coli* from a human patient in Athens, Greece, named Temoneira (hence the designation TEM). They are found amongst Gram-negative bacilli (for example, *E. coli*). TEM-1 is the most common of these, and the ESBLs derived from TEM-1, TEM-2 or SHV-1 only differ by as few as one amino acid from their progenitor. Well over 100 TEM-type beta-lactamases have been described to date (Paterson, 2006).

CTX-M, the second largest group of ESBLs, with about 40 members, is a plasmid-encoded ESBL commonly found in *K. pneumoniae, E. coli*, and other *Enterobacteriaceae* around the world. There is evidence that the enzyme was derived from a bacterium of paramount human

pathogenic importance, the *Kluyvera spp*., through horizontal gene transfer, as opposed to other class A ESBL members like TEM-3, which were derived from TEM or SHV (Rawat & Nair, 2010). The potent hydrolytic activity of the group of beta-lactamases is reflected in the name CTX, and organisms producing the CTX-M- type beta-lactamases could also harbor SHV-type ESBLs and AmpC-type beta-lactamases, altering the antibiotic resistance phenotype (Yan et al., 2000). Genes encoding CTX-M- enzymes are usually found on mobile genetic elements like insertion sequences (ISEcp1) and transposable elements (Tn402-like transposons), which can be captured by large plasmids giving rise to a wide range of dissemination. Hence, CTX-M enzymes are the most prevalent ESBL worldwide (Ye et al., 2018). The CTX-M producing *Enterobacteriaceae,* mostly *E. coli,* have been isolated from the community, causing urinary tract infections (Rawat & Nair, 2010).

SHV group of ESBL enzymes is also another large group of beta lactamase. SHV (sulfhydryl variable) has been found in a wide range of *Enterobacteriaceae*. The first $bla_{\text{SHV-1}}$ gene was identified from *E.coli* in the 1970s (Pitton, 1972). In recent years, they have been found in several environmental niches and outside of their most common clinical hosts- *E. coli* and *K. pneumoniae* members of the *Enterobacteriaceae*. Over the years, more than 189 known SHV allelic variants with developed resistance to cephalosporin, monobactams, and carbapenems, have been identified. Generally, the SHV beta-lactamases are divided into three groups, based on their functional properties or molecular characteristics: 1) subgroup 2b having 37 variants, 2) subgroup 2br with 7 variants; and 3) and subgroup 2be with 46 variants. Of these SHV variants, there are at least 46 known SHV-ESBL genes together with more than 150 non-ESBL or unclassified alleles to date. The SHV-ESBLs are more frequently found in clinical isolates than any other ESBL-type. Members of the subgroup 2be, which are made up of the ESBLS, have the

ability to hydrolyze one or more oxyimino b-lactams (cefotaxime, ceftazidime, and aztreonam), and of these, seventeen variants are exclusively associated with clinical isolates of *K. pneumoniae*, which are *bla*SHV-6, *bla*SHV-13, *bla*SHV-16, *bla*SHV-18, *bla*SHV-23, *bla*SHV-45, *bla*SHV-64,*bla*SHV-66, *bla*SHV-86, *bla*SHV-90, *bla*SHV-91, *bla*SHV-98, *bla*SHV-99, *bla*SHV-100,*bla*SHV−104,*bla*SHV-105, and *bla*SHV-134. SHV variants are usually located on plasmids belonging to the replicon types: A/C, F, HI2, I1, L/M, N, X3), ColE, K, P, and R. These variants have been seen globally and found in food animals such as poultry and swine, and in clinical isolates (Liakopoulos et al., 2016).

1.6 Colistin and carbapenem resistance in Nigeria

In most developing countries, including Nigeria, there are poor antimicrobial prescribing practices, no regulation of use, antibiotics are bought over the counter without prescription, leading to an increased risk of antimicrobial resistance (Chukwuani et al., 2002; Morency-Potvin et al., 2017). Not only is this high antimicrobial resistance seen in humans, but also in animals. In 2015, the global antimicrobial use in food animal was estimated at 63,000 tonnes annually and was expected to rise by about 70% in livestock, with the most significant increase expected to be seen in developing countries such as Nigeria, Indonesia, and Myanmar (Van Boeckel et al., 2015). In Nigeria, AMU in animals is usually for prophylactic, therapeutic, and growth promotion purposes (Adebowale et al., 2016). There is a high risk of AMR transfer from animals to humans as farmers and other farmworkers in Nigeria are often in close contact with the animals (Umeokonkwo et al., 2019). A report of alarming antibiotic use in the ICU has also been made in Nigeria. All (100%) of the patients on ICU admission in four health institutions across the nation were placed on at least one antibiotic (Oduyebo et al., 2017).

Colistin usage is commonly seen in food animals in Nigeria, especially in poultry production, accounting for 72% of its total use. Colistin is usually used therapeutically (57.3%) or in prophylaxis (42.2%), either alone or in combination with other antimicrobials (Bachiri et al., 2018; Tanfous et al., 2018). Several variants of *mcr* (*mcr-1.1*, *mcr-5,* and *mcr-8.1*) have also been reported in *Enterobacteriaceae* of human and animal origin in Nigeria. However, there have been no reports of the *mcr*-2 to *mcr*-7 and *mcr*-9 genes in African countries (Ngbede et al., 2020a). A significant widespread of ESBL-producing Gram-negative bacteria has also been seen in all six geopolitical zones in Nigeria, but more studies have been carried out in the Southern part compared to the Northern part of Nigeria. *E. coli* and *K. pneumoniae* were the most commonly identified isolates and the most reported ESBL enzymes were TEM, SHV, and CTX-M; in decreasing order of prevalence (Tanko et al., 2020).

While the insurgence and wide dissemination of ESBL producing and carbapenem-resistant bacteria is a global issue, the problem was more severe in developing nations (Desalegn, 2013). In 2019, A 35% occurrence of ESBL producing *E. coli* was reported among hospitalized patients in Enugu, Northern Nigeria, with the $bla_{\text{CTX-M}}$ gene was most frequently seen. Other reports have also been made in Maiduguri, and bla_{SHV} was the predominant gene reported (36.4%), followed by bla_{TEM} (31.4%) and $bla_{\text{CTX-M}}$ (27.3%) (Nwafia et al., 2019).

1.7 Colistin and carbapenem resistance in the USA

In the United States, the prevalence of MDR *E. coli* among community isolates increased by more than 56% from the 1950s to the 2000s (Tadesse et al., 2012). Infections due to pan-drug resistant bacteria were observed in 60% of participants during a 2011 national survey conducted by IDSA Emerging Infections Network (Spellberg & Gilbert, 2014). Despite laws and policies

regulating the use of antibiotics in developed nations and the US as an example, the incidence of carbapenem-resistant *Enterobacteriaceae* is a call for urgent public health measures, with the CRE resistant to almost all classes of antimicrobials (Schwaber & Carmeli, 2008). Carbapenem-resistant *K. pneumoniae* constitute 92% of all the CRE, while the most common resistance mechanism found in these isolates is the bla_{KPC} (CDC, 2015), the ESBL producing *Enterobacteriaceae* has also been frequently reported amongst clinical isolates.

In the US, generally within the year 2007 to 2011, the incidence of ESBL infections has increased, with a higher occurrence for ESBL *Klebsiella* compared to ESBL *E. coli* (Sheu et al., 2018). The incidence rate of ESBL producing *Klebsiella pneumoniae*, increased from 3.2% to 34.7%, and that of *E.coli* ESBL changed from 5.2% to 20.1%, with the highest rates reported in the West North Central Region and the highest rates in the Mid-Atlantic region (Tamma & Villegas, 2017). Meanwhile, in 2011-2013, the *Klebsiella* isolates exhibiting ESBL phenotypes increased to 15% from isolates obtained from 79 US hospital 13, and an increase of 12% among *E.coli* isolates obtained from hospitals in the US (Pana & Zaoutis, 2018).

Colistin has not been used in food animals in the United States, and the prevalence of *mcr* gene has also been low, with most human infections associated with international travel (Henig et al., 2019). Despite its lack of use in animals, *mcr* genes have been isolated from pigs, poultry, and cattle in addition to environmental sources such as seas, rivers, and hospital sewage, and are frequently located on a wide range of plasmids such as IncI2, IncHI2, IncX4, IncP, IncF, and IncY (Jeannot et al., 2017). The first report of a *K. pneumoniae* harboring coresistance to colistin and carbapenem in the US was isolated from patients under long-term acute care (LTAC) in 2009 (Marchaim et al., 2011).

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Chapter 2

Genetic mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae*

2.1 Introduction

Antimicrobial resistance is a pressing issue of global importance. Of more concern, however, is the occurrence of resistance to antibiotics of last resort, including colistin and carbapenem. While carbapenem has not been approved for animal use, the presence of carbapenem-resistant *Enterobacteriaceae* in animals could be an indication of transmission between humans and animals in close contact. The drugs of last resort are reserved for treating infections caused by multidrug resistant bacteria. Resistance to these drugs could result in pan drug resistance, necessitating the need for urgent development of new drugs to combat infections caused by multidrug resistance. This highlights the importance of understanding the prevalence of resistant bacteria and understanding the mechanisms of resistance in both developed and developing nations**.**

2.2 Materials and Methods

2.2.1 Sample collection

Between January 2016 and April 2019, animal, human clinical, and hospital environmental samples were collected for isolation of antibiotic resistant *Enterobacteriaceae*. Environmental samples (n=30) and human clinical samples: stool from outpatients presenting with chief complaint of diarrhea (n=60) and urine samples from catheterized inpatients in the intensive care unit (ICU) (n=40) were collected from two hospitals at Makurdi, Benue State,

Northcentral Nigeria. The stool and urine samples were from patients not previously treated with colistin and no international travel history.

Over the same time frame, 835 rectal/cloacal swabs were collected from cattle (n=70), dogs (n=110), pigs (n=220), and poultry (n=435), as well as 50 liver samples from sick chickens, from Benue State, North-central Nigeria. The poultry samples were from 32 backyard poultry farms, the pig samples were from 15 farms (n=100) and a slaughterhouse (n=110), and the dog samples were from 60 households. The cattle samples were from two slaughterhouses. In addition, 104 rectal swabs from camels were collected at three live camel markets in Jigawa and Katsina States, Northwest Nigeria. These samples were used for the analysis of colistin and carbapenem resistance in Nigeria throughout this study.

Between July 2020 and August 2020, a total of 85 fecal swab samples were collected from pigs raised commercially in the Auburn University Research Facility farm, AL, USA. Fecal swabs were collected into sterile Eppendorf tubes containing 400 µl 1X phosphate buffer solution and were transported to the research lab within 3 hours of sample collection. Fecal swab samples were directly stored at -20℃ until nucleic acid extraction. Fecal swabs were collected from pigs of different ages, ranging between 7.5weeks-22weeks old and including males and females (Table 2.10).

2.2.2 Bacterial isolation and antimicrobial susceptibility testing

All samples were processed using standard bacteriological techniques for isolation of *Enterobacteriaceae*-like bacteria within 4 hours of collection. Briefly, the collected samples were inoculated into 5 ml Tryptic Soy Broth (Oxoid, UK). After incubation at 37°C for 24 hours, a loopful of the enrichment broth was streaked onto MacConkey agar plates supplemented with

2µg/ml colistin (except for the camel, dog samples which were inoculated onto MacConkey agar without colistin) and incubated for 24 hours at 37°C.

