Molecular Characterization of Carbapenem Resistant Extended Spectrum Beta-Lactamase (ESBL) Producing *Enterobacteriaceae*

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Dissertation accepted in fulfilment of the requirements for the degree *Master of Science in Biology* at the North West University

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DECLARATION

I, Lungisile Tshitshi, declare that the dissertation entitled **Molecular Characterization of Carbapenem Resistant Extended Spectrum Beta-Lactamase (ESBL) Producing *Enterobacteriaceae***, submitted for the Master of Science in Biology (Molecular Microbiology) degree at the North-West University, Mafikeng Campus, has not previously been submitted by me to this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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DEDICATION

I dedicate this work to my late father Mr. P.M Tshitshi and my mother Mrs. V.N Tshitshi who laid the strong and solid foundation to all that I am today. I also dedicate the work to my siblings whom I am grateful for their undying love and support. My nephews Zuko, Mosuli, Xabiso, Putuma, Luthando, Khaka, Ndomelele, Avukile and Mayine and My nieces Sibulela, Sinemihlali, Somila, Silindokuhle, Ngazibini, Lusanele, Zimi, Ndalo and Unabantu. I pray that, you will develop a passion for education and remember that the sky will never be your limit through prayer, hard work and dedication. I love you all so much.

"Education is the most powerful weapon which you can use to change the world"

Dr Nelson R Mandela

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I thank God that He made all things come together for my benefit, truly in Him I have a good, good Father.

ABSTRACT

Carbapenem resistance amongst the Extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* is increasing worldwide, leading to the Centre for Disease Control and Prevention declaring them an urgent public health threat. Animal species are believed to be a source of most disease causing pathogens in humans, this being the case, despite all the preventative measures in place. Carbapenem-resistant *Enterobacteriaceae* (CRE) are also believed to have originated from animals although, Carbapenems have not been approved for Veterinary use anywhere in the world. Carbapenem-resistant *Enterobacteriaceae* confer resistance to most clinically prescribed antibiotics, making it almost impossible to treat diseases they cause. This, together with the lack in newly developed antibiotics has led to use of previously phased out antibiotics associated with toxicity and efficacy in humans, such as fosfomycin, polymyxins and aminoglycosides. In this study Carbapenem-resistant extended spectrum β-lactamase producing *Enterobacteriaceae* from cattle faeces were isolated and characterized. Samples were collected from commercial and communal farms in the North West Province of South Africa.

Three biochemical tests namely the oxidase test, Simmon's Citrate test and Triple Sugar Iron test were used as preliminary tests for screening isolates belonging to the *Enterobacteriaceae* family. The preliminary isolates were phenotypically tested for resistance to Carbapenem antibiotics using the disk diffusion test and also their growth on Brilliance ™ Carbapenem Resistance *Enterobacteriaceae* agar. Modified Hodge Test was performed on all isolates that were either resistant or intermediate to Imipenem antibiotic determining their ability to produce Carbapenemase enzyme. Extended spectrum β-lactamase (ESBL) production amongst carbapenem resistant isolates was phenotypically tested based on chromogenic behaviour on Brilliance ™ Extended Spectrum β-Lactamase (ESBL) agar. Multi drug resistance profiling was determined amongst the isolates using 13 antibiotics namely Piperacillin, Ticarcillin, Amoxicillin, Amoxicillin-clavulanate, Cephalothin, Cefoxitin, Cefuroxime, Cefotaxime,

Ceftazidime, Ceftiofur, Cefepime, Aztreonam and Ciprofloxacin. The isolates were characterized as either *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* species or *Proteus mirabilis* using species specific primers. Presence of both extended spectrum β-lactamase and Carbapenem resistance encoding genes was tested on all the resistant isolates.

The 233 samples collected yielded 314 isolates of which a total of 280 presumptive isolates were obtained after the biochemical tests were performed. Some 67.5% (189) of the presumptive isolates were resistant to at least one Carbapenem antibiotic with Meropenem resistance at 68.57%, Imipenem 67.86%, Ertapenem 66.07% and Doripenem at 63.57%. The results on Brilliance™ Carbapenem Resistance *Enterobacteriaceae* agar however, showed a resistance of 69.29% (194), proving the unreliability and human error possibilities associated with phenotypic tests. Of these 53.10% were from the *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) group, 35.03% as *Escherichia coli*, 8.25% as Non-*Enterobacteriaceae* and 3.61% belonging to *Proteus*, *Morganella* and *Providencia* group. Only 19.14% (31) of the isolates tested with Modified Hodge test were positive for Carbapenemase production. Of these isolates 151(77.84%) were positive for Extended Spectrum β-Lactamase (ESBL) production, when cultured on Brilliance ™ Extended Spectrum β-Lactamase (ESBL) agar. Of these 54.97% belonged to *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) group, 33.77% were *Escherichia coli*, 7.95% *Salmonella*, *Acinetobacter* and other and 3.31% were *Proteus*, *Morganella* and *Providencia* group. About 94.04% of the Carbapenem resistant Extended spectrum β-lactamase producing *Enterobacteriaceae* were multi-drug resistant where CTX was at 59.60%, ATM 54.30%, CXM 47.68%, PRL 43.71%, FOX 42.38%, KF 41.06%, CAZ 41.06%, EFT 39.74%, A 39.74%, TC 39.07%, CPM 19.87%, CIP 12.58%, and AMC 9.27% resistance.

Some 66 (43.17%) of the isolates were identified as *Klebsiella pneumoniae*, 52 (33.77%) as *Escherichia coli*, 7 (4.6%) as *Salmonella* species and 2 (1.32%) as *Proteus mirabilis* using species specific Polymerase Chain Reaction (PCR). The most prevalent Carbapenem Resistant *Enterobacteriaceae* gene

in the current study was bla_{KPC} 35.76%, followed by bla_{NDM} at 20.53%, bla_{GES} at 17.88%, bla_{OXA-48} at 10.60%, bla_{VIM} at 6.62% and bla_{OXA-23} with 3.31%. 8 (5.29%) of the isolates were found to harbour multiple genes with the combination of bla_{KPC}+ bla_{OXA-23}, bla_{KPC}+ bla_{NDM} and bla_{GES}+ bla_{OXA-48} at 2.65%, 1.32% and 1.32% respectively. The most prevalent extended spectrum β-lactamase gene amongst the isolates was bla_{SHV} at 33.11%, followed by bla_{TEM} with 22.52%, then bla_{CTX-M} with 20.53% and bla_{OXA} at 11.26%. 19 (12.58%) of the isolates harboured multiple genes, where 7.28% had a bla_{OXA}+ bla_{CTX-M} combination and 5.30% had the bla_{SHV}+ bla_{TEM} combination.

Similar to clinical cases *Klebsiella pneumoniae* has been found to be the most prevalent species in Carbapenem resistant extended spectrum β-lactamase producing *Enterobacteriaceae* isolated in this study. The isolation of multiple genes for both extended spectrum β-lactamase and Carbapenem resistant *Enterobacteriaceae* genes has been recorded in clinical isolates in South Africa and the current study proved their existence in animals also. Cattle from both commercial and communal farms were found to be carriers of multi-drug resistant Carbapenem resistant Extended spectrum β-lactamase producing *Enterobacteriaceae* in the current study. These multi drug resistant Carbapenem resistant Extended spectrum β-lactamase producing *Enterobacteriaceae* were successfully characterised and the genes they harbour identified from the commonly isolated species.

TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENTS iii

ABSTRACT iv

TABLE OF CONTENTS vii

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF APPENDICES xiv

DEFINITION OF CONCEPTS xv

LIST OF ABBREVIATIONS xix

CHAPTER 1 1

INTRODUCTION AND BACKGROUND 1

1.1 Background 1

1.2 Problem Statement 4

1.3 Study Aim 6

1.4 Study Objectives 6

CHAPTER 2 7

LITERATURE REVIEW 7

2.1 The Family *Enterobacteriaceae* 7

2.2 Antibiotics and their Mechanisms of Action 10

2.3 Classification and Mechanisms of antimicrobial resistance 11

2.4 Beta-Lactam antibiotics 13

2.5 *Enterobacteriaceae* resistance to β-lactam antibiotics 14

2.5.1 Target site mutations or alterations 15
2.5.2 Efflux Pumps 15
2.5.3 Beta-lactamase production 15
2.6 Extended Spectrum Beta-Lactamase producing *Enterobacteriaceae* 16
2.6.1 Classification of Beta-lactamases 17
2.6.2 Types of ESBL's 19
2.6.3 Treatment options for ESBL infections 20
2.7 Carbapenem Antibiotics 21
2.7.1 Clinically prescribed Carbapenems 22
2.8 Carbapenem Resistant *Enterobacteriaceae* 23
2.8.1 Mechanism of resistance 24
2.8.2 Classification of Carbapenemase Producing *Enterobacteriaceae* (CPE) 25
2.8.3 Prevalence of Carbapenem Resistant *Enterobacteriaceae* in South Africa 26
2.8.4 Treatment options for carbapenem resistant *Enterobacteriaceae* (CRE) infections 27
2.9 Use of antibiotics in animal species and transmission of resistant organisms to humans 29
CHAPTER 3 31
3.0 MATERIALS AND METHODS 31
3.1 Sampling Information 31
3.1.1 Sample size determination 31
3.1.2 Sampling Sites 32
3.1.3 Collection of faecal samples from cattle 32
3.2 Ethical Clearance 33
3.3 Control Strains 33
3.4 Sample Processing and Bacterial Isolation 33
3.4.1 Purification of Bacteria Isolates 33

3.5 Bacterial Identification by Cellular Morphology 34
3.6 Preliminary Identification of *Enterobacteriaceae* Isolates 34
3.6.1 Oxidase Test 34
3.6.2 Triple Sugar Iron (TSI) Test 34
3.6.3 Simons Citrate Test 37
3.7 Phenotypic detection of carbapenem resistant *Enterobacteriaceae* (CRE) 37
3.7.1 Disk Diffusion Test to Detect Carbapenem Resistant Isolates 37
3.7.2 Detection of Carbapenemase Production Using the Modified Hodge Test (MHT) 38
3.7.3 Culture on Brilliance™ Carbapenem Resistant *Enterobacteriaceae* (CRE) Agar 39
3.8 Phenotypic Detection of Extended Spectrum Beta-Lactamase (ESBL) Producing Isolates 40
3.8.1 Culture on Brilliance Extended Spectrum Beta-Lactamase Agar 40
3.8.2 Antimicrobial Resistance Profiles of the Isolates 40
3.9 Molecular Characterisation of *Enterobacteriaceae* 43
3.9.1 DNA Extraction 43
3.9.2 Determination of the quality and quantity of DNA extracted 43
3.9.3 Amplification of 16S rRNA gene fragments 43
3.9.4 Genus Specific PCR to Confirm Identities of *Enterobacteriaceae* 46
3.10 Molecular Detection of the ESBLs Encoding Genes in *Enterobacteriaceae* 46
3.10.1 Detection of Bla_{CTX-M}, Bla_{OXA}, Bla_{SHV}, and Bla_{TEM} genes 46
3.11 Molecular Detection of Carbapenem Resistant *Enterobacteriaceae* 47
3.11.1 Detection of Carbapenemase Resistance Genes in isolates using Multiplex PCR 47
3.12 Agarose Gel Electrophoresis and Visualization 51
CHAPTER 4 52
RESULTS AND INTERPRETATION 52
4.1 Identification of *Enterobacteriaceae* Isolates 52

4.1.1 Colony and Cellular Morphology 52

4.1.2 Preliminary Biochemical Tests 52

4.2 Proportion of *Enterobacteriaceae* that possessed Carbapenem Resistance phenotypes 53

4.2.1 Proportion of isolates with resistance to Carbapenem antibiotics 53

4.2.2 Detection of Carbapenemase producing phenotypes among *Enterobacteriaceae* using the Modified Hodge test 54

4.2.3 Brilliance™ Carbapenem Resistant *Enterobacteriaceae* Agar 55

4.3 Proportion of *Enterobacteriaceae* with ESBL and Carbapenem producing traits 56

4.3.1 Brilliance™ ESBL Agar 56

4.3.2 Antimicrobial Resistance Profile 57

4.4 Molecular Characterization of ESBL producing Carbapenem Resistant *Enterobacteriaceae* 59

4.4.1 16S rRNA Gene PCR analysis 59

4.4.3 Genus Specific PCR 60

4.4.4 Detection of CRE resistant genes in isolates using Multiplex PCR analysis 63

4.4.5 Proportion of isolates with ESBL genes 66

CHAPTER 5 69

DISCUSSIONS AND INTERPRETATION 69

CHAPTER 6 82

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS 82

6.1 Conclusion 82

6.2 Limitations of the study 84

6.3 Recommendations 85

BIBLIOGRAPHY 86

APPENDICES 112

LIST OF TABLES

Table 3.1: Sample stations and the total number of samples collected during the study 32

Table 3.2: Ingredients of the Triple Sugar Iron Agar and Reactions Interpretation 36

Table 3.3: Characteristics of the *Enterobacteriaceae* Family Species on the Triple Sugar Iron Test 36

Table 3.4: Expected results for members of the family *Enterobacteriaceae* based on the Simons Citrate agar 37

Table 3.5: Antibiotics that were used to screen for Carbapenem Resistant *Enterobacteriaceae* (CRE) 38

Table 3.6: Morphological characteristics of *Enterobacteriaceae* on Brilliance™ CRE agar 39

Table 3.7: Morphological characteristics of *Enterobacteriaceae* on Brilliance ESBL agar 40

Table 3.8: Details of the different antibiotics used in the study for antibiotic susceptibility test of *Enterobacteriaceae* 42

Table 3.9: Oligonucleotide primers used for 16S rRNA amplification and PCR conditions 45

Table 3.10: Oligonucleotide primers used for genus specific PCR amplification and amplification conditions 48

Table 3.11: Oligonucleotide primers used for the detection of ESBL genes and multiplex-PCR amplification conditions 49

Table 3.12: Oligonucleotide primers used for the detection of Carbapenemase resistance genes and multiplex-PCR amplification conditions 50

Table 4.1 Biochemical Tests Results of the Presumptive Isolates 53

Table 4.2: Distribution of carbapenem resistant isolates according to collection location. 54

Table 4.3: Modified Hodge Test results for isolates susceptible or intermediate resistant to Imipenem 55

Table 4.4: Colour Production as Observed on the Brilliance™ CRE Agar 55

Table 4.5: Different colour morphology of bacteria observed on the Brilliance™ ESBL Agar 57

