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Molecular epidemiology and mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae* from clinical isolates, the environment and porcine samples in Pretoria, South Africa

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Molecular epidemiology and mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae* from clinical isolates, the environment and porcine samples in Pretoria, South Africa

by

DINEO BOGOSHI

Submitted in partial fulfilment of the requirements for the degree

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DECLARATION

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree, MSc (Medical Microbiology), and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed.....……on this day……of……2020

"Trust in the LORD with all your heart and lean not on your own understanding; In all your ways acknowledge Him, And He shall direct your paths."

Proverbs 3: 5-6

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LIST OF ABBREVIATIONS

Abbreviations

AmpC	Ampicillin-resistance gene group C
AST	Antimicrobial susceptibility testing
bp	Base pairs
BMD	Broth microdilution
CHDLs	Carbapenem-hydrolysing class D β-lactamase
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
CLSI	Clinical Laboratory Standard Institute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EMB	Eosin methylene blue
ESBLs	Extended-spectrum β-lactamases
GES	Guiana extended-spectrum enzyme
IMP	Imipenemase metallo-β-lactamase
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharides
LRTI	Lower respiratory tract infections
MBC	Minimum bactericidal concentration
MBLs	Metallo-β-lactamases
MIC	Minimum inhibitory concentration
mcr-1	Mobile colistin resistance-1
MDR	Multiple drug resistance/multidrug-resistant

MH	Mueller-Hinton broth
M-PCR	Multiplex polymerase chain reaction
NHLS	National Health Laboratory Service
NCP-CRE	Non-carbapenemase producing CRE
NDM	New Delhi metallo-β-lactamase
OXA-48	Oxacillinase-48 enzyme
PBPs	Penicillin binding proteins
PEA	Phosphoethanolamine
PCR	Polymerase chain reaction
SA	South Africa
SAHPRA	South African Health Products Regulatory Authority
ST	Sequence type
µg	Microgram
µL	Microlitre
USA	United States of America
UTI	Urinary tract infections
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole-genome sequencing
XDR	Extensively drug-resistant

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

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Molecular epidemiology and mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae* from clinical isolates, the environment and porcine samples in Pretoria, South Africa

by

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DEGREE: MSc (Medical Microbiology)

DISSERTATION SUMMARY

Introduction: Carbapenems and colistin are currently the last-line antibiotics of choice for treating Gram-negative bacterial infections; however, resistance to these antibiotics is increasingly being reported in clinical settings, the environment and animals. This study describes the molecular epidemiology and resistance mechanisms of colistin and carbapenems in clinical, veterinary and environmental Enterobacterales isolates in Pretoria, South Africa.

Method: One hundred VITEK®-2-confirmed colistin- and carbapenem-resistant clinical isolates were collected from the departmental isolate bank at the National Health Laboratory Service. A total of 88 porcine (stool) and 11 environmental (effluents) samples were collected in November 2018 and again in March 2019 from a Winterveldt farm in Pretoria. Both the porcine and environmental samples were screened using Eosin-methylene blue agar with colistin and ertapenem disks. Antimicrobial susceptibility testing of all isolates was performed using the MicroScan® WalkAway system. Colistin-resistant isolates were then confirmed using the broth microdilution method. All phenotypic-resistant carbapenem and confirmed colistin-resistant isolates were characterised using multiplex polymerase chain reaction (M-PCR), single-plex PCR and whole genome sequencing. Phylogenetic trees were constructed to determine the isolates' relatedness in human, animals and the environment.

Results: In total, 275 Gram-negative isolates were identified from the clinical (100), environmental (57) and veterinary (118) samples. Of the Enterobacterales family, *Escherichia coli* (*E. coli*) was the predominant species in animals (76%) and environmental (52%) isolates, while *Serratia marcescens* (*S. marcescens*) was common in clinical samples (46%). Carbapenem resistance was higher in clinical (93%) and environmental (52%) isolates, whereas in animal isolates colistin resistance (19%) was lower. Analyses by M-PCR demonstrated no mobile colistin-resistant (MCR) genes (*mcr-1*, *-2*,*-3*,*-4* and *-5*) among clinical, animal and environmental isolates; however, carbapenemases (bla_{VIM-1}, bla_{NDM-1} and bla_{OXA-48}) were detected in clinical, animal and environmental isolates; bla_{OXA-48} (78%) was the most frequently detected carbapenemase, followed by bla_{NDM} (17%). Multiple-resistance genes from several antibiotics were found to mediate resistance among the isolates. Of these antibiotics, β-lactamases were commonly identified with the CTX-M family and variants, being the most predominant genes. In two clinical (C80 and C84) isolates, the colistin-resistance gene *mcr-9* was detected. SW10B (porcine), SG003 (porcine) and C052 (human) were phylogenetically related. EB008 (environment) and SW10B (porcine) shared a common ancestry. The linages of these two strains branched into clades consisting of strains C080 and C084; both human. In addition, strains in this study were phylogenetically related to strains from (Durban) South Africa, the United States of America, Canada, China, Russia and Australia.

Conclusion: This study demonstrated multiple-resistance genes to mediate resistance among the Enterobacterales isolates sampled from humans, animals and the environment. Combinations of resistance genes among isolates limits treatment options of infections caused by Enterobacterales. The presence of carbapenem resistance in environmental isolates and the detection of the *mcr-9* colistin gene raises a public health concern. Strain relatedness of the Enterobacterales species within the study; clinical (human and animals) and environmental isolates and with strains across the globe demonstrated common ancestry, the ability of Enterobacterales to persist and evolve as well as share resistant genes through horizontal transfer. Larger molecular-based One Health approach studies are necessary to mitigate the paucity of prevalence data and commit to resolving antibiotic resistance in Enterobacterales species in South Africa.

CHAPTER 1

INTRODUCTION

Colistin and carbapenem resistance in Enterobacterales is increasingly becoming a worldwide clinical, veterinary, and environmental concern, owing to the limited treatment options available for such organisms. Enterobacterales are a family of Gram-negative bacteria broadly disseminated in animals, humans and the environment (Oliveira & Reygaert, 2020). Gram-negative bacteria are present in the normal flora and tend to colonise rather than cause an infection (Paterson, 2011). However, when an opportunity is present diseases associated with Gram-negative bacteria have the ability to disseminate in almost all the body systems such as the gastrointestinal tract thus causing life threatening infections (Paterson, 2011). Enterobacterales cause infections such as bacteraemia, gastroenteritis, meningitis, peritonitis, pneumonia and urinary tract infections (Jenkins *et al*., 2014). Common pathogenic Enterobacterales include *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae (K. pneumoniae)* and *Salmonella enterica* (*S. enterica*), whereas *Citrobacter freundii*, *Proteus mirabilis* and *Raoutella planticola* are rarer ones (Potter *et al.*, 2016). Enterobacterales infections are often successfully treated with carbapenems, fluoroquinolones, polymyxins (colistin) and/or tigecycline antibiotics. However, an increase in antibiotic resistance among Gram-negative bacteria is being reported (Tamma *et al.*, 2012; Zowawi *et al.*, 2015; Potter *et al.*, 2016).

The ability of microorganisms to resist the effects of an antibiotic can result from natural selection and/or genetic mutations that enable the microorganism to change or adapt to the potency of the antibiotic, leading to antibiotic resistance (Gillings *et al.*, 2017). Antibiotic resistance may be intrinsic or acquired, and the mechanisms of resistance may be physiological or genetic (Exner *et al.*, 2017). The physiological mechanisms include: (1) bacterial production of enzymes that inactivate the antibiotic; (2) cell membrane alteration that decreases the permeability of the cell membrane to the antibiotic; (3) target protein alteration, which inhibits antibiotic binding to the target site, e.g., by reducing the target's antibiotic affinity; (4) active efflux of the antibiotic; and (5) alternative metabolic pathway development to avoid the action of the antibiotic (Habibi & Pezeshki, 2013).

The genetic mechanisms include mutations and transferable drug resistance (Ghasemian *et al.*, 2018). Mobile genetic elements such as integrons, plasmids and/or transposons that act as

vectors of resistance genes can be shared through transduction, transformation and/or the conjugation processes (Akrami *et al.*, 2017), leading to horizontal gene transfer among bacterial species *(*Amos *et al.*, 2018). Integrons acquire resistance genes in the form of gene cassettes (Kaushik *et al.*, 2018) and allow spreading of resistance genes from one bacterium to the other on plasmid vectors (Deng *et al.*, 2015). Five classes of integrons are commonly associated with Enterobacterales resistance genes, namely classes 1, 2, 3, 4 and 5, of which classes 1 and 2 are associated with human and animal isolates harbouring resistance genes, while class 3 is commonly associated with environmental isolates (Chainier *et al.*, 2017; Kaushik *et al.*, 2018).

Gram-negative bacteria can acquire resistance to more than one class of antibiotic more commonly than Gram-positive bacteria (Exner *et al.*, 2017). Thus, generally, single Gram-negative bacterial species may be associated with resistance to more than one class of antibiotic, such as β-lactams, aminoglycosides and fluoroquinolones (Ojdana *et al.*, 2015; Cerqueira *et al.*, 2016; Duin & Doi, 2017). This may result in multidrug resistance (MDR) in Gram-negative bacteria (Exner *et al.*, 2017).

Carbapenems are a class of β-lactam antibiotics that were used as a last line of defence for treating Enterobacterales infections until recently when carbapenem resistance emerged (Vigil *et al.*, 2016). Doripenem, ertapenem, imipenem and meropenem are the common clinically used carbapenems (Camargo *et al.*, 2015). Carbapenems kill bacteria by inhibiting cell wall synthesis through the introduction of an acyl group to penicillin-binding proteins (PBPs). Altered PBPs prevent the process of transpeptidation, i.e. the cross-linking of peptidoglycan chains to form a rigid cell wall, causing cell lysis and death (Mlynarcik *et al.*, 2016). Gram-negative bacterial resistance to carbapenems occurs in either of the following two mechanisms: (1) hydrolysis of the β-lactam ring by carbapenemases, which is by far the commonest mechanism; and (2) efflux hyperexpression and/or reduction in porins, which results in reduced membrane permeability, combined with expression of extended spectrum β-lactamases or chromosomally/acquired β-lactamases (ESBLs/AmpCs) (Potter *et al.*, 2016).

Classification of β-lactamases is based on either molecular or functional classification. Molecular classification, which is based on the enzyme's amino acid sequence, categorises β-lactamase enzymes into four classes, viz. A-D (Bush & Jacoby, 2010). Classes A, C and D enzymes use serine for β-lactams hydrolysis and class B metallo-β-lactamases (MBLs) use active zinc ions to facilitate β-lactam hydrolysis (Bush & Jacoby, 2010). Functional

classification is based on the substrate and inhibitor profiles of the enzymes, which can enable phenotypic correlation of the enzymes in clinical isolates (Bush & Jacoby, 2010). The current classification system is the updated functional classification, based on available information in the public domain, and aligns structural and functional classifications (Bush & Jacoby, 2010).

The functional classification divides β-lactamases into three groups. Group 1 is the cephalosporinases categorised into class C enzymes; group 2 is the ESBLs, broad-spectrum, inhibitor-resistant and serine carbapenemases categorised into classes A and D; and group 3 is the MBLs categorised into class B enzymes (Bush & Jacoby, 2010). The frequently identified carbapenemases in the above-mentioned classes are *K. pneumoniae* carbapenemase in class A, New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and imipenemase metallo-β-lactamase (IMP) in class B, and oxacillinase-48 (OXA-48) in class D (Laxminarayan *et al.*, 2013). The increasing prevalence of carbapenem-resistant Enterobacteriaceae (CRE) in the world has limited antibiotic treatment options (Bassetti *et al.*, 2015), hence the reintroduction of colistin use clinically to treat CRE infections (Potron *et al.*, 2015).

Colistin is a polypeptide cationic antibiotic from the family of polymyxins, consisting of cyclic peptides with a long hydrophobic tail (Brown & Dawson, 2015). Colistin was initially isolated in 1949 and then became clinically available in 1959; however, its use in human beings was stopped in 1979 owing to neurotoxic and nephrotoxic side effects (Falagas *et al.*, 2005). Colistin has since then, been predominantly used for prophylactic treatment of Enterobacterales infections in animals owing to its high bactericidal and anti-endotoxin effect, particularly in smaller animals such as piglets and chickens maintained under intensive farming conditions in combination with a low level of absorption (Catry *et al.*, 2015).

Currently, in clinical settings, colistin is used as the last-line antibiotic, administered intravenously as colistin sulphate and/or colistin methanesulphate to treat MDR Gram-negative bacteria that have become resistant to carbapenems in humans (Poirel *et al.*, 2016). Colistin's mechanisms of action in Gram-negative bacterial infections include rapid bactericidal and potent anti-endotoxin activities (Anandan *et al.*, 2017). During rapid bactericidal activity, colistin binds to the lipopolysaccharide (LPS) outer membrane of Gram-negative bacteria and initiates potent anti-endotoxin activity by binding to negatively charged phosphate groups of lipid A within LPS (Sun *et al.*, 2018). These binding mechanisms neutralise the LPS, thus

preventing the pathophysiologic effects of the endotoxins in the plasma (Paterson & Harris, 2016).

Colistin resistance in some Gram-negative bacteria has recently been reported (Yousfi *et al.*, 2019). Liu and colleagues reported that colistin resistance, encoded by the novel colistin resistance gene, *mcr-1*, was plasmid-mediated (Liu *et al.*, 2016). Various factors have been implicated as drivers of colistin resistance, including improper dosage, use of the drug at low doses widely as a growth promoter, inappropriate use of colistin in animals and improper administration of antibiotics, which ultimately affect the environment (Rhouma *et al.*, 2016; Newton-Foot *et al.*, 2017). Since Liu's report, more *mcr* genes (*mcr-2* to *mcr-10*) have been identified: *mcr-2* in *E. coli* described in Belgium from animal sources, *mcr-3* in *E. coli* from pigs in China, *mcr-4* in *E. coli* and *S. enterica* from Italy, Spain and Belgium, and *mcr-5* in *Salmonella* Paratyphi B dTa-+ from poultry in Germany (Rebelo *et al.*, 2018). The pHNSHP45 plasmid, harbouring the *IS*ApI insertion sequence on the 5' region of the *mcr-1* gene, was reported in 2015 by Liu *et al.* in an *E. coli* strain (Liu *et al.*, 2016). It is believed that this insertion sequence might be triggering rapid mobilisation of the *mcr-1* gene in the plasmid (Petrillo *et al.*, 2016).

The use of antibiotics, particularly polymyxins and carbapenems, is increasing worldwide (Messina *et al.*, 2018). This increase correlates with the increases in antibiotic resistance (Potron *et al.*, 2015) and MDR Gram-negative pathogens (Messina *et al.*, 2018), raising concern about global health because of the limited antibiotics available to treat MDR Gram-negative infections. Mobile genetic elements facilitate the spread of these antibiotic genes, leading to increased morbidity and mortality (Syal *et al.*, 2017). Although colistin was not clinically used in humans for some years, its extensive use continued in agriculture and in animal production (Webb *et al.*, 2017). In South Africa, colistin was used as a growth enhancer (promoter) and as a prophylactic antibiotic in animals due to its poor oral bioavailability. Not surprisingly it found its way into the environment through faeces and feed (Webb *et al.*, 2017). According to Messina and colleagues (2018), the most prevalent Gram-negative pathogens associated with colistin resistance clinically are *K. pneumoniae* (39.2%), *Pseudomonas aeruginosa* (*P. aeruginosa*) (20.1%) and *Acinetobacter baumannii* (*A. baumannii*) (9.0%) (Messina *et al.*, 2018). The use of colistin in South Africa is restricted because access is only through limited approval under the control of Section 21 of the Medicines and Related Substances Control Act, Act 101 of 1965, as administered by the South African Health Products Regulatory Authority (SAHPRA)

of the South African National Department of Health (Messina *et al.*, 2018). *Klebsiella. pneumoniae, A. baumannii* and *E. coli* are the most prevalent carbapenem-resistant Gram-negative bacteria in South Africa (Osei Sekyere *et al.*, 2016). The distribution and prevalence of resistance genes, such as ESBLs genes and $bla_{\text{NDM-1}}$ in Enterobacterales, point to the ease with which they can spread (Laxminarayan *et al.*, 2013).

Diagnostic methods commonly used for the detection of Enterobacterales in microbiology laboratories include phenotypic or molecular methods (Syal *et al.*, 2017). Phenotypic methods such as culture-based tests, biochemical tests and antimicrobial susceptibility testing are the primary diagnostic techniques and help to identify bacteria and their antimicrobial sensitivity (Das *et al.*, 2014; Castro-Escarpulli *et al.*, 2015). The methods are inexpensive and routinely used, although they are laborious, time-consuming and delay treatment. Molecular methods such as polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing are used for characterising bacteria (Das *et al.*, 2014). Molecular methods are highly specific and sensitive compared to phenotypic methods and provide rapid turn-around time. However, they are expensive and require expertise (Castro-Escarpulli *et al.*, 2015).

Antimicrobial susceptibility testing (AST) for Enterobacterales may be done through diffusion tests such as E-test disk and/or dilution tests such as broth micro-dilution tests (BMD) (Mlynarcik *et al.*, 2016). Detection of colistin- and carbapenem-resistant Enterobacterales is recommended by the Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Doyle *et al.*, 2012). According to the CLSI and EUCAST, BMD is the standard reference technique for AST for colistin in Enterobacterales (Syal *et al.*, 2017). The recommendations include clinical breakpoints for minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (Syal *et al.*, 2017). The CLSI does not provide colistin breakpoints for Enterobacterales; however, EUCAST recommended MICs of ≤ 2 ug/mL as susceptible and ≥ 2 ug/mL as resistant to colistin (Simar *et al.*, 2017). The EUCAST carbapenem resistance breakpoint for Enterobacterales is $\geq$ 2µg/mL for ertapenem and doripenem and ≥ 4 µg/mL for meropenem or imipenem (Potter *et al.*, 2016).

Antimicrobial resistance is increasing at a faster rate than that at which new antibiotics are being produced (Laxminarayan *et al.*, 2013). The increase in colistin and carbapenem resistance poses a threat to healthcare systems, particularly in developing countries such as South Africa.

Therefore, a One Health investigational approach is important in South Africa, due to the paucity of data on colistin- and carbapenem-resistant Enterobacterales. Furthermore, there is uncertainty about whether humans, animals and/or the environment are the reservoirs for colistin resistance. Carbapenem resistance is increasingly becoming a concern in animals, as indicated by several studies (Osei Sekyere *et al.*, 2016; Webb *et al.*, 2016; Haenni *et al.*, 2017; Piedra-Carrasco *et al.*, 2017).

To understand the phenomenon of antimicrobial resistance better, multidisciplinary studies in humans, animals and the environment need to be undertaken. The purpose of this study was to describe the molecular epidemiological and resistance mechanisms of colistin and carbapenems in clinical, veterinary, and environmental Enterobacterales isolates in Pretoria, South Africa. The study thus determined the epidemiological association of resistance patterns between animals, humans and the environment in a One Health approach and identified the main reservoir of the *mcr* colistin resistance gene and carbapenemases in Pretoria.

AIM

The aim of this study was to describe the molecular epidemiology and resistance mechanisms of colistin and carbapenems in clinical, veterinary and environmental Enterobacterales isolates in Pretoria, South Africa.

OBJECTIVES

The objectives of this study were:

1. To isolate colistin- and carbapenem-resistant Enterobacterales from clinical (human), animal and environmental samples after screening on Eosin methylene blue agar through streak and lawn culture techniques.
2. To determine the MICs of the isolated drug-resistant Enterobacterales against routinely tested antibiotics including carbapenems and colistin using the MicroScan® WalkAway System (BeckMan Coulter, USA) and the BMD method respectively.
3. To characterise carbapenemase-producing Enterobacteriaceae (CPE) and *mcr-1/2/3/4/5* producers in Pretoria using multiplex PCR assay.
4. To determine the molecular relatedness of *mcr-1* and CPE gene types in the clinical, veterinary and environmental Enterobacterales isolates compared to national and international isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Earlier studies on antimicrobial resistance focused more on Gram-positive bacteria compared to Gram-negative bacteria because of prevalence observed. This led to "neglect" of studies of possible resistance among Gram-negative bacteria (Codjoe & Donkor, 2018). To date, antimicrobial resistance in Gram-negative bacteria, particularly among the Enterobacterales family, has become one of the biggest challenges encountered in the healthcare, environmental and veterinary sectors globally (Al-Tawfiq *et al.*, 2017). Infections commonly associated with Enterobacterales are urinary tract infections (UTIs), gastrointestinal infections, lower respiratory tract infections and bloodstream infections, and the pathogens commonly isolated include *Klebsiella*, *Escherichia* and *Enterobacter* genera (Francisco *et al.*, 2012).

Carbapenems (ertapenem, doripenem, meropenem and imipenem) are reserve antibiotics of choice for treating MDR Gram-negative bacterial infections (Jenkins *et al.*, 2014). These drugs are classified into the β-lactam family whose mode of action involves inhibiting penicillin-binding proteins, involved in the cell wall synthesis of the bacteria, from intercalating peptidoglycan chains, resulting in cell death (Pilmis *et al.*, 2014). Nevertheless, resistance in carbapenems is increasingly being reported worldwide. The mechanisms of resistance include hyperexpression of chromosomally/acquired β-lactamase (AmpC/ESBLs) and deficiency of porins in the outer membrane, efflux and carbapenemases, of which production of carbapenemases is most common (Samuelsen *et al.*, 2017). The phenotypic resistance mediated by the production of ESBLs/AmpCs and carbapenemases has become one of the major public health problems because of the ease with which these genes can spread. Consequently, carbapenem-resistant Enterobacteriaceae (CRE) infections are associated with increased mortality and morbidity globally (Potron *et al.*, 2015).

Therefore, health care practitioners are opting for broader spectrum antibiotics such as aminoglycosides, tigecycline and colistin (Hamzan *et al.*, 2015). Colistin is used as the last therapeutic drug of choice for treating CRE infections following unsuccessful treatment with carbapenems (Banerjee & Humphries, 2017). Although, clinically, colistin was banned in the 1970s because of its neuro- and nephron-toxicity, there has been continued usage in the

veterinary sector (Codjoe & Donkor, 2017). Resistance to colistin is increasingly being reported and the resistance can be attributed to horizontal gene transfer, though hitherto colistin's mechanism of resistance was thought to be chromosomally mediated (Wang *et al.*, 2017). In 2015, in China, Liu *et al.* reported that increasing spread of colistin resistance occurred through a plasmid-borne mobile colistin resistance-1 (*mcr-1*) gene (Liu *et al.*, 2016). Although resistance to antibiotics is often observed in clinical settings, antibiotic usage also occurs in the veterinary and environmental sectors (Yang *et al.*, 2017). In addition, reports have demonstrated that more antibiotic usage occurs in the veterinary sector compared to the clinical sector resulting in the rapid spread in antimicrobial resistance in the environment (Agga *et al.*, 2015; Laxminarayan *et al.*, 2013) The presence of antimicrobials in the environment and food-producing animals has devastating outcomes because food security becomes threatened, affecting human health (Cunningham *et al.*, 2017).