Antimicrobial susceptibility profiles of the isolates were determined by the disk diffusion method (CLSI, 2020) using 12 antimicrobial agents: Amoxycillin (10 µg), amoxicillin/clavulanic acid (30 µg), cefoxitin (30µg), cefotriaxone (30µg), ciprofloxacin (5µg), chloramphenicol (10µg), gentamicin (10 µg), doxycycline (30µg), erythromycin (15µg), enrofloxacin (5µg), florfernicol (30µg), sulfamethaxozole-trimethoprim (25µg). The results were interpreted according to the guidelines of the CLSI (Weinstein et al., 2020).

2.2.3 DNA extraction

In Nigeria, genomic DNA (gDNA) was extracted from overnight cultures of the isolates using the Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's recommendations. The gDNA was used for molecular identification of bacterial species, PCR detection of the nine *mcr* genes, and the WGS.

At Auburn University, the High-Pure PCR Template Preparation Kit (Roche Diagnostics, USA) was used to extract total nucleic acids from fecal swabs according to the manufacturer's instructions and as described before (Li et al., 2017; Poudel et al., 2020). Briefly, 400 µl of fecal samples suspension was mixed with 400 µl of binding buffer followed by homogenization. 80 µl of proteinase K was added for protein digestion. Cell lysis was carried out by incubation at 72 for 20 minutes with shaking at 600 rpm in a thermomixer (Eppendorf, Hamburg, Germany). Afterwards, 200 µl of isopropanol (Avantor Performance Materials, Center Valley, PA) was added to the mixture and vortexed thoroughly. The kit filter was then inserted into a 2ml collection tube and the aqueous solution was transferred into the filtered collection tube,

followed by centrifugation at 8,000g x1minute. The flow-through was discarded and 500 ul of inhibitor removal buffer was added, followed by centrifugation at 8,000g x1minute. The flow-through liquid was again discarded and the 1000 µl washing buffer was added to the filter in two washing steps. In the first washing step, the 600 µl washing buffer was added to the filter and centrifuged at 8,000g x1minute. This was followed by the addition of 400 µl of washing buffer to the filter, and centrifuged at 13,000g x1minute. The filter column was thereafter inserted into a new 2 ml collection tube. The genomic DNA was eluted with 100 µl prewarmed elution buffer in two steps of 50 µl per elution step, by addition of 50 µl of elution buffer directly to the middle of the filter, incubated for 5minutes and centrifuged at 8,000g x1minute. The extracted DNA was used for the molecular identification of bacterial species, PCR detection of the nine *mcr* genes and the WGS.

2.2.4 Identification of bacterial species

Speciation of the resistant bacterial isolates was performed by PCR amplification of the full length of the 16S rRNA (Patel, 2001) followed by DNA sequencing and BLASTn.

2.2.5 Determination of colistin minimum inhibitory concentration (MIC)

One presumptive colony from each plate was purified on Tryptic Soy Broth (Oxoid, UK), and the MIC of colistin was determined using the broth macrodilution method based on the recommendation of the Clinical and Laboratory Standard Institute Guideline (Clinical and Laboratory Standards Institute, 2018). Briefly, 2 – 3 colonies of each bacteria isolate from overnight culture on Nutrient agar were used to prepare suspensions of the inoculum equivalent of 0.5 MacFarland standard. The suspension (20 µl) was then dispensed into 2 ml of Mueller

Hinton broth containing the different concentrations of colistin sulfate, incubated at 35°C for 24hours. The reference strain *E. coli* ATCC 25922 and broths without antibiotics were used for quality control purposes. The resistance breakpoint was defined using the Clinical and Laboratory Standard Institute Guideline with isolates exhibiting colistin MIC≥4 µg/ml categorized as resistant (Clinical and Laboratory Standards Institute, 2018). The colistin-resistant isolates were stored on tryptone soya agar slants at 4°C until required for further analyses.

At Auburn University, frozen fecal samples suspended in PBS were thawed at room temperature. A loopful of this suspension was streaked on MacConkey agar supplemented with 3.5ug/mL of colistin, and on CHROMagar™ COL-APSE incubated at 37°C for 24 hours. Presumptive positive colonies were subcultured on MacConkey agar supplemented with 3.5 µg/mL colistin and CHROMagar to obtain pure, isolated colonies of colistin resistant bacteria.

A loopful of the feces and PBS suspension was also streaked on MacConkey agar supplemented with 2 ug/ml and 4.0 ug/mL of meropenem respectively and incubated at 37°C for 24 hours, for isolation of carbapenem resistant bacteria.

In total, 15/110 isolates were randomly selected for MIC determination based on different morphological characteristics on the MacConkey agar + 3.5ug/mL colistin. Antibiotic susceptibility testing was performed using the VITEK®2, (Biomerieux, Inc., Hazelwood, MO). The following antibiotics were tested: ampicillin, ampicillin-clavulanic acid, cefpodoxime, cephalexin, ceftofur, cefovecin, ceftazidime, chloramphenicol, doxycycline, imipenem, amikacin, gentamicin, ciprofloxacin, marbofloxacin, enrofloxacin, polymyxin B, trimethoprim-sulfamethoxazole, and nitrofurantoin. Supplemental testing using the Kirby–Bauer disk diffusion method was employed for colistin testing on Mueller Hinton agar. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and

Enterococcus faecalis ATCC 29212 were used as quality controls in MIC determinations. Zones of inhibition were read and interpreted as susceptible, intermediate, or resistant. Minimum inhibitory concentrations (MICs) were interpreted according to the Clinical and Laboratory Standards Institute guidelines for *Enterobacterales* (CLSI, 2018) guideline. Isolates exhibiting MIC ≥4 μg/ml were considered as resistant to colistin while those with MIC below this breakpoint were categorized as susceptible.

2.2.6 Detection of *mcr-1* to *mcr-9* by PCRs

Real-time fluorescence resonance energy transfer (FRET)-PCRs were performed as described to detect *mcr-1* (Zhang et al., 2018), *mcr-2* (Zhang et al., 2018), *mcr-3* (Zhang et al., 2018), *mcr-4* (Chen et al., 2018) and *mcr-5* (Chen et al., 2018) in the extracted DNA of the colistin-resistant isolates. In addition, previously published methods were used to detect *mcr-6* (R. Wang et al., 2018), *mcr-7* (Yang et al., 2018), *mcr-8* (Nabti et al., 2020) and *mcr-9* (Yuan et al., 2019). The primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

E. coli NCTC 13846 isolate was used as the positive control in the *mcr-1* PCR. The positive controls for *mcr-2* to *mcr-5* PCRs were from previous studies (Chen et al., 2018; Zhang et al., 2018). In addition, the nucleotide fragments corresponding to the PCR amplicons of *mcr-6* to *mcr-9* were synthesized and inserted into the pUC57 cloning vector (GenScript, Nanjing, Jiangsu, China), and the four resulting plasmids were linearized with Sac I (Takara Biotechnology, Dalian, China) and quantified using the PicoGreen DNA fluorescence assay (Molecular Probes, Eugene, OR, USA) for preparation of positive controls used in this study. The PCR thermal conditions used were denaturation at 95 °C for 2 min; three high stringency

step down cycles followed by 30 cycles of 95 °C for 15 s, 56 °C for 78 s, and 72 °C for 10 s. All PCRs positive for *mcr* were further verified by electrophoresis of PCR products through 2% agarose gels, purification using the QIAquick Gel Purification Kit (QIAGEN, Valencia, CA, USA), and sequencing with forward and reverse primers (ELIM Biopharmaceuticals, Hayward, USA), followed by BLAST search of existing nucleotide database.

2.2.7 Determination of carbapenem MIC

Carbapenem (meropenem, ertapenem, and imipenem) MIC were determined using the broth dilution method recommended by the Clinical and Laboratory Standards Institute (Weinstein et al., 2020). Briefly, colonies of each isolate from overnight growth on tryptone soya agar were suspended in 5 ml normal saline to make an inoculum the equivalent of a 0.5 MacFarland standard. 20 µL volume of the inoculum was dispensed into 2 ml of Mueller–Hinton broth containing different concentrations of the respective carbapenems and incubated at 35°C for 24 h. *E. coli* ATCC25922 and *Pseudomonas aeruginosa* were used as controls. Isolates were categorized as resistant based on the CLSI clinical breakpoints for resistance (meropenem and imipenem = ≥ 4 µg/ml, ertapenem = ≥ 2 µg/ml ertapenem)(Weinstein et al., 2020).

2.2.8 Phenotypic assay for carbapenemase production

The Carbapenem Inactivation Method (CIM), was used to screen the isolates for carbapenemase production as previously described (Van Der Zwaluw et al., 2015). Briefly, a loopful (≈ 10 µl) of the isolate was suspended in 400 µl water before a 10 µg meropenem disk (Oxoid, UK) was immersed in the suspension and incubated for 2 hours at 35°C. The disk was removed from the suspension with an inoculation loop and placed on a Mueller-Hinton agar p

late inoculated with a 0.5 McFarland standard *E. coli* strain ATCC 29522 (a susceptible indicator strain) using a sterile cotton swab and subsequently incubated at 35°C for 24 hours. Carbapenemase production by the isolate inactivates meropenem in the disc allowing uninhibited growth of the susceptible indicator strain (positive result), while meropenem discs incubated in suspensions of isolates that do produce carbapenemases yielded a clear inhibition zone of the susceptible indicator strain (negative result).

2.2.9 Conjugation experiment

Conjugative transferability of the colistin and carbapenem resistance determinants was investigated with the solid mating conjugation assay utilizing the sodium azide-resistant *E. coli* J53 as the recipient cell as previously described (Ojo et al., 2016). Briefly, overnight cultures of each donor and the recipient mixed in 1:4 ratio in tryptone soy broth were centrifuged at 14,000 x *g* for 1 minute and the pelleted cells resuspended in 15 µl of 0.85% NaCl. The cell suspension was spotted onto Mueller-Hinton agar and incubated at 37℃ for 20-24 hours. Cell mixtures growing on the Mueller-Hinton agar were resuspended in 1 ml 0.85% NaCl and a 100µl aliquot was serially diluted with each dilution placed on brain heart infusion agar (BHIA) supplemented with sodium azide (150 µl/ml) + colistin (2 µg/ml), meropenem (2 µg/ml) + sodium azide (150 µl/ml), colistin (2 µg/ml) + meropenem (2 µg/ml) + sodium azide (150 µl/ml) for selection of transconjugant. The conjugation frequencies were calculated as the number of transconjugants per recipient cell.