LIST OF FIGURES

Figure: 2.1: Gram negative bacteria cell wall, showing bacterial outer membrane, periplasmic space and peptidoglycan and plasma membrane 9

Figure 2.2: Methods of antibiotic resistance acquisition. A- Bacterial transformation, B- Bacterial transduction and C- Bacterial conjugation. 13

Figure 4.1: Susceptibility test results for different Carbapenem antibiotics 54

Figure 4.2: The presence of Carbapenem resistant *Enterobacteriaceae* as observed on the Brilliance™ CRE agar 56

Figure 4.3: The persistence of Carbapenem resistant *Enterobacteriaceae* isolates on Brilliance ESBL agar 57

Figure 4.4: Percentage antibiotics resistance of isolates tested with Disk Diffusion test 59

Figure 4.5: Agarose gel (1% w/v) image showing universal 16S rRNA gene fragments (1420 bp) amplified from the selected isolates and control strains. Lane 1= 1 kilo base pairs O'GeneRuler, Lane 2= *K. pneumoniae* (ATCC 13883) control strain, Lane 3= *E. coli* (ATCC 25922) control strain, Lane 4 - 23 = gene fragments of the tested isolates. 60

Figure 4.6: Agarose gel (1% w/v) image showing PCR products for *uidA* (556bp) gene fragments. Lane M = 100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1= *uidA* gene fragments amplified from *E. coli* (ATCC 25922) and lanes 2-19= *uidA* gene fragments amplified from *E. coli* isolates. 60

Figure 4.7: Agarose gel (1% w/v) image showing PCR products for *ntrA* (90bp) gene fragments amplified. Lane M = 100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1= *ntrA* gene fragments amplified from *K. pneumoniae* (ATCC 13883) and lanes 2-17 = *ntrA* gene fragments amplified from isolates. 61

Figure 4.8: Agarose gel (1% w/v) image showing PCR amplification for *tuf* (240bp) gene fragments of the tested isolates. Lane 1=100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 2 = *tuf* gene

fragments amplified from *P. mirabilis* (ATCC 29930) and Lanes 3 – 4 = *tuf* gene fragments amplified from isolates. 62

Figure 4.9: Agarose gel (1% w/v) image showing PCR products for *invA* (284bp) gene fragments amplified from isolates. Lane 1 =100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 2= *invA* gene fragments amplified from *S. enterica* (ATCC 12325) and lanes 3-5 and 6-10= *invA* gene fragments amplified from *Salmonella* isolates. Lane 6= Negative control reaction. 62

Figure 4.10: Agarose gel (1% w/v) image showing multiplex PCR for CRE genes, KPC (798 bp), NDM (621 bp), OXA-23 (501 bp). Lane M=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1,2,5,6,11 and 12= KPC gene fragments, Lane 3 and 15= KPC and OXA gene fragments, Lane 8= KPC and NDM gene fragments, and Lane 13 and 14= NDM gene fragment. Lane 4, 7, 9 and 10= No gene fragments amplified. 65

Figure 4.11: Agarose gel (1% w/v) image showing multiplex PCR for CRE genes, OXA-48 (438bp), VIM (390bp), and GES (371bp). Lane M=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1,2, 5-7, 9-11, 13-14 and 16=OXA-48gene fragments, Lane 3 = GES gene fragment, Lane 4 and 15= VIM gene fragment, Lane 8 and 12= No gene fragments amplified. 65

Figure 4.12: Agarose gel (1% w/v) image showing multiplex PCR for ESBL genes OXA (619bp), CTX-M (550 bp), SHV (822 bp) and TEM (753 bp). Lane 1=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lanes 2-7 = bla_{OXA} gene from isolates, Lane 8-9= bla_{CTX-M} gene fragments, Lane 10, 12-14= bla_{SHV} gene fragments, and Lane 11 = bla_{TEM} gene fragment from isolates. 68

LIST OF APPENDICES

APPENDIX 1: Culture Media and Gels used in this study. 112

APPENDIX 2: Chemicals used in this study 118

APPENDIX 3: Stains and Kits used in this study 120

APPENDIX 4: Antibiotics discs used in this study 122

APPENDIX 5: Antibiogram Data of all Isolates Tested with the 13 Antimicrobial Agents and Areas of Collection 123

DEFINITION OF CONCEPTS

Annealing: A PCR step in which the primers attach, to the DNA template.

Antibiotic: A drug that is capable of destroying or weakening certain microorganisms, especially bacteria or fungi, which cause infections or infectious diseases.

Antibiotic resistance: An ability of a given bacterium to survive the exposure to a defined concentration of an antimicrobial agent.

Amplification: A massive replication of genetic material and especially of a gene or DNA sequence.

Beta-Lactam Antibiotics: a class of broad-spectrum antibiotics, consisting of all antibiotic agents that contain a beta-lactam ring in their molecular structures.

Beta-Lactamase: A bacterial enzyme capable of inactivating the activity of beta-lactam antibiotics by hydrolysing the beta-lactam ring portion of the molecule.

Carbapenems: A class of beta-lactam broad spectrum antibiotics which are known to be most effective against gram negative infections.

Carbapenemase: Carbapenem-hydrolyzing beta-lactamases that confer resistance to a broad spectrum of beta-lactam substrates, including carbapenems.

Chromogenic Medium: Microbiological media suitable for incubation, differentiation, or selection of many microorganisms by using chromogenic substrates and resulting in colour production.

Combination Therapy: The use of multiple drugs that work by different mechanisms to treat a single disease.

Commercial Farms: Farming for a profit, where food is products generated in the farm are sold to the public.

Confidence level: The percentage of all possible samples that can be expected to include the true population parameter.

Crush Pens: A passage of fence with one narrow end that is used to handle large domestic animals, such as cattle.

Denaturing: Destroying the characteristic properties of a protein or other biological macromolecule by heat, acidity, or other effect which disrupts its molecular conformation.

Enzymes: Are protein molecules in cells which work as catalysts by speeding up chemical reactions, but do not get used up in the process.

Extension: PCR step in which the hydrogen bonds holding the complementary strands of DNA together are broken.

Fermentation: Is a metabolic process that consumes sugar in the absence of oxygen.

Gene: a sequence of DNA or RNA which codes for a molecule that has a specific unique function

Genus: A class of things which have common characteristics and which can be divided into subordinate kinds.

Genotypic: The genetic makeup of an organism or group of organisms with reference to a single trait, set of traits, or an entire complex of traits.

***In-vitro*:** A test procedure outside a living organism, in a test tube or culture dish.

Monotherapy: The treatment of a disease with a single drug.

Morphology: A particular specific form, shape, or structure.

Multiplex PCR: A variant method of PCR in which more than one locus is simultaneously amplified in the same reaction.

Mutation: the changing of the structure of a gene, resulting in a variant form which may be transmitted to subsequent generations, caused by the alteration of single base units in DNA, or the deletion, insertion, or rearrangement of larger sections of genes or chromosomes.

Normal flora: Microorganisms including bacteria, protozoa, and fungi that are found on or in specific areas of the body**.** They are either harmless, or beneficial to the host in their usual sites, and they inhibit the growth of pathogens.

Phenotypic: The physical and biochemical characteristics of an organism as determined by the interaction of its genetic constitution and the environment.