Antimicrobial resistance is increasing at a faster rate than that at which researchers can produce or even modify newer antibiotics. Coupled with limited treatment options, it is already clear that the health system is under threat (Gillings *et al.*, 2017). Therefore, it is essential that antimicrobial studies in humans are also conducted alongside veterinary and environmental disciplines. This will enable researchers to gain better understanding of public health problems, which have the potential to affect the overall livelihood of humans (Destoumieux-Garzón, 2018).

2.2 Classification of Enterobacterales

Antimicrobial resistance is increasingly becoming a global problem that is crippling the health, environmental and veterinary sectors. Added to this phenomenon is the increasing resistance in Enterobacterales, a family of Gram-negative bacteria (Paterson, 2011). The Enterobacterales are classified into over 200 species and over 50 genera (Figure 2.1). They share similar characteristics such as fermenting glucose, reducing nitrates to nitrites, testing negative for oxidase, being non-spore formers and motile, excluding *Klebsiella*, *Shigella* and *Yersinia* genera (Das *et al.*, 2014). The virulence factors entail the ability to colonise, adherence, production of various toxins, biofilm formation that has been associated with horizontal gene transfer through plasmids, and invasion of tissues (Das *et al.*, 2014). Enterobacterales are ubiquitous in nature, being found in the soil, water, animals, vegetation and humans (Exner *et al.*, 2017). In humans and animals, Enterobacterales form part of the normal flora and are thus found in the intestinal tract of humans and animals without causing any harm to the source

(Mittal *et al.*, 2016). However, in immunocompromised hosts, both human and animal, these Gram-negative bacteria become harmful and cause infection, which can ultimately lead to death. The latter is especially a problem in animals maintained in high stress environments such as commercial intensive farming units.

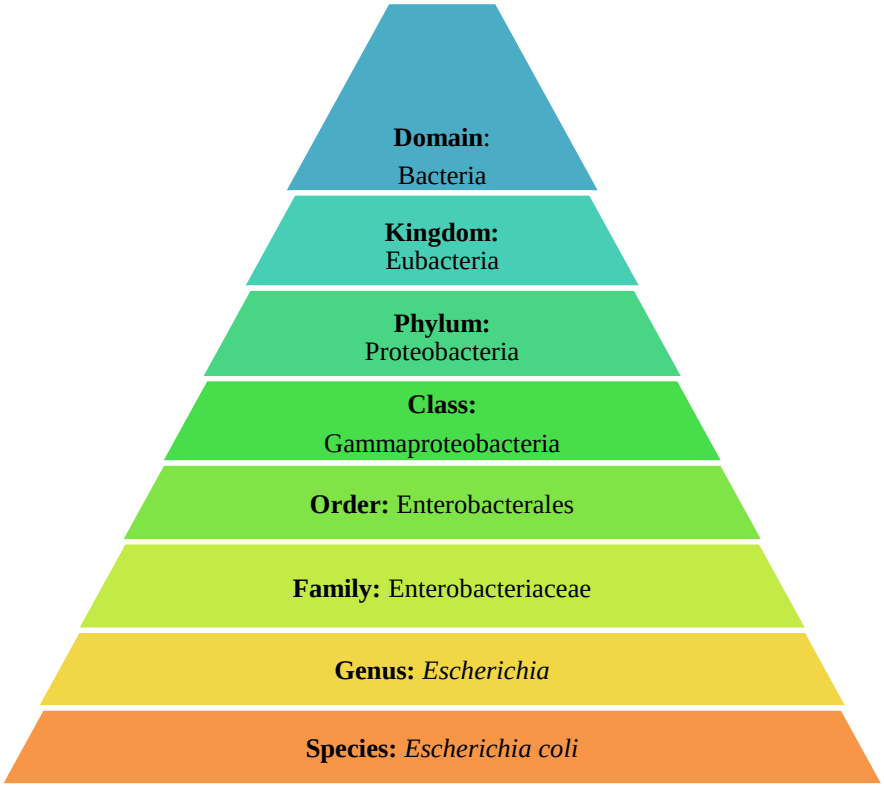

Figure 2.1: Hierarchy representing classification of Enterobacterales (inspired by (Berman, 2012)).

Infectious-disease causing pathogens are divided into two categories: opportunistic pathogens such as *Escherichia coli*, *Proteus* and *Serratia* species and primary intestinal pathogens such as *Salmonella*, *Shigella* and *Yersinia* species (Mittal *et al.*, 2016). Infections commonly associated with these Gram-negative bacteria are UTIs, lower respiratory tract infections and bloodstream infections. Among these infections, lower respiratory tract infections and bloodstream infections are most fatal (Paterson, 2011). In South Africa (SA), common organisms causing bloodstream infections are *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*), and it has been reported that one in four of infections caused by both of these organisms are resistant to third-generation cephalosporins, while one in 12 of the latter is resistant to carbapenems (National Department of Health, 2018).

Several transmission routes are involved in transmitting pathogenic Enterobacterales to the host, depending on the infection caused. Transmission of intestinal infections occurs via the faecal-oral route through direct contact with animals or their environment, person-person contact and consumption of contaminated food or water (Berman, 2012). When an opportunity is present in the normal flora, transmission through the endogenous pathway occurs when the bacteria from the gut travel into the blood (Jenkins *et al.*, 2014).

2.3 Antimicrobial resistance

Antimicrobial resistance is defined as the ability of microorganisms to resist the effects of an antibiotic, which can result from natural selection and/or genetic mutations that enable the microorganism to change or adapt to the potency of the antibiotic (Gillings *et al.*, 2017). The genomes of an organism evolve in their natural environment through mutation, recombination, horizontal transfer and hybridisation (Luiz *et al.*, 2016). The genetic entities respond differently to selective environmental factors and some genotypes are selected. Consequently, the bacterium may express new phenotypes and colonise new hosts and can also cause damage to the hosts they colonise, thus becoming pathogens (Webb *et al.*, 2017).

Antibiotic resistance may be intrinsic or acquired, and the mechanisms of resistance may be physiological or genetic (Exner *et al.*, 2017). Physiological mechanisms include: (1) bacterial production of enzymes, which inactivate the antibiotic; (2) cell membrane alteration that decreases the permeability of the antibiotic, (3) target protein alteration, which inhibits antibiotic binding, e.g, by reducing the target's antibiotic affinity, (4) active efflux of the antibiotic; and (5) alternative metabolic pathway development to avoid the action of the antibiotic (Habibi & Pezeshki, 2013).

The genetic mechanisms include mutations and transferable drug resistance (Exner *et al.*, 2017). Mobile genetic elements such as integrons, plasmids and/or transposons that act as vectors of resistance genes can be shared through transduction, transformation and/or conjugation processes (Akrami *et al.*, 2017), leading to horizontal gene transfer among bacterial species (Amos *et al.*, 2018). Integrons acquire resistance genes in the form of gene cassettes (Chainier *et al.*, 2017) and allow spreading of resistance genes from one bacterium to the other on plasmid vectors (Deng *et al.*, 2015). There are five classes of integrons commonly associated with Enterobacterales resistance genes, namely classes 1, 2, 3, 4 and 5, of which classes 1 and 2 are

associated with human and animal isolates harbouring resistance genes, while class 3 is commonly associated with environmental isolates (Chainier *et al.*, 2017; Kaushik *et al.*, 2018).

Gram-negative bacteria can acquire resistance to more than one class of antibiotic more commonly than Gram-positive bacteria (Exner *et al.*, 2017). Thus, generally, single Gram-negative bacterial species isolates may be associated with resistance to more than one class of antibiotic, such as β-lactams, aminoglycosides and fluoroquinolones (Nordmann & Poirel, 2016). This may result in multidrug resistance (MDR) in Gram-negative bacteria. Currently treatment of Gram-negative bacterial infections involves combination or monotherapy of drugs such as carbapenems, colistin and tigecycline. However, there is increasing reports of resistance among these antibiotics (Potron *et al.*, 2015).

Increasing reports of MDR Gram-negative bacteria are limiting treatment options for infections caused by Enterobacterales (Del Bianco *et al.*, 2018). Accounting for antimicrobial resistance is the overuse of these drugs in both the human and veterinary sectors, which results in selection pressure and resistance dissemination (Falagas *et al.*, 2014). Although the resistance mechanisms of antibiotics are complex, the genes conveying resistance are either chromosomally or plasmid-borne within mobile genetic elements such as transposons and insertion elements, which influence the ease with which these resistance genes can travel or be transferred between species and strains, ultimately playing a pivotal role in the dissemination of MDR bacteria (Brink *et al.*, 2011; Kaushik *et al.*, 2018).

2.4 The practice of antimicrobial use in South Africa

In SA, the Medicines and Related Substances Control Act 101 of 1965 recognises only prescribable antimicrobials for use (Theobald *et al.*, 2019). Administration of antimicrobials in private and public hospitals varies; in the public sector administration of antimicrobials is restricted by standard treatment guidelines in the Essential Medicines List, while in the private sector use is unrestricted, though influenced by financial considerations (Schellack *et al.*, 2017). Only clinicians, nurses (Nursing Act 33 of 2005 section 56(6)) and pharmacists (Act 53 of 1974) registered with the Health Professions Council of South Africa can administer antimicrobials. Nurses and pharmacists are limited to the primary care level (Schellack *et al.*, 2017).

The National Department of Health and the Department of Agriculture, Land Reform and Rural Development (DALRRD) regulate the controlled use of antimicrobials in animals as over the counter products (Schellack *et al.*, 2017). Initially only anti-parasitic substances were allowed for use in livestock; however, about 30 compounds of antimicrobials, in particular growth promoters, have since been included. This is supported by the Fertilizers, Farm, Feeds, Agricultural and Stock Remedies Act 36 of 1947, making provision for some antimicrobials to be purchased over the counter without a prescription, such as tetracycline antibiotic used for the treatment of parasitic diseases such as heartwater (*Ehrlichia ruminantium*) (Eagar *et al.*, 2012). These products are referred to as Stock Remedies.

Antimicrobials registered as stock remedies, and used in food-producing animals, tend to be used in an unrestricted manner, be it therapeutic to treat sick animals; prophylactic to prevent infections such as diarrhoea and coli-septicaemia in animals and at time metaphylactic to simultaneously treat sick and healthy animals in a herd/flock to limit animal mortalities (van den Honert *et al.*, 2018). Antibiotic use in South Africa is poorly controlled and there is no control of stock remedies because records are not kept (Hayley *et al.*, 2012). In terms of usage, the split in antimicrobial use in SA was estimated to be 23-36% and 74-77% in animal health and human health respectively between 2012 and 2017. Data collected indicated that higher consumption occurred among humans in SA, compared to reports in the United States of America (USA), China and India, where animal consumption formed the larger proportion (2014-2015 data) (National Department of Health, 2018).

Consumption of antimicrobials in SA, estimated through import data from 2012-2016, illustrates an average import of 5.66 tons per annum of antibiotics. In 2014, 4.35 tonnes of antimicrobials were imported, with 23% estimated for animal use and 77% for human use. Worryingly, in 2015 there was an estimated increase of 58% in animal imports and 38% in human imports. These numbers contrast with those of developed countries, although they are similar to those of developing countries, excluding China and India (National Department of Health, 2018).

According to the 2018 South African surveillance for antimicrobial resistance report, use of growth promoters are to be reported to the authorities. In spite of this categorisation of antibiotics as growth promoters, their use is at the discretion of the pharmaceutical companies because currently, there is no clear definition of the antibiotic classes that fall into this category.

In addition, the DALRRD, in partnership with the South African Animal Health Association, has reported that antibiotics used as growth promoters in the veterinary sector is the most common group of antibiotics used. These include antibiotics that are not currently in use for human health care, such as the ionophores, flavophospholipol, olaquindox, zinc bacitracin and tylosin (Hayley *et al.*, 2012). Among these antimicrobials, only tetracycline and tylosin are registered by the Fertilizers, Farm Feeds, Agriculture Remedies and Stock Remedies Act 36 of 1947 for growth promotion. However, the ionophores and zinc bacitracin are used as growth promoters, depending on the interpretation by the pharmaceutical industry (National Department of Health, 2018).

2.5 Classification of carbapenem antibiotics

Carbapenems are antibiotics from the family of β-lactams, which are bactericidal antibiotics known to be effective against ESBL-producing bacteria. The antibiotics are used to treat both Gram-positive and Gram-negative bacteria, as well as anaerobes (Codjoe & Donkor, 2017). Carbapenem antibiotics are classified by two ways: Ambler and Bush (molecular), Jacoby and Madeiros (functional) classification. Classification according to the Ambler method comprises four classes, classes A-D (Codjoe & Donkor, 2017). Classes A, C and D are the serine β-lactamases because serine is used for β-lactams hydrolysis, while active zinc ions are used to facilitate β-lactam hydrolysis in class B, also known as the metallo-β-lactamases (MBLs) (Banerjee & Humphries, 2017). Enzymes belonging to class A are *Klebsiella*-producing carbapenemase (KPC) and Guiana extended-spectrum (GES) enzymes. Those belonging to class B comprise Verona integron-encoded metallo-β-lactamase (VIM), imipenemase metallo-β-lactamase (IMP) and New-Delhi metallo-β-lactamase (NDM) enzymes and class D enzymes comprise oxacillinase -48- (OXA-48) like enzymes (Potron *et al.*, 2015).

The substrate and inhibitor profiles of the enzymes have the ability to enable phenotypic correlation of the enzymes in clinical isolates and are thus used for functional classification (Bush & Jacoby, 2010). This classification scheme is divided into three groups (groups 1-3): Group 1 is the cephalosporinases categorised into class C enzymes; Group 2 includes ESBLs, broad-spectrum, inhibitor-resistant and serine carbapenemases categorised into classes A and D; and Group 3 is the MBLs categorised into class B enzymes (Bush & Jacoby, 2010). Oxacillinases are class D β-lactamases grouped in a heterogenous class of enzymes with respect to their structural or biochemical properties (Banerjee & Humphries, 2017). Class D β-lactamases have a broad substrate spectrum, with some hydrolysing expanded-spectrum

cephalosporins and most of the class D β-lactamase, known as extended-spectrum oxacillinases, being point mutant derivatives of narrow-spectrum β-lactamases (Jeong *et al.*, 2016). In addition, certain class D β-lactamase, such as OXA-48-like ones, have intrinsic carbapenemase activity, hence the term carbapenem-hydrolysing class D β-lactamases (CHDLs) (Potron *et al.*, 2015).

Enterobacterales' expression of ESBLs comprises class A β-lactamases such as Temoniera [TEM] and sulfhydryl variable β-lactamase [SHV] genes, which confer resistance on ampicillin, amoxicillin as well as other penicillin antibiotics and cephalosporins (Mathlouthi *et al.*, 2016). These ESBLs (*bla*$_{SHV}$ and *bla*$_{TEM}$) have been identified on both plasmids and chromosomes and are widely reported to spread among the Enterobacterales (Potron *et al.*, 2015). Enterobacterales pathogens may also express ESBLs that are not closely related to TEM/SHV-related genes, for instance CTX-M and OXA-type ESBLs (Mathlouthi *et al.*, 2016). These are plasmid-mediated β-lactamases and genes encoding these ESBL types are widely spread among the Enterobacterales (Jeong *et al.*, 2016). Extended spectrum β-lactamases that hydrolyse carbapenems should be distinguished from other β-lactamases because they have broader spectrum activity that covers carbapenems and extended spectrum cephalosporins (Mathlouthi *et al.*, 2016).

2.5.1 Class A carbapenemases

Class A carbapenemases consist of six major families, which include *Serratia marcescens* (*S. marcescens*) enzyme (SME), GES, KPC, sulfhydryl variable β-lactamase (SHV), imipenemase/non-metallocarbapenemase-A (IMI/NMC-A) and *Serratia fonticola* carbapenemase (SFC) (Sawa *et al.*, 2020). Among these families, *bla*$_{SME-1}$ enzyme was the first reported carbapenemase in 1990 in a *S. marcescens* isolate from the United Kingdom (Potter *et al.*, 2016). Reports of other carbapenemases (*bla*$_{GES-2}$, *bla*$_{IMI/NMC-A}$, *bla*$_{KPC}$ and *bla*$_{SFC-1}$) then followed. In this class, KPC and GES enzymes are the most clinically identified class A carbapenemases. The KPC (*bla*$_{KPC}$) and GES (*bla*$_{GES-2}$) enzymes are plasmid-borne, while *bla*$_{SME-1}$ and *bla*$_{IMI/NMC-A}$ are chromosomally encoded (Sawa *et al.*, 2020). Class A carbapenemases are inhibited *in vitro* by β-lactamase inhibitors such as clavulanic acid and tazobactam and confer resistance on extended-spectrum cephalosporins, carbapenems, penicillins and aztreonam (Jayol *et al.*, 2016). The most common class A carbapenemase is the KPC-type, which plays an important role in controlling the characteristics of CRE. KPC is a plasmid- and chromosomally mediated carbapenemase. It is these characteristics that facilitate

its rapid appearance in non-*K. pneumoniae* Enterobacterales because the genes can be transmitted among different species of the same genera such as Enterobacterales (Potter *et al.*, 2016).

Klebsiella pneumoniae is the most commonly isolated species, followed by *E. coli* in both public and private sector hospitals in SA and globally (Coetzee & Brink, 2011). KPC was first reported in the USA from a *K. pneumoniae* isolate in 2001. Since then, it has become the most common carbapenemase identified globally, causing carbapenem resistance (CR) in CRE infections (Huang *et al.*, 2018). Resistance is conferred only by carbapenemase production and not from membrane permeability changes or ESBL expression, thus setting it apart from the other serine β-lactamases such as NMC as SME (Oteo *et al.*, 2016). In SA, the prevalence of ESBL-producing *K. pneumoniae* is between 66-70% and affects the susceptibility of *K. pneumoniae* to all cephalosporins, therefore limiting the use of cephalosporins as a therapeutic option (Coetzee & Brink, 2011).

The chromosomal transmission is associated with clones such as ST258, which is the most commonly reported clone in the USA (Tijet *et al.*, 2014). A study in five Colombian hospitals had differing results concerning KPC belonging to ST258 because 62% of the *K. pneumoniae* isolates resistant to carbapenems were found not to belong to the ST258 sequence type (ST) (Codjoe & Donkor, 2017). Through whole genome sequencing (WGS), analysis of ST258 revealed two clades (cps1 and cps2) due to divergence. The analysis demonstrated cps1 to be associated with KPC-2 and cps2 with KPC-3 (Potter *et al.*, 2016). This suggests that transmission of KPC chromosomally is due to point mutations, which influence KPC variations and ultimately the spread, hence reports of other carbapenem-resistant *K. pneumoniae* ST isolates belonging to various STs, such as ST11 found in Taiwan, ST23 found in the USA and ST197 found in China (Rojas *et al.*, 2017). Horizontal gene transfer occurs through transposon *Tn*4401 as well as plasmid pKp28. Plasmid pKp28 is also associated with ST258.

The GES enzymes are commonly identified clinically and 37 variants have been reported to date (Bonnin *et al.*, 2017). These enzymes have extended spectrum activity towards carbapenems (although not all), cephamycins, cephalosporins and or monobactams (Naas *et al.*, 2016). GES-1 was first reported in *K. pneumoniae* isolates from French-Guiana in 1998 and is characterized as an ESBL due to lack of the hydrolytic activity (Hishinuma *et al.*, 2018). GES-2 is the first example of GES enzyme with reduced susceptibility to inhibitors and extended

activity to carbapenems (Bonnin *et al.*, 2017). Some GES variants display hydrolytic activity against carbapenems i.e. GES-2, -4, -5, -6, -14, -15, -16 -18, -20, and -24 (Bonnin *et al.*, 2017). The GES-3 and GES-4 - are common in Japan and GES-5,-6 and -7 are common in Greece (Naas *et al.*, 2016). Although dissemination of GES genes worldwide is rare compared to KPCs, reports of frequent association with single occurrences have been reported in Africa (SA), South America, Europe and Asia (Naas *et al.*, 2016). Reports of outbreaks have also been detailed in *P. aeruginosa* isolates from South Africa, in *K. pneumoniae* isolates from Korea, Portugal and Brazil and in *S. marcescens* isolates in Netherlands (Naas *et al.*, 2016).

The Brazilian *Klebsiella* carbapenemase-1 (BKC-1) is a class A carbapenemase found in Brazil in 2008 from *K. pneumoniae* ST1781 isolates resistant to carbapenems. It has broad activity against penicillins, cephalosporins, carbapenems and monobactams. The BKC-1 enzyme shares 63% sequence identity with an environmental β-lactamase (Potter *et al.*, 2016). Seven years later, after the report of BKC-1, France discovered French imipenemase-1 (FRI-1) enzyme in 2015. This class A β-lactamase was discovered from *Enterobacter cloacae* (*E. cloacae*) that produced a tazobactam inhibitable carbapenemase negative for KPC, GES, SFC-1, IMI and NMC-A. FRI-1 has broad activity against carbapenems and aztreonam except broad spectrum cephalosporins (Codjoe & Donkor, 2017). A plasmid is suggested to cause transfer of this carbapenemase (Potter *et al.*, 2016). Chromosomally integrated NMC-A was identified from a hospital in Italy in August 2014 from *Enterobacter ludwigii* (Duin & Doi, 2017). This β-lactamase was found to be associated with IMI and NMC-A carbapenemase in other members of clonally complexed *E. cloacae*, suggesting possible dissemination throughout Enterobacterales (Bush & Jacoby, 2010).

2.5.2 Class B carbapenemases

Class B carbapenemases**,** also referred to as MBLs, were first reported in the 1960s however sparked attention in the 1990s due to nosocomial outbreaks associated with the MBL enzymes (Logan & Bonomo, 2016). MBLs use zinc-mediated hydrolysis of β-lactams, thus hydrolyses all β-lactams including carbapenemases; however, exclude azetronam (Everett *et al.*, 2018). Metal chelators such as ethylenediamine tetra acetic acid (EDTA) and dipicolinic acid inhibits MBLs, but clavulanic acid or clinically used β-lactamase inhibitors cannot inhibit MBLs (Thomas & Duse., 2018). Metallo β-lactamases have a broad spectrum and when combined with ESBL production and membrane permeability changes, they confer a high level of resistance on all other β-lactams, excluding monobactams (Potron *et al.*, 2015).

Metallo-β-lactamases (MBLs) are further sub-classed into B1, B2 and B3 according to sequence identity and dependence of zinc ion (Sawa *et al.*, 2020). Most clinically important MBL enzymes (IMPs, VIMs and NDMs) belong to B1 subclass (Logan & Bonomo, 2016). The genes for MBLs reside on mobile genetic elements such as integrons, plasmids and transposons, which facilitate transfer of genetic material between bacterial species (Sawa *et al.*, 2020). Currently identified class B β-lactamases include IMP, VIM, Sao Paulo MBL, Germany imipenemase, NDM and Florence imipenemase (Sawa *et al.*, 2020). Among these MBLs, VIM and IMP types were commonly reported globally until the emergence of NDM types in 2008 (Everett *et al.*, 2018).