2.2.10 Whole-genome sequencing (WGS) and MLST

Fifty isolates co-resistant to colistin and carbapenem were subjected to WGS. Briefly, Illumina libraries were prepared from the extracted gDNA and sequenced using next-generation sequencing on an Illumina MiSeq platform (OE Biotech, Shanghai, China) followed by *de novo* assembly of the generated sequences using SeqMan Pro v.11.2.1 (DNASTAR, Madison, WI, USA). Species identity of the isolates was further confirmed from the WGS data using the MLST (Clausen et al., 2018; Larsen et al., 2014). Multilocus sequence types (ST) of each isolate were predicted using the pubMLST (PubMLST, 2010), while the assignment of phylogroups for the *E. coli* isolates was carried out using the online Clermont tool (Beghain et al., 2018; Clermont et al., 2019). *In silico* serotyping was performed using the SeroFinder (Joensen et al., 2015) and Kaptive (Wick et al., 2018) for *E. coli* and *Klebsiella,* respectively. The ResFinder database ResFinder v4.0 (Bortolaia et al., 2020) and the comprehensive antimicrobial resistance database (CARD) (Alcock et al., 2020) were used to detect the existing and putative new antibiotic resistance genes mediating resistance to extended-spectrum and/or carbapenem (CARB/ESBL), tetracyclines (TET), quinolones (QUIN), and sulfamethoxazole and trimethoprim (SMX/TMP) in the bacterial genomes). Plasmids were identified using PlasmidFinder (Carattoli et al., 2014) and MGEFinder (Johansson et al., 2021), while virulence genes were identified using the VirulenceFinder (Liu et al., 2019). The nucleotide sequences for *mcr* genes identified by WGS in this study were deposited in GenBank with accession numbers (Table 2.3)

In silico MLST of *K. pneumoniae* and *E. coli* isolates was determined based on allelic numbers of 7 housekeeping genes in *K. pneumoniae* (*gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*) and *E. coli* (*adk, fumC, gyrB, icd, mdh, purA, recA*) using MLST 2.0 (Voldby Larsen, 2014). In addition, the *E. coli* O:H typing (Larsen et al., 2012) and *K. pneumoniae* O and K antigens typing (Joensen et al., 2015) was done on these isolates (Table 2.1; Table 2.2).

At Auburn University, two (2) of the *mcr* positive isolates carrying *mcr-4* and 9 and *mcr-9* by PCR, were randomly chosen and analyzed for resistance genes using WGS (South Texas Center for Emerging Infectious Diseases, the University of Texas at San Antonio).

2.2.11 Detection of mutations in genes related to colistin and carbapenem resistance

Mutations in the genes previously identified to encode for resistance to colistin (*mgrB, pmrAB, phoPQ, arnT, ccrB*) and carbapenem (*ompC, ompF, ompK/35/36/37, marR, acrR, ramR*) were investigated by alignment with wild type reference genomes of *Escherichia coli* strain K-12 substrain MG1655 (NC_000913.3) and *Klebsiella pneumonia* subspecies *pneumoniae* MGH 78578 (NC_009648.1). PROVEAN v1.1.3 was employed to predict the possible role/effect of observed amino acid substitutions (mutation/alteration) on the protein functions i.e., colistin or carbapenem resistance (Choi & Chan, 2015). The standard PROVEAN cutoff score of $\leq$ -2.5 and > - 2.5 was used to categorize the mutation to have a deleterious and neutral effect on protein function respectively (Choi et al., 2012; Choi & Chan, 2015).

2.2.12 Statistical analysis

Chi-squared test was used to compare the prevalence of antimicrobial resistance in different groups. $P < 0.05$ was considered significantly different.

2.3 Results

2.3.1 Bacterial isolation from colistin and carbapenem-resistant *Enterobacteriaceae* in Nigeria

A total of 583 non-duplicate isolates were recovered from 1,119 samples collected in this study. Overall, 17.0 % of the bacterial isolates (99/583), including *E. coli* (67/99), *K. pneumoniae* (30/99), *Citrobacter werkmanii* (1/99), and *Alcaligenes faecalis* (1/99), were found to be resistant to colistin with the colistin MICs between 8 and >64 µl/ml (Table 2.3). The percentage of the human isolates resistant to colistin was 40.4% (40/99) (CI: 30.7-51%) was significantly higher (P<0.01) than the percentages of resistant isolates from cattle (7/36, 19.4%; CI: 8.2-36%), pigs (12/65, 18.4%; CI: 11.6-34%), poultry (34/290, 11.7%; CI:8.3-16%), camels (3/40, 7.5%; CI: 1.6-20%), human hospital swabs (1/15, 6.7%; CI: 0.2-32%), and dogs (2/42, 4.8%; CI: 0.6-16). Colistin-resistant bacteria were detected in at least one bird in 17 (53.1%) out of the 32 poultry farms sampled and 12 (10%) out of the 110 pigs at the slaughterhouse. However, no colistin-resistant bacteria were detected in all 100 samples collected from the 15 pig farms (Table 2.3).

Of the 583 isolates tested, 110 (18.9%) were resistant to at least one of three carbapenems: meropenem (98.0 %; 109/583), ertapenem (16.8%; 98/583) and imipenem (18.2%; 106/583) while 53 (9.1%; 53/583) were resistant to both colistin and carbapenem (Table 2.4). There was a significantly higher prevalence (P=0.0016) of carbapenem-resistant isolates being resistant to colistin when the colistin supplemented media (45/77, 58.4%) was used compared to when a non-supplemented media was used (8/33, 24.2%). The minimum inhibitory concentrations of the carbapenems ranged between 2-32 µg/ml for meropenem, 2-16 µg/ml for ertapenem, and 2-12 µg/ml for imipenem (Table 2.5, Table 2.6).

The susceptibility testing result showed the majority of the isolates were multidrug resistant (resistant to at least 3 antibiotics of different classes) with the dominant resistant profile identified as: AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX (n=5) (Table 7, Table 2.8). All the isolates, except one *E. coli,* were resistant to Amoxycillin (100%) and amoxicillin-clavulanic acid (100%). Resistance to other critical antimicrobial agents was; fluoroquinolones 78% (*Klebsiella,* 13/20; *E. coli,* 26/30) and aminoglycosides 48% (*Klebsiella* 9/20; *E. coli,* 15/30).

2.3.2 Colistin and carbapenem resistance mechanisms

In total, only 21.2% of the colistin-resistant isolates (21/99) were found to harbor *mcr*: *mcr-1* (n=13), *mcr-8* (n=5), both *mcr-1* and *mcr-5* (n=1), and both *mcr-1* and *mcr-8* (n=2) (Table 2.3). The complete nucleotide sequences of *mcr-1* identified in this study (Deposited in GenBank with the accession number: MT070404) were identical to each other and the *mcr-1.1* variant. The identified complete nucleotide gene of *mcr-8* (GenBank accession number: MT070402) in this study was determined to be *mcr 8.1* based on the BLASTn. The partial *mcr-5* sequence (271-bp) identified in this work (GenBank accession number: MT070403) showed 100% similarity to the *mcr-5* sequence a *Salmonella* Typhimurium isolate from Germany (MK360096.1).

While none of the colistin-resistant bacteria from hospital environments and the rectal swabs of camels and dogs were found to carry the *mcr* gene, *mcr*-1 was identified in one swine *A. faecalis* and one bovine *E. coli* isolate. The *mcr-1* and *mcr-8* genes were found in human isolates of *K. pneumoniae* (n=8) and *E. coli* (n=1), and three *mcr* genes (*mcr-1*, *mcr-5*, *mcr-8*) were detected in poultry isolates of *K. pneumoniae* (n=1), *E. coli* (n=8) and *C. freundii* (n=1).

The *mcr* was seen in only 8 out of the 17 farms positive for colistin-resistant bacteria. The *K. pneumoniae* isolates harboring the *mcr-8* gene exhibited a higher colistin MIC compared to those harboring the *mcr-1* gene while in the case of *E. coli*, the strain carrying two *mcr*-genes (*mcr-1* and *mcr-8*) exhibited a higher MIC (Table 2.3). No point mutations associated with colistin resistance (previously reported or novel) were detected in the *pmrABC* and *phoPQ*. Alterations in the *mgrB* were detected in all *E. coli* (29/29), *Klebsiella* (20/20), and *Citrobacter* (1/1). Novel substitutions were also found in some of the *E. coli* isolates, including V336M and T411I in the *etpA* and A278T, T157A, L485F, and W100M in the *arnT*. Similarly, the majority of the *Klebsiella* isolates harbored the N195S amino acid substitution (18/20) and some the N141I/H (5/20) and W140L/S (5/20) substitutions in the *ccrB* and R256G in *pmrB,* previously reported to mediate colistin resistance (Aires et al., 2016; Cheng et al., 2016). Finally, a novel mutation G164S in *arnT* and the M1_L56del deletion that mediates colistin resistance were detected in one *Klebsiella* isolate (Table 2.5, Table 2.6).

Meanwhile, WGS data of the carbapenem-resistant isolates were in agreement with the negative carbapenemase inhibition assay for carbapenemase production, confirming that none of the isolates harbored any known carbapenemase-producing enzyme genes. However, sequence analyses of known genes involved in carbapenem resistance revealed several genetic alterations and polymorphisms in comparison to the wild-type references strains (*Escherichia coli* MGH1655 and *Klebsiella pneumoniae* MGH 78578). These included novel deletions M1_V24del in the *ompC* of all the *E. coli* isolates (29/29), and insertion M233_T234insHYTH in the *ompK37* mediating carbapenem resistance in half the *Klebsiella* species (10/20). Additionally, previously known substitutions mediating carbapenem resistance were identified: D192G/K (17/29) and N47D (5/29) in the *ompC* of the *E. coli* isolates; A217S (15/20) and

N218H (6/20) in *ompK36* of *Klebsiella* and substitutions I70M (20/20); I128M (20/20), N230G (10/20), T261A (1/20) in *ompK37* of *Klebsiella* species. No mutation associated (existing or novel) with carbapenem resistance was detected in the *ompF*. Multiple substitutions were detected in the global regulator protein *marR* and *ramR*, but not regulators *AcrR*, *MarA, RamA* and *SoxR*. However, only the M1V substitution in *E. coli marR* and *Klebsiella pneumoniae ramR* was associated with deleterious effect i.e. carbapenem resistance (Table 2.5, Table 2.6).

2.3.3 WGS identified IncX4 harboring *mcr-1.1* and other concurrent carbapenem-colistin resistance mechanisms

The IncX4 replicon was present in each of the nine *mcr-1* positive isolates selected for WGS. The *mcr-1* was located in a unique contig with the same sequence as the IncX4 plasmid backbone, indicating that this plasmid carries the colistin resistance gene. Interestingly, no other resistance genes were found on the IncX4 plasmid, which harbored *mcr-1*.

The WGS of these bacterial isolates also identified several chromosomal genes regulated by a two-component system PhoP/Q and PmrA/B which mediates the resistance to colistin when they are over-expressed (Laurent Poirel et al., 2015). However, no mutation was identified in *pmrA*, *pmrB* and *mgrB* that are essential for the overexpression of those genes (Jayol et al., 2014).

Also, several other genes mediating resistance to β-lactams (*TEM*, *SHV*, *CTX-M* and *CMY*), tetracyclines (*tetA*, *tetC* and *tetG*), quinolones (*oqxB,* and *qnrS*), and sulfonamides (*sul1*, *sul2,* and *sul3*) were identified by WGS. The MLST of colistin-resistant *E. coli* and *K. pneumoniae* did not identify clonal relationships between *mcr* positive bacteria (Table 2.1; Table 2.2) as has been previously reported (Abiola Olumuyiwa Olaitan et al., 2015; Wick et al., 2018).

However, a possible clonal relation was found in two poultry *E. coli* isolates which had the same O-antigen (O15) and H-antigen (H45) and an identical MLST type (2485) (Table 2.1; Table 2.2). In addition, analysis of the WGS data also revealed that concurrent carbapenem-colistin resistance was strongly associated with carriage of the M1V mutation on the *mgrB* (100%; 50/50), I70M and I128M substitutions in the *ompK37* of *Klebsiella* species (100%), presence of the M1_V24del deletion on the *ompC* in the *E. coli* isolates (100%), and presence of the M1V mutation in the efflux pump regulators: *ramR* in *Klebsiella* (100%) and *marR* in *E. coli* (100%).