Plasmid: A circular DNA molecule that can replicate independently from the chromosome and promote lateral transfer among different species of bacteria through the conjugation process.

Primer: A short strand of RNA or DNA that serves as a starting point for DNA synthesis.

Species: A collection of bacterial cells which share an overall similar pattern of traits in contrast other bacteria whose pattern differ significantly.

Strains: An isolate or group of isolates that can be distinguished from isolates of the same genus or other genus of the same species either by phenotypic characteristics and/or genotypic characteristics.

LIST OF ABBREVIATIONS

ALP:	Alkaline Phosphatase
AmpC:	Class C Beta-Lactamase
ATCC:	American Type Culture Collection
BIBLI:	Beta-Lactam-Beta-Lactamase Inhibitors
CDC:	Centre for Disease Control
CLSI:	Clinical and Laboratory Standards Institute
CPE:	Carbapenemase Producing *Enterobacteriaceae*
CRE:	Carbapenem Resistant *Enterobacteriaceae*
CTXM:	Cefotaximase-Munich
DHP:	Dehydropeptidase
DNA:	Deoxyribonucleic Acid
EDTA:	Ethylene diamine Tetra-acetic Acid
ESBL:	Extended Spectrum Beta-lactamase
EtBr:	Ethidium bromide
FDA:	Food and Drug Administration
GES:	Guiana Extended-Spectrum β-lactamases
GIM:	German Imipenemase Metallo-β-lactamases
ICAAC:	Interscience Conference on Antimicrobial agents and Chemotherapy
IMI:	Imipenem Hydrolyzing β-Lactamase
IRT:	Inhibitor-resistant TEM
KESC:	*Klebsiella, Enterobacter, Serratia* and *Citrobacter*
KPC:	*Klebsiella Pneumoniae* Carbapenemases

LPS:	Lipopolysaccharides
MBL:	Metallo-β-lactamases
MDRTB:	Multi Drug Resistant *Mycobacterium tuberculosis*
NCTC:	National Collection Type Culture
NDM:	New Delhi MBL
NICD:	National Institute for Communicable Diseases
NMC-A:	Not Metalloenzyme Carbapenemases
OMP:	Outer Membrane Proteins
OXA:	Oxacillin
PBP:	Penicillin-Binding Proteins
PMP:	Proteus, Morganella and Providencia
rRNA:	ribosomal Ribonucleic acid
SHV:	Sulfhydryl variable
SIM:	Seoul Imipenemase Metallo-β-Lactamases
SME:	*Serratia Marcescens* Enzyme
TEM:	Temoniera
UV:	Ultraviolet
VIM:	Verona Integron-encoded MBL

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Background

Enterobacteriaceae is a family of Gram-negative rod-shaped bacilli that are normal inhabitants of the gastrointestinal tract of both humans and animals (Jesse *et al.*, 2013). These organisms were previously considered to be non-pathogenic but some strains are pathogenic and cause a variety of severe health complications in humans and animals (Valentin *et al.*, 2014). Some species within the family *Enterobacteriaceae* are currently known to be the most common cause of both community-acquired and hospital-acquired infections, specifically infections of the urinary and gastrointestinal tracts, pneumonia, peritonitis, meningitis, sepsis, as well as medical device-associated infections in humans (Hrabák *et al*., 2014). Although some of these complications can be self-limiting in some patients, infections such as salmonellosis, shigellosis, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) have been reported to progress to renal failure with life-threatening implications on patients (Humphries and Linscott, 2015). *Enterobacteriaceae* are easily spread through contaminated hands and the consumption of contaminated food and water. This makes it extremely difficult to eliminate these bacterial contaminants in the food chain.

Beta-lactam antibiotics especially penicillin and its derivatives (penams), Cephalosporins (cephems), Monobactams and Carbapenems are the most commonly used antibiotics in the treatment of infections caused by members of the *Enterobacteriaceae*. These groups of antibiotics possess a common β-lactam ring in their structure which binds to and inactivates the penicillin-binding proteins (PBP's) that are responsible for the formation of bacterial cell wall structure (Meletis, 2016). Beta-lactam antibiotics thus interfere with transpeptidase enzymes responsible for the formation of the cross-links between

peptidoglycan strands and impair the development of bacterial cell wall, which leads to cell death (Meletis, 2016). However, as an adaptative mechanism the organisms have developed the ability to produce β-lactamases, which are hydrolytic enzymes that are capable of inactivating β-lactam antibiotics (Abraham and Chain, 1940; Bradford, 2001). This inactivation mechanism has been expanded to derivatives of beta-lactam antibiotics comprising first, second and third generation cephalosporins as well as aztreonam (Paterson and Bonomo, 2005). Given that organisms' ability to produce the enzyme beta-lactamases results in resistance against beta-lactam antibiotics and their derivatives (Geser *et al*., 2012) coupled with the fact that these antibiotics are inactivated even before they reach the penicillin binding proteins (PBP) on the bacterial cytoplasmic membrane (Falagas and Karageorgopoulos, 2009), proper detection of the occurrence of resistant strains is of great epidemiological importance.

Among the *Enterobacteriaceae*, species belonging to the genera *Escherichia*, *Klebsiella*, *Salmonella* and *Shigella* are frequently isolated in infections and they are capable of producing Extended spectrum beta-lactamases (ESBLs) (Bradford, 2001). ESBL-producing *Enterobacteriaceae* were previously known to exhibit resistance to various beta-lactam antibiotics such as 2nd-, 3rd- and 4th-generation cephalosporins and monobactam (aztreonam) except for carbapenems (Bradford, 2001; Pitout *et al.*, 2005; Efsa, 2011). Carbapenems were therefore the only practicable therapeutic treatment option for severe community-acquired and hospital acquired infections caused by multi-drug resistant ESBL producing *Enterobacteriaceae* (Liao *et al.*, 2014). This is because they present fewer adverse effects and safer than other last line drugs (Meletis, 2016). However, in recent years a number of cases of Carbapenem Resistant *Enterobacteriaceae* (CRE) have been reported worldwide (Nordmann, Dortet and Poirel, 2012; Schultsz and Geerlings, 2012; Thirapanmethee, 2012; Birgy *et al.*, 2012; Hawser *et al.*, 2012; Baroud *et al.*, 2013; Liao *et al.*, 2014; Thaden *et al.*, 2014; Zurawski, 2014; Falagas, Lourida, *et al.*, 2014; Barbarini *et al.*, 2015; Iweriebor *et al.*, 2015; Meletis, 2016) as well as in South Africa (Coetzee and Brink, 2011; Lowman *et al.*, 2011; Brink *et al.*, 2012, 2013, 2014; Rubin *et al.*, 2014; Chibabhai and Perovic, 2014;

Singh-moodley, Ekermans and Perovic, 2015; De Jager *et al.*, 2015; Gqunta *et al.*, 2015; Jacobson *et al.*, 2015; Malande *et al.*, 2016; Perovic *et al.*, 2016; D. Annear, 2017).