IMP-1 was first identified in Japan in 1991 from an *S. marcescens* isolate found on HI2 conjugatable plasmid (Potter *et al.*, 2016). It is common in Asian countries such as Japan and China, as well as in Australia. IMP genes are common among MBL-producing *Pseudomonas aeruginosa* although rare in Enterobacterales, they can be found associated with *K. pneumoniae*, *E. coli* and *Enterobacter* spp. (Matsumura *et al.*, 2017b). Currently over 41 IMP and its variants have been reported (Mlynarcik *et al.*, 2016) and among the IMP variants, IMP-4 is most commonly isolated (Potter *et al.*, 2016). In Australia *E. cloacae* is a common organism identified with IMP enzyme and *Raoutella ornithinolytica* (*R. ornithinolytica*) is reported to commonly harbouring both IMP and KPC in China (Huang *et al.*, 2017).

In 1997, Italy reported VIM-1 for the first time and since then more than 39 VIM variants have been reported globally (Mlynarcik *et al.*, 2016; Matsumura *et al.*, 2017a). VIM is endemic to Europe particularly to Greece and Italy (Exner *et al.*, 2017).VIM-1 has the closest amino acid sequence identity to NDM-1 and has been detected in nine Enterobacterales species from mostly *K. pneumoniae* with STs; ST147, ST36 and ST383 (Potter *et al.*, 2016). Similar to IMP carbapenemases, VIM genes are associated with Enterobacterales species such as *K. pneumoniae*, *E. coli* and *Enterobacter spp*. however are commonly isolated from MBL-producing *Pseudomonas aeruginosa* (Matsumura *et al.*, 2017a). The variant VIM-39 hydrolyses meropenem, doripenem and imipenem more efficiently than VIM-1 and confers greater resistance on carbapenem in *E. coli* DH5α (Potter *et al.*, 2016). In the USA, a combination of VIM-4 and CMY-4 in an *E. coli* isolate was reported on an A/C replicon 188 kb plasmid (Mediavilla *et al.*, 2016). Such resistance gene combinations in pathogens result in complicated therapeutic treatments and illustrate the dissemination of these enzymes among Enterobacterales.

Several years after the report of IMP-1, NDM-1 was reported in India in 2008 from a Swedish patient and since then has become responsible for sporadic outbreaks around the globe although it remains endemic in parts of Asia (India and China) (Khan *et al.*, 2017). Currently, NDMs comprise several variants (n=16) identified on a variety of plasmid types; this explains the diversity in Enterobacterales species because plasmids allow transfer of genetic material between bacterial species (Ghasemian *et al.*, 2018). However, globally, the incidence of NDM is low, i.e., 0.5% (n=38 266 isolates) out of 34/40 countries with high dissemination (Potter *et al.*, 2017).

NDM-1 is the most diverse common variant among geographical countries with NDM-associated infections (Mathlouthi *et al.*, 2016). The co-presence of ESBLs and NDMs or NDMs with other resistance genes is increasingly being reported (Osei Sekyere, 2016a) and increasing population migration may be contributing to the spread of CRE. To exemplify, NDM was detected in combination with CMY (plasmid-mediated class C AmpC β-lactamase) on IncAlc conjugative plasmid in extremely-drug resistant (XDR) *S. enterica* serovar Senftenberg isolate in India in 2012 (Ghasemian *et al.*, 2018). Strains associated with NDM include ST10 found in China in 2014 and ST11 found in Poland between 2012 and 2014. Organisms commonly isolated with NDMs include *K. pneumoniae*, *E. coli*, *A. baumanii*, *E. cloacae*, *E. lwoffii*, *E. hormaechei* and *S. enterica* (Potter *et al.*, 2016). In 2013 in Nepal, NDM-13, a chromosomally mediated NDM variant, was detected from an *E. coli* isolate. This NDM shares a similar spectrum of action to the NDM-1 variant; however, with increased cefotaxime affinity due to D95N and M154L mutations (Potron *et al.*, 2015).

The plasmids associated with NDMs include A/C, IncF, IncL/M, IncH, IncN, IncX and IncHI and transposons *Tn*3000 and Tn125 (Ghasemian *et al.*, 2018). IncF plasmid is most associated with NDM-1 however, IncX-3 is reported to make the spread more effective (Khan *et al.*, 2017). Transposon *Tn*125 has been reported to be responsible for the dissemination of bla_{NDM-1} among *Acinetobacter* species (Wailan *et al.*, 2016). This is because, *Tn*125 transposon is found in *A. baumannii* and bla_{NDM-1} gene is carried on *Tn*125 transposon flanked by *ISAba*125 elements (Khan *et al.*, 2017). It is these mobile genetic elements that regulate horizontal gene transfer of these carbapenemases therefore enabling easy spread of the resistance genes among Enterobacterales and Gram-negative organisms.

2.5.3 Class D β-lactamases

Class D β-lactamases are also known as oxacillinases because they cleave oxacillin in addition to penicillin (Banerjee & Humphries, 2017). Oxacillinase-23 was first characterised as an ESBL in 1985 from the United Kingdom, conferring resistance against imipenem in *A. baumannii* isolates. In Enterobacterales, OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like and OXA-48-like family members, discovered since 2001, are referred to as CHDLs and are increasing in incidence globally, but appear to be endemic in African and Asian continents rather than the class A and class B carbapenemases (Dortet *et al.*, 2016).

Oxacillinase-48 (OXA-48) is commonly reported in *K. pneumoniae*, with the first case being reported from an infection caused by an unique carbapenem-resistant *E. cloacae* ST89 producing OXA-48 carbapenemase in Central Europe (Poland) (Ojdana *et al.*, 2015). It was first detected in Turkey from a *K. pneumoniae* isolate in 2001 (Bradford *et al.*, 2016). To date, the appearance of *E. cloac*ae-producing OXA-48 family carbapenemase has been reported in Turkey, Senegal, Morocco, France, Belgium, the United Kingdom and Germany (Majewski *et al.*, 2018). The first report of OXA-48 in South Africa was reported from a *K. pneumoniae* isolate in 2012 (Brink *et al.*, 2013). The spread of OXA-48 from *K. pneumoniae* to *P. mirabilis* has been identified in Moscow, Spain, Morocco, Greece, Taiwan and Turkey in *K. pneumoniae*, *E. coli* and *Rautella planticola* (*R. planticola*) (Huang *et al.*, 2018., Pitout *et al.*, 2020). Although commonly isolated in clinical settings, OXA-48 enzymes have also been reported in animals (Pitout *et al.*, 2020). Reports suggest OXA-48 is most prevalent in Spain in *K. pneumoniae* ST11 (Potter *et al.*, 2016; Huang *et al.*, 2018).

Oxacillinase-48 is capable of hydrolysing carbapenems and penicillins, but unlike KPC and NDM, is unable to hydrolyse cephalosporins. It is through plasmids that OXA-48 is able to spread to Enterobacterales bacteria and it is commonly associated with *K. pneumoniae*, hence the term OXA-48-producing *Klebsiella pneumoniae* (Doi & Paterson, 2015). In addition, OXA-48-producing *Klebsiella pneumoniae* has the ability to co-produce ESBLs, therefore making the organism resistant to all ß-lactams (Bradford *et al.*, 2016).

A chromosomally mediated OXA-54, isolated from *Shewanella oneidensis* (*S. oneidensis*), an environmental bacterium, shares 92% amino acid similarity with OXA-48 and has catalytic activity against imipenem (Potter *et al.*, 2016). Both OXA-163- and OXA-48-like families have little or no activity against ceftazidime and cefotaxime. However, in isolates co-expressing

CHDLs and cephalosporins that cleaves β-lactamases, resistance to cephalosporins can occur (Huang *et al.*, 2018). OXA-4- like families weakly cleave carbapenems (Pitout *et al.*, 2020).

2.6 Mechanisms of carbapenem resistance

According to CLSI guidelines, CR is defined as MICs of ≥2 ug/mL against ertapenem and ≥4 ug/mL against doripenem, meropenem and imipenem. Several mechanisms confer CR, which includes production of carbapenemases to hydrolyse the β-lactam ring and change in the membrane permeability as a result of mutations in the efflux pumps and/or porins with ESBLs hyper-expression (Ghasemian *et al.*, 2018).

The mechanisms of resistance for carbapenems can be divided into two groups, namely non-carbapenemase-producing CRE (NCP-CRE) and carbapenemase-producing CRE (CP-CRE). The NCP-CRE resistance mechanism occurs without expression of carbapenemases and the isolates have reduced susceptibility to the carbapenems, especially to ertapenem, owing to expression of an acquired class A or class B β-lactamase with weak carbapenem hydrolytic activity. Examples include ESBLs or AmpC enzymes coupled with permeability defects such as outer membrane porin mutation or loss (Haenni *et al.*, 2017). The ESBLs and AmpCs commonly contribute to the reduced carbapenem susceptibility of NCP-CRE (Banerjee & Humphries, 2017).

Another method of resistance is through multiple antibiotic resistance protein A (marA) (Haenni *et al.*, 2017), an AraC-type transcriptional regulator (TR) responsible for primarily controlling the outer membrane permeability in Enterobacterales, causing antibiotic resistance by coordinating with two other AraC-type TRs (Potter *et al.*, 2016). The change in membrane permeability through altering efflux pumps and porin expression can confer CR either alone or synergistically with ESBLs in the absence of carbapenemase (Potter *et al.*, 2016). In Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, resistance-nodulation-division (RND) efflux pumps are the most common resistance mechanism found. Although RND efflux pumps inhibition holds promise as synergistic treatment against MDR Gram-negative bacteria, increased resistance against ertapenem and meropenem has been reported in *K. pneumoniae*, *E. coli* and *Enterobacter spp*. (Codjoe & Donkor, 2017).

Single nucleotide polymorphism (SNP) analysis has found that increased CR may occur as a result of natural phylogenetic variation between CR and non-carbapenem-producing *E.*

cloacae. However, SNPs in ampD that leads to increased ampC expression, combined with mutations that lower the expression of ompC, ompF or both, increases CR as well (Potter *et al.*, 2016). In 2015, an intermediate carbapenem-susceptible *K. pneumoniae* isolate in the USA and Israel, lacking carbapenemase genes, was found to have more frequent porin nonsense mutations (ompK-36, -36 and -37) and greater ESBL expression compared to *K. pneumoniae* with carbapenemase genes (Potter *et al.*, 2016). Loss of porin mutation expression (ompK-35 and -36) reported in Taiwan in 2013 was found to be associated with a 4-, 8- and 32-fold increase in doripenem, meropenem and ertapenem resistance respectively; however, only ertapenem was beyond clinical breakpoints. In addition, loss of ompK 35 and 36, combined with CTX-M-15 ESBL/plasmid-mediated DHA-1 and its regulator, ampR, led to clinical resistance against all carbapenems (Potter *et al.*, 2016). This combination is believed to have contributed to *K. pneumoniae* CR dissemination.

Carbapenemase producing-CRE isolates express an acquired carbapenemase gene that specifically hydrolyses the carbapenem β-lactam ring. The carbapenemase could belong to the Ambler class A, B and C groups of β-lactamases (McMullen *et al.*, 2017). The genes are typically found on acquired plasmids but may also be found on other transmissible genetic elements inserted into the chromosome. This mechanism is the main cause of CR (Pilmis *et al.*, 2014).

2.7 Plasmids associated with carbapenemases

Mobile genetic elements such as plasmids play a pivotal role in spreading resistance genes. Class A, B and D carbapenemases are harboured on mobile genetic elements within IncL/M, IncX3, IncF and IncA/C plasmids (Sugawara *et al.*, 2017). IncL/M plasmids are a self-transmissible replicon common in Enterobacterales found to harbour β-lactamases from classes A-D (Potter *et al.*, 2016). Reports demonstrated that OXA-48 β-lactamases can be transferred with 40-fold higher efficiency compared to NDM-1 β-lactamases. Although originally endemic to the Mediterranean areas, it has been disseminating globally (Ghasemian *et al.*, 2018). The IncX3 plasmid is commonly associated with global dissemination of bla_{NDM-1} and influences the genetic exchanges during horizontal gene transmission between Enterobacterales (Wailan *et al.*, 2015). Of the IncF family, IncFII- type is the most common and is often associated with NDM-type carbapenemase. A/C plasmids frequently indicate genetic exchange with non-Enterobacterales members. Other plasmids include ColE1-like, IncQ-like, BJ01-like and IncT

(Potter *et al.*, 2016). The presence of multiple carbapenemase genes in one isolate is concerning, given the possibility of different carbapenemase genes crossing onto the same plasmid.

2.8 Carbapenems in South Africa

According to CR-reporting publications (2000-2016) in SA, 2315 estimated carbapenemases cases were reported (Osei Sekyere, 2016a). Gauteng and KwaZulu-Natal were the leading provinces, while Limpopo and Mpumalanga had fewer reported cases (Osei Sekyere, 2016a). The most common organisms isolated were *K. pneumoniae*, *Acinetobacter baumanii* (*A. baumanii*), *Enterobacter cloacae* (*E. cloacae*) and *Serratia marcescens* (*S. marcescens*) and the most common carbapenemases detected were NDMs and OXA-48 (Lowe *et al.*, 2019). In SA, OXA-48 was first detected in 2011 and increasing reports were received a year later in 2012. Yet, OXA-like enzymes, VIM, IMP and GES carbapenemases were less frequently reported compared to NDM and KPC carbapenemases (Brink *et al.*, 2013).

According to CR reports over a nine-year period (2000-2016), CR in SA has been increasing, suggesting that overuse of carbapenems occurs in the country. Unlike the colistin resistance gene (*mcr-1*) that was previously isolated from poultry in *E. coli*, carbapenemases have been detected in clinical isolates in more than 10 Gram-negative bacteria species (Osei Sekyere, 2016a). Previously, CR had not been reported in animals and agriculture, and was attributed to the absence of use of carbapenems in veterinary practice and agriculture (Coetzee & Brink, 2011). However, lately, CR in animals and agriculture has been reported (Brink *et al.*, 2012; Köck *et al*, 2018). Reports of surveillance of CRE in animals or the environment are unavailable in SA (Coetzee & Brink, 2011). Thus, approach resistance in a One Health manner studies in SA is important as antibiotic resistance was not only observed in clinical settings and therefore data from the clinical setting does not represent the overall CR burden.

CRE infections are a global health problem, including in SA, leading to high mortality and morbidity (Raible *et al.*, 2017). The overall transmission and risk are high because of the undetected and persistent nature of CRE, as well as the association of CRE with intensive care units and invasive medical procedures (Francisco *et al.*, 2012). Furthermore, detection of CR in a *Salmonella* Typhimurium (*S.* Typhimurium) isolate from a hospital in Port Elizabeth indicated the possibility of carbapenem transmission via contaminated food and water (Coetzee & Brink, 2011). It is thus essential that the concern of CR is not only focussed on SA, but also

on Africa and Europe, because of the increasing migration of individuals across the continents (Osei Sekyere, 2016a).

Treatment options for antimicrobial resistance infections are limited in SA (Brink *et al.*, 2011). This factor is influenced by the age of the patients, the economy and social status of the patients. Consequently, to treat these infections, clinicians opt for drug combinations that may be toxic (Brink *et al.*, 2013).

2.9 Re-introduction of colistin as a treatment option

Polymyxin antibiotics are cationic, cyclic peptides with a synthesised non-ribosomal hydrophobic tail (Liu *et al.*, 2016). The polymyxins consist of five subtypes: A, B, C, D and E, of which subtypes E (colistin) and B are the clinically important polymyxins (Al-Tawfiq *et al.*, 2017). These two subtypes are used extensively in the veterinary as well as environmental sectors because of their high bactericidal and endotoxin effects, particularly in smaller animals such as piglets and chickens maintained under intensive farming conditions (Bulman *et al.*, 2017). Polymyxin B was originally produced from a Gram-negative bacterium called *Paenibacillus polymyxa* (*P. polymyxa*) in 1946 and became clinically available in 1959 in the USA (Catry *et al.*, 2015). However, in the 1970s, because of its neuro- and nephrotoxicity, it was ultimately abandoned (Falagas *et al.*, 2005). Owing to the emergence of MDR Gram-negative bacteria, the use of colistin has resumed as the last therapeutic agent for the treatment of MDR Gram-negative bacteria resistant to carbapenems.

Clinically, colistin is administered intravenously as colistin sulphate and/or colistin methanesulphate and is often used in combination with other drugs such as tigecycline, meropenem, gentamicin and/or fosfomycin to reduce its toxicity and ultimately, resistance (Liu *et al.*, 2016). Colistin acts by binding to the lipopolysaccharide (LPS) outer membrane of Gram-negative bacteria and initiates potent anti-endotoxin activity by binding to negatively charged phosphate groups of lipid A within LPS (Huang *et al.*, 2017). These binding mechanisms neutralise the LPS, thus preventing the pathophysiologic effects of the endotoxins in the plasma (Liu *et al.*, 2016).

Several mechanisms mediate resistance to polymyxin (colistin and polymyxin E) and these mechanisms modify lipid A L-amino-arabinose and attachment of phosphoethanolamine (PEA) of the 1-4’-phosphate group in lipid A of LPS, which is the main target for polymyxin

(Bernasconi *et al.*, 2016). The resistance mechanisms can be classified into intrinsic and acquired resistance. Intrinsic resistance mechanisms are associated with chromosomal mutations, efflux pumps or regulators of efflux pumps and capsule formation. This mechanism is commonly observed in *Proteus spp.*, *Providencia spp.*, *Serratia spp.* and *Morganella spp*. (Sun *et al.*, 2018), whereas acquired resistance mechanisms are mediated by mobile genetic elements such as plasmids. Although commonly reported in *E. coli*, there have been increasing reports from diverse Enterobacterales such as *Klebsiella spp.*, *Salmonella spp*. and *Enterobacter spp*. (Rojas *et al.*, 2017).

Intrinsic colistin resistance mechanisms are mainly due to addition of L-ara4N to lipid A, which targets the 4-phosphate group, 1-phosphate group or Kdo. However, mutations in the two component systems, PhoPQ and PmrAB, commonly confer colistin resistance and are mediated by *mgrB, eptA, phoP, phoQ* and/or *pmrA-pmrB* genes (Yin *et al*., 2017; Rojas *et al*., 2017). Enterobacterales species that are intrinsically resistant to colistin are increasing in prevalence as a result of increased colistin use and are associated with high mortality rates (Codjoe & Donkor, 2018).

Acquired resistance to colistin occurs mainly in response to horizontal gene transfer mediated by plasmids (Jayol *et al.*, 2016). The genes associated with acquired resistance are MCR genes (*mcr-1 to mcr-10*), including the recently discovered MCR subtypes (*mcr-1.2* to *mcr-1.12*) (Sun *et al*., 2018). The first MCR gene (*mcr-1*) was discovered in 2015 in Southern China by Liu and colleagues (Liu *et al.*, 2016). The gene was detected from *E. coli* strains isolated from pigs and meat and was harbouring the IncI-2 type plasmid pHNSHP45, hence the current knowledge that these genes are plasmid-mediated and confer low-level resistance (Liu *et al.*, 2016). However, a report from the same group indicated resistance from colistin as early as the 1980s, while reports from France described resistance in 2005 and from the Netherlands in 2009. This resistance mechanism is commonly reported in *E. coli* strains and has been found to be more prevalent in animals compared to human and environmental samples. Various factors have been implicated as drivers of colistin resistance, including improper dosage, inappropriate use of colistin in animals and improper administration of antibiotics, which ultimately affect the environment (Betts *et al.*, 2014). Since Liu's report, more *mcr* genes together with their variants have been reported around the world. The reported pHNSHP45 plasmid, harbouring the *IS*ApI insertion sequence on the 5' region of the *mcr-1* gene, is believed to be triggering the rapid mobilisation of the *mcr-1* gene in the plasmid (Bernasconi *et al.*, 2016).

2.10 Mobile genetic elements associated with colistin resistance

Since the first *mcr-1* report, more than10 types of plasmids have been described to harbour the *mcr-1* gene, including IncP, IncHI1, IncFII, IncFI, IncFIB, F18: A–: B+, IncY, IncK and phage-like plasmids, with the predominating replicon types comprising IncI-2, IncX-4 and IncHI2 (Kieffer *et al*., 2017). Genomic sequencing discovered that these plasmids are highly similar, suggesting that most of them have been circulating in multiple Enterobacterales species globally, especially the IncX-4 plasmid (Sun *et al.*, 2018). In addition, it should be noted that plasmids have been reported in diverse host strains from different geographic locations (Zhi *et al.*, 2016). The *E. coli* ST131 clone bearing *mcr-1* genes, recently reported in Denmark and Spain, seems to be an epidemic high-risk clone (Del Bianco *et al.*, 2018). There is increasing detection of clinical isolates that simultaneously harbour carbapenemases such as NDM-5 and KPCs and MCR enzymes; infections with multiple resistance mechanisms appear to cause the most complicated sequelae and treatment becomes challenging (Codjoe & Donkor, 2017).

The dissemination of genetic *mcr* (*mcr-1* to *mcr-10*) and variants (Table 2.1), discovered around the world, indicates the occurrence of selection pressure from humans on animals and the environment. According to epidemiological studies, *mcr-1* has been detected in no more than 10 Enterobacterales species, namely *Cronobacter sakazakii*, *E. coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *K. pneumoniae*, *Kluyvera spp*., *Raoutella ornithinolytica*, *Shigella sonnei*, *Salmonella enterica* and *Moraxella* spp. (Wang *et al.*, 2018a). Although biochemical evidence is lacking, *Moraxella spp.* identified through genetic and bioinformatics analyses were thought to be the potential source of *mcr-1* and *mcr-2* genes (Kieffer *et al.*, 2017).

Table 2.1: Summary of the first detection of MCR genes and their variants (Ling *et al.*, 2020)

Gene	Variants	Host	Organism	Plasmid/Chromosomal	Country	Reference
mcr-1	*mcr-1.1-1.22*	Pigs	*E. coli*	Plasmid	China	(Liu *et al.*, 2016)
mcr-2	*mcr-2.1-2.3*	Porcine & bovine	*E. coli*	Plasmid	Belgium	(Xavier *et al.*, 2016)
mcr-3	*mcr-3.1-3..30*	Porcine	*E. coli*	Plasmid	China	(Yin *et al.*, 2017)

mcr-4	*mcr-4.1-4.6*	Pigs	*S. enterica* *E. coli*	Plasmid	Italy Spain & Belgium	(Carattoli *et al.*, 2017)
mcr-5	*mcr-5.1-5.4*	Poultry & food	*S.* paratyphi BdTa+	Plasmid	Germany	(Borowiak *et al.*, 2017)
mcr-6	*mcr-6.1.*	Pigs	*Moraxella spp.*	Chromosomal	Britain	(AbuOun *et al.*, 2018)
mcr-7	*mcr-7.1*	Chickens	*K. pneumoniae*	Plasmid	China	(Yang *et al.*, 2018)
mcr-8	*mcr-8.1-8.2*	Swine	*K. pneumoniae*	Plasmid	China	(Wang *et al.*, 2018b)
mcr-9	*mcr-9.1*	Human	*S. typhimurium*	Plasmid	USA	(Carroll *et al.*, 2019)
mcr-10	*mcr-10.1*	Human	*E. roggerkampii*	Plasmid	China	(Wang *et al.*, 2020)

The mobile colistin-resistant-2 (*mcr-2*) gene was first detected in Belgium and shares approximately 80% identity with *mcr-1* (Liassine *et al.*, 2016). It is carried by IncX-4 plasmids Pkp37-BE, flanked by insertion sequence *IS*Ec69 and together with *mcr-1*, it is annotated as lipid A-4'-PEA transferase (Liassine *et al.*, 2016). Mobile colistin resistance-3 (*mcr-3*), together with *mcr-4* and *mcr-5,* was discovered in *E. coli* and *Salmonella* species (Sun *et al.*, 2018). According to phylogenetic analysis, *mcr-3, mcr-4* and *mcr-5* appear distinctly different from *mcr-1* and *mcr-2*. The genes *mcr-4* and *mcr-5* were found to be located on ColE-type plasmids (Schwarz *et al.*, 2019). The genetic diversity of *mcr-3* indicates the presence of the gene in humans and animals (Schwarz *et al.*, 2019). Unlike *mcr-4* and *mcr-5*, *mcr-3* is carried by IncHI2 plasmid, while its variants (*mcr-3.2-5* & *mcr-3.7*) are carried by a *Tn*AS3-like transposon on a self-transmissible IncP plasmid (Schwarz *et al.*, 2019).