2.3.4 Conjugation experiment

Twenty of 21 isolates (95.2%) harboring *mcr* genes successfully transferred their resistant genes to the recipient *E. coli* J53 AZ^R as indicated by broth mating assay. The trans-conjugants all grew on MacConkey plates supplemented with 2µg/ml colistin and 300µg/ml sodium azide, and MICs of the transconjugants were ≥2µg/ml. The *K. pneumoniae* strain that could not transfer the resistance harbored both *mcr-1* and *mcr-8*. DNA sequencing was performed on the plasmid after conjugation to ensure a successful transfer. None of the isolates were found to transfer the carbapenem resistance determinant.

2.3.5 Detection of *mcr* genes from colistin-resistant bacteria at Auburn University

A total of 110 colistin-resistant bacteria isolates were recovered from 85 fecal swab samples. Altogether, only 34.5% (n=38/110) of all colistin resistant isolates were *mcr*-positive. Of these, most of the isolates were found to carry *mcr-2*, (n=28), and others were *mcr-4* (n=1), *mcr-6*(n=2), *mcr-9* (n=4), *mcr 4* and *mcr-9*(n=1), *mcr-2* and *mcr-6* (n=2). The 16S rRNA PCR

and sequencing of the *mcr* positive isolates identified *E. coli* 70.8% (n=17/24), *K. pneumoniae* 25% (6/24), and *Salmonella enterica* 4.2% (n=1/24).

All 15 isolates analyzed for MIC determination were susceptible to colistin and imipenem (0.5- ≤0.25 ug/mL). However, 6/15 (40%) of the isolates, all *K. pneumoniae,* were shown to be multidrug resistant, showing resistance to Ampicillin + Nitrofurantoin+ Sulfamethoxazole, with the MIC range between ≥32 – 128 ug/mL. All other isolates, *E. coli* (8/15) and Salmonella enterica (1/15) showed susceptibility to all the drugs tested, with an exception of two E. coli isolates, showing intermediate resistance to chloramphenicol (16ug/mL) and nitrofurantoin(64 ug/mL). Of all 85 samples tested, no carbapenem resistant isolate was isolated from the selective media containing carbapenem.

2.3.6 WGS analysis of colistin-resistant bacteria at Auburn University

WGS analysis of the two *mcr* positive isolates failed to confirm the presence of *mcr* genes in them. However other resistance genes were identified using CARD, ARG-ANNOT (Gupta et al., 2014) and NCBI (Table 2.11). Both isolates were identified as *E. coli* and were both found to express resistance genes (*marA, ampC, pmrC, pmrE, pmrF,* and bla_{EC}) known to confer multiple antibiotic resistance. These had greater than 95% coverage and identity to the wild type. WGS also identified $bla_{AMPH,}$ a class C beta lactamase, conferring resistance to cephalosporins, in only one of the isolates, having a greater than 95% coverage and identity to the wild type strain. Members of the multiple antibiotic resistant (*mar*) genes, are known to confer resistance to quinolones, β-lactams, and tetracyclines. *marA* is known to activate the ACRAB efflux system and to also activate the *waaY* gene, which acts by altering the LPS, thereby changing sensitivity to cationic peptides (Sundaramoorthy et al., 2019). In *E.coli*, *pmrC*

and *pmrE* are known to mediate the addition of PEtN to the phosphate group of lipid A, This modification is regulated by *pmrA/pmrB.* This addition confers resistance to polymyxins, including colistin (Aghapour et al., 2019; Abiola O. Olaitan et al., 2014). Samples were however not analyzed for mutations in these genes.

2.4 Discussion

The last two decades have witnessed a significant rise in infections caused by multidrug-resistant *Enterobacteriaceae* with a resultant increase in the use of carbapenems and colistin as last resort drugs (Peyclit et al., 2019). Concurrent resistance to last-resort drugs represents a severe health concern globally (Chaudhary, 2016; Serwecinska, 2020). This study has provided evidence of high levels of concurrent resistance to colistin and carbapenem in *Enterobacteriaceae* in Nigeria. This of concern in a low/middle-income country with minimal antimicrobial surveillance such as Nigeria as it indicates the emergence and establishment of pan drug-resistant strains and the resultant limitations in the antimicrobials available to control infections.

Despite growing information on the global spread and distribution of *mcr*, very few studies have looked into the prevalence of *mcr* mediated colistin resistance in African countries. This study shows that *mcr* harboring bacteria are prevalent in people and animals in Nigeria. The human *mcr*-positive isolates were mainly *K. pneumoniae* and they had a high colistin MIC. In contrast, animal *mcr*-positive isolates were mainly *E. coli* and had a lower value of colistin MIC, indicating different epidemiology in humans and animals. It has to be taken into account that most human strains were of clinical origin while most animal strains were commensals. The high detection rate of the *mcr-1* in *E. coli* and the *mcr-8* in *K. pneumoniae* in this study is consistent with reports from various parts of the world (Anyanwu et al., 2020; Bonnin et al., 2020; Nabti et al., 2020). In agreement with previous studies, most *mcr-8* positive isolates expressed high colistin MIC (Bonnin et al., 2020; X. Wang et al., 2019). Similarly, a previous report (Long et al., 2019) showed that all three isolates that harbored more than one *mcr* gene in the present study had a high colistin MIC (>64 µg/mL).

This study has also provided the first evidence of the *mcr-1* in *Alcaligenes faecalis.* This bacterium is a human commensal that is present in hospital environments. Recently, clinical isolates of *A. faecalis* carrying genes mediating ESBLs and carbapenemases were reported in Pakistan (Masseron et al., 2019). This is also the first report of *mcr-5* and *mcr-8* in Nigeria and the first description of human hospital patients in Nigeria harboring *K. pneumoniae* and *E. coli* with *mcr*. It is noteworthy that these patients had no history of colistin treatment or international travel, suggesting that colistin resistance was probably community-acquired.

The presence of the *mcr-1* in plasmids and the *pmrAB* mutations in chromosomes are the main reported mechanisms for colistin resistance (Sun et al., 2018). The WGS of the isolates revealed that the *mcr-1* gene detected from the isolates originating from humans, poultry, and pig is located on the self-transmissible IncX4 plasmid. Other studies have also reported the IncX4 plasmids bearing *mcr-1* in different bacterial species and different geographic locations (Bai et al., 2018; Manageiro et al., 2019; Sun et al., 2018; Wang et al., 2017). Probably this plasmid is the key vehicle for dissemination of *mcr-1* gene amongst *Enterobacteriaceae* in Nigeria and probably in the whole of Africa. Nevertheless, the majority of the strains identified in this study did not carry one of the *mcr*. Only 21.2% of the colistin-resistant isolates (21/99) harbored the *mcr,* suggesting other resistance mechanisms may be involved. Chromosomal mutations in regulators of the two-component systems including *mgrB, phoP/phoQ, pmrA, pmrB, pmrC* and *crrABC* (Laurent Poirel et al., 2015) are often linked to high colistin MIC among *mcr*-negative *K. pneumoniae*.

Of the chromosomal genes analyzed, alterations/mutations were detected in the *mgrB, ccrB, pmrB,* (for colistin resistance) and *OmpC, OmpK36, OmpK37* (for carbapenem resistance) which is consistent with previous reports on their role in colistin/carbapenem resistance (Cheng et al., 2016; Olaitan et al., 2016; Poirel et al., 2015). Multiple novel mutations/alteration associated

with colistin resistance were identified: *arnT* (W100M, T157A, G164S, A278T, L485F), *etpA* (V336M, T411H), *pmrB* (G164S, R256G), *ccrB* (M1_L56del, M1_KA52del, M1_A78del, M1_M59del). However, the possibility that the high colistin MIC among *mcr*-positive *K. pneumoniae* in this study is due to chromosomal mutations, or other unknown resistant genes remains to be elucidated. There were no mutations in any of the genes mentioned above in these 14 *mcr*-positive colistin-resistant isolates including *K. pneumoniae* isolates on which there was WGS information.

Surprisingly, no evidence was found for carbapenemase-producing genes in both the phenotypic and genomic investigations (WGS). Although some studies on resistance in the region have shown carbapenemase genes such as bla_{NDM} and $bla_{OXA-181}$ are widespread (Jesumirhewe et al., 2017; Olalekan et al., 2020; Olowo-okere et al., 2020), there are also reports from Nigeria which agree with the current study and show carbapenemase genes were not present in 85% (57/67) (Ogbolu & Webber, 2014) and 63% (12/19) (Olowo-okere et al., 2020) of carbapenem-resistant isolates (with high MIC) from human clinical samples in South-west and Northwest Nigeria, respectively. These studies, however, did not ascertain the mechanisms of resistance. Novel mutations associated with carbapenem resistance were also identified in: *ompC* (M1_V24del, K173T, N228_T229insGSYTSNGV, A231_Y232insNGYGER, A230_A231insYYISNGVAR), ompK37 (K27Q, D28Q, G29V, N30G, K31S, D33T, M1_Y25del, M233_T234insHYTH.

While transferable mobile genetic elements such as *mcr*- and carbapenemase genes have generated much interest and are the target of most studies investigating the mechanism of colistin and carbapenem resistance, the results obtained from this study show that the concurrent carbapenem–colistin resistance in the *E. coli* and *K. pneumoniae* isolates is linked to previously

reported and novel mechanisms. These include chromosomal mutations/disruptions affecting regulatory and non-regulatory genes controlling efflux pumps and membrane permeability. Such mutations/ disruptions may play a greater role in resistance than previously suspected.

The hypothesis is that the concurrent carbapenem–colistin resistance observed resulted from a combination of the chromosomal mutations/alterations in the *mgrB* (M1V), *ompC* (M1_V24del), *ompK37* (I70M, I128M), and the regulatory efflux pump genes (*marR* (M1V) and *ramR* (M1V)). Other studies have reported that mutations in the efflux system, in particular the global regulator *marR* and *ramR*, β-lactamase production, and porin deficiency could play major roles in carbapenem resistance (Chetri et al., 2019; Findlay et al., 2012; Girlich et al., 2009; Lerner et al., 2013). However, expression level analyses including transcriptomics and complementation assays are required to determine the significance of the mutations and enable a full understanding of the impact they have on the MIC and resistance to carbapenem and colistin.

Of note in this finding, is that there was a high probability for an isolate that is colistin resistant to also be carbapenem resistant, and vice versa. This may not be unconnected to the potential for isolates resistant to any of the two agents to develop pan drug resistance. Most of the isolates expressed multidrug resistance profiles, including high resistance to amoxicillin-clavulanic acid (100%), fluoroquinolones (78%) and gentamicin (48%) which are widely used in the treatment of infections in Nigerian hospitals. The high resistance rates recorded are in accordance with data in other reports on human and animal isolates from Nigeria and sub Saharan Africa (Aworh et al., 2019; Chah et al., 2018; Ojo et al., 2016; Olalekan et al., 2020). These resistance profiles emphasize the important roles the carbapenems and colistin play as last resort drugs and the challenges in successfully treating common and life threatening *Enterobacteriaceae* infections in the region. Most of the isolates also carried the $bla_{\text{CTX-M-15}}$

which is consistent with the increasing reports of the *bla* genotype in Nigeria (Chah et al., 2018; Okpara et al., 2018; Olowo-okere et al., 2020). There is thus growing evidence that the genotype is expanding rapidly and might become the dominant cause of resistance to extended spectrum β-lactams.