The emergence of Carbapenem Resistant *Enterobacteriaceae* has resulted in limited mono therapeutic treatment options available against infections caused by these pathogens. However, Fosfomycin, Colistin, Aminoglycosides and Tigecyclines are commonly used for treatment of these infections despite their toxic nature (Coetzee and Brink, 2011; Schultsz and Geerlings, 2012). Some studies have suggested that a combination therapy could produce more reliable outcomes when compared to a monotherapy (Liege, 1991; Georgopapadakou, 1993; Meletis, 2016). Given that most disease causing pathogens associated with human infections are believed to have originated from animal species (Geser *et al*., 2012; Valentin *et al.*, 2014), strategies designed to limit the occurrence of complications in humans must be centered around reducing zoonotic transfer of the bacterial strains. Unfortunately, the precise origin of the genes encoding for carbapenem resistance is not fully understood, although it is suspected that they may have escaped from environmental bacterial strains into bacterial species that are of clinical relevance (Carfi *et al.*, 1995; Concha *et al.*, 1996; Lee *et al.*, 2015).

Despite the fact that carbapenems have not been approved as valid recognised drugs for use in veterinary medicine anywhere in the world (Webb *et al.*, 2016), recent patterns of resistance to the antimicrobial agents have provided opportunities leading to suggestions that farmers either use them for the treatment, control and prevention diseases, or as a growth promoters (Livermore, 1995; Poirel *et al.*, 2004; Wang and Quinn, 2010; Guerra *et al*., 2014). This explains why carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae* have received a lot of attention in recent years. This study aims to investigate the presence of Carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae* in cattle and further characterise the isolates for the presence of resistant determinants using PCR analysis.

1.2 Problem Statement

Several control measures such as proper hygiene and sanitation have been put in place to minimize contamination of meat and meat products with pathogens. In South Africa meat and meat products importation and exportation to establish meat safety schemes is regulated by the Meat Safety Act No. 40 of 2000. Despite all these measures, contamination of carcasses and their associated meat products have been an issue of severe public health concern (Rani *et al.*, 2017). The natural hosts of bacteria particularly members belonging to the family *Enterobacteriaceae* are animals and this gives room for pathogenic strains to be transmitted between animals and humans through handling or consumption of undercooked contaminated food products (Ateba and Mbewe, 2011). Contamination with these pathogens has even more severe impacts on consumers especially if they harbour antibiotic resistant determinants such as ESBLs (Rani *et al.*, 2017).

Over the past 60 years, β-lactam antibiotics have been widely used in the treatment of serious infections caused by *Enterobacteriaceae* (Shah and Isaacs, 2003; Shaikh *et al.*, 2015; Tamma and Rodriguez-Baño, 2017). This was due to the fact that they are excellently efficient, safe and easily tolerated by a number of individuals (Shah and Isaacs, 2003). Beta-lactams such as penicillin and their derivatives (penams), Cephalosporins (cephems), Monobactams and Carbapenems were therefore the most commonly used antibiotics in the treatment of infections caused by members of the family *Enterobacteriaceae* until the emergence of extended spectrum beta-lactamase producing *Enterobacteriaceae*. Carbapenems were later approved as the most suitable drugs of choice for the treatment of infections caused by extended spectrum beta-Lactamase producing *Enterobacteriaceae* (Liao *et al.*, 2014). However, the emergence of carbapenem-resistant *Enterobacteriaceae* presents severe challenges to public health concerns to humans (Da Costa *et al*., 2013). In addition, the prevalence of carbapenem-resistant *Enterobacteriaceae* has increased worldwide over the last decade

especially in health care settings, and a number of cases have also been reported in South Africa (Bamford *et al.*, 2011; Brink *et al.*, 2012, 2013, 2014; Perovic *et al.*, 2014; Rubin *et al.*, 2014; Chibabhai and Perovic, 2014; Lowman *et al.*, 2014a; De Jager *et al.*, 2015; Gqunta *et al.*, 2015; Singh-Moodley and Perovic, 2016a; Makena *et al.*, 2016; Malande *et al.*, 2016; Schellack *et al.*, 2017). This indicates the need for constant monitoring.

Carbapenemase-producing *Enterobacteriaceae* (CPE) complicate therapeutic processes by limiting the options of antibiotics that could be used for treatment (Falagas *et al.*, 2014; Tzouvelekis *et al.*, 2014). With this in mind, clinicians are left with no option but to use drugs that have since been phased out as a result of their high toxicity to humans. The constant increase in the prevalence of CRE in many developing and developed countries prompted the Centre for Disease Control and Prevention's decision to declare these pathogens as severe public health threat agents (Kallen *et al*., 2013). This also highlights the sudden increase in the number of studies designed to determine the occurrence of CPE and also assess their resistant profiles. However, despite the fact that there has been a number of reports in which CRE strains were isolated from humans particularly in clinical settings (Benenson *et al.*, 2009; Carmeli *et al.*, 2010; Falagas, Lourida, *et al.*, 2014; Iweriebor *et al.*, 2015; Ventola, 2015; Hijazi *et al.*, 2016; Webb *et al.*, 2016; Tacconelli *et al.*, 2017; NICD, 2018), very little information is available on the persistence of CRE in meat producing animals such as cattle (Poirel *et al.*, 2004, 2012; Wang and Quinn, 2010; Tramuta *et al.*, 2011; Xu *et al.*, 2015; Harada *et al.*, 2017). It is therefore not clear which *Enterobacteriaceae* are commonly resistant to carbapenems as well as produce ESBL and which resistant genes they carry in animal cases. As mentioned above several research has been conducted for human cases and similar knowledge would be beneficiary for veterinary studies. This study was therefore designed to isolate ESBL's that are also resistant to Carbapenems from cattle and characterise the isolates using genotypic techniques.

1.3 Study Aim

The aim of this study was to isolate, identify and characterize Carbapenem Resistant Extended Spectrum Beta-Lactamase producing *Enterobacteriaceae* isolated from cattle faeces.

1.4 Study Objectives

The specific objectives of the study were:

- To isolate *Enterobacteriaceae* from cattle faeces and confirm their identities using PCR assays
- To determine antibiotic resistance profiles of all *Enterobacteriaceae* isolated
- To determine the production of extended spectrum beta lactamase and carbapenemase enzymes by the resistant isolates
- To determine the presence of extended spectrum beta-lactamase and carbapenemase resistance genes in the resistant isolates using PCR analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 The Family *Enterobacteriaceae*

There is no single family in the δ class of the phylum Proteobacteria with a greater impact on medicine, public health, molecular genetics and phylogeny, pathogenesis, gene structure, regulation, and function or microbial ecology and physiology than the *Enterobacteriaceae* (Goldman and Green, 2015). This family contain more than 50 genera and over 200 species and the most frequently isolated species belong to the genus *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, *Proteus* and *Yersienia* (Prescott and Klein, 2002; Rosenberg *et al.*, 2013; Willey *et al*., 2014). Members of this family are gram-negative, rod shaped, non-spore forming and facultative anaerobic bacteria. Despite the fact that some members are non-motile, most of them possess peritrichous flagella. Organisms in the different genera of this family are collectively called coliform bacilli, due to the fact that they are normal commensals of the intestines of both humans and animals and have a number of properties in common except for *Proteus* species (Guentzel, 1996).