Although the prevalence of *mcr-1* appears to be low**,** *mcr* genes have been reported in over 40 countries on five continents, indicating the rapid dissemination of these genes (Zhi *et al.*, 2016). Suggested origins of *mcr-1* include humans, animals, the environment, wildlife, poultry and food (Wise *et al.*, 2018). Plasmid diversity (over 10 plasmids) harbouring *mcr-1* genes suggests that most of these plasmids have circulated in multiple species of Enterobacterales worldwide. The IncX4 plasmid is the most prevalent subtype of the six IncX groups and can be traced back

to the pre-antibiotic era. Because of its capability of being self-transferable at high frequencies (10^{-1}- 10^{-4}), it represents promiscuous vectors contributing to the intercontinental spread of the *mcr-1* gene (Sun *et al.*, 2018).

There are several hybrid versions of *mcr-1*-positive plasmids, namely IncX3, IncX-4 and IncI2-FIB, suggesting genetic recombination events among different circulating plasmids (Al-Tawfiq *et al.*, 2017). Detection of different MCR-bearing plasmid types in the same isolate or strain implies that the dissemination of plasmid-borne *mcr-1* might be driven by selective pressure from the overuse of colistin in livestock production. Despite this, it appears that jumping of *mcr-1* from plasmids though transposal events into the chromosome is rare (Wang *et al.*, 2018a).

Large scale use at low doses of colistin in food production as a growth promoter seems to be the driving force behind the spread of the *mcr-1* resistance gene. For example, countries such as China and Vietnam have detected *mcr-1* from chickens and pigs (Paterson & Harris, 2016). However, the colistin resistance gene has also been detected in food animals (0.1-10% prevalence) in the USA and Europe even though colistin is not registered for use as a growth promoter in feeds. High detection rates of *mcr-1* in *E. coli* have been reported in some European countries such as Portugal and Italy. This increased rate can be attributed to the frequent use of colistin metaphylactically and/or therapeutically in the management of post weaning diarrhoea (Sun *et al.*, 2018). Wildlife also seems to have the ability to spread *mcr-1* because *mcr-1* was detected in Magellanic penguins and migratory birds in Brazil, as well as Asia, Europe and South America (Al-Tawfiq *et al.*, 2017).

Increasing reports on carbapenem-resistant Enterobacterales worldwide have led clinicians to opt for colistin to treat CRE (Brink *et al.*, 2013). In 2015, SA detected the colistin resistance gene *mcr-1* in 19/20 *E. coli* strains. The strains originated from six geographically distant broiler operations throughout SA, suggesting a locally widespread distribution (Poirel *et al.*, 2016; Theobald *et al.*, 2019). This report led to the withdrawal of registration of registered colistin products in January 2016, which was later followed by a ruling by the South African Veterinary Council further restricting veterinary use of colistin in food-producing animals, unless the consulting veterinarian could justify the use of colistin as a last resort to treat valuable diseased animals after sensitivity tests (Schellack *et al.*, 2017). A ruling by the Registrar of Stock

Remedies further preventing any registration of colistin for registration as a Stock Remedy to bypass the restriction placed by actions previously mentioned.

Although colistin resistance is rare in human non-typhoidal *Salmonellae* isolates, *mcr-1* has been detected in diverse *Salmonella spp.* strains, including human and animal strains, thus suggesting a possible zoonotic origin (Rule *et al.*, 2019). The use of polymyxins or other antimicrobials in animals is possibly associated with the prevalence of colistin resistance. Co-selection of MDR-strains could be a factor associated with the use of other antimicrobials, resulting in increased colistin resistance prevalence in animals (Kieffer *et al.*, 2017). Specifically, a study in China reported on the detection of *mcr-1* and $bla_{\text{NDM-5}}$ simultaneously in an *E. coli* isolate from a domestic cat, thus indicating dissemination among humans and animals (Zhi *et al.*, 2016).

2.11 Identification and characterisation of resistance genes in Enterobacterales

Identification of Enterobacterales species and characterisation of resistance genes are essential tools in the treatment of Gram-negative bacterial infections. Early diagnoses encourage initiation of early treatment and the use of correct treatment ultimately has an impact on the reduction of mortality and morbidity. Identification of Enterobacterales and characterisation of resistance genes can be by phenotypic and molecular methods. Phenotypic methods, such as culture, have the ability to identify the organism to species level based on the biochemical reaction of the species (Syal *et al.*, 2017). However, the tests are laborious, require at least 24-hour culture and have a two-to-four-day turn-around time, causing delay in treatment that may lead to the death of the patient (Das *et al.*, 2014; Castro-Escarpulli *et al.*, 2015).

Antimicrobial susceptibility testing (AST) is used to determine the susceptibility profile of organisms. AST measures the bacterial growth in the presence of antibiotics using turbidity or fluorescence and can be automated (MicroScan® WalkAway and Vitek-2) or not automated (disk diffusion, E. test, Sensititre and broth microdilution [BMD]) (Mlynarcik *et al.*, 2016). This testing is crucial because it yields guidelines on the correct antibiotics for treatment of bacterial infections. The recommendations include clinical breakpoints for MICs and minimum bactericidal concentration (MBC) (Syal *et al.*, 2017) from CLSI and EUCAST. However, CLSI does not provide colistin breakpoints for Enterobacterales. Nonetheless, EUCAST recommended MIC of ≤ 2 ug/mL as susceptible and ≥ 2 ug/mL as resistant to colistin (Simar *et*

al., 2017). The EUCAST carbapenems resistance breakpoint for Enterobacterales is ≥ 2 µg/mL for ertapenem and doripenem and ≥ 4 µg/mL for meropenem or imipenem (Potter *et al.*, 2016).

Broth microdilution is the gold standard for phenotypic susceptibility testing for colistin (Syal *et al.*, 2017) while for carbapenems, Vitek-2 is commonly preferred in most studies (Hernández-durán *et al.*, 2017). These methods differ in their sensitivity and specificity. For example, studies have indicated that MicroScan® WalkAway has increased specificity for detecting carbapenemases compared to Vitek-2. However, Vitek-2 is more sensitive (Quesada *et al.*, 2010). For colistin susceptibility testing MicroScan® WalkAway (automated BMD) is becoming a standard method; however, results are to be confirmed using phenotypic BMD according to CLSI and EUCAST guidelines (Syal *et al.*, 2017).

Molecular methods such as PCR and whole-genome sequencing are based on identifying and characterising the genomic information of the organism and have the ability to identify the bacteria to species level and in less time compared to phenotypic methods (Das *et al.*, 2014). The resistance gene profiles are used to demonstrate antimicrobial resistance determinants spread between genetically unrelated bacterial hosts. These methods are highly specific and sensitive compared to the phenotypic methods and provide rapid turn-around time. In addition, the ability of Gram-negative bacteria to survive biofilms formation makes these methods ideal to use compared to culture-based methods, which may be difficult to detect (Rojas *et al.*, 2017).

Characterisation of resistance genes through molecular techniques such as PCR, WGS and genotyping techniques enables the detection of specific genes mediating resistance in Enterobacterales such as MCR genes or carbapenemases. Although PCR is commonly used, it can often not detect all genes and other resistance mechanisms such as efflux pumps and chromosomal mutations (Oviaño *et al.*, 2017). Whole-genome sequencing is the ultimate method for both identification and characterisation of resistance genes in Enterobacterales. The method provides all the genomic information about an organism, from species identification to several resistance mechanisms mediating resistance in that organism, as well as species relatedness (Tagini & Greub, 2017). However, there is no standardization, it requires a reference genome, is expensive and needs expertise (Ellington *et al.*, 2017). In low-resourced countries, use of WGS is preferably applied in epidemiological studies, referral laboratories and surveillance programmes.

Although studies are finding ways to characterise resistance genes from direct specimens such as blood culture and urine (Oviaño *et al.*, 2017; Meier & Hamprecht, 2019), many of these genomic methods are dependent on cultured isolates. Nevertheless, in low-resourced countries, phenotypic methods in diagnostic microbiology laboratories are preferred to molecular methods because molecular techniques are expensive (Banerjee & Humphries, 2017). Regardless of the method used to identify and characterise resistance genes in Enterobacterales, the methods should provide correct diagnoses with a short turnaround time.

2.12 Treatment and prevention of Enterobacterales infections

The current therapeutic options are based on *in vitro* synergism studies on old drugs or between different drugs for which CRE isolates exhibit good *in vitro* susceptibility. Polymyxin, tigecycline, fosfomycin and aminoglycoside antibiotics (particularly gentamicin) are the few remaining drugs of choice for treating CRE infections (Dickstein *et al.*, 2016; Exner *et al.*, 2017). *In vitro* studies have shown combination therapy to be superior to monotherapy when using these antibiotics (Tamma *et al.*, 2012; Betts *et al.*, 2014). For example, colistin is more effective when combined with other effective drugs (carbapenems, tigecycline) compared to when used alone, thus increasing patient survival rates (Tamma *et al.*, 2012). Although no clinical data is available, it has been suggested that colistin and rifampicin in combination could be a therapeutic option for colistin-resistant KPC-producing *K. pneumoniae* (Durante-Mangoni *et al.*, 2013). Targeted treatment with gentamicin was reported to be associated with lower patient mortality compared to other drugs, with a strong correlation being observed between gentamicin MIC levels and mortality (Falagas *et al.*, 2014). Fosfomycin, combined with carbapenems, colistin, tigecycline and netilmicin, also shows good synergistic effects against CRE infections and thus serves as a good therapeutic option (Yu *et al.*, 2017). However, there are studies that disagree with the finding recommendation (de Maio Carrillho *et al.*, 2017; Ghafur *et al.*, 2017). In addition, most widely used antimicrobial combinations were formulated from clinical observation of effectiveness but not comprehensively analysed for the mechanistic basis of synergy (Potter *et al.*, 2016).

Synergistic effect in antimicrobial combinations have the ability to promote occurrence of different mechanisms for microbial killing (Tamma *et al.*, 2012). To illustrate, combination therapy using β-lactams and aminoglycoside to treat enterococcal endocarditis in both *in vivo* and *in vitro* studies demonstrated penicillin to increase aminoglycoside uptake (Tamma *et al.*, 2012). A synergistic effect was observed in colistin and tigecycline combination therapy to treat

CRE infections (Betts *et al.*, 2014). The effect emphasizes the use of combination therapy compared to monotherapy. Nevertheless, there is strong evidence, such as increasing resistance observed in colistin and tigecycline monotherapies, for combination antimicrobial use for treating CRE infections.

While empiric antibiotic therapy has led to the development of clinically useful combinations, most notably trimethoprim-sulfamethoxazole and β-lactam/β-lactamase inhibitor combinations, the immediate benefits from synergy may be countered by quicker evolution of resistance to these pairings (Tangden & Giske, 2015). Beta-lactamase inhibitors have extended the spectrum and efficacy of several β-lactam antibiotics and a few β-lactam/β-lactamase inhibitor combinations have gained widespread clinical use (Tangden & Giske, 2015). Unfortunately, current β-lactamase inhibitors are ineffective against class B β-lactamases, which is concerning, considering the global dissemination of NDM-positive Enterobacterales and regional prevalence of VIM and IMP enzymes (Potter *et al.*, 2016). The reappearance of polymyxins as treatment option for CRE infections has motivated further study of other antibiotics with historically limited use, such as chloramphenicol (Nordmann & Poirel, 2016). The potency of therapeutic antibiotics can be altered when these are combined (Brown & Dawson, 2015). Thus, understanding how commonly used antibiotics affect one another and identifying new combinations are necessary to optimise CRE treatment (Del Bianco *et al.*, 2018).

In SA, options to treat antimicrobial-resistant infections are limited, particularly in neonates, mainly because of their age (Messina *et al.*, 2018). The consequences of these infections include the use of toxic combination drugs and increased hospital stay, which ultimately affect the economy negatively (Schellack *et al.*, 2017). Without clinical trials, no optimal therapeutic management option can be established. Combination therapies with effective drugs are effective in treating CRE infections (de Maio Carrillho *et al.*, 2017). Studies recommend combination therapies compared to monotherapies because combination therapies are considered superior and increase the chances of survival when treating CRE infections (Falagas *et al.*, 2014).

Ceftazidime-avibactam displays good *in vitro* activity against carbapenem-resistant Enterobacteriaceae isolates that produce KPC and AmpC and partially against OXA-48-positive CREs; however, it is not active against MBL-positive CREs with VIM, NDM and IMP

carbapenemases. Moreover, clinical data provides limited evidence of the efficacy of ceftazidime-avibactam in severe CRE infections (Perovic *et al.*, 2016). Carbapenems can serve as a good therapeutic option in combination with other effective drugs (Gugliandolo *et al.*, 2017).

Therapies for XDR and pan-drug resistant Enterobacterales are currently limited and are poorly investigated (Duin & Doi, 2017). At present, antimicrobials that have *in vitro* activity against CREs are in different clinical trial stages; these include: (1) avibactam combined with β-lactams, e.g ceftaroline fosamil-avibactam and azetreonam-avibactam; (2) carbapenems combined with β-lactamase inhibitor, e.g meropenem-vaborbactam and impinem/cilastatin-relebactam; and (3) plazomicin and eravacycline. However, none of these antibiotics is effective against all carbapenemases, as they target specific enzymes (Trecarichi & Tumbarello, 2017).

2.13 The need for One Health approach studies

The emergence and re-emergence of infectious and non-infectious diseases are linked to rapid environmental changes. To understand disease dynamics better and drive public policies, it is essential to be knowledgeable about the causes and consequences of human and ecosystem behaviours (Cleaveland *et al.*, 2017). The One Health concept was created in 2004 with the aim of incorporating the human, veterinary and environmental sectors to constitute a global strategy highlighting the need for a transdisciplinary approach (Cunningham *et al.*, 2017).

This approach is essential because it provides a way of looking at complex systems and approaching processes leading to undesirable effects, such as the emergence of disease. One Health encourages and promotes the interdependence, coexistence and evolution of organisms and their environment, which is constantly transforming (Rostal *et al.*, 2018). Initially, introduction of the concept brought about collaboration between human medicine and veterinary medicine. However, it resulted in unavoidable research bias toward zoonotic diseases, temporarily ignoring the important question of chronic non-infectious diseases, which also leads to global human mortality (Rhouma *et al.*, 2016). Nevertheless, few studies now combine these three sectors (human, animal and the environment) in one study; resulting in limited data. (Agga *et al.*, 2015; Shen *et al.*, 2020)

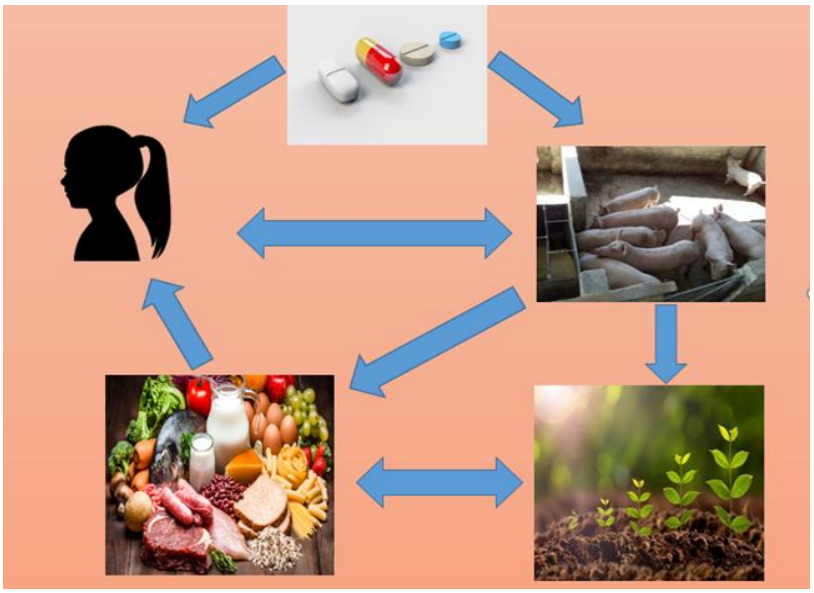

Figure 2.2: Pictorial demonstration of One Health concept relating to antimicrobial resistance. Overuse of antimicrobials by humans in animals and in the environment directly or indirectly has an effect on other sectors (inspired by McEwen and Collignon, 2018).

Although a few antibiotics such as isoniazid and inophores are exclusively used for humans and animals respectively, many classes of antibiotics (cephalosporins, polymyxins, aminoglycosides and macrolides) are used in both human and veterinary medicine (McEwen & Collignon., 2018). The outcome of antibiotics overuse (Figure 2.2) in the human and veterinary sectors promotes selection pressure, which in turn enhances the acquisition and expression of resistance genes, ultimately leading to sharing of resistance genes among bacterial species (Cunningham *et al.*, 2017; Destoumieux-Garzón, 2018). These, consequently, reach the environment through waste mismanagement from farms and industrial sites (Ryu *et al.*, 2017).

Colistin and cephalosporins are good indicators of overuse of antimicrobials in both humans and animals (particularly in food-producing groups) (McEwen & Collignon, 2018). Colistin was initially used to treat cystic fibrosis infections in humans; however, because of neuro- and nephro-toxicity, usage in humans was discontinued, though it continued to be used for treating animals (Falagas *et al.*,2005; Kasiakou & Saravolatz, 2005). As a result, MCR genes (*mcr-1*) were first reported in China in late 2015, although the first identification of colistin resistance dates back to the 1980s (Liu *et al.*, 2016; Shen *et al.*, 2016). To date, MCR genes (*mcr-1* to *mcr-10*) and variants have been disseminated globally and have been identified in clinical, veterinary and environmental isolates (Shen *et al.*, 2016; Wang *et al.*, 2018b; Wang & Feng,

2020). Increasing reports of colistin resistance is a health problem because currently colistin is the last therapeutic drug used to treat CRE infections. Cephalosporins (third- and fourth-generation) and colistin are used in both humans and animals. In humans these are used to treat nosocomial infections and community-associated infections such as UTIs and in animals to treat bacterial infections, administered per individual animal or in groups (McEwen & Collignon, 2018).

Historically, domesticated animals indirectly mediated the transfer of infectious agents between wildlife and humans (Al-Tawfiq *et al.*, 2017). Most emerging infectious diseases are considered to be significant because 70% of infections in humans are thought to be zoonotic in origin and almost a third quarter originate in wild animals (Madec & Haenni, 2018). The industrialisation of agriculture and farming is also responsible for widespread and often abusive use of pesticides, fertilizers and antibiotics, causing problems such as on the one hand resistance to insecticides in mosquitoes that transmit pathogens, and on the other hand resistance to antibiotics in bacteria (Exner, 2017). This threatens global health, food security and development (Cunningham *et al.*, 2017).

Antimicrobial resistance is a global health crisis with multiple dimensions and reservoirs of resistance. The reservoirs may be the animals, or the environment. Resistance occurs where antimicrobials are misused or overused thus creating an environment that favours selection pressure resulting in bacterial resistant strains (van den Honert *et al.*, 2018). The One Health approach emphasises the direct or indirect impact of antimicrobial use on humans, animals and the environment (Chainier *et al.*, 2017). Therefore, applying a One Health approach is urgently needed to effectively manage antimicrobial resistance.

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CHAPTER 3

Molecular epidemiology and mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae* from clinical isolates, the environment and porcine samples in Pretoria, South Africa

The style of Clinical Microbiology and Infection journal was followed in this chapter.

Abstract

Introduction: Carbapenems and colistin are the last-line antibiotics for treating Gram-negative bacterial infections. However, increasing reports of resistance to these antibiotics is being reported in clinical settings, the environment and in animals. In this paper, we describe the molecular epidemiology and resistance mechanisms of colistin and carbapenem resistance in clinical, veterinary, and environmental Enterobacterales isolates in Pretoria, South Africa.

Method: One hundred VITEK®-2-confirmed colistin and carbapenem-resistant clinical isolates were collected from the departmental isolate bank at the National Health Laboratory Service. A total of 88 porcine (stool) and 11 environmental (effluents) samples were collected in November 2018 and again in March 2019 from a farm in Pretoria. Both the porcine and environmental samples were screened using Eosin methylene blue agar with colistin and ertapenem disks. All isolates were identified and a minimum inhibitory concentration of colistin and carbapenems was determined using the MicroScan® WalkAway system. Isolates resistant to colistin were confirmed by the broth microdilution method. Isolates phenotypically resistant to colistin and carbapenems were selected for whole genome sequencing to determine the resistome and phylogenetic trees were drawn to determine the relatedness of isolates.

Results: A total of 275 Gram-negative isolates were identified from the clinical (100), environmental (57) and veterinary (118) samples using the MicroScan® WalkAway system. The MicroScan® WalkAway system's minimum inhibitory concentration results for clinical isolates revealed 88% and 93% resistance to colistin and carbapenems, respectively. BMD was found to be more reliable in all isolates, and it recorded higher MICs (increased resistance) than the MicroScan® WalkAway system. Overall, colistin susceptibility was higher among animal isolates compared to the clinical and environmental samples. Genomic analysis identified several resistance genes associated with resistance among the isolates and the CTX-M family

were the dominant resistance genes. Phylogenomic analysis demonstrated closer evolutionary relationship between EB008 (environment), SW10B (animals), and C080 and C084 (both humans) strains as well as with strains from the United States of America, Canada, China, Russia and Durban (South Africa).

Conclusion: The study established multiple resistance genes from different antibiotics to mediate resistance in Enterobacterales isolates from humans, animals and the environment. The presence of carbapenemases in animals is alarming and poses a public health concern. Strains EB008 (environment), SW10B (animals) and C080 and C084 (both human) were phylogenetically related with strains from the United States of America, China and Durban (South Africa) more commonly. Therefore, One Health approach studies are significant to ascertain colistin and carbapenem transmission from human to animals/the environment and vice versa to combat increasing resistance in Enterobacterales.

Keywords: Enterobacterales, colistin, carbapenem, resistance, Gram-negative bacteria; South Africa.