The significant clonal diversity, i.e., polyclonality observed amongst the isolates is consistent with previous reports, particularly those of carbapenem and colistin resistant isolates in Nigeria where the population structure is diverse (Ngbede et al., 2020b; Olalekan et al., 2020). Some of the animal and human isolates in this study belong to the "high-risk" clones, e.g., K. *pneumoniae* ST11, ST17, ST45, ST340 (human isolates) and *E. coli* ST58, ST744, ST410 (animal isolates). These are known for their global dissemination, ease of transmission between different hosts, ability to cause disease and acquire genetic determinants such as virulence factors, epidemic plasmids and antibiotic resistance that provide them with a competitive advantage over other bacterial clones. These "high-risk" clones are implicated in the global spread of bla_{KPC}, bla_{NDM}, bla_{OXA} and $bla_{CTX\text{-}M\text{-}15}$ (Feng et al., 2019; C. R. Lee et al., 2016; Nadimpalli et al., 2019; Patiño-Navarrete et al., 2020; Roer et al., 2018). In particular the ST11 and ST340 detected in human isolates in this study are closely related to ST258 as they belong to the same clonal complex CC258 which is responsible for outbreaks, pandemics and mass dissemination of KPC *K. pneumoniae* (Netikul & Kiratisin, 2015; Sui et al., 2018). ST11 has been implicated in multiple outbreaks in China (Fu et al., 2019; Zhao et al., 2019) while the ST307 we detected is a known virulent clone that is thought will replace the dominant epidemic ST258 in many countries in the near future (Cienfuegos-Gallet et al., 2019). The dominant conjugative IncF and IncX plasmids detected are known to facilitate the spread and acquisition

of resistance determinants by non-clonal strains and the associated *traT* is consistently associated with urinary tract infections and sepsis in humans, as was the case in this study.

The high incidence of *mcr-2* in swine in this study is in agreement with a previous report (Zhang et al., 2018). It is, however, surprising to note that isolates carrying more than one *mcr* gene had low MIC in this study as opposed to previous reports, which have associated multiple *mcr* genes with high MIC values (Long et al., 2019). A solid conclusion cannot be made from this since not all *mcr* positive isolates were tested in this study, it is probable that there could be other *mcr* isolates with high MIC above the breakpoint if tested. *E. coli* 70.8% (n=17/24), constituted the higher percentage of the population of the bacteria isolated from these samples, while *K. pneumoniae* only constituted 25% (6/24). This finding agrees with our study in Nigeria, with *E. coli* most commonly isolated from animals and *K. pneumoniae* from humans.

While carbapenem is not approved for animal use anywhere in the world, the occurrence of CRE has been reported in livestock across the globe, including Africa, Americas, Asia and Europe (Hernandez et al., 2002; Manageiro et al., 2019; Nandi et al., 2013; Ojo et al., 2016; Singh et al., 2012; Vieira et al., 2010). In the USA, CRE has been reported in fecal samples obtained from diary cows, albeit with a very low burden (3/323 isolates) (Webb et al., 2016). Of the 85 fecal swab samples tested in this study, no carbapenem-resistant isolate was recovered from MacConkey supplemented with meropenem. This result is in agreement with a previous study in the country (Mollenkopf et al., 2017), which tested swine fecal samples and environmental samples, but failed to detect any CRE in any of the fecal samples. The low incidence of carbapenem and colistin resistance obtained from the samples in the USA are different from Nigeria, with a high occurrence of concurrent colistin and carbapenem resistance. This difference could be attributable to the stringent measures in place for antimicrobial use in

developed countries, as opposed to its lack thereof in developing nations. Although, no carbapenem resistant isolate was observed in this study, of concern is the incidence of colistin resistance among the swine.

Conclusions

This study shows evidence for the widespread occurrence of multidrug-resistant *Enterobacteriaeceae* with concurrent carbapenem-colistin resistance due to novel and previously known genetic determinants not previously described in animal and human isolates in Nigeria and Saharan Africa. It also demonstrates the presence of internationally recognized high-risk clones and epidemic IncF plasmids that have the potential to impact human and animal health negatively. Combining diverse drug resistance genes and sequence types highlights the considerable genome plasticity and polyclonality that characterize the population structure of both clinical and non-clinical colistin and carbapenem-resistant *Enterobacteriaceae* in Nigeria. The polyclonality might create considerable problems during outbreak tracing and source attribution. Furthermore, swine fecal samples obtained from the USA in this study identified colistin resistant isolates carrying *mcr* genes, but no carbapenem resistant isolate was recovered. As such, results from this research observed that there is a high prevalence of concurrent colistin and carbapenem resistance in Nigeria, but this is contrary to results from the USA, where this concurrent resistance is seldom found.

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Table 2.1: MLST of colistin-resistant *K. pneumoniae* isolated from humans and poultry

Isolate	host	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*		MLST	O-Ag	K-Ag
H6	human	3	3	1	1	1	1	4		11	O3b	KL38
H2	human	2	3	1	1	1	4	42		501	O1v2	KL8
H7	human	2	1	2	1	7	1	7		36	O2v2	KL102
H39	human	2	1	1	1	4	4	4		17	OL104	KL107
H45	human	3	3	1	1	1	1	18		340	O4	KL15
L18	poultry	1	6	13	1	1	1	1		627	O1v2	KL54

Table 2.2: MLST of colistin-resistant *E. coli* isolated from humans and poultry

Isolate	Host	*adk*	*fumC*	*gyrB*	*icd*	*Mdh*	*purA*	*recA*	MLST	O-Ag	H-Ag
L23	poultry	6	4	159	140	112	1	17	2485	O15	H45
L26	poultry	6	NA*	14	16	NA	8	14	NA	O155	H51
L39	Poultry	83	331	32	246	1	1	2	2954	O160	H9
L40	Poultry	6	29	5	18	11	8	41	191	O128	H20
L29	poultry	6	4	159	140	112	1	17	2485	O15	H45
L36	poultry	6	29	5	18	11	8	41	191	O128	H20
H31	human	?	1012	193	804	636	561	139	NA	O9	NA

* Due to the insufficient coverage for the whole genome sequencing, there is not enough to get the definite information for some MLST analysis

Table 2.3: Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

Isolate ID	Species identity	Source of sample hospital 1 = fmc, 2= bsuth	Host	Type of sample	Health status of host	Year	State of origin	Colistin MIC (µg/ml)	*mcr*-gene detected (ND= *mcr*-gene not detected)
L1	*E. coli*	Backyard farm 1	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L2	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	8	ND
L3	*E. coli*	Backyard farm 1	Poultry	Cloacal swab	Healthy	2019	Benue	> 64	ND
L4	*E. coli*	Backyard farm 1	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L5	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	8	ND
L6	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	> 64	ND
L7	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	8	ND
L8	*E. coli*	Backyard farm 2	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L9	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	8	ND
L10	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	8	ND
L11	*E. coli*	Backyard farm 5	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L12	*E. coli*	Backyard farm 5	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND
L13	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	32	ND
L14	*E. coli*	Backyard farm 8	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L15	*K. pneumoniae*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	> 64	ND
L16	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	16	ND
L17	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2019	Benue	16	ND
L18	*K. pneumoniae*	Backyard farm 9	Poultry	Cloacal swab	Healthy	2019	Benue	16	*mcr-8*
L19	*E. coli*	Backyard farm 9	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L20	*E. coli*	Backyard farm 9	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND

Table 2.3 (cont'd): Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

L21	*E. coli*	Backyard farm 12	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L22	*E. coli*	Backyard farm 12	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND
L23	*E. coli*	Backyard farm 12	Poultry	Cloacal swab	Healthy	2019	Benue	16	*mcr-1*
L24	*E. coli*	Backyard farm 13	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L25	*E. coli*	Backyard farm 13	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND
L26	*E. coli*	Backyard farm 13	Poultry	Cloacal swab	Healthy	2019	Benue	16	*mcr-1*
L27	*E. coli*	Backyard farm 18	Poultry	Cloacal swab	Healthy	2019	Benue	16	*mcr-1*
L28	*E. coli*	Backyard farm 18	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND
L29	*E. coli*	Backyard farm 18	Poultry	Cloacal swab	Healthy	2019	Benue	> 64	*mcr-1, mcr-5*
L30	*E. coli*	Backyard farm 18	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L31	*E. coli*	Backyard farm 18	Poultry	Cloacal swab	Healthy	2019	Benue	16	ND
L32	*E. coli*	Backyard farm 19	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L33	*E. coli*	Backyard farm 19	Poultry	Cloacal swab	Healthy	2019	Benue	8	*mcr-8*
L34	*E. coli*	Backyard farm 25	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L35	*E. coli*	Backyard farm 25	Poultry	Cloacal swab	Healthy	2019	Benue	8	ND
L36	*E. coli*	Backyard farm 26	Poultry	Liver	Sick birds	2019	Benue	16	*mcr-1*
L37	*E. coli*	Backyard farm 27	Poultry	Liver	Sick birds	2019	Benue	8	ND
L38	*C. werkmanii*	Backyard farm 28	Poultry	Liver	Sick birds	2019	Benue	16	*mcr-1*

Table 2.3 (cont'd): Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

L39	*E. coli*	Backyard farm 29	Poultry	Liver	Sick birds	2019	Benue	16	*mcr-1*
L40	*E. coli*	Backyard farm 30	Poultry	Liver	Sick birds	2019	Benue	16	*mcr-1*
L41	*E. coli*	Backyard farm 31	Poultry	Liver	Sick birds	2019	Benue	> 64	ND
L42	*E. coli*	Backyard farm 32	Poultry	Liver	Sick birds	2019	Benue	8	ND
L43	*E. coli*	Backyard farm 32	Poultry	Liver	Sick birds	2019	Benue	16	ND
H2	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	> 64	*mcr-1, mcr-8*
H3	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
H4	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H5	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H6	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	> 64	*mcr-8*
H7	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	*mcr-8*
H8	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	*mcr-1*
H9	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H10	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
H21	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
H22	*K. pneumoniae*	Hospital 1	Environment	ICU Wash hand basin		2019	Benue	32	ND
H23	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	32	ND
H24	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND

Table 2.3 (cont'd): Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

H25	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	32	ND
H26	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H27	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H28	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
H29	*E. coli*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	16	ND
H30	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	16	ND
H31	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	> 64	*mcr-1, mcr-8*
H32	*E. coli*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	*mcr-1*
H33	*E. coli*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H34	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	16	ND
H35	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	> 64	ND
H36	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	16	ND
H37	*E. coli*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H38	*E. coli*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H39	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	*mcr-8*
H40	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H41	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	> 64	ND

H42	*E. coli*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND

Table 2.3 (cont'd): Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

H43	*E. coli*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	8	ND
H44	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	*mcr-1*
H45	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	> 64	*mcr-1*
H46	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H47	*K. pneumoniae*	Hospital 1	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H48	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H49	*K. pneumoniae*	Hospital 2	Human	Urine	In patient ICU	2019	Benue	> 64	ND
H50	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	16	ND
H51	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
H52	*K. pneumoniae*	Hospital 2	Human	Stool	Out patient	2019	Benue	8	ND
B6	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	8	ND
B7	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	8	ND
B8	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	8	ND
B10	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	8	*mcr-1*
B14	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	16	ND
B16	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	8	ND
B22	*E. coli*	At slaughter	Cattle	Rectal swab	Healthy	2018	Benue	16	ND
C8	*E. coli*	Live camel market	Camel	Rectal swab	Healthy	2016	Jigawa	8	ND
C14	*E. coli*	Live camel market	Camel	Rectal swab	Healthy	2016	Jigawa	8	ND
C40	*E. coli*	Live camel market	Camel	Rectal swab	Healthy	2016	Katsina	> 64	ND