Gram-negative bacteria have a unique cell envelope structure which is critical in maintaining integrity of the cell wall and composed of an outer membrane, the peptidoglycan cell wall and cytoplasmic or inner membrane. The outer membrane appears as a lipid bilayer which is about seven (7) nanometers (nm) thick. The main function of outer membrane includes acting as a coarse molecular sieve and retaining certain enzyme while preventing entrance of some toxic substances in the bacteria. Lipopolysaccharides (LPS) also called lipoglycans and endotoxins are the major components of the outer leaflet of outer membrane in most Gram negative bacteria (Wang and Quinn, 2010). LPS helps the bacterium to avoid host defense as well as in stabilizing the bacterial membrane structure (Prescott and Klein, 2002). The

structure of LPS consist of Lipid A, core polysaccharides and O antigen repeats (or O side chain), and in many bacteria these components are toxic. LPS functions include activation of the innate immune system and are also essential for bacterial survival (Caroff and Karibian, 2003). The core polysaccharide and O antigen repeats are displayed on the surface of bacterial cell wall while Lipid A is embedded in the membrane. Lipid A is a major constituent of the outer membrane (Prescott and Klein, 2002). It is an immune-modulator which induces non-specific resistance to bacterial and viral infections and is responsible for toxic effects of infection with gram negative bacteria (Wang and Quinn, 2010). O antigen's composition varies between different bacterial strains (Willey *et al*., 2014).

The phospholipids are located on the inner layer of the outer membrane. Proteins are divided into β-barrel proteins which are transmembrane proteins and Lipoproteins. Lipoproteins contain lipid moieties attached to an amino-terminal cysteine residue. Most integral transmembrane proteins of the outer membrane assume a β-barrel conformation, and they are collectively called outer membrane proteins (OMPs). Some OMPs such as porins, OmpF and OmpC function to allow the passive diffusion of small molecules such as mono- and disaccharides and amino acids across the outer membrane *(J.Silhavy et al*., 2010). Braun's lipoprotein is the most abundant membrane protein in gram negative bacteria (Prescott and Klein, 2002).

The peptidoglycan layer determines the bacterial cell shape because of its rigidity. It is made up of repeating units of the disaccharide N-acetyl glucosamine-N-acetylmuramic acid, which is cross linked by pentapeptide side chains (Silhavy *et al*., 2010). A family of enzymes called Penicillin Binding Proteins (PBPs) are responsible for the assembly, maintenance, and regulation of peptidoglycan structure (Massova and Mobashery, 1998). The cytoplasmic or inner membrane is a phospholipid bilayer which performs all the functions of eukaryotic organelles. All membrane proteins that are responsible for energy production, lipid biosynthesis, protein secretion as well as transportation are stored in the cytoplasmic membrane (Silhavy *et al*., 2010). Some Gram-negative bacteria are also known to possess a large

genetic element called a resistance plasmids (R plasmid), which usually encodes for putative virulence and antibiotic resistance genes (Tang *et al*., 2014).

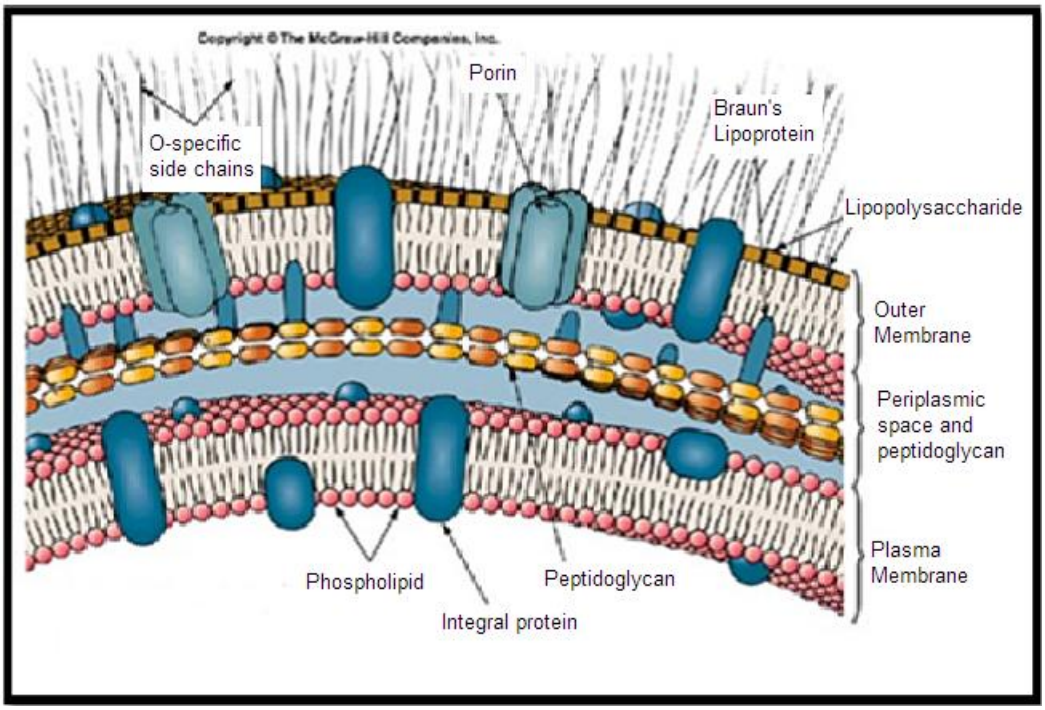


Figure: 2.1: Gram negative bacteria cell wall, showing bacterial outer membrane, periplasmic space and peptidoglycan and plasma membrane (copyright Mc Graw-Hill).

Based on their nutritional requirements, bacteria in this family are known to grow optimally on MacConkey agar supplemented with crystal violet and salt. Phenotypic identification of these isolates is based on their ability to ferment a variety of carbohydrates aerobically and anaerobically, and they are able to reduce nitrate to nitrite.

Some strains of *Enterobacteriaceae* are opportunistic pathogens that take advantage of a weakened immune system. They colonize and cause a variety of different diseases in their hosts, and therefore a lot of attention has been focused on these organisms (Korzeniewska and Harnisz, 2013). Infections caused by these organisms range from simple diarrhoea that is usually self-limiting to bloody diarrhoea that may progress to severe forms of disease including salmonellosis, shigellosis, hemorrhagic colitis (HC), hemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) and renal failure. These organisms are amongst the most common human pathogens and are a source of many

community- and hospital-acquired infections (Nordmann *et al*., 2012; Korzeniewska and Harnisz, 2013) including infections of the urinary and gastrointestinal tracts, pneumonia, peritonitis, meningitis, sepsis, as well as medical device-associated infections in humans (Hrabák *et al*., 2014).

Escherichia coli (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) are among the most common *Enterobacteriaceae species* implicated both in community- and hospital-acquired infections worldwide (Baroud *et al*., 2013). *Enterobacteriaceae* spread easily between humans and animals, through hand carriage, contaminated food and water as they can acquire genetic material through horizontal gene transfer, mediated mostly by plasmids and transposons (Nordmann *et al*., 2011). These organisms can also spread through faecal material and wastewater in different environments, including soil, vegetables, and other sources (Korzeniewska and Harnisz, 2013; Ben Said *et al*., 2015).

2.2 Antibiotics and their Mechanisms of Action

Antibiotics are low molecular weight compounds which may be natural products or synthetic chemicals designed to block crucial processes in pathogenic cell development. The danger however with antibiotics is that there is no specificity as they antagonize both disease causing organisms as well as normal flora which the body needs. They are designed to either inhibit the growth or kill microbial organisms using different mechanisms of action (Etebu and Arikekpar, 2016). Those that kill bacteria are termed bactericidal and those that inhibit growth are bacteriostatic.