3.1 Introduction

Constant reports of increasing resistance in Gram-negative bacteria, in particular among the Enterobacterales family, are of great concern (Gillings *et al.*, 2017). Enterobacterales are Gram-negative bacteria that are ubiquitous in nature; they are present in soil, water and the environment and even form part of the normal flora of both humans and animals (Liu *et al.*, 2018). When the environment is suitable, some of these species (*E. coli* and *S. marcescens*) become pathogenic and thus cause infections such as pneumonia, meningitis and urinary tract infections (UTIs) (Pilmis *et al.*, 2014). Drugs such as carbapenems (imipenem, doripenem, ertapenem and meropenem) are used to treat Gram-negative bacterial infections. These drugs act by binding to penicillin binding proteins and inhibiting cell wall synthesis, leading to cell death (Farrington, 2012). However, antibiotic resistance in carbapenems is increasingly being reported, leading to multi-drug resistance (MDR) among Gram-negative bacteria (Potter *et al.*, 2016).

Resistance mechanisms to carbapenems are diverse. The production of carbapenemases is most common (Francisco *et al.*, 2012; Theobald *et al.*, 2019). Common carbapenemases isolated are *Klebsiella*-producing carbapenemase (KPCs), New-Delhi metallo-β-lactamases (NDMs), imipenemase metallo-β-lactamases (IMPs), Verona integron-encoded metallo-β-lactamases (VIMs), Guiana extended-spectrum beta-lactamase and oxacillinases (OXA-types) families (Tijet *et al.*, 2014). The prevalence of these genes differs per geographical location across the world. Consequently, clinicians have resorted to old drugs such as colistin to treat MDR Gram-negative bacterial infections (Labuschagne *et al.*, 2016). Colistin is a polymyxin drug that was abandoned in the 1970s owing to its toxicity, although continued use was observed in the veterinary sector as a prophylactic drug (Del Bianco *et al.*, 2018). The mode of action of colistin is to modify the lipid A, which alters the lipopolysaccharide of the bacterial cell, thus disrupting the outer membrane and causing cell death (Hamzan *et al.*, 2015). However, colistin resistant (*mcr-1*) mediated by a plasmid has now spread globally (Liu *et al.*, 2016).

Increasing resistance to last-line therapeutic drugs used to treat MDR Gram-negative bacterial infections leaves limited treatment options, increasing morbidity and mortality, particularly in developing countries such as South Africa (Brink *et al.*, 2016). Although antimicrobial resistance is mostly reported in clinical settings, the concern continues into the veterinary and environmental sectors (Yang *et al.*, 2017). It is believed that increased resistance to colistin in the veterinary sector is due to overuse of the antibiotic as a growth promoter, which may have spread into the environment (Zhi *et al.*, 2016). In addition, human behaviour (environmental

contamination, poor sanitation and poor infection control), along with the ever changing environment due to global warming (Rodríguez-Verdugo *et al.*, 2020), may have influenced the spread and adaptation of Enterobacterales species, hence the carriage of resistance genes among these species (Cunningham *et al.*, 2017). Carbapenem drugs are not registered for use in animals in South Africa by the local regulatory body South African Health Products Regulatory Authority (SAHPRA) to treat Gram-negative infections. However, carbapenem resistance has been reported in isolates from animals in some parts of the world (Laxminarayan *et al.*, 2013; Köck *et al.*, 2018). This indicates that mobile genetic elements such as transposons, integrons and plasmids, in which most of these genes are detected, play a role in spreading these resistance genes (Hosseini *et al.*, 2016; Potter *et al.*, 2016).

The delay in antibiotic therapy is one of the major causes of high mortality in patients Gram-negative bacterial infections (Exner, 2017). Enterobacterales infections from MDR bacteria result in death twice as often compared to death from susceptible antibiotic infections, particularly in neonates and immunocompromised patients (Skov & Monnet, 2016). In addition, available clinical data does not reflect the overall problem because the veterinary and environmental sectors, which are affected as well, are not well studied (Osei Sekyere, 2016). It is thus essential that One Health studies fully incorporating humans, animals and the environment are undertaken to combat the paucity of data, as well as provide knowledge that will aid in combating one of the major health, veterinary and environmental problems facing the 21st century (Bassetti *et al.*, 2015). We thus aimed to determine the epidemiological association between animals, humans and the environment in a One Health approach as well as identify the main reservoir of the *mcr* colistin resistance genes in Pretoria.

3.2 Materials and Methods

3.2.1 Questionnaire

Prior to sample collection, a questionnaire was explained to the owner of the farm who then answered the questions posed in the questionnaire (Appendix 1).

3.2.2 Isolates and samples collection, and screening

The study was a descriptive and quantitative study that aimed to describe the molecular epidemiology and resistance mechanisms of colistin and carbapenem resistance in clinical, veterinary and environmental Enterobacterales isolates in Pretoria, South Africa. The study was conducted at the Department of Medical Microbiology, Prinshof Campus, University of Pretoria. Three different sample types were collected. They were clinical isolates (n=100),

porcine faeces (n=88) and effluents (n=11). Animal and environmental samples were collected on one occasion in each month of November 2018 and March 2019. Forty-four animal samples were collected in both November and March while for environmental samples, three 500 mL and five 500 mL samples were collected in November and March, respectively. The samples were collected in sterile containers, transported to the laboratory at 4 ºC within 6 hours of collection and processed immediately for further experiments. After screening all isolates were stored at -20 ºC (short period) and -80ºC (longer period) until ready for use.

3.2.3 Clinical isolates

In this study, previous routinely characterised 100 Vitek®-2 system-confirmed colistin- and carbapenem-resistant Gram-negative non-duplicate bacteria isolated between 2017 and 2018 were collected from the Tshwane Academic Division of the National Health Laboratory Service (NHLS) isolate bank in Pretoria, South Africa. These isolates were cultured from clinical specimens received by the NHLS from three hospitals across Pretoria and included various specimen types such as blood culture, urine, tissue culture, swabs, blood, and catheter tips. The isolates were cultured on blood agar for further experiments.

3.2.4 Animal samples

Simple random sampling of porcine faecal samples was undertaken from four different housing sites (sow, farrow, weaner and grower) per collection at a single farm in Pretoria. Immediately after collection, the faeces samples were homogenised using saline and the supernatant was lawn-cultured on Eosine-methylene blue (EMB) agar (Appendix 2). Ten micrograms (10 µg) of colistin sulphate and ertapenem disks of 10 µg were placed on the agar. For colistin-resistant isolates, the samples were considered to be positive when a zone of inhibition for colistin-resistant colonies was not present around the antibiotic disks. For ertapenem resistance, Clinical Laboratory Standard Institute (CLSI) breakpoints were used to interpret the zones of inhibition and infer carbapenem-resistant colonies. The samples were re-cultured on MacConkey agar and then repeatedly on blood agar until pure colonies were obtained.

3.2.4 Environmental samples

Purposive, grab sampling was adopted for these samples. Thus, for first collection, effluent samples were sampled at three different housing channels of the pig farm. In the second collection, samples were collected as per first collection and at five sites within the main effluent collection point at a farm in Pretoria, South Africa (Yang *et al.*, 2017). Immediately after collection, serial dilution of the effluents was performed. The supernatant was lawn-

cultured on EMB agar. Ten micrograms (10 µg) of colistin sulphate and 10 µg ertapenem disks were placed on the agar. For colistin-resistant isolates, the samples were considered to be positive when a zone of inhibition was present around the antibiotic disks. For ertapenem resistance, CLSI breakpoints were used to interpret the zones of inhibition and infer carbapenem-resistant colonies. The samples were re-cultured on MacConkey agar and then repeatedly on blood agar until pure colonies were obtained (Appendix 2).

3.2.5 Species identification and antimicrobial susceptibility testing

Identification of the species and antimicrobial susceptibility was performed using the MicroScan® WalkAway system (Beckman Coulter, USA). The antimicrobials tested for included the following antibiotics: ampicillin, amikacin, Augmentin, aztreonam, ceftazidime, cephalothin, cefotaxime, K clavulanate, cefuroxime, colistin, ciprofloxacin, cefepime, chloramphenicol, ertapenem, nitrofurantoin, fosfomycin, gentamicin, imipenem, levofloxacin, meropenem, nitrofurantoin, norfloxacin, piperacillin, sulbactam, sulphonamide, tazobactam, trimethoprim, tigecycline and tobramycin. The susceptibility results were interpreted and recorded according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint. Colistin-susceptible and -resistant isolates were confirmed using the broth microdilution (BMD) (Appendix 2) method and interpreted using EUCAST breakpoints.

3.2.6 Polymerase chain reaction assay for colistin and carbapenem resistance gene amplification

DNA extraction was performed on all isolates resistant to colistin and carbapenem using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, USA) commercial kit according to the manufacturer's instructions (Appendix 2). The total DNA of strains phenotypically resistant to colistin were screened for *mcr-1*,*-2*,*-3*,*-4*,*-5* genes using the primers synthesized by Inqaba Biotechnical Industries indicated in Table 3.1 (Rebelo *et al.*, 2018). The multiplex PCR consisted of of 12.5 uL Thermo Scientific DreamTaq Green PCR master mix, 0.5 uL of forward and reverse primers, 5.5 uL nuclease free water and 2 uL of DNA template to make up a 25 uL reaction. The thermocycling conditions for the amplification of MCR genes were: 94 °C for 15 min, followed by 25 cycles of 94 °C for 30 s, annealing at 58 °C for 1 min and 30 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Ertapenem-resistant strains were screened for carbapenemase-encoding genes bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{KPC} and bla_{OXA-48} using primers by Inqaba Biotechnical Industries indicated in Table 3.2 (Poirel *et al.*, 2011; Doyle *et al.*, 2012). The total DNA was then used to perform multiplex and singleplex polymerase chain reaction (PCR) for the detection of carbapenemase genes. Multiplex (M-

PCR) was performed for detection of *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{OXA\text{-}48}$ while singleplex (S-PCR) was performed for the detection of *bla*$_{IMP}$ and *bla*$_{KPC}$. The PCR reactions consisted of 1 µL of DNA template, 12.5 µL of MyTaq HS mix (Bioline), 0.4 µM of each primer and nuclease-free water (Qiagen) added to make up the volume to 25 µL in each PCR tube. The M-PCR conditions were as follows: 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 57 °C for 45 s and 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The S-PCR conditions were as follows: 94 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 50 s and a final extension step at 72 °C for 5 min. The amplicons were visualised using a 1.2% Seakem agarose gel (Whitehead Scientific) run at 90 volts for 90 minutes for both colistin and carbapenem resistance genes; the gels were stained with Ethidium bromide. A 100 bp Plus DNA Ladder (Thermo Fisher Scientific, USA) for colistin and 100 bp DNA Ladder (Celtic Molecular Diagnostics, USA) for carbapenem resistance genes were used as a reference gene ladder (Mlynarcik *et al.*, 2016; Rebelo *et al.*, 2018).

Table 3.1: Primer sequences used for M-PCR assay for the detection of colistin resistance genes (*mcr-1*, *-2*, *-3*, *-4* and *-5*)

Target gene	Primer sequences	Sequence (5'-3' direction)	Amplicon size (bp)	Reference
Colistin resistance genes				
mcr-1	Forward	AGTCCGTTTGTTCTTGTGGC	320	(Rebelo *et a.l*, 2018)
	Reverse	AGATCCTTGGTCTCGGCTTG		
mcr-2	Forward	CAAGTGTGTTGGTCGCAGTT	715	(Rebelo *et a*l., 2018)
	Reverse	TCTAGCCCGACAAGCATACC		
mcr-3	Forward	AAATAAAAATTGTTCCGCTTATG	929	(Rebelo *et al.*, 2018)
	Reverse	AATGGAGATCCCCGTTTTT		
mcr-4	Forward	TCACTTTCATCACTGCGTTG	1116	(Rebelo *et al.*, 2018)
	Reverse	TTGGTCCATGACTACCAATG		
mcr-5	Forward	ATGCGGTTGTCTGCATTTATC	1644	(Borowiak *et al.*, 2017)
	Reverse	TCATTGTGGTTGTCCTTTTCTG		

Table 3.2 Primer sequences for M-PCR and S-PCR assays used for the detection of carbapenem resistance genes (*bla* VIM, *bla* OXA-48, *bla* NDM, *bla* IMP and *bla* KPC)

Target	Gene	Primer sequence (5' to 3' direction)	Amplicon size (bp)	Reference
Carbapenem resistance genes				
IMP	*bla*$_{IMP}$ (Forward)	GGAATAGAGTGGCTTAAYTCTC	232	(Poirel *et al.*, 2011)
	bla$_{IMP}$ (Reverse)	GGTTTAAYAAAACAACCACC		
KPC	*bla*$_{KPC}$ (Forward)	TGTCACTGTATCGCCGTC CTCAGTGCTCTACAGAAAACC	900	(Doyle *et al.*, 2012)
	bla$_{KPC}$ (Reverse)	TTGTCATCCTTGTTAGGCG		
VIM	*bla*$_{VIM}$ (Forward)	GATGGTGTTTGGTCGCATA	390	(Poirel *et al.*, 2011)
	bla$_{VIM}$ (Reverse)	CGAATGCGCAGCACCAG		
OXA	*bla*$_{OXA-48}$ (Forward)	GCGTGGTTAAGGATGAACAC	438	
	bla$_{OXA-48}$ (Reverse)	CATCAAGTTCAACCCAACCG		
NDM	*bla*$_{NDM-1}$ (Forward)	GGTTTGGCGATCTGGTTTTC	782	
	bla$_{NDM-1}$ (Reverse)	CGGAATGGCTCATCACGATC		

3.2.8 Whole-genome sequencing and molecular analysis

Whole-genome sequencing (WGS) for 24 representative isolates was performed using the Oxford Nanopore MinION. DNA was extracted from all representative isolates resistant to both colistin and carbapenems. DNA libraries were prepared using the Rapid Barcoding Sequencing kit (SQK-RBKOO4) according to the manufacturer's protocol. Briefly, DNA was fragmented using the Fragmentation Mix RB to attach a pair of barcodes simultaneously to the fragments. The barcoded samples were pooled, and the sequencing adapters were attached to the DNA ends. The flow cell was primed, and the DNA library was loaded into the flow cell. Sequencing was run using the MinION software and analysed using the EPI2ME software. The resistance genes of the isolates were obtained using the ResFinder-3.2 Server from the Centre for Genomic Epidemiology.

3.2.7 Phylogenetic analysis

The raw fast5 reads were quality-trimmed and converted into Fasta using MinION. The Fasta files were run through the CGE databases such as ResFinder to determine the resistance profiles of the isolates. The species of the isolates were further confirmed using average nucleotide identity at National Centre for Biotechnology Information (NCBI) (GenBank). The genomes of

the isolates, together with genomes of the species downloaded from PATRIC, were used to draw a neighbour-joining tree using RAXmL. The trees were annotated using Figtree.

CHAPTER 4

RESULTS

4.1 Response to questionnaire

The outcome from the questionnaire was that veterinary medicines were administered to animals via injection. The veterinary medicines administered were Sulfatrim, Litter guard, Farrowsure, Respisure, Dectomax, Dexiron 200 and Rucenta vitamin B complex. In Table 4.1 the ingredients of each medicine administered are listed.

Table 4.1: List of ingredients per medicine administered to the animals on the farm

Medicine	Ingredients
Sulfatrim	Sulfamethoxazole and Trimethoprim
Litter guard	*E. coli* (enterotoxigenic strain) and *C. perfringens* type C
Farrowsure	Inactivated porcine parvovirus, *Erysipelas rhusiopathiae, Leptospira bratislava, L. canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae and L. pomona* vaccine
Respisure	*Mycoplasma hyopneumoniae*
Dectomax	Doramectin 1 % m/v (macrocydic lactone)
Dexiron 200	Iron
Rucenta vitamin B complex	Thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), biotin, folic acid and the cobalamins (vitamin B12).

These medications are administered three weeks before farrowing, three days after birth to the piglets, two weeks before weaning and every six months to the boars. Administration of the medicine was for the treatment of illnesses, to improve fertility and to prevent infections.

4.2 Identification of isolates

Of 199 clinical (100), veterinary (88) and environmental (11) samples collected, 275 Gram-negative isolates, making up 26 genera, were screened using the MicroScan® WalkAway system (Beckman Coulter Inc, California, USA). Among the 275 Gram-negative isolates identified, 79% (n=217) were categorised as belonging to the Enterobacterales family, representing 12 out of 26 genera (Figure 4.1). The genera in the Enterobacterales family included *Providencia* (n=4), *Enterobacter* (n=15), *Klebsiella* (n=25), *Hafnia* (n=1), *Proteus*

(n=23), *Escherichia* (n=86), *Serratia* (n=48), *Morganella* (n=4), *Shigella* (n=1), *Yersinia* (n=5), *Salmonella* (n=1), *Citrobacter* (n=1) and *Cedecea* (n=3).

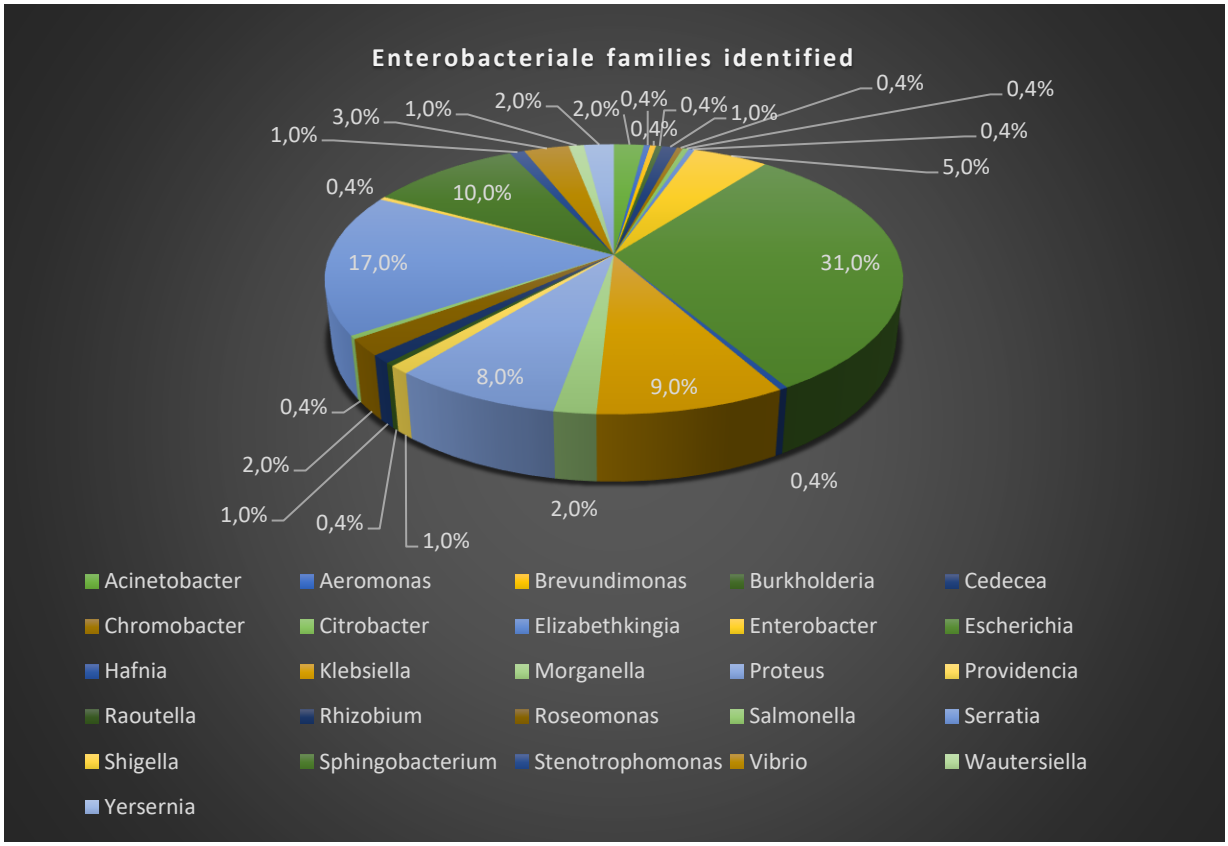

Figure 4.1: Illustration of Gram-negative bacterial isolates (n=275) from human, animal, and environmental samples classified into 26 genera of the Enterobacterales family. Identification of the families was achieved using by MicroScan® WalkAway automated system.

The most prevalent species identified clinically were *Serratia marcescens* (*S. marcescens*) (46%) followed by *Proteus mirabilis* (*P. mirabilis*) (19%). Among the porcine samples, *Escherichia coli* (*E. coli*) (76%), followed by *Enterobacter cloacae* (*E. cloacae*) (4%), was common. *E. coli* (52%) was predominant, followed by *E. cloacae* with 26% among environmental isolates (Figure 4.2).

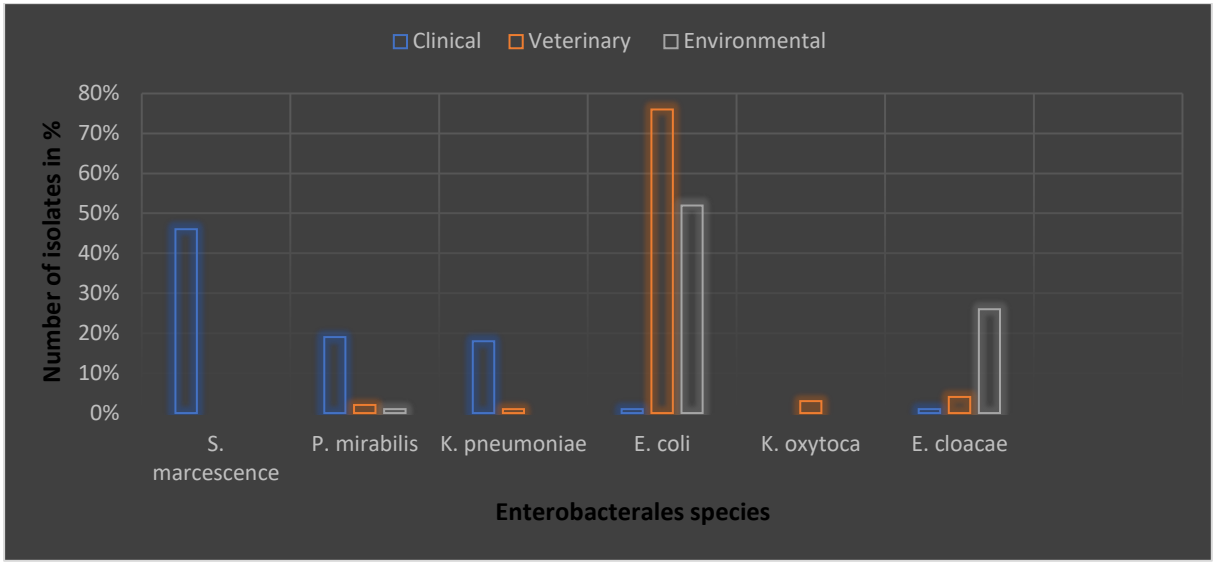

Figure 4.2: Distribution of the common identified Enterobacterales species among human, animals, and environmental samples.