D4	*E. coli*	Household	Dog	Rectal swab	Healthy	2017	Benue	8	ND
D8	*E. coli*	Household	Dog	Rectal swab	Healthy	2017	Benue	8	ND

Table 2.3 (cont'd): Distribution of *mcr* genes in colistin resistant *Enterobacteriaceae* recovered from Nigeria

E12	*A. faecalis*	At slaughter	Pig	Rectal swab	Healthy	2016	Benue	> 64	*mcr-1*
E41	*E. coli*	At slaughter	Pig	Rectal swab	Healthy	2016	Benue	64	ND
E56	*E. coli*	Backyard farm	Poultry	Cloacal swab	Healthy	2016	Benue	> 64	ND

Table 2.4: Distribution of carbapenem and colistin resistant isolates recovered from humans and animals in Nigeria

	No of isolates recovered on			Ranges of MIC (µg/ml)
	Colistin supplemented media	Non-supplemented media	Total	
Carbapenem resistant (n=583)	77	33	110 (18.9%)	2 – 32
Colistin resistant (n=583)	86	15	99 (17%)	8 - > 64
Co-resistance to colistin & carbapenem (n=583)	45	8	53 (9.1%)	

Table 2.5: Characteristics and genotypic profile of *Enterobacteriaceae* of human origin expressing concurrent resistance to carbapenem and colistin from Nigeria

ID	Sample type	MLST	Phylo-group	MIC (µg/ml)				Mutations and Alterations Mediating								
								Colistin resistance				Carbapenem resistance				
				Col	Mer	Ert	Imp	*mgrB*	*ccrB*	*pmrB*	*arnT*	*ompK36*	*ompK37*	*ompC*	*ramR*	*marR*
H2	Stool	ST 501	NA	>64	32	2	2	MIV	**N195S**	-	-	**A217S, N218H**	**I70M, I128M**	-	MIV	-
*H34	Stool	ST156	B1	16	16	8	12	M1V	-	-	-	-	-	M1_V24del	-	M1V
*H35	Stool	ST2006	B1	> 64	2	2	2	M1V	-	-	-	_	_	M1_V24del, D192G	-	MIV
H36	Stool	ST 252	NA	16	16	2	8	MIV	M1_A52del, **W140L, N141I**		-	**A217S**	**I70M, I128M**	-	MIV	-
*H41	Stool	ST182	E	>64	8	16	8	M1V	-	-	-	-	-	M1_V24del, R267L	-	M1V
H6	Stool	ST 11	NA	>64	32	2	4	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
H50	Stool	ST 925	NA	16	16	16	12	MIV	M1_M59del , **W140S N141H, N195S**		-	**A217S, N218H**	**I70M, I128M**	-	MIV	-
H4	Urine	ST 45	NA	>64	32	2	8	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
H5	Urine	ST 45	NA	>64	32	16	12	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
H7	Urine	ST 36	NA	>64	32	16	12	MIV	**N195S**	-	G164S	-	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
H23	Urine	ST 307	NA	32	8	16	12	MIV	**N195S**	-	-	-	**I70M, I128M, N230G,**	-	MIV	-

												M233_T234insHYTH				
H25	Urine	ST 307	NA	32	32	16	12	MIV	**N195S**	-	-	-	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
H26	Urine	ST 307	NA	>64	6	16	8	MIV	**N195S**	-	-	-	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	-
*H29	Urine	ST210	B1	16	4	2	2	M1V	-	-	-	-	-	M1_V24del	-	M1V
**H30	Urine	ND	NA	16	32	16	12	MIV	M1_K78del, W140L, N141M, **N195S**		-	**A217S, N218H**	**I70M, I128M**	-	MIV	-
H31	Urine	ST 200	NA	>64	8	8	12	MIV	**N195S**	G164S	-	**A217S, N218H**	**I70M, I128M**	-	MIV	-
H39	Urine	ST 17	NA	>64	16	16	2	MIV	**N195S**		-		**I128M, I70M, T261A**	-	MIV	-
H40	Urine	ST 2617	NA	>64	32	16	12	MIV	M1_A52del, **W140L, N141I**		-	**A217S, N218H**	**I128M, I70M**	-	MIV	-
H45	Urine	ST 340	NA	>64	32	16	12	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	_
H46	Urine	ST 340	NA	>64	32	16	8	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	_
H47	Urine	ST 340	NA	>64	16	16	12	MIV	**N195S**	R256G	-	**A217S**	**I70M, I128M, N230G,** M233_T234insHYTH	-	MIV	_
H48	Urine	ST 3271	NA	>64	16	16	12	MIV	**N195S**		-	**A217S**	M1_Y25del, K27Q, D28Q, G29V, N30G, K31S, D33T, **I70M, I128M**	-	MIV	-

*H49	Urine	ST58	B1	>64	32	2	12		**M1V**	-	-	-	-	M1_V24del, D192G, A231_Y232insNGYGER	-	M1V
*H50	Urine	ST 925	NA	16	16	16	12	MIV	M1_M59del, **W140S N141H, N195S**	-	-	**A217S, N218H**	**I70M, I128M**	-	MIV	-
**H22	Sink	ST 3266	NA	32	32	16	12	MIV	**N195S**	-	-	**A217S**	**I70M, I128M**	-	MIV	-

Bold: Mutations/alterations previously reported by other authors **Escherichia coli, **Klebsiella quasipneumoniae, others: Klebsiella pneumoniae*

Table 2.6: Characteristics and genotypic profile of *Enterobacteriaceae* of animal origin expressing concurrent resistance to carbapenem and colistin, Nigeria

ID	Host	Sample Type	MLST	Phylo-group	Minimum inhibitory concentration (µg/ml)				Mutations and Genetic Alterations Mediating								
									Colistin resistance				Carbapenem resistance		*ompK36*	*ompK37*	*ramR*
					Col	Mer	Ert	Imp	***mgrB***	***ccrB***	***etpA***	***arnT***	***OmpC***	***marR***	-	-	-
B14	Cattle	Rectal swab	ND	B1	16	32	16	12	**M1V**	-	-	**A278T**	M1_V24del, G29S, D39N, G40A, L41K, D46S, Q54T, Y74F	M1V	-	-	-
B22	Cattle	Rectal swab	ST215	A	16	32	16	12	**M1V**	-	-	-	M1_V24del	M1V	-	-	-
C40	Camel	Rectal swab	ST46	A	>64	32	8	8	**M1V**	-	V336M	-	M1_V24del, D192G	M1V	-	-	-
E41	Pig	Rectal swab	ST4977	A	>64	8	16	8	**M1V**	-	-	-	M1_V24del, D192G	M1V	-	-	-
L6	Pig	Rectal swab	ST410	C	>64	4	2	2	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L13	Pig	Rectal swab	ST410	C	32	32	16	4	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L15	Pig	Rectal swab	ST3113	C	>64	8	16	12	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L16	Pig	Rectal swab	ST226	A	16	32	8	4	**M1V**	-	-	-	M1_V24del, D192G, N228_T229insGSYTSNGV	M1V	-	-	-
L17	Pig	Rectal swab	ST224	B1	16	16	16	12	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L20	Pig	Rectal swab	ST2179	B1	16	32	16	8	**M1V**	-	T411I	**T157A**	M1_V24del	M1V	-	-	-
L3	Poultry	Cloacal swab	ST196	B1	>64	32	2	8	**M1V**	-	-	-	M1_V24del	M1V	-	-	-
*L18	Poultry	Cloacal swab	ST627	NA	16	8	16	8	**M1V**	M1_L56del, W140S, N141H, **N195S**	-	-	-	-	**A217S, N218H**	**I70M, I128M**	MIV
L22	Poultry	Cloacal swab	ST224	B1	16	32	8	8	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L23	Poultry	Cloacal swab	ST2485	D	16	32	16	12	**M1V**	-	-	-	M1_V24del, N47D, **D192K**	M1V	-	-	-
L25	Poultry	Cloacal swab	ST6707	D	16	16	16	12	**M1V**	-	-	-	M1_V24del, N47D, **D192K**	M1V	-	-	-
L26	Poultry	Cloacal swab	ND	B1	16	32	16	4	**M1V**	-	-	**L485F**	M1_V24del	M1V	-	-	-
L27	Poultry	Cloacal swab	ST744	A	16	32	16	4	**M1V**	-	-	-	M1_V24del, D192G, N228_T229inGSYTSNGV	M1V	-	-	-
L28	Poultry	Cloacal swab	ST1673	B1	16	32	2	4	**M1V**	-	-	-	M1_V24del, **D192G**	M1V	-	-	-
L29	Poultry	Cloacal swab	ST2485	D	16	8	16	12	**M1V**	-	-	-	M1_V24del, N47D, **D192K**	M1V	-	-	-
L31	Poultry	Cloacal swab	ST6061	A	16	32	2	4	**M1V**	-	-	-	M1_V24del	M1V	-	-	-
L36	Poultry	Viscera-liver	ST191	A	16	32	4	8	**M1V**	-	-	-	M1_V24del	M1V	-	-	-
**L38	Poultry	Viscera-liver	ST259	NA	16	32	16	12	**M1V**	-	-	-	-	-	-	-	-

L39	Poultry	Viscera-liver	ST2954	E	16	4	2	4	**M1V**	-	-	-	M1_V24del, N47D	M1V	-	-	-
L40	Poultry	Viscera-liver	ST191	A	16	16	16	4	**M1V**	-	-	-	M1_V24del, A230_A231insYYISNGVAR	M1V	-	-	-
L41	Poultry	Viscera-liver	ND	A	16	8	8	8	**M1V**	-	-	**W100M**	M1_V24del, K173T, N176_T183del, G190E, **D192G,** D225W	M1V	-	-	-
L43	Poultry	Viscera-liver	ST929	B2	64	32	4	12	**M1V**	-	-	-	M1_V24del, N47D, **D192G**	M1V	-	-	-

Bold: Mutations/alterations previously reported by other authors *: *Klebsiella pneumoniae, **: Citrobacter werkmanii*

Table 2.7: Phenotypic and genotypic resistance profile of *Enterobacteriaceae* of human origin expressing concurrent carbapenem-colistin resistance from Nigeria