When investigating antibiotics molecular structure, and their mode of action is the most important aspects to consider. Antibiotics are classified based on their mechanisms of action as follows (Kohanski *et al*., 2010; Soares *et al*., 2012; Etebu and Arikekpar, 2016):

- Inhibitors of bacterial cell wall synthesis (e.g. β-lactams, Cephalosporins etc.)
- Agents that interfere with nucleic acid structure and function (e.g. Quinolones, Novobiocin etc.)

- Inhibitors of protein synthesis (affecting function of both 30S and 50S ribosomal subunits) (e.g. 30S: Tetracycline, Gentamycin etc. 50S: Chloramphenicol, Erythromycin etc.)
- Those that interfere with the cell membrane of bacteria (e.g. Polymyxins, Daptomycin etc.)
- Those that block the key metabolic pathways (e.g. Sulfonamides, Trimethoprim etc.).

In the present study only those antibiotics that inhibit cell wall synthesis are looked at in more detail as they are the main focus of this study. The gram negative bacterial cell envelope as well as the function of the different structural layers including the peptidoglycan layer were previously discussed. The peptidoglycan layer maintenance is critical for the bacterium's survival as it maintains osmotic pressure. This is achieved by transglycosylase and transpeptidase enzyme (Penicillin Binding Proteins) activities, which add disaccharide penta peptides to extend the glycan strands of the layer and also cross-linking adjacent peptide strands (Kohanski *et al*., 2010).

The peptidoglycan layer is the main target for most antibiotics as they inhibit its synthesis and maintenance (Soares *et al.*, 2012). They achieve this by blocking the cross-linking of the peptidoglycan units by inhibiting the peptide bond formation which is catalyzed by penicillin binding proteins (Etebu and Arikekpar, 2016). Due to the antibiotic conformation being similar to the D-alanine-D-alanine portion of the N-acetylmuramic moiety the antibiotic is able to act as a substrate for the enzyme during the acylation phase of cross-link formation. This will then result in new peptidoglycan chains that cannot cross-link and lack tensile strength, leading to cell rupture due to osmotic lysis (Kohanski *et al*., 2010; Soares *et al.*, 2012; Etebu and Arikekpar, 2016).

2.3 Classification and Mechanisms of antimicrobial resistance

Bacterial resistance to antibiotics dates back as early as 1950s, which was approximately 10 years after antibiotics were first prescribed (Alanis, 2005). Antimicrobial resistance is the ability of bacteria to survive exposure to defined concentration levels of a certain antimicrobial substance known to be able to kill that bacteria (Acar and Röstel, 2001). Antibiotic overuse, incorrect prescription, extensive use in the

agricultural sector, and development of few new antibiotics are identified as contributors to bacterial resistance (Ventola, 2015). Bacterial cell resistance to an antibiotic can either be a natural property or acquired, co-operation between these mechanisms often generates high levels of resistance towards antibiotics (Acar and Röstel, 2001).

Antimicrobial resistance can be classified as intrinsic resistance, mutational resistance or acquired resistance (Alekshun and Levy, 2007). **Intrinsic resistance** is an inherent resistance whereby the microorganism is naturally resistant to the antibiotic. **Mutational resistance** leads to production of a genetically-altered bacterial population resistant to antibiotics they were previously susceptible to due to chromosomal mutation. **Acquired resistance** is due to horizontal transfer of genetic element encoding antibiotic resistance from one organism (resistant to the antibiotic) to another (susceptible to the antibiotic). Acquired resistance is the more common for transferring antibiotics resistance genes and can be achieved through the following ways:

- **Transduction**: a process where exogenous DNA is transferred from one bacterium to another by the intervention of a bacteriophage and incorporating the resistant genetic material into bacterial genome (Alanis, 2005; Alekshun and Levy, 2007; Soares *et al.*, 2012).
- **Transformation**: a process by which bacteria acquired naked DNA segments of dead bacterial cells that are in the environment and incorporate it into its genome (Alekshun and Levy, 2007; Soares *et al.*, 2012; Willey *et al*., 2014).
- **Conjugation:** a process where genetic material is passed from cell to cell through sex pilus or a bridge via a plasmid containing the resistance genes (Alanis, 2005; Soares *et al.*, 2012; Willey *et al*., 2014).

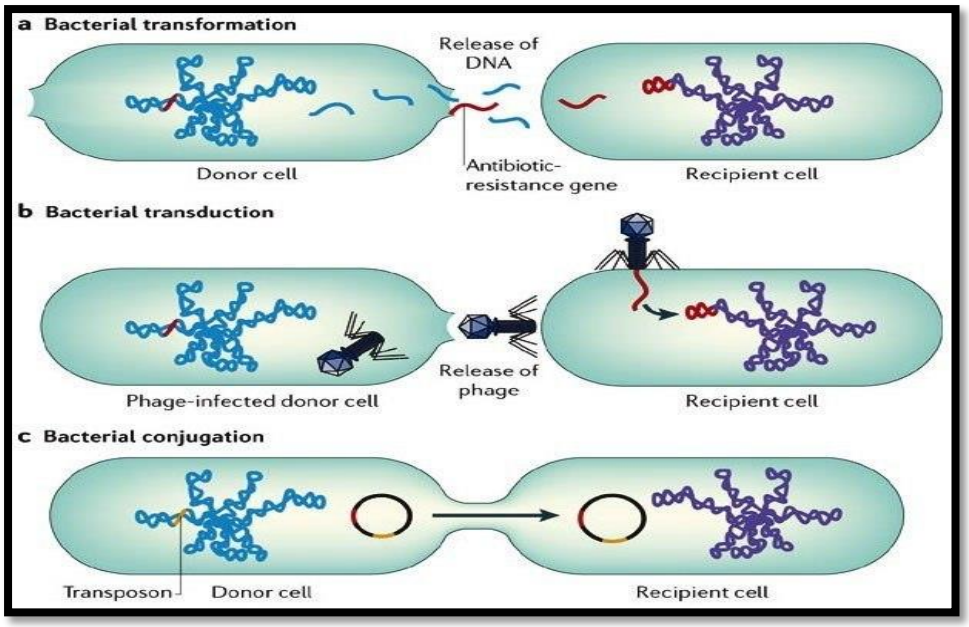


Figure 2.2: Methods of antibiotic resistance acquisition. A- Bacterial transformation, B- Bacterial transduction and C- Bacterial conjugation (Furuya and Lowy, 2006)

Generally micro-organisms use three common mechanisms to gain resistance to different antibiotics (Soares *et al.*, 2012; Tang *et al*., 2014; Willey *et al*., 2014):

- Preventing the drug from reaching its target: impairing entry of antibiotic to bacteria outer membrane
- Alteration of the target: mutation or alteration to binding proteins decreasing affinity to antibiotic
- Inactivating the antibiotics: achieved by producing inactivation enzymes.