Overall, *E. coli* and *S. marcescens* were the dominant species identified. The distribution of different Enterobacterales species of the isolates identified is summarised in Table 4.2.

Table 4.2: Identified Enterobacterales and their species using MicroScan® WalkAway system from human, animal, and environmental isolates.

Genera	Species
Klebsiella (n=26)	*K. pneumoniae*(n=19) *K. ozaenae* (n=2) *K. oxytoca* (n=3) *K. ascorbata* (n=2)
Hafnia (n=1)	*Hafnia alvei* (n=1)
Proteus (n=23)	*P. mirabilis* (n=21) *P. penneri* (n=1) *P. vulgaries* (n=1)
Providencia (n=4)	*P. stuartii* (n=1) *P. rettegeri* (n=1) *P. rustigianii* (2)
Enterobacter (n=15)	*E. cloacae* (n=15)
Escherichia (n=84)	*E. coli* (n=84)
Serratia (n=47)	*S. marcescens* (n=44) *S. odorifera* (n=3)
Morganella (n=4)	*M. morganii* (n=4)
Shigella (n=1)	*Shigella* (n=1)
Yersinia (n=5)	*Y. enterolitica* (n=1) *Y. ruckeri* (n=1) *Y. pseudotb* (n=3)
Salmonella (n=1)	*Salmonella enterica* (n=1)
Citrobacter (n=1)	*C. farmeri* (n=1)
Cedecea (n=3)	*Cedecea* sp.3 (n=2) *C. davisae* (n=1)

Among the porcine isolates, 118 isolates were identified as Gram-negative bacteria from both the first and second collection. *Escherichia coli* (76%) was the most common organism, followed by *Klebsiella* (8%) species (*K. oxytoca*, *K. ozaenae*, *K. pneumoniae* and *K. ascorbata*) and *E. cloacae* (4%). In the case of environmental isolates, 57 of the isolates were identified as Gram-negative bacteria, of which 47% (27/57) were Enterobacterales. *Escherichia coli* (*E. coli*) was the most common organism with 52%, followed by *E. cloacae* (26%), with the least common species identified being *P. mirabilis, P. penneri, P. vulgaris* and *Providencia rustigianii* (*P. rustigianii*). Overall, *Proteus* and *Providentia* species made up 4% of isolated organisms. From clinical samples *S. marcescens* was the most frequently identified species (46%), followed by *P. mirabilis* (19%) and *K. pneumoniae* (18%). The organism identified least often was *E. coli*. However, *E. coli* was dominant among both porcine and environmental samples at 75% and 52% respectively. A summary of Enterobacterales species isolated from clinical, porcine, and environmental sites is given in Table 4.3.

Table 4.3: The frequency of Enterobacterales organisms identified in clinical, animal and environmental isolates.

Species	**Clinical (n=95)**		**Porcine (n=95)**		**Environmental (n=27)**	
	No.	**Percentage %**	**No.**	**Percentage %**	**No.**	**Percentage %**
C. farmeri	0	0	1	1.1	0	0
C. davisae	0	0	0	0	1	3.7
Cedecea sp.3	0	0	2	2.1	0	0
E. cloacae	4	4.2	4	4.2	7	25.9
E. coli	1	1.1	71	74.7	14	51.9
Hafnia alvei	1	1.1	0	0	0	0
K. ascorbata	0	0	2	2.1	0	0
K. oxytoca	0	0	3	3.2	0	0
K. ozaenae	0	0	2	2.1	0	0
K. pneumoniae	18	18.9	1	1.1	0	0
M. morganii	4	4.2	0	0	0	0
P. mirabilis	18	18.9	2	2.1	1	3.7
P. penneri	0	0	0	0	1	3.7
P. rettgeri	1	1.1	0	0	0	0
P. rustigianii	0	0	1	1.1	1	3.7
P. stuartii	1	1.1	0	0	0	0
P. vulgaries	0	0	0	0	1	3.7
S. marcescens	44	46.3	0	0	0	0

S. odorifera	3	3.2	0	0	0	0
Salmonella enterica	0	0	1	1.1	0	0
Shigella	0	0	1	1.1	0	0
Y. enterolitica	0	0	1	1.1	0	0
Y. pseudotb	0	0	2	2.1	1	3.7
Y. ruckeri	0	0	1	1.1	0	0

The antimicrobial susceptibility profiles for colistin and carbapenems per isolate among clinical, porcine and environmental isolates are summarised in Appendix 3. Colistin resistance in all three groups of isolates was observed in *E. coli* and *P. mirabilis*. For other species, such as *K. pneumoniae* and *Y. pseudotubeculosis,* it were observed in two sites, while in the remaining organisms, such as *S. marcescens* and *K. oxytoca*, resistance was observed in one site, whether clinical, porcine or environmental. Carbapenem phenotypic resistance was observed only in *P. mirabilis* from all three groups. In other species, such as *E. coli* and *E. cloacae,* it was observed in two sites, while in the remaining organisms, such as *K. pneumoniae*, resistance was observed in one site, whether clinical, porcine, or environmental.

Table 4.4: Antimicrobial resistance profile of clinical, porcine and environmental Enterobacterales isolates using the automated MicroScan® WalkAway system.

Sample type	Colistin			Ertapenem		
	Resistant	**Susceptible**	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**
Clinical	84/95=88%	7/95=7%	0/95=0%	88/95=93%	3/95=3.2%	6/95=6.3%
Porcine	18/95=19%	67/95=71%	0/95=0%	13/95=14%	72/95=76%	3/95=3%
Effluents	9/27=33%	9/27=33%	0/27=0%	14/27=52%	9/27=33%	2/27=7%

Colistin resistance was predictably highest in clinical samples (88%), followed by environmental isolates (33%), with the least resistant isolates being from porcine samples (19%). Carbapenem-resistant clinical isolates (93%) were most frequent, followed by environmental isolates (52%), while the least resistant isolates were from porcine samples

(14%) (Table 4.4). BMD was found to be more reliable in all isolates, and it recorded higher MICs (increased resistance) than the MicroScan® WalkAway system (Table 4.5).

Table 4.5: Antimicrobial resistance profile (for colistin) of clinical, porcine and environmental Enterobacterales isolates using manual broth microdilution

	MicroScan® WalkAway system		**Broth microdilution method**	
	Resistant	**Susceptible**	**Resistant**	**Susceptible**
Clinical isolates	88% (n=96)	7%	90% (n=84)	10%
Porcine isolates	20% (n=89)	15%	83% (n=5)	16%
Environmental isolates	35% (n=25)	35%	100% (n=9)	0%

4.3 Polymerase chain reaction analysis

MCR genes (*mcr-1/2/3/4/5*) were not detected among the clinical, porcine or environmental Enterobacterales isolates. However, carbapenemase genes were detected in Enterobacterales isolates from all three sample sources (clinical, porcine and environmental).

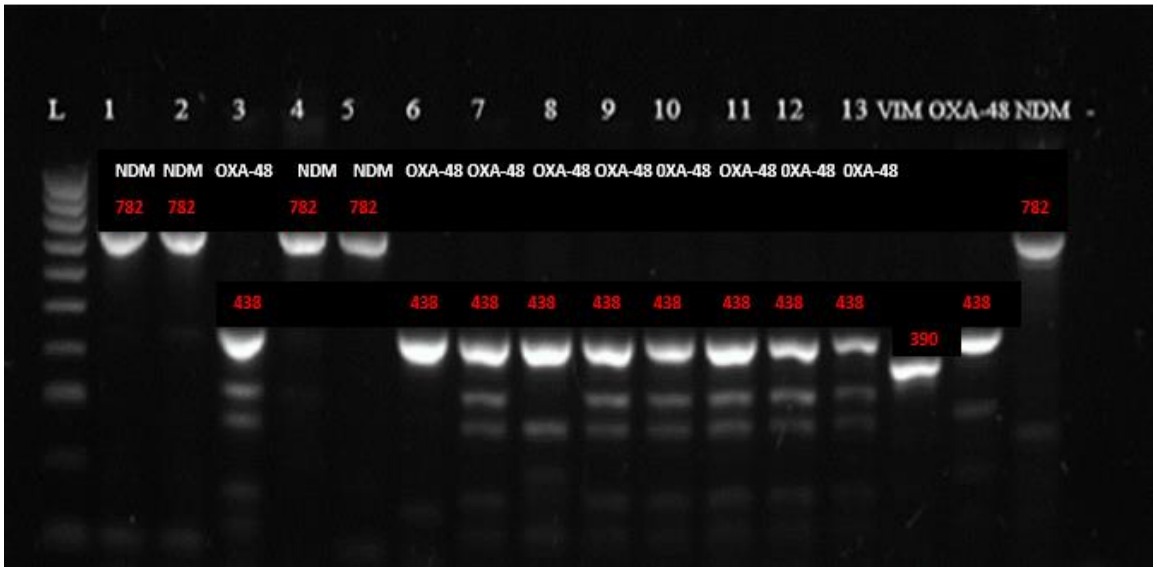

Figure 4.3: Representative gel electrophoresis image of M-PCR for carbapenemase genes (*bla*$_{VIM}$, *bla*$_{OXA-48}$ and *bla* $_{NDM}$). The expected amplicon sizes in base pairs (bp) for *bla*$_{VIM}$, *bla*$_{OXA-48}$ and *bla* $_{NDM}$ are 390 bp, 438 bp and 390 bp respectively. The ladder used is the 100 bp DNA ladder (Celtic Molecular Diagnostics, USA).

The carbapenemases detected were more prevalent among clinical isolates compared to porcine and environmental isolates, with *bla*$_{OXA-48}$ (78%) being the most detected carbapenemase followed by *bla*$_{NDM-1}$ (17%). However, no KPC genes (*bla*$_{KPC-2}$ and *bla*$_{KPC-3}$) and IMP-1 variant were detected in this study (Figures 4.3 and 4.4). The non-specific bands obtained were not expected because the genes in which they target were not the focus of the study although the bands represent other carbapenemase genes such as *bla*$_{SPM}$. This may be due to similar sequences of the targeted genes found in the other bands.

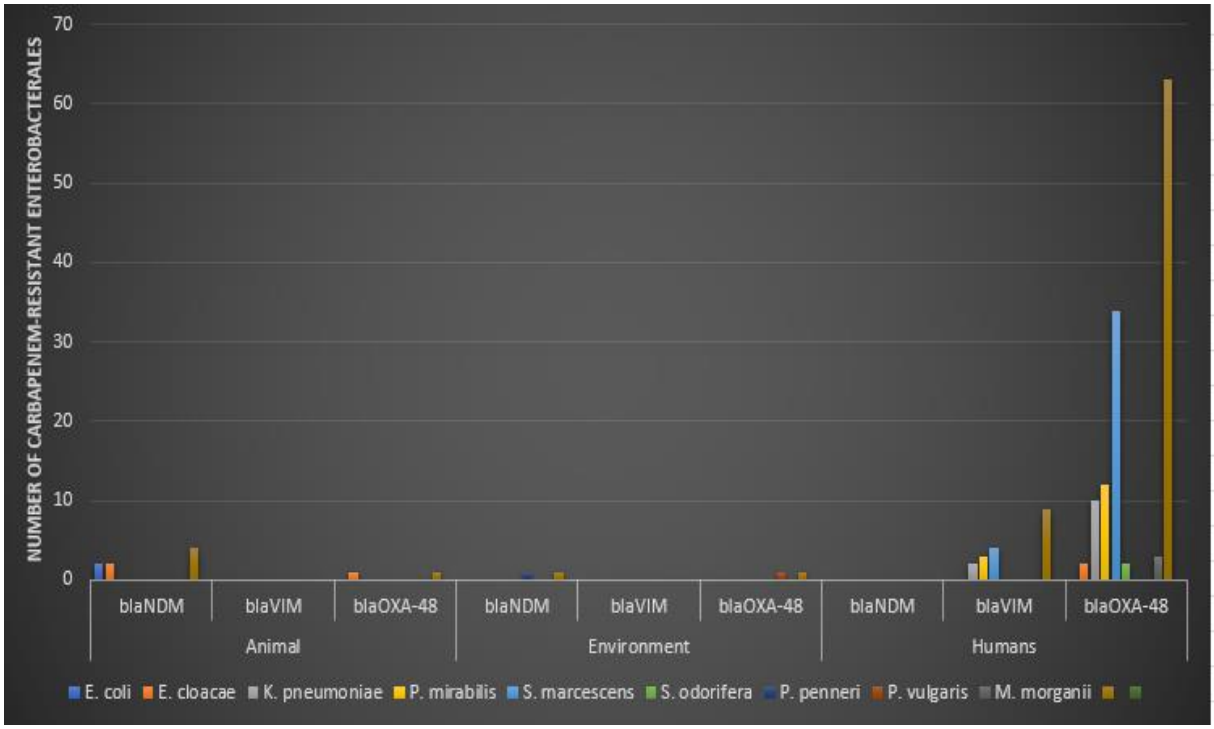

Figure 4.4: Carbapenemases detected in Enterobacterales isolated from pigs, pig farm effluents and humans.

4.4 Genomic analysis

4.4.1 Antimicrobial resistance gene analysis

The resistome from the 24 representative isolates demonstrated resistance in 20 of the isolates. The resistance genes obtained belonged to the following antibiotics: aminoglycosides, beta-lactams, colistin, fluoroquinolone, fosfomycin, lincosamide, macrolide, phenicol, quinolone, rifampicin, sulphonamide, tetracycline and trimethoprim. The resistance genes associated with each antibiotic are listed in Table 4.6 below.

Table 4.6: Resistance genes associated identified in 24 representative isolates.

Antibiotics	Resistance genes
Aminoglycosides	*aadA1, aadA24, ant (6)-19, aph (3')-IIc, aph (3'')-Ib* and *aph (6)-Id*
Beta-lactams	bla_{OXA}, bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{SRT-2}, bla_{DHA}, bla_{L-1}, bla_{NDM} and variants
Colistin	*mcr-9*
Fluoroquinolone	*aac (3)-I*, aac *(6')-Ib*, *aac (6')-Ic* and variants
Fosfomycin	*FosA* and *FosB*
Lincosamide	*Inu (G)*)
Macrolide	*mdfA*
Phenicol	*cat and catB3*
Quinolone	*OqxA, qnrB, qnrD1, qnrS1* and variants
Rifampicin	*AAR-3*
Sulphonamide	*sul1* and *sul2*
Tetracycline	*tet(A)*
Trimethoprim	*dfrG*

Beta-lactams were the leading resistance genes and the CTX-M family (n=37) and its variants were the most commonly identified among the isolates. Of the 24 isolates, PCR vs WGS demonstrated the following about the resistance genes. The same resistance genes (bla_{OXA-48} and bla_{NDM-1}) were reported in five isolates, nine isolates harboured different resistance genes (e.g PCR reported bla_{OXA-48} and WGS reported bla_{NDM-1}) and 18 isolates reported resistance genes from the same family, though with different variants (e.g PCR reported bla_{OXA-48} and WGS reported bla_{OXA-5}). Two of the isolates (C080 and C084) identified as *S. marcescens* species demonstrated a colistin resistance gene *mcr-9*. Both these isolates with *mcr-9* were isolated from clinical isolates and in addition harboured β-lactamase genes (bla_{OXA-5} and bla_{NDM-19}). In four isolates (MEC, SB009, C045 and C030) no resistance profiles were obtained. Five isolates (C022, C023, C026 and C027) reported to have bla_{VIM-1} using PCR but were not confirmed with WGS; instead, other carbapenemases, such as bla_{OXA-48} and bla_{NDM-19}, were demonstrated among the isolates.

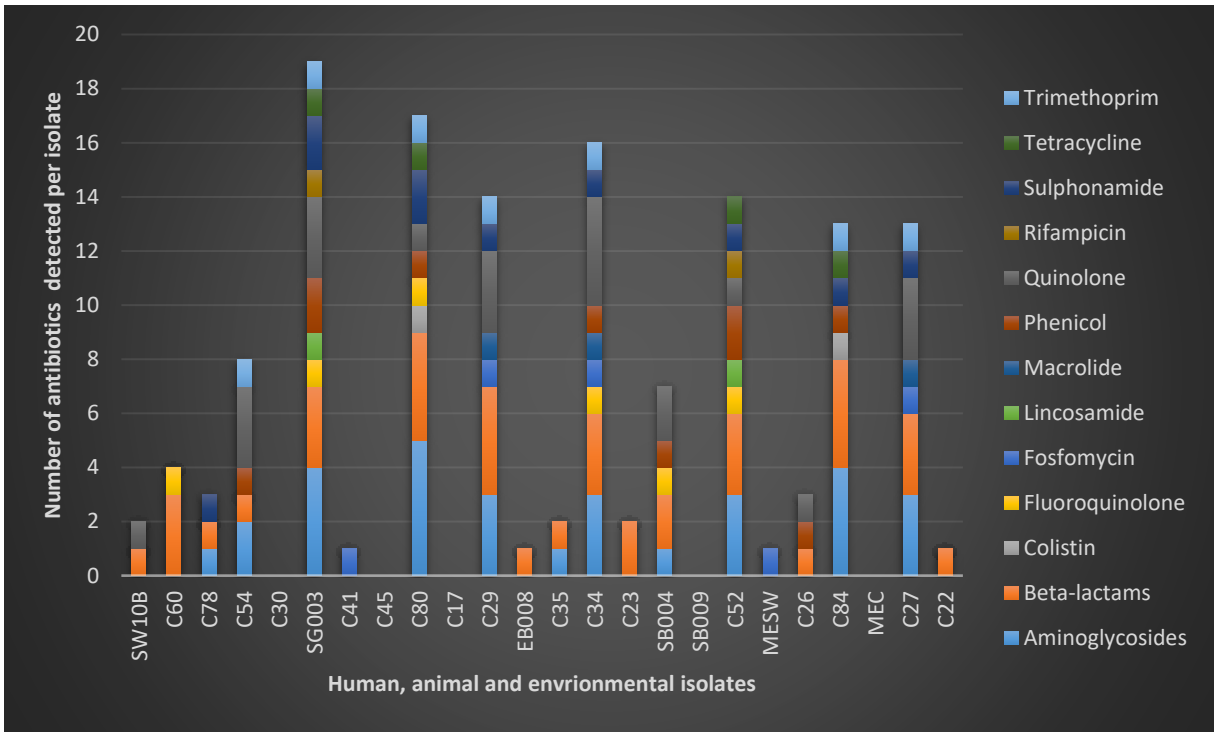

Figure 4.5: Distribution of resistance genes identified per Enterobacterales isolates from human, animal and the environmental samples. Symbols (C, SW/SG/SB and EB/ME) represent the source of samples as follows: C=clinical (human), SW/SG/SB= porcine and EB/ME= effluents.

4.5 Phylogenetic analysis of the representative isolates

The phylogenetic analysis was carried out to establish the evolutionary relationship between clinical, porcine, and environmental isolates resistant to colistin and carbapenems. The genomes sizes of the 24 isolates ranged from 4.5-5 Mb. Five trees were generated, representing the species identified. The analysis of the strains; EB008 (environment), SW10B (animal) and C080 and C084 (both humans) demonstrated the strains to be closely related with strains from the United States of America, Canada, China, Russia and Durban (South Africa). The bacterial species divergence within the trees may demonstrate horizontal gene transfer either within the same species and/or within Enterobacterales family.

4.5.1 Phylogenomic analysis of *P. mirabilis* strains

In this study (Figure 4.6), *P. mirabilis* C078, was found to be closely related with other *P. mirabilis* strains: strain 50664164 from Norway isolated from the urine of a patient in 2012, strain PmBC1123 isolated from a swine in China in 2017, PmPHII strain from France isolated from a human in 2012 and strain SCDR1 isolated from a human in Saudi Arabia in 2015. The C078 strain shared the clade with strains from the United States of America (USA) (FDAAROOS_284 and CRKOO56), from China (BC11-24) and strain SAS71 with an

unknown origin, as well as a South African strain PM005 isolated from a human in Pretoria. This strain was found to be resistant to ESBLs, of which WGS found many frameshifted proteins. The South African strain (PM005) was closely related to a Uruguayan strain, Pr2921. No other strains from porcine and environmental isolates were demonstrated in this tree. This phylogeny demonstrated that the majority of the strains were related to strains isolated in the USA (35 strains) followed by France (26 strains). Unlike the other phylogenies, only *Proteus* species were generated in this tree.

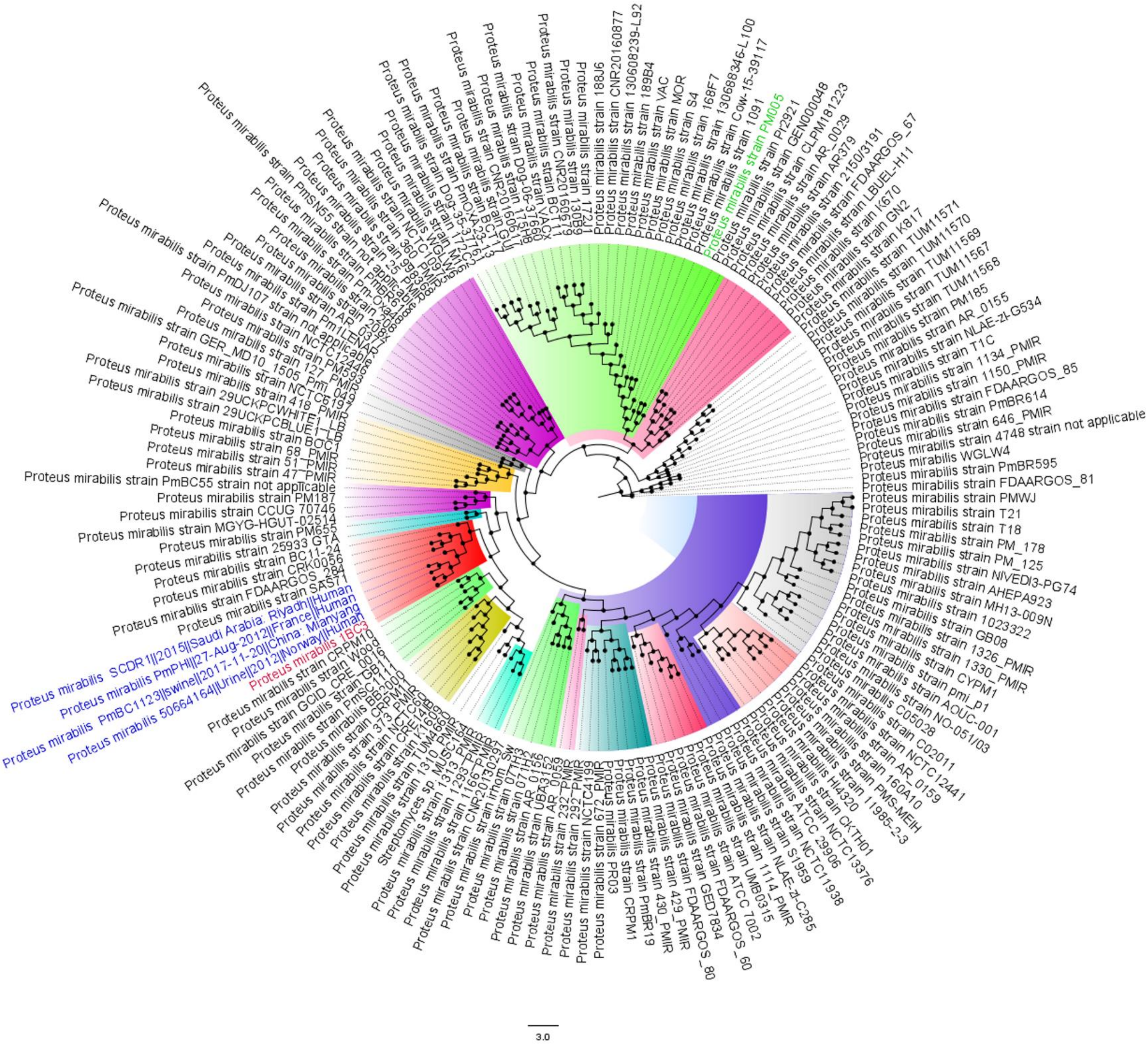

Figure 4.6: Phylogenetic representation of *P. mirabilis* strains globally. Red represents strains of this study; green represents other South African strains and blue represents strains within the same clade.