	ID	Phenotypic Resistance Profile	β-lactamases	Aminoglycosides	Fluoroquninolones	Sulphamethazoxle -Trimethoprim	Tetracycline	Phenicol
Human clinical sample (stool)	H2	AMC AMX CEF GEN	SHV-11	-	*acrR*, oqxA, oqxB*	-	-	-
	H34	AMC AMX CHL CIP CEF DOX ERY ENR FOR STX	CTX-M-15, TEM-1B	*aac(6')-Ib-cr, aac(6')-Ib3*	*gyrA*, parC*, parE**	*sul1, dfrA1*	*tetB*	*catA1, catB3*
	H35	AMC AMX CHL CIP CEF ERY	CTX-M-15	*aadA1*	*qepA4, gyrA*, parE**	*sul1, sul2, dfrA12*	*tetB*	*catA1, cmlA1*
	H36	AMC AMX CEF DOX ERY STX	SHV-1	-	*acrR*, oqxA, oqxB*	-	-	-
	H41	CEF COL DOX ERY	-	-	-	-	-	
	H6	AMC AMX CHL CIP CEF GEN ERY STX	TEM-1B, SHV-11	*aac(3)-IId, aadA1, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrS1*	*sul2, dfrA14,*	*tetA, tetD*	*catA2*
	H50	AMC AMX CEF DOX ERY STX	OKP-B-8	-	*acrR*, oqxA, oqxB*	-	-	-
Human clinical sample (urine)	H4	AMC AMX CHL CIP CEF DOX ERY ENR FOX STX	SHV-1	-	*acrR*, oqxA, oqxB*	-	-	-
	H5	AMC AMX CEF DOX ERY ENR STX	TEM-1B, DHA-1, SHV-1	*aph(3'')-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrB4*	*sul1, sul2, dfrA1, dfrA14,*		
	H7	AMC AMX CEF DOX ERY ENR	SHV-11	*aadA1*	*acrR**	*sul2, dfrA5*		
	H23	AMC AMX CIP CEF DOX GEN ERY ENR SXT	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14*	*tetA*	
	H25	AMC AMX CIP CEF DOX GEN ERY ENR STX	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6')-Ib-cr*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14,*	*tetA*	*catB3*
	H26	AMC AMX CIP CEF GEN ERY ENR STX	CTX-M-15, OXA-1, TEM-1B, SHV-28	*aac(3)-IIa, aac(6')-Ib-cr*	*acrR*, oqxA, oqxB, qnrB1*	*sul2, dfrA14,*	*tetA*	*catB3*
	H29	AMC AMX CEF ERY FLOR	-	-	-	*sul2, dfrA15*		-
	H30	AMC AMX CEF ERY FLOR MER	OKP-B-7	-	*acrR*, oqxA, oqxB*	-	-	-
	H31	AMC AMX CEF DOX ERY ENR FLOR STX	SHV-1	-	*acrR*, oqxA, oqxB*	-	-	-
	H39	AMC AMX CEF DOX ERY	TEM-1C, SHV-1	*aadA1*	*oqxA, oqxB*	*sul1, dfrA1*	*tetA*	
	H40	AMC AMX CEF ERY		*aph(3')-Ib, aph(6)-Id*	*acrR*, oqxA, oqxB*			
	H45	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2*	*acrR*, oqxA, oqxB*	*sul1, sul2 dfrA1*	*tetD*	*catA2*
	H46	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2, ant(2'')-Ia, aph(3')-Ia, aph(3')-Ib*	*oqxA, oqxB*	*sul1, sul2 dfrA10, dfrA12*	*tetD, tetJ*	
	H47	AMC AMX CHL CIP CEF GEN ERY STX	CTX-M-15, TEM-1B, SHV-11	*aac(3)-IId, aadA2, aph(3')-Ib*	*acrR*, oqxA, oqxB*	*sul1, sul2 dfrA12*	*tetD*	*catA2*
	H48	AMC AMX CEF ERY FOX STX	TEM-1B, SHV-11	-	*acrR*, oqxA, oqxB*	*sul2 dfrA26*	-	-

	H49	AMC AMX CEF ERY	TEM-1B, DHA-17	*aph(3")-Ib, aph(6)-Id, aadA1*	*sul1*, *sul2*	*dfrA1*, *dfrA5*	*tetA*	*catA2*
Hospital environment (sink)	H22	AMC AMX CEF DOX ERY ENR STX	OKP-B-5	*aph(3")-Ib, aph(6)-Id*	*acrR**, *oqxA*, *oqxB*			

Table 2.8: Phenotypic and genotypic resistance profile of *Enterobacteriaceae* of animal origin expressing concurrent carbapenem-colistin resistance from Nigeria

	ID	Phenotypic Resistance Profile	β-lactamases	Aminoglycosides	Fluoroquninolones	Sulphamethazoxle-Trimethoprim	Tetracycline	Phenicol
Cattle rectal swab	B14	AMC AMX CEF DOX GEN ERY ENR STX	CTX-M-15, TEM-1B	*aph(3")-Ib, aph(6)-Id, aac(3)-IId*	*qnrS1, qepA1, gyrA**	*sul2, dfrA14, dfrA17*	*tetA, tetB*	-
	B22	AMC AMX CEF ERY ENR STX	CTX-M-15	*aph(3")-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*		-
Camel rectal swab	C40	AMC AMX CEF ERY ENR	CTX-M-15		*qnrS1*		*tetA*	-
Pig rectal swab	E41	AMC AMX CEF STX	CTX-M-15, TEM-1B	*aph(3")-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*	*tetA*	-
	L3	AMC AMX CEF DOX GEN ERY STX	CTX-M-55	*aac(3)-IId*	*qnrS1*	*sul2, dfrA14*	*tetA*	
	L18	AMC AMX CHL CEF DOX GEN ERY ENR FLOR SXT	TEM-1B	*armA, aadA1, aadA2b, aac(3)-IIa, aph(3')-Ia, aph(6)-Ic,*	*acrR*, qnrB1, oqxA, oqxB, qnrB17*	*sul1, sul3, dfrB4,*	*ramR*, tetD, tetM*	*cmlA1*
	L6	AMC AMX CIP CEF DOX ERY ENR STX	CTX-M-15, OXA-1	*aac(6')-Ib-cr, aadA5*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
	L13	AMC AMX CIP CEF DOX ERY ENR STX	CTX-M-15, OXA-1	*aac(6')-Ib-cr*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
	L15	AMC AMX CEF ERY ENR	CTX-M-15, OXA-1	*aac(6')-Ib-cr*	*gyrA**	*sul1, dfrA17*	*tetA*	*CatB3*
	L16	AMC AMX CEF COL DOX ERY ENR MER STX	CTX-M-15, TEM-1B	*aph(3")-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*	*tetA*	
	L17	AMC AMX CHL CIP CEF DOX GEN ERY ENR STX	CTX-M-15	*aadA2, aadA1*	*gyrA**	*sul2, dfrA12*	*tetA*	*cmlA1*
Poultry cloacal swab	L20	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR	CTX-M-65	*aac(3)-IId*	*qnrS13*		*tetA*	*floR*
	L22	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX	-	*aac(3)-IIa, aph(3')-Ia, aph(3")-Ib, aadA1*	*gyrA**	*sul3, dfrA1*	*tetA*	*floR*
	L23	AMC AMX CHL CEF DOX GEN ERY ENR	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	
	L25	AMX AMC CIP CEF ENR STX	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	
	L26	AMC AMX CHL CEF DOX ERY ENR FLOR MER STX	TEM-1B	*aph(3')-Ia, aph(3")-Ib, aadA1*	*gyrA*, qnrS1, parC**	*sul3, dfrA1*	*tetA*	*floR*
	L27	AMC AMX CHL CEF COL GEN ERY ENR FLOR STX	TEM-1B	*aac(3)-IIa, aadA1, aph(3")-Ib*	*gyrA**	*sul3, dfrA1*	*tetA*	*floR*
	L28	AMC AMX CIP CEF DOX GEN ERY ENR	TEM-1A	*aac(3)-IId, aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	-
	L29	AMC AMX CEF DOX GEN ERY ENR STX	TEM-1B	*aac(3)-Via*	*gyrA*, qnrS1*	*sul1, sul3, dfrA14*	*tetA*	-
	L31	AMC AMX CEF DOX GEN ERY ENR	CTX-M-55	*aph(3')-Ia, aph(3")-Ib, aph(6)-Id*	*gyrA*, parC**	*sul2*	*tetB*	-
Poultry clinical sample (liver)	L36	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX	TEM-1B	*aac(3)-IId, aph(3')-Ia, aph(3")-Ib, aph(6)-Id*		*sul2, dfrA14*	*tetA*	*floR*
	L38	AMC AMX CHL CEF DOX GEN ERY ENR FOX STX	CMY-98, TEM-1	*aph(3')-Ia, aph(6)-Id, aadA2b*	*qnrB19, qnrB34*	*sul1, dfrA12*	*tetB, tetM*	*cmlA1*
	L39	AMC AMX CIP CEF DOX ENR STX	TEM-1B	*aadA5*	*qnrS1*	*sul2, dfrA17*	*tetA*	-

	L40	AMC AMX CHL CIP CEF DOX GEN ERY ENR FLOR STX	TEM-1B	*aac(3)-IId, aph(3')-Ia, aph(3")-Ib, aph(6)-Id*	*qnrS1*	*sul2, dfrA14*		*floR*
	L41	AMC AMX CEF DOX GEN ERY ENR		*aac(3)-IIa, aph(6)-Id*	*qnrS13, gyrA*, parC*,*	*sul2*	*tetA*	-
	L43	AMC AMX CIP CEF GEN ERY ENR	TEM-1B	-	*qnrS1*	-	-	-

AMC: Amxycyline-clavulanic acid, AMX: Amoxycillin, CEF: Ceftriaxone, CIP: Ciprofloxacin, CHL: Chloramphenicol, DOX: Doxycycline, GEN: Gentamicin, ERY: Erytromycin, ENR: Enrofloxacin, FOX: Cefoxitin, FLOR: Florfernicol, STX: Sulfamethaxaxole-Trimethoprim

Table 2.9: Plasmid replicon profile of colistin and carbapenem co-resistant *Enterobacteriaceae*

Isolates	**Plasmid Replicon Types (No. of isolates harboring the plasmid)**
Klebsiella pneumonia	IncFIB(mar) (n=1); IncHIIB (n=1); IncFII(K) (n=10); IncFIB (pKPH51) (n=1); IncFIB(K) (n=15); IncR (n=10); IncQI (n=1); IncFIA(HI1) (n=5); IncFII (n=2); ColMGD2) (n=1); Col4401 (n=8); Col3M (n=2); ColRNAI (n=1)
Klebsiella quasipneumoniae	IncFII (n=1); IncFII(K) (n=1); IncFIB(K) (n=1); IncR (n=1); Col4401 (n=2)
Escherichia coli	IncFIA (n=9); IncFIB (n=1); InFIB(pLF82) (n=1); IncHIIB(CIT) (n=1); IncFII(PHN7A8) (n=1); IncFIB(K) (n=3); IncFIB(AP001918) (n=17); IncFII (pAMA1167-NDM-5) (n=1); IncY (n=4); IncQI (n=3); IncFII (n=9); IncFIC(FII) (n=6); IncI1 (n=2); IncI2 (n=1); IncII-1(Gamma) (n=1); p0111 (n=5); IncN (n=1); IncX1 (n=7); IncX4 (n=8); Col3M (n=1); Col156 (n=5); Col4401 (n=1)
Citrobacter werkmanii	Col4401 (n=1); IncQ1 (n=1)

Table 2.10: Detection of *mcr* genes from swine fecal swabs in USA

Sl. No.	**Pig #**	**Age**	**Sex**	***Identity***	***mcr* genes detected**			
					mcr-2	***mcr-4***	***mcr-6***	***mcr-9***
1	7001	7.5 weeks	F		ND	ND	ND	ND
2	6903	7.5 weeks	F		ND	ND	ND	*mcr-9*
3	6806	7.5 weeks	F		ND	ND	ND	ND
4	5714	7.5 weeks	F		ND	*mcr-4*	ND	*mcr-9*
5	5908	7.5 weeks	F		ND	ND	ND	ND
6	7002	7.5 weeks	F	Escherichia coli	ND	ND	ND	*mcr-9*
7	8607	7.5 weeks	F		ND	ND	ND	ND
8	5614	7.5 weeks	F		ND	ND	ND	ND
9	6915	7.5 weeks	F		ND	ND	ND	*mcr-9*
10	5812	7.5 weeks	F	Klebsiella pneumoniae	ND	ND	ND	*mcr-9*
11	5314	12.5 weeks	F		ND	ND	ND	ND
12	5008	12.5 weeks	F		ND	ND	ND	ND
13	5409	12.5 weeks	F		ND	ND	ND	ND
14	5311	12.5 weeks	F		ND	ND	ND	ND
15	5205	12.5 weeks	F		ND	ND	ND	ND
16	5410	12.5 weeks	F		ND	ND	ND	ND
17	5210	12.5 weeks	F		ND	ND	ND	ND
18	5206	12.5 weeks	F		ND	ND	ND	ND
19	5106	12.5 weeks	F		ND	ND	ND	ND
20	5208	12.5 weeks	F		ND	ND	ND	ND
21	3703	17 weeks	F		ND	ND	ND	ND
22	4007	17 weeks	F		ND	ND	ND	ND
23	3205	17 weeks	F		ND	ND	ND	ND
24	3411	17 weeks	F		ND	ND	ND	ND
25	3912	17 weeks	F		ND	ND	ND	ND
26	3413	17 weeks	F		ND	ND	ND	ND
27	3506	17 weeks	F		ND	ND	ND	ND
28	3916	17 weeks	F		ND	ND	ND	ND
29	4107	17 weeks	F		ND	ND	ND	ND

30	3204	17 weeks	F		ND	ND	ND	ND
31	6013	11 weeks	F		*mcr-2*	ND	ND	ND
32	7001	11 weeks	F		ND	ND	ND	ND
33	6806	11 weeks	F		*mcr-2*	ND	ND	ND
34	6014	11 weeks	F		ND	ND	ND	ND
35	7002	11 weeks	F		*mcr2*	ND	ND	ND
36	4102	22 weeks	M		ND	ND	ND	ND
37	3602	22 weeks	M		*mcr-2*	ND	ND	ND
38	3701	22 weeks	M		*mcr-2*	ND	*mcr-6*	ND
39	4002	22 weeks	M		ND	ND	ND	ND
40	3601	22 weeks	M		ND	ND	ND	ND
41	6106	11 weeks	F		*mcr-2*	ND	ND	ND
42	6208	11 weeks	F		*mcr-2*	ND	ND	ND
43	6104	11 weeks	F		ND	ND	ND	ND
44	5311	16 weeks	F		*mcr-2*	ND	ND	ND
45	6215	11 weeks	F		*mcr-2*	ND	ND	ND
46	5409	16 weeks	F		ND	ND	ND	ND
47	5205	16 weeks	F		*mcr-2*	ND	ND	ND
48	6701	11 weeks	F		ND	ND	ND	ND
49	4910	16 weeks	F		ND	ND	ND	ND
50	4404	16 weeks	M		ND	ND	ND	ND
51	5306	16 weeks	M		ND	ND	ND	ND
52	5101	16 weeks	M		*mcr-2*	ND	ND	ND
53	4601	16 weeks	M		*mcr-2*	ND	ND	ND
54	4603	16 weeks	M		*mcr-2*	ND	ND	ND
55	5404	16 weeks	M		*mcr-2*	ND	ND	ND
56	4008	22 weeks	F		*mcr-2*	ND	*mcr-6*	ND
57	3603	22 weeks	F		*mcr-2*	ND	ND	ND
58	4006	22 weeks	F		*mcr-2*	ND	ND	ND
59	3913	22 weeks	F		*mcr-2*	ND	ND	ND
60	3811	22 weeks	F		ND	ND	*mcr-6*	ND
61	4203	22 weeks	F		*mcr-2*	ND	*mcr-6*	ND
62	3917	22 weeks	F		*mcr-2*	ND	ND	ND
63	3111	22 weeks	F		*mcr-2*	ND	ND	ND
64	3910	22 weeks	F		ND	ND	ND	ND

65	3915	22 weeks	F		ND	ND	ND	ND
66	3413	22 weeks	F		ND	ND	ND	ND
67	3204	22 weeks	F		*mcr-2*	ND	ND	ND
68	3411	22 weeks	F		*mcr-2*	ND	ND	ND
69	3504	22 weeks	F		*mcr-2*	ND	ND	ND
70	4105	22 weeks	F		*mcr-2*	ND	ND	ND
71	4107	22 weeks	F		*mcr-2*	ND	ND	ND
72	3506	22 weeks	F		*mcr-2*	ND	ND	ND
73	3916	22 weeks	F		*mcr-2*	ND	ND	ND
74	4304	22 weeks	F		*mcr-2*	ND	ND	ND
75	4109	22 weeks	F		ND	ND	ND	ND
76	4412	16 weeks	F		ND	ND	ND	ND
77	4606	16 weeks	F		*mcr-2*	ND	ND	ND
78	4409	16 weeks	F		*mcr-2*	ND	ND	ND
79	4307	16 weeks	F		*mcr-2*	ND	ND	ND
80	5008	16 weeks	F		ND	ND	ND	ND
81	6210	11 weeks	F		ND	ND	ND	ND
82	6012	11 weeks	F		ND	ND	ND	ND
83	5907	11 weeks	F		ND	ND	ND	ND
84	6309	11 weeks	F		ND	ND	ND	ND
85	6105	11 weeks	F		ND	ND	ND	ND

Table 2.11: Identification of resistance genes in two *E. coli mcr*- positive isolates in USA

Isolate ID	Pig ID	Gene	Coverage (%)	Identity (%)	Database
20013	5714	*marA*	98.96	98.7	CARD
		pmrF	98.97	98.25	CARD
		pmrE	98.72	95.98	CARD
		pmrC	99.51	98.72	CARD
		ampC	98.24	96.56	CARD & ARG-ANNOT
		bla_{EC}	98.24	97.88	NCBI
		bla_{AMPH}	98.01	96.72	ARG-ANNOT
20026	6915	*marA*	99.22	99.22	CARD
		ampC	97.97	97.88	CARD
		blaEC	97.97	97.88	NCBI
		pmrC	99.7	99.39	CARD
		pmrF	98.97	98.87	CARD
		pmrE	99.14	97.18	CARD

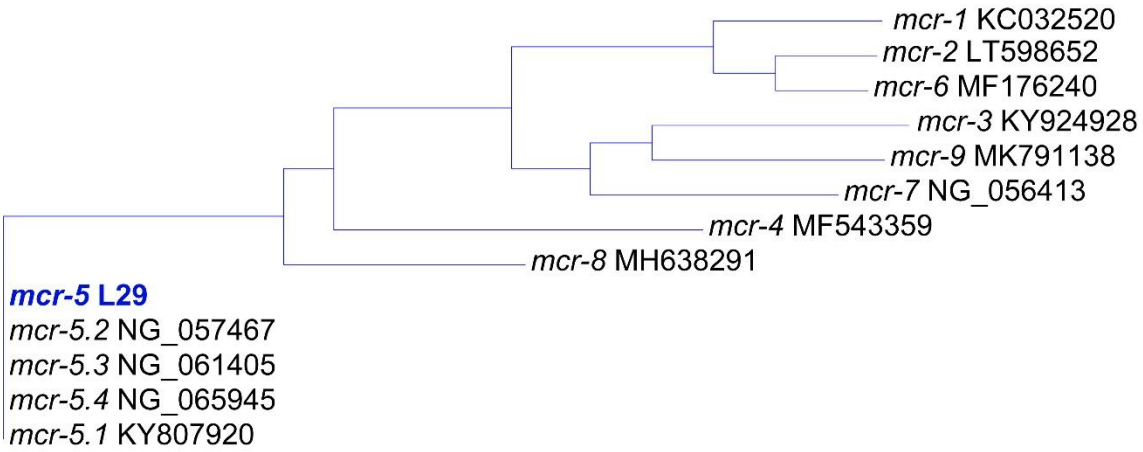

Figure 2.1: Phylogenetic analysis of *mcr-5* identified in this study with other *mcr* genes. The nucleotide sequences of *mcr-5* (271 bp from isolate L29) identified in this study (in boldface) is compared with representative sequences of other *mcr* genes and different mcr-5 variants of *mcr-5*. The evolutionary history was inferred using the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic comparison demonstrated the *mcr-5* identified in this study is identical to four other *mcr-5* variants but showed significant dissimilarities to other *mcr* genes (similarities with *mcr-1*: 37%; *mcr-2*: 36%; *mcr-3*: 32%; *mcr-4*: 49%; *mcr-6*: 35%; *mcr-7*: 39%; *mcr-8*: 62%; *mcr-9*: 31%).

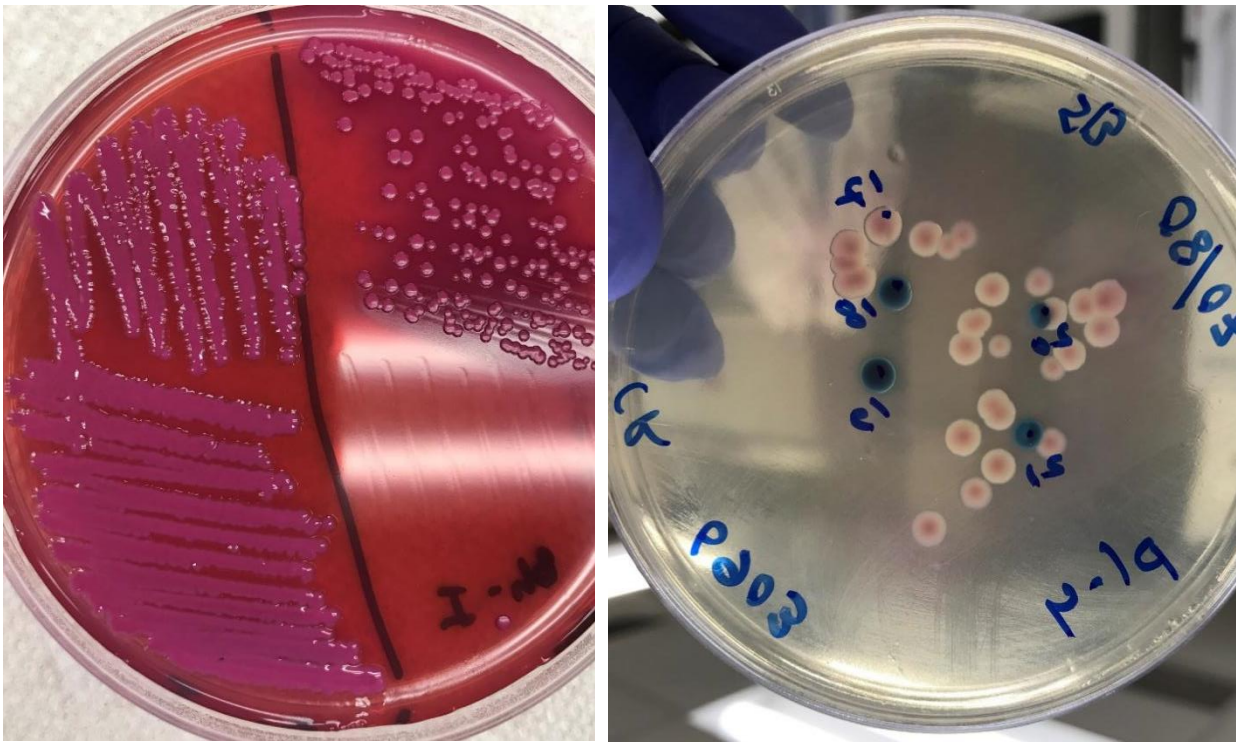

Figure 2.2: Morphological characteristics of colistin-resistant *Enterobacteriaceae* isolated in USA on MacConkey agar (Left) and CHROM^{TM}agar COL-APSE (Right). Left: *E. coli* (pink, round colonies without mucoid appearance); *K. pneumoniae* (pink, round colonies without mucoid appearance); Right: *E. coli* (pink, round colonies); *K. pneumoniae* (metallic blue, round colonies).