4.5.2 Phylogenomic analysis of *P. rettgeri* strains

The *Providencia rettgeri* strains (Figure 4.7) in this phylogeny were SW10B, SG003 and C052. Strain SW10B and SG003 were from porcine isolates, while strain C052 was isolated from a human. All three strains shared the same clade and occurred closely related with strains from Malaysia (strain MGF104), isolated in 2015, Spain (Pret_2032), isolated in 2015, and China (Pr-15-2-50), isolated in 2015, all from human specimens. The three strains shared clades with strains from the USA, Madagascar, and China, with the majority of strains originating from the USA. The strains were isolated from human, the environment and animals. Three other South African strains (PROO2, APW139-51 and PM005) were found within this clade. However, only strains PROO2 and APW139-51 shared the same clade with strains in this study. Apart from *Providencia* species the phylogeny demonstrated *Klebsiella*, *Serratia*, *Citrobacter*, *Escherichia*, *Pseudomonas* and *Proteus* species. Unlike other phylogenies, seven main clades were demonstrated in this tree and two smaller clades within the clades. The South African *P. mirabilis* strain PM005, *Pseudomonas aeruginosa* strain N15-01092 and *Streptomyces sp.* strain MUSC164 did not share lineages with the seven clades within the phylogeny. However, the *Streptomyces sp.* strain MUSC164 appears to be the main ancestor from which the seven clades originated.

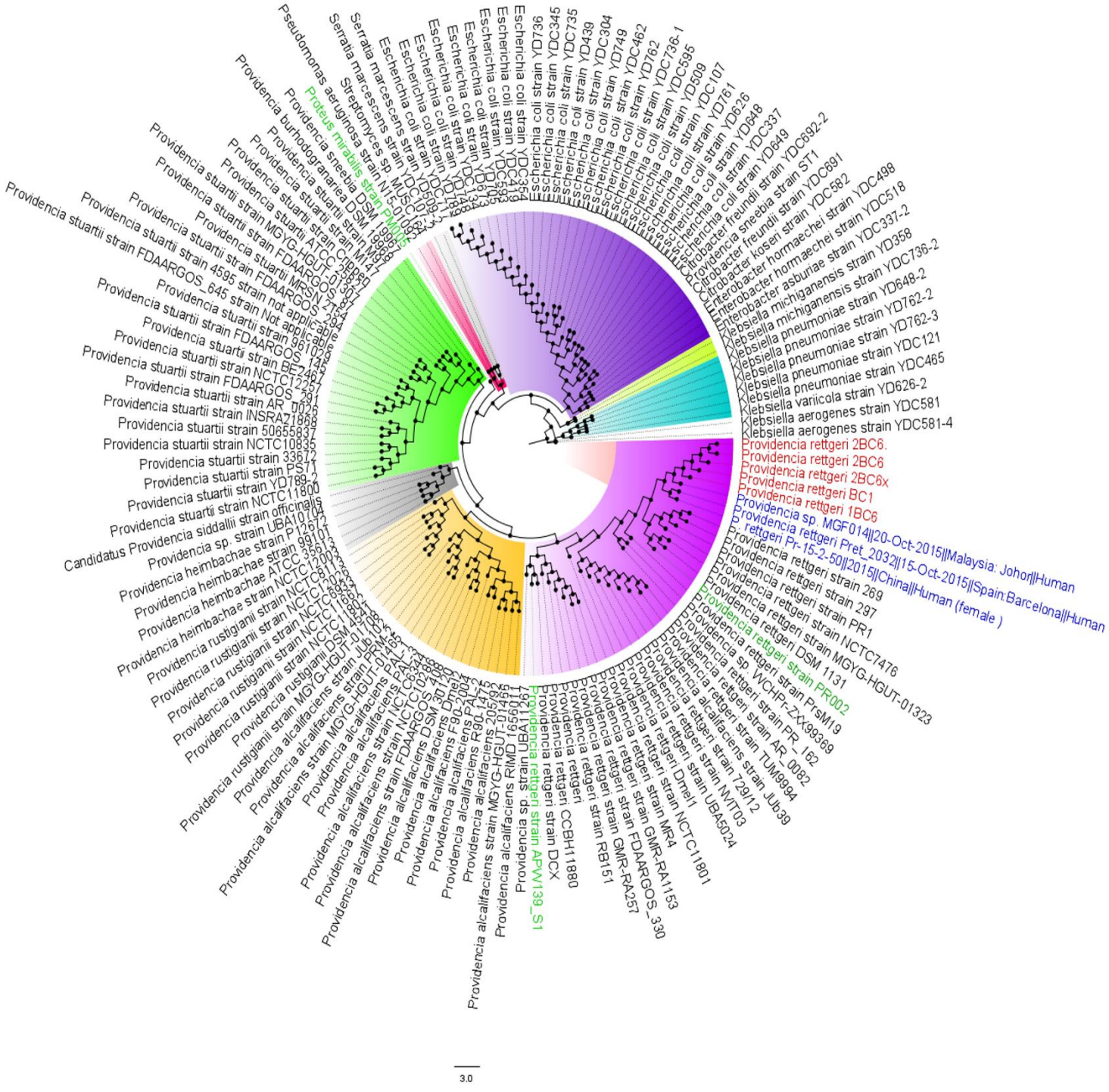

Figure 4.7: Phylogenetic representation of *Providencia rettgeri* strains. Red represents strains of this study; green represents other South African strains and blue represents strains within the same clade.

4.5.3 Phylogenomic analysis of *S. marcescens* strains

In this phylogeny 11 main clades were demonstrated with two smaller clades within the main clades (Figure 4.8). *Serratia marcescens* strains C080 and C084 are closely related strains and share a close lineage with strains 333 and 464, both isolated from a human in the USA. These two strains, although not closely related, share the clade with strains from the USA, Germany, Australia and Italy. Strain C030 also formed part of this clade; however, it was furthest from the two strains (C080 and C084) although this strain shares a close linage with the WVU-005 strain from the USA isolated from a human. Strains EB008 and SW10B, despite not forming part of a clade, shared a common ancestry. *Serratia marcescens* strain C078 was closely related to strains SK33y33t and NIG-Kp-4-18, both isolated from *Klebsiella pneumoniae*, strain NIG-Ec-5-18 from *Escherichia coli* and strain C022 from this study. The strains were isolated from humans in the USA, United Kingdom, Italy, Egypt and Brazil respectively. The US strains were the most common strains in the clade. The strains C054, SG003, C026, MEC, C035, C060 and C017 shared a clade. The clade demonstrated that C026 and C054, C017 and SG003 are closely related. No other South African strains were observed here.

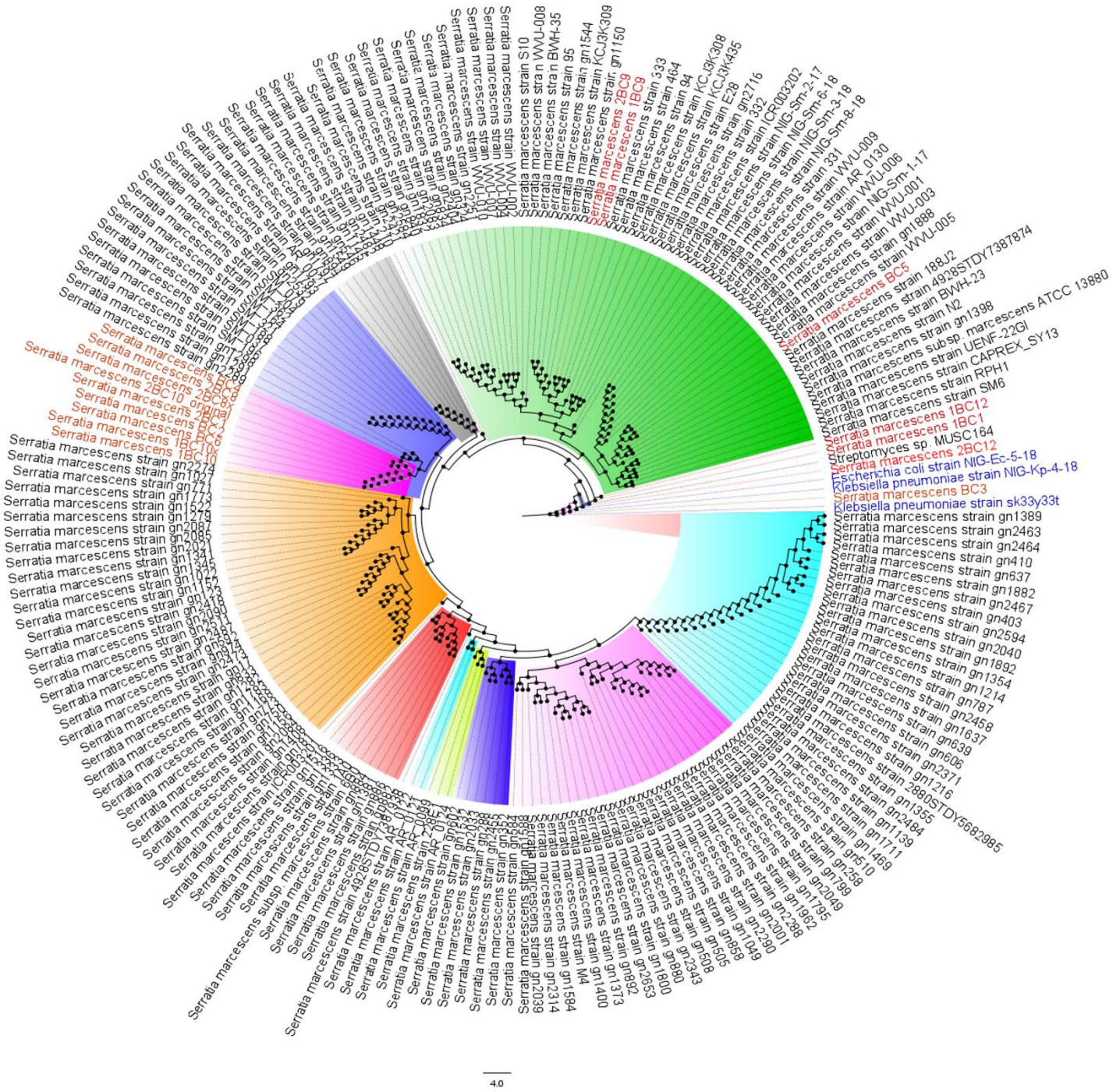

Figure 4.8: Phylogenetic representation of *Serratia marcescens* strains. Red represents strains of this study; green represents other South African strains and blue represents strains within the same clade.

Unlike the other trees, this tree (Figure 4.9) demonstrated *Serratia marcescens*, *E. coli* and *Klebsiella pneumoniae* strains. Strains EB008 (*S. marcescens*) isolated from an environment source in this study and CRE94 (*S. marcescens*) isolated in the USA from a human source appeared to be closely related. Strain C078 (*S. marcescens*) shared a clade with strains YD762-

3, YD648-2, YD762-2 isolated from humans in the USA. *S. marcescens* strains SW10B and C022, although not closely related to the YD strains, shared the same clade. *S. marcescens* strain C030 was closely related to *S. marcescens* strain AH0650_sm1 isolated from a human specimen from Australia. Strains C080 and C084 (both *S. marcescens*) were closely related and shared a clade with isolated stains 333 from the USA, 950172946/950172838 strain from South Africa, Durban and ML2637 from Gauteng (South Africa). The strains C080 and C084

appeared closely related to the ML2637 a *S. marcescens* strain. Eighteen main clades and four smaller clades were demonstrated.

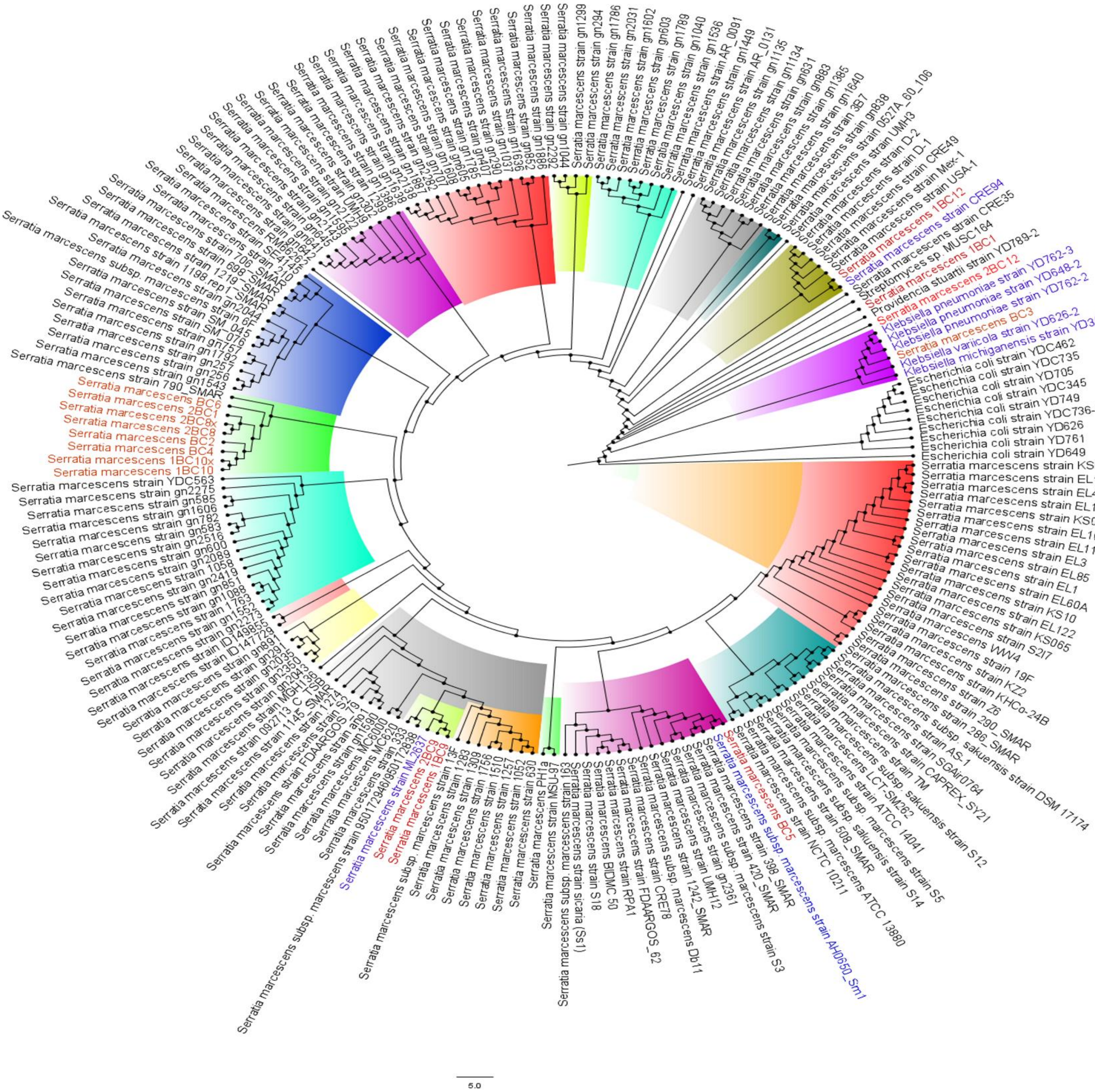

Figure 4.9: Phylogenetic representation of *Serratia marcescens*, *E. coli* and *Klebsiella pneumoniae* strains. Red represents strains of this study; green represents other South African strains and blue represents strains within the same clade.

In Figure 4.10, *Serratia marcescens* SW10B and EB008 strains were not closely related; however, they shared a common ancestry. *S. marcescens* strain C078 is paraphyletic to a clade with *Klebsiella pneumoniae* strains YDC465 and YDC121, *Klebsiella michiganensis* strain YDC736-, *Serratia marcescens* CQMUS2 strain from China, *Enterobacter hormaechei* strains YDC498, YDC519 and YDC337- in the USA; all were isolated from a human host. Strains C080 and C084 (*S. marcescens*) were closely related strains and shared clades with strains (950196656, 950164094, 950005607, 9501453777, 950174583, 950165859, 946252515, 950163386 and 950166381) all isolated from Durban, South Africa from a human host. The strains SG003, C022, C045, the ID147991 strain from Canada and RCE05_sm isolated from Russia shared a clade. The strains C060, C054, SW10B and strains from Canada (ID148587, ID1148696 and ID149856) isolated from a human host shared a clade and were closely related. Strain C030 shared a clade with strains originating commonly from the USA (UMH1, UMH10, UMH11, 1241-SMAR, 374-SMAR, 454-SMAR, 370-SMAR, 410-SMAR, and 395-SMAR), all isolated from a human host, as well as with strains from Germany (S13) and South Africa, North West province.

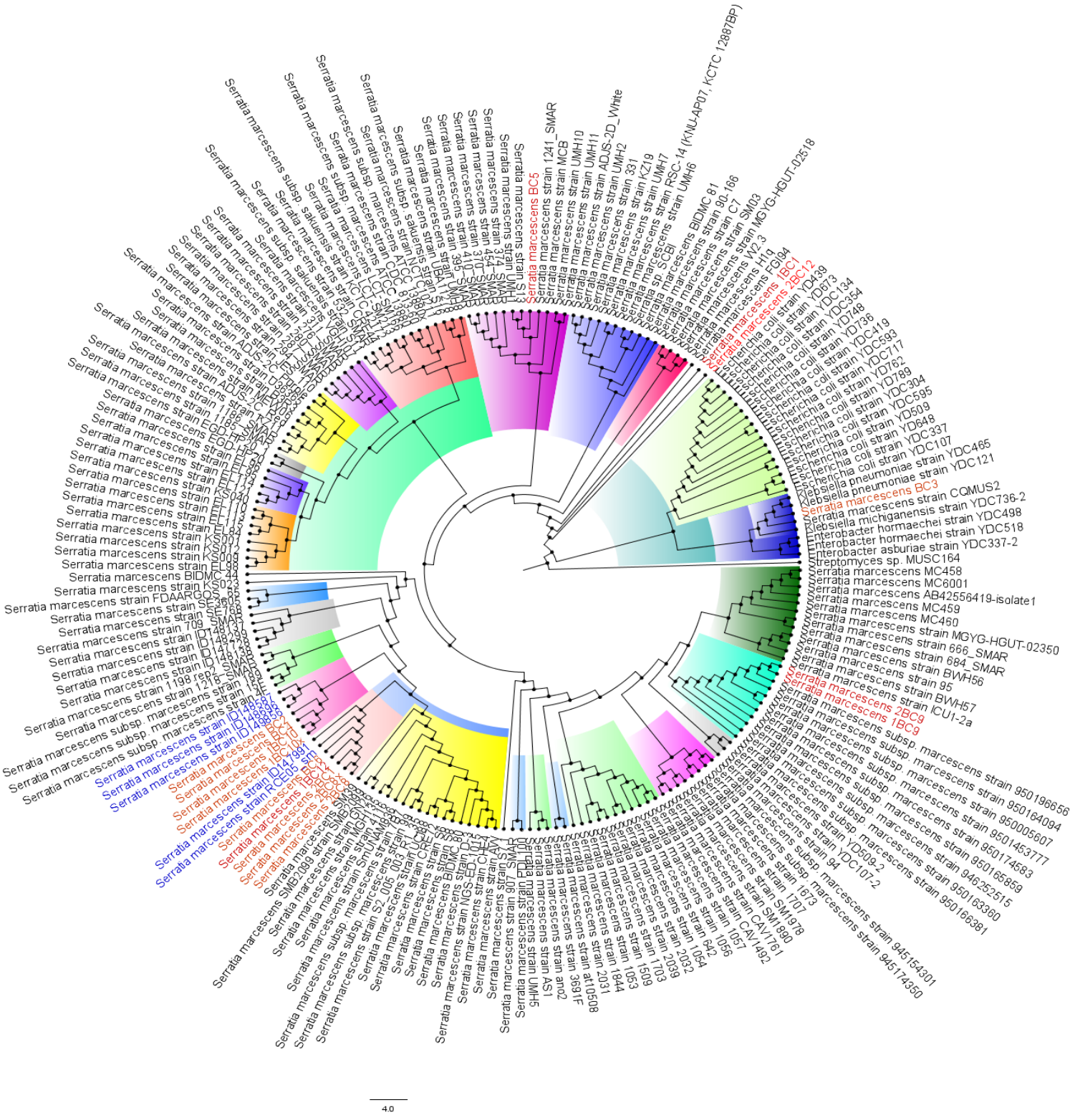

Figure 4.10: Phylogenetic representation of *Serratia marcescens* strains. Red represents strains of this study; green represents other South African strains and blue represents strains within the same clade.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

Resistance mechanisms associated with isolates in the study were found to confer resistance to diverse antibiotics and included both plasmid and chromosomally mediated resistance. There are few studies on the One Health approach to AMR. (Ribeiro *et al.*, 2016; Rousham *et al.*, 2018; Seni *et al.*, 2018). According to the researcher's knowledge, this is the first study in this setting that aimed to describe the molecular epidemiology and resistance mechanisms of colistin and carbapenem through a One Health approach.

In the study, *S. marcescens* and *P. mirabilis* were the most frequently identified Enterobacterales among the clinical samples, although previous studies have commonly identified *K. pneumoniae* and *E. coli* species, which were found to be associated with bloodstream infections (Perovic *et al.*, 2016; Mbelle *et al.*, 2020). In contrast, in both porcine and environmental isolates, *E. coli* was the most frequently identified among the Enterobacterales. Understandably, *E. coli* is one of the leading organisms affecting animal and human health. Clinical isolates were isolated from different specimen sites such as blood, superficial swabs, and catheter tips, with isolates from blood culture being most common. Demographically, most isolates were from males and the median age was 45 years.

Phenotypic resistance for both colistin and carbapenems was much more frequently observed in clinical isolates compared to veterinary and environmental isolates; the lowest incidence was found in environmental samples. Carbapenems in South Africa are not registered by SAHPRA for use in the veterinary sector and in 2015, colistin was restricted as a growth promoter in South Africa after the detection of the *mcr-1* gene (Poirel *et al.*, 2016). In addition, previous studies focused mainly on clinical isolates than environmental and animal samples. The incidence of phenotypic resistance of porcine and environmental samples are similar because both samples were collected from the same farm. Antibiotic use in animals influence the presence of antibiotic residues and resistance genes in the environment (McEwen & Collignon, 2018). Although our findings report that carbapenem resistance in animals and the environment is reportedly low, reports on carbapenem resistance in animals and the environment are increasingly reported globally (Piedra-Carrasco *et al.*, 2017; Köck *et al.*, 2018).

At all three sampling sites, no colistin resistance genes (*mcr-1*/-2/-3/-4/-5) were detected in any Enterobacterales isolates; this could be owing to the ban on colistin use in animals in South Africa that requires a veterinarian's approval based on evidence of susceptibility or a low incidence of use in veterinary medicine. In addition, in humans, colistin may only be used when all carbapenems are resistant. Nonetheless, colistin resistance has a low prevalence in South Africa. The identification of the *mcr*-1 gene, particularly in clinical isolates, has been reported (Snyman *et al.*, 2020). Three of the five carbapenemases screened for were identified and combinations included NDM and OXA-48 or VIM and OXA-48 genes. Of these carbapenemases, bla_{NDM} and bla_{OXA-48} were the most frequently identified from human sources. Although, bla_{VIM} was identified in some human isolates, the presence of bla_{VIM} carbapenemase in Enterobacterales appears rare in South Africa (Brink *et al.*, 2016; Singh-moodley & Perovic, 2016).

In the study, WGS identified *mcr-9* in two clinical *S. marcescens* isolates. Recently *mcr-9* has been reported following reports of *mcr-1* to *mcr-8*. MCR-9 gene was first reported in *Salmonella enterica* serotype Typhimurium (*S*. Typhimurium) in the USA (Carroll *et al.*, 2019). It is thought to commonly occur in *Enterobacter* spp., followed by *Klebsiella* spp., *Salmonella* spp., *Escherichia* spp., *Citrobacter* spp., *Leclercia* spp., *Cronobacter* spp., *Raoultella* spp. and *Phytobacter* spp. (Ling *et al.*, 2020). In South Africa it was first reported by Osei Sekyere and colleagues (2020) in *Enterobacter hormaechei* (*E. hormaechei*) strains, thus indicating its presence in South Africa (Osei Sekyere *et al.*, 2020). To the researcher's knowledge, this study is the second to report on *mcr-9* gene in South Africa and the first to be identified in *S. marcescens* isolates. This finding further demonstrates the diversity of *mcr-9* gene among the Enterobacterales family.

Due to the methodology adopted to detect MCR genes, only *mcr-1* to *mcr-5* genes were targeted and would explain the absence of *mcr-9* using PCR. The presence of *mcr-9* confirms the increasing reports of *mcr* genes, since to date *mcr-1* to *mcr-10* have been identified (Liu *et al.*, 2015; Xavier *et al.*, 2016; Borowiak *et al.*, 2017; Carattoli *et al.*, 2017; Yin *et a*l., 2017; AbuOun *et al.*, 2018; Wang *et al.*, 2018; Yang *et al.*, 2018; Carroll *et al.*, 2019; Wang *et al.*, 2020). Although the number of *mcr-1* gene reports in the country is low, reports of *mcr-9* raise concern; therefore, the risk of colistin resistance in South Africa cannot be ignored. Appropriate measures should be taken to mitigate this risk as it impacts on treatment and food security.

The absence of plasmid-mediated resistance genes among phenotypically resistant colistin isolates does not eliminate colistin resistance because resistance to colistin is not only plasmid-mediated. Therefore, it is possible that the isolates found to be resistant phenotypically may be chromosomally mediated or mediated by other unknown mechanisms (Sun *et al.*, 2018). Resistance to colistin in porcine isolates is largely associated with its use for prophylactic, metaphylactic and growth-promoting purposes for productivity and the health of the animals (van den Honer*t et al*., 2018). As a result, over use of colistin and use of antibiotics at low dosage promote selection pressure, disseminating resistance genes among animals (Rhouma *et al*., 2017). However, there has been a recent decline in resistance to colistin, which can be attributed to the colistin ban imposed in several countries such as China, USA, UK and South Africa (Lekagul *et al.*, 2019). This may also explain the low colistin resistance observed in the porcine isolates in this study. Resistance among CRE was commonly mediated by bla_{NDM} and $bla_{OXA\text{-}48}$, in agreement with previous studies (Coetzee & Brink, 2011; Singh-Moodley & Perovic, 2016;Singh-Moodley & Perovic., 2018).

In addition, WGS identified several other resistance genes in the Enterobacterales isolates that confer resistance to diverse antibiotic classes. These were aminoglycosides, beta-lactams, colistin, fluoroquinolone, fosfomycin, lincosamide, macrolide, phenicol, quinolone, rifampicin, sulphonamide, tetracycline, and trimethoprim. The β-lactamase enzymes were the most frequent; however, unlike M-PCR analysis, the CTX-M family of genes ($bla_{CTX\text{-}M\text{-}15}$) was commonly identified. Currently, β-lactam antibiotics such as carbapenems are a common causes of antibiotic resistance among Enterobacterales species (Iredell *et al.*, 2016). According to the WGS resistome, the isolates (n=15) harboured more than one group of resistance genes such as $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{OXA} genes in clinical, animal, and environmental isolates. The common combinations included $bla_{CTX\text{-}M}$ and bla_{OXA} genes, which was also noted in a local study by Ashika and colleagues (Singh-Moodley & Perovic, 2016). In developing countries such as South Africa, resistance genes combinations in all three sectors are concerning as the occurrence limit drugs used to treat these infections. When present in food producing animals and the environment, these genes threaten food security. Multiple resistance genes conferring resistance among Enterobacterales have become common in recent years and the reports are not confined to humans; the veterinary and environmental sectors have also been reporting on the trend (Singh-moodley & Perovic, 2016; Webb *et al.*, 2016; Ludden *et al.*, 2017).

Noting that carbapenems are not used in veterinary medicine and agricultural production in South Africa, resistance in porcine and environmental isolates is concerning. In this study, environmental isolates demonstrated increased resistance compared to porcine isolates, thus contradicting studies that demonstrated that resistance in animals is higher than environmental isolates. Antimicrobial resistance reports in animals without comparison to other environments such as non-agricultural environments, suggest that animals are the sole source of antimicrobial resistance (Collignon & McEwen, 2019); however, most of the antibiotics used are several years old and have an environmental origin. Moreover, the environment harbours bacterial strains with natural resistant traits (van den Honer*t et al*., 2018). Therefore, pressure is exerted on the environment such that in animals it promotes antimicrobial resistance, which then can be transmitted to other animals or humans (Agga *et al.*, 2015). Although humans and animals are not present around waste water, their excreta ends up in wastewater and are therefore indirectly present in the wastewater. (Collignon & McEwen, 2019). Therefore, our findings further demonstrate that the environment can serve as a reservoir for antimicrobial resistance and that the environment within animal farms could be the dominating reservoir for antimicrobial resistance.

Isolates reported to harbour bla_{VIM-1} by M-PCR analysis did not contain the gene in WGS analysis; instead the isolates demonstrated another carbapenemase gene (bla_{OXA-48}). The discrepancies between PCR and WGS analysis emphasize the superiority of WGS to PCR, because although PCR is a widely used method in most laboratories because of its ability to detect resistance genes, it is limited by its inability to detect resistance genes caused by other mechanisms such as efflux pumps and chromosomal mutations (Tagini & Greub, 2017). It thus offers limited information about the organism. Contrarily, WGS offers a wide range of information about the organism and low sequencing depth, as observed in this WGS analyses, could also account for the absence of this gene in the WGS sequences. Further, lack of standardization for WGS makes facilitation of antimicrobial resistance genes detection challenging (Ellington *et al.*, 2017).

The phylogenetic trees demonstrated evolutionary relatedness amongst Enterobacterales isolates from three different sites, signifying the influence the environment, animals and humans have on one another with regard to infection transmission, species survival and adaptation trends (Destoumieux-Garzón, 2018). To illustrate, the environment strain (EB008) and the porcine strain (SW10B), both identified as *S. marcescens* species, shared a common

ancestry. The strains C054, SG003, C026, MEC, C035, C060 and C017 also shared a clade. The strains in the study commonly shared clades with strains from the USA, as well as strains from different regions in South Africa. Strains C080 and C084 were closely related and shared a clade with isolated stains (333) from the USA, 950172946/950172838 strain from Durban (South Africa) and a strain ((ML2637) from Gauteng (South Africa). The strains (C080 and C084) were also closely related to strains (50196656, 950164094, 950005607, 9501453777, 950174583, 950165859, 946252515, 950163386 and 950166381, all isolated from Durban, South Africa, from a human host. The strains SG003, C022, C045, and ID147991 from Canada and RCE05_sm isolated from Russia shared a clade.

The common ancestry of different Enterobacterales isolates from different parts of the country and the world emphasize horizontal gene transfer and clonal expansion, which influence dissemination of these resistance genes among Enterobacterales. The findings of this study confirm the paucity of data on One Health studies in the current setting by focusing on humans, animals, and the environment. Colistin resistance in the environment and animals appears low and this could be owing to the ban in 2015 of antibiotics in agriculture and livestock. Although not emphasized in the study, carbapenem resistance in animals and the environment is concerning. More studies involving all three domains is recommended to give a full picture on the challenge of antimicrobial resistance in the country.

5.2 Concluding remarks

In this study, Enterobacterales organisms from clinical isolates, the environment and porcine samples from South Africa were examined using a One Health approach. They were phenotypically confirmed to be resistant to colistin and/or carbapenems. No *mcr* genes were detected by PCR; however, the *mcr-9* gene was identified in two clinical isolates by WGS. Colistin resistance is not only plasmid-mediated; thus, it is possible that the isolates found to be phenotypically resistant could be investigated for other resistance mechanisms such as chromosomally mediated mechanisms or other unknown mechanisms. Nonetheless, the presence of carbapenem resistance in the environmental isolates raises a public health concern because carbapenems are not used in veterinary medicine and agricultural production. WGS is a good tool to confirm and highlight the significance of One Health approach studies to highlight strains circulating in different geographical regions effectively.

5.4 Future research

Future research through One Health approach should be inclusive of humans, animals, and the environment to investigate antimicrobial resistance transmission patterns and the influence of mobile genetic elements such as plasmids on horizontal gene transfer. Although we managed to determine the resistance mechanisms between humans, animals, and the environment, we recommend the following for future research: 1) increased sample size and bigger geographical coverage inclusive of more farms, environmental sources, and hospitals. Direct comparison of these three sectors is recommended because humans, the environment, and animals are interrelated as they directly and/or indirectly have an effect on each other. Subsequently, this shall promote knowledge on the reservoir and epidemiology of resistance genes and infections arising from humans, the environment, and animals. Inadequate information on this menace is one of the driving factors of overuse of antimicrobials, which increases selection pressure from antibiotics, thus influencing antimicrobial resistance. 2) For clinical (human and animal) samples, association of variables such as age, gender, diagnosed condition and prior antibiotics used should be considered to aid better treatment by promoting appropriate antibiotics treatment and implementation of prevention strategies; ultimately, morbidity and mortality rates shall reduce. 3) Food animals sources can be looked into as well because although antibiotics administration may be reduced, resistance genes detected in the environment may contribute to resistance gene in foods. This is because wastewater may be used for irrigating crops, which may then be contaminated and used as food for pigs. Lastly, continued surveillance of resistance, as well as antimicrobial stewardship, are essential. These programmes create awareness and demonstrates the trends in antimicrobial resistance, which informs the veterinary and health sectors on better treatment, infection control, and antibiotic usage.

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5.6 Ethical approval

Ethics approval for this study was granted by the University of Pretoria Faculty of Health Sciences Research Ethics Committee (381/2019) and the Animal Ethics Committee of the University of Pretoria Ethics (H010-18).

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APPENDICES

Appendix 1: Antibiotic use survey questionnaire

Part B: Antibiotic use survey questionnaire

1. Do you give medicines/antibiotics to your animals?

Yes ☒

No ☐

1.a) If yes, what are the names of these medicine/antibiotics you give?

Sulphrim, Lithagard, Farrowsure, Respisure
Doctamox Vitamin B complex Iron

2. How are these medicines/antibiotics given to the animals? (Tick one/more answer(s)).

Food ☐

Water ☐

Injection ☒

Other ☐

If other, please explain below.

3. At what age of the animals are these medicines/antibiotics given? (Tick one/more answer(s)).

Week(s) ☒ Please indicate

3 weeks before Farrowing, 3days after birth piglets, 2week before wa

Month(s) ☒ Please indicate below

every month dewarm boars

Year(s) ☐ Please explain below.

N/A

3.a. How many times are these medicines/antibiotics given?

It depended on the State of animals e.g if it sick or pregnant

4. What are the reasons for giving these medicines to the animals? (Tick one/more)

Growth ☐

Illness ☒

Fertility ☒

Other ☐ Please explain below.

Thank you for your participation.

Appendix 2: Laboratory methods

Homogenisation of stool samples

1. Label all Durham tubes and plates.
2. Add 2 mL sterile saline into Durham tubes using a Pasteur pipette.
3. Inoculate a pea-sized portion of stool into a Durham tube containing saline.
4. Vortex tube for 2-4 seconds.
5. Swirl the supernatant using a cotton swab and lawn culture on the EMB agar.
6. Sterilise the forceps and cool in 70% ethanol.
7. Select an antibiotic disk using the forceps and place the disk onto the lawn-cultured EMB agar plate.
8. Incubate the EMB plate at 35ºC for 18 hours.

Eosine methylene blue agar preparation

1. Weigh 37.5 g of Eosine methylene blue (EMB) agar powder (Sigma Aldrich, South Africa) on a scale.
2. Suspend in a 1 L Erlenmeyer glass flask and add 1 L of distilled water.
3. Vigorously shake the Erlenmeyer flask and autoclave at 121ºC for 15 minutes.
4. Allow the EMB agar to cool at 60ºC.
5. After cooling, place the petri dishes in the petri dish filter.
6. Insert a tube from the petri dish filter into the Erlenmeyer flask to allow suspension of the agar onto the sterile petri dishes (18 mm x 20 mm); to oxidise the agar shake the EMB and suspend the precipitate.
7. Pour a volume of 10-12 ml into the petri dishes.
8. Cool until the EMB agar solidifies, then thoroughly label and store at 4 to 8ºC for a maximum of two months.

Serial dilution of effluents samples

1. Label all tubes (1-10)
2. Add 1 mL of effluent to 9 mL of saline solution.
3. Take 1 mL of the solution and add into a new tube.
4. Repeat step 2 with all 10 tubes.
5. Swirl the supernatant using a cotton swab and and lawn culture on the EMB agar.
6. Sterilise the forceps and cool in 70% ethanol.

7. Select an antibiotic disk using the forceps and place the disk onto the lawn-cultured EMB agar plate.
8. Incubate the EMB plate at 35ºC for 18 hours.

Oxidase testing: Filter method

1. Dissolve 0.1g Naphtyl-(1)-amin in 10 mL 96% ethanol (solution 1: Oxidase 1).
2. Dissolve 01g P-aminodimethylalinine in 10 mL deionized water (solution 2: Oxidase 2).
3. Mix equal volume (2 mL) of Oxidase 1 and Oxidase 2.
4. Pick an isolated colony with a stick and smear onto reagent-dampened filter paper.

Table 3.10: Antimicrobial resistance profile of the representative isolates Table 3.10: Antimicrobial resistance profile of the representative isolates

DNA extraction

1. Put 2000 µL brain heart infusion broth (BHI) into Eppendorf tube (2 mL).
2. Centrifuge at 5 000 g or RPM for 5 minutes.
3. Discard the supernatant and add 200 µL phosphate-buffered saline and mix using a pipette.
4. Put 200 µL of the mixture into a ZR BashingBead^{TM} lysis tube.
5. Then add 600 µL of the lysis solution to the same tube.
6. Secure in a bead beater fitted with a 2 mL tube holder assembly and process at maximum speed for ≥ 5 minutes.
7. Centrifuge the ZR BashingBead^{TM} lysis tube in a microcentrifuge at ≥ 10 000x g for 1 minute.
8. Transfer 400 µL supernatant to a Zymo-Spin^{TM} IV spin filter in a collection tube and centrifuge at 7000x g for minute.
9. Add 1 200 µL of fungal/bacterial DNA binding buffer to the filtrate in the collection tube.
10. Transfer 800 µL of the mixture from step 9 to a Zymo-Spin^{TM} IV Spin IIC column in a collection tube and centrifuge at 10 000x g for 1 minute.
11. Discard the flow through the collection tube and repeat step 10.
12. Add 200 µL DNA pre-wash buffer to a Zymo-Spin^{TM} IV Spin IIC column in a new collection tube and centrifuge at 10 000x g for 1 minute.

13. Add 500 µL fungal/bacterial DNA wash buffer to the Zymo-Spin™ IV Spin IIC column and centrifuge at 10 000x g for 1 minute.
14. Transfer the Zymo-Spin™ IV Spin IIC column to a clean 1.5 mL microcentrifuge tube and add 100 µL (minimum 35 µL) DNA elution buffer directly to the column.
15. Centrifuge at 10 000x g for 30 seconds to elute the DNA.

Store DNA in triplicate (two tubes in -80 ºC freezer and one tube in -20ºC).

Appendix 3: Colistin and carbapenem resistance profiles in Enterobacterales

MicroScan ®WalkAway colistin resistance profile in Enterobacterales

		Colistin antibiotic	
Species	**Source**	**n**	**% Resistance**
E. coli	Clinical	1	100
	Porcine	9	12.7
	Environmental	14	35.7
P. mirabilis	Clinical	18	100
	Porcine	2	100
	Environmental	1	100
E. cloacae	Clinical	NR	0
	Porcine	NR	0
	Environmental	NR	0
P. rustigianii	Clinical	0	0
	Porcine	1	100
	Environmental	0	0
K. pneumoniae	Clinical	18	88.9
	Porcine	1	0
	Environmental	0	0
Y. pseudotubeculosis	Clinical	0	0
	Porcine	2	100
	Environmental	1	100
K. ascorbata	Clinical	0	0
	Porcine	2	0
	Environmental	0	0
K. oxytoca	Clinical	0	0
	Porcine	3	66.7
	Environmental	0	0
K. ozaenae	Clinical	0	0
	Porcine	2	50
	Environmental	0	0
C. farmeri	Clinical	0	0
	Porcine	1	0
	Environmental	0	0
C. davisae	Clinical	0	0
	Porcine	0	0
	Environmental	1	100
***Cedecea* sp.3**	Clinical	0	0
	Porcine	2	66.7
	Environmental	0	0
Hafnia alvei	Clinical	1	100
	Porcine	0	0
	Environmental	0	0

M. morganii	Clinical	4	100
	Porcine	0	0
	Environmental	0	0
P. penneri	Clinical	0	0
	Porcine	0	0
	Environmental	1	100
P. rettegeri	Clinical	1	100
	Porcine	0	0
	Environmental	0	0
P. stuartii	Clinical	1	100
	Porcine	0	0
	Environmental	0	0
P. vulgaries	Clinical	0	0
	Porcine	0	0
	Environmental	1	100
S. marcescens	Clinical	44	88.6
	Porcine	0	0
	Environmental	0	0
S. odorifera	Clinical	3	100
	Porcine	0	0
	Environmental	0	0
Salmonella enterica	Clinical	0	0
	Porcine	1	0
	Environmental	0	0
Shigella	Clinical	0	0
	Porcine	1	100
	Environmental	0	0
Y. enterolitica	Clinical	0	0
	Porcine	1	0
	Environmental	0	0
Y. ruckeri	Clinical	0	0
	Porcine	1	0
	Environmental	0	0

MicroScan ®WalkAway colistin resistance profile in Enterobacterales

Carbapenem antibiotics			
Species	**Source**	**n**	**% Resistance**
E. coli	Clinical	1	0
	Porcine	9	9.8
	Environmental	14	35.7
P. mirabilis	Clinical	18	94.4
	Porcine	2	100
	Environmental	1	100
E. cloacae	Clinical	NR	50
	Porcine	NR	25
	Environmental	NR	100

P. rustigianii	Clinical	0	0
	Porcine	1	0
	Environmental	0	0
K. pneumoniae	Clinical	18	88.9
	Porcine	1	0
	Environmental	0	0
Y. pseudotb	Clinical	0	0
	Porcine	2	50
	Environmental	1	0
K. ascorbata	Clinical	0	0
	Porcine	2	0
	Environmental	0	0
K. oxytoca	Clinical	0	0
	Porcine	3	0
	Environmental	0	0
K. ozaenae	Clinical	0	0
	Porcine	2	0
	Environmental	0	0
C. farmeri	Clinical	0	0
	Porcine	1	100
	Environmental	0	0
C. davisae	Clinical	0	0
	Porcine	0	0
	Environmental	1	0
***Cedecea* sp.3**	Clinical	0	0
	Porcine	2	50
	Environmental	0	0
Hafnia alvei	Clinical	1	100
	Porcine	0	0
	Environmental	0	0
M. morganii	Clinical	4	75
	Porcine	0	0
	Environmental	0	0
P. penneri	Clinical	0	0

	Porcine	0	0
	Environmental	1	100
P. rettgeri	Clinical	1	100
	Porcine	0	0
	Environmental	0	0
P. stuartiii	Clinical	1	0
	Porcine	0	0
	Environmental	0	0
P. vulgaries	Clinical	0	0
	Porcine	0	0
	Environmental	1	100
S. marcescens	Clinical	44	97.7
	Porcine	0	0
	Environmental	0	0
S. odorifera	Clinical	3	100
	Porcine	0	0
	Environmental	0	0
Salmonella enterica	Clinical	0	0
	Porcine	1	0
	Environmental	0	0
Shigella	Clinical	0	0
	Porcine	1	100
	Environmental	0	0
Y. enterolitica	Clinical	0	0
	Porcine	1	100
	Environmental	0	0
Y. ruckeri	Clinical	0	0
	Porcine	1	0
	Environmental	0	0

Appendix 4 Research ethics

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

13 November 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 381/2018
Title: Molecular epidemiology and mechanisms of colistin and carbapenem resistance in Enterobacteriaceae from clinical isolates, the environment and porcine samples in Pretoria, South Africa.

Dear Miss D Bogoshi

The **Annual Renewal** as supported by documents received between 2019-10-17 and 2019-11-06 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-11-06.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-11-13.
- Please remember to use your protocol number (381/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 1

PROJECT TITLE	**Molecular epidemiology and mechanisms of colistin and carbapenem resistance in *Enterobacteriaceae* from clinical isolates, the environment and porcine samples in Pretoria, South Africa**
PROJECT NUMBER	**H010-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**D Begoshi**

STUDENT NUMBER (where applicable)	**U_12236102**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES/SAMPLES	**Pigs**
NUMBER OF ANIMALS	**84**
Approval period to use animals for research/testing purposes	**March 2019 – March 2020**
SUPERVISOR	**Prof. NM Mbelle**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 5 March 2019
CHAIRMAN: UP Animal Ethics Committee	Signature

S428S-15