2.4 Beta-Lactam antibiotics

These are a broad class of antibiotics whose structure consists of a 3-carbon and 1-nitrogen ring as well as a common β-lactam ring. The most common and highly prescribed β-lactam antibiotics are Penicillin derivatives (penams), Cephalosporins (cephems) Monobactams, and Carbapenems. Beta-lactam (β-lactam) antibiotics have been widely prescribed to treat serious bacterial infections for over 60 years owing to their excellent efficacy, safety and tolerability profiles (Shah and Isaacs, 2003). These characteristics are the motivation behind the frequent use of these antibiotics also in veterinary medicine

(Seiffert *et al.*, 2013). Penicillin, Cephalosporin, Monobactam and Carbapenems are bactericidal antibiotics (Willey *et al*., 2014). They inactivate key enzymes of the bacterial cell wall synthesis and repair pathways, leading to defective cell walls and, eventually, cell death (Concha *et al.*, 1996; Kohanski *et al*., 2010; Soares *et al.*, 2012; Etebu and Arikekpar, 2016). However, due to widespread use of these antibacterial agents, there has been a strong selection for microorganisms which are resistant to β-lactams (Escobar *et al*., 1991).

2.5 *Enterobacteriaceae* resistance to β-lactam antibiotics

Use of any antibiotic whether in animals, humans, plants or food processing technology has a potential to lead to bacterial resistance at a later point in time. This is especially true for β-lactam antibiotics as they have been widely used to treat infections for over 70 years (Acar and Röstel, 2001; Shah and Isaacs, 2003; Seiffert *et al.*, 2013). Resistance in bacteria that are important pathogens of humans and the spread of resistance from hospital environment into communities are increasingly perceived as great threats in public health (Acar and Röstel, 2001). *Enterobacteriaceae* resistance to β-lactam antibiotics, coupled with the void in the discovery of new antimicrobials created a major setback in medical advances (Tang *et al*., 2014). The past two decades have seen a marked increase in resistance, especially in relation to β-lactams (Bush, 2010a).

In *Enterobacteriaceae* β-lactam antibiotics traverse across the bacterial outer membrane in order to reach their target site of action. Any modulation that restricts entry (such as porin loss), increased extrusion (efflux pumps) will confer multi-antibiotic resistance as other drugs share the entry channels (Rawat, D. and Nair, 2010; Tang *et al*., 2014) coupled with Beta-lactamase production (Escobar *et al*., 1991; Pitout *et al.*, 2005; Schultsz and Geerlings, 2012).

2.5.1 Target site mutations or alterations

Enterobacteriaceae may be resistant to β-lactam antibiotics due to mutation which causes structural changes in the outer membrane proteins (OMP) which are the target for the antibiotics entry into the cell (Paterson and Bonomo, 2005). These structural changes and mutation in genes coding for porin proteins like OmpF and OmpC families (responsible for taking up β-lactam antibiotics) impair the entry of hydrophobic penicillins into the cells, thereby increasing the minimal inhibitory concentration of the antibiotic for the organism (Soares *et al.*, 2012). Antibiotic sensitivity is decreased in cases of mutation at a gatekeeping loop or central channel in the porin proteins, a loss of porin expression or a shift in the type of porins found in the outer membrane (Poole, 2004; Nordmann *et al*., 2012). Production of altered OMPs with reduced affinity for β-lactam antibiotics has been seen in organisms that are resistant to β-lactam antibiotics (Poole, 2004) although alteration occurs less frequently in gram negative bacteria than in gram positive bacteria (Tang, *et al*., 2014).

2.5.2 Efflux Pumps

Efflux pumps are energy-dependent transporters that excrete various drugs and other toxic compounds out of the bacterial cell (Guilhelmelli *et al.*, 2013). If the antibiotic is pumped out of the cell at the same or at a greater rate than its uptake into the cell then the intracellular concentration of the drug is reduced (Soares *et al.*, 2012). The tripartite efflux pump system which is composed of a protein transporter of the cytoplasmic membrane, a periplasmic connective protein and an outer membrane are the mediators of active expulsion of β-lactams (Meletis, 2016). Over expression of genes encoding efflux pump system lead to resistance to Penicillins, Carbapenems, Quinolones, Cephalosporins and Aminoglycosides as they are all common efflux pump substrates (Soares *et al.*, 2012; Meletis, 2016).

2.5.3 Beta-lactamase production

Beta-lactamase is an enzyme which hydrolyzes β-lactam antibiotics, thereby destroying the drug before it binds to PBPs on the bacterial cytoplasmic membrane (Thirapanmethee, 2012). Production of this enzyme by *Enterobacteriaceae* is the predominant cause of resistance to β-lactam antibiotics (Escobar

et al., 1991; Hall and Barlow, 2005; Pitout *et al*., 2005; Wilke *et al*., 2005; Babic *et al*., 2006; Dallenne *et al*., 2010; Schultsz and Geerlings, 2012; Soares *et al*., 2012). According to Concha *et al*., (1996) production of β-lactamases was due to evolutionary pressure as certain organisms produced antibiotics (β-lactam) in order to gain advantage over their competitors in the environment. The second population in the environment produced β-lactamases in order to overcome the challenge of β-lactam antibiotics exuded into the environment by their competitors.

Beta-lactamase can either be chromosomal or plasmid encoded, they can either be produced through inductive or constitutive processes (Soares *et al*., 2012). They compromise of four major groups based on substrate specificities: Penicillinases, AmpC-type Cephalosporinases, ESBL's and Carbapenemases, of which ESBL's and Cephalosporinases comprise the largest group (Bush, 2010b). A few β-lactamases use zinc ions to disrupt the β-lactam ring, but a far greater number operate via the serine ester mechanism.

2.6 Extended Spectrum Beta-Lactamase producing *Enterobacteriaceae*

Over the years more and more β-lactam antibiotics designed to resist the hydrolytic action of β-lactamases have been developed. However, with each developed new class being introduced, new β-lactamases emerged causing resistance to the new class. In the 1980's the oxyimino-cephalosporins became widely used for infections due to Gram-negative bacteria (Bradford, 2001). Resistance to oxyimino-cephalosporins emerged immediately after that, and these enzymes were called Extended Spectrum Beta-Lactamases (Huang *et al*., 2012). ESBL's are resistant to Penicillins, broad-spectrum Cephalosporins and Monobactams and they are inhibited by clavulanic acid and tazobactam (Berg *et al*., 2008; Huang *et al*., 2012; Al-Mayahie, 2013).

2.6.1 Classification of Beta-lactamases

Beta-lactamases are either classified based on their enzyme functional properties (Bush-Jacoby-Medeiros classification scheme) or molecular classification based on their primary structure (Ambler classification scheme). Of the two, the Bush-Jacoby-Medeiros classification scheme is the most popular (Ghafourian *et al.*, 1995; Sah and Hemalatha, 2015; Sar, 2015).

ESBLs belong to Class A according to Ambler and 2be according to Bush-Jacoby-Medeiros classification. Table 2.1 indicates classification of beta-lactamases with both schemes (Ghafourian *et al.*, 1995)

Table 2.1: Classification of Beta-Lactamases by Ambler Classification and Bush-Jacoby-Medeiros Classification

Ambler Classification	Bush-Jacoby-Medeiros	Characteristics of β-lactamases	Number of enzymes
C	1	Often chromosomal enzymes in gram-negatives but some are Plasmid-coded. Not inhibited by clavulanic acid	51
A	2a	Staphylococcal and enterococcal penicillinases	23
	2b	Broad spectrum β-lactamases including TEM-1 and SHV-1, mainly occurring in gram-negatives	16
	2be	Extended spectrum β-lactamases (ESBL)	200
	2br	Inhibitor-resistant TEM (IRT) β-lactamases	24
	2c	Carbenicillin-hydrolysing enzymes	19
	2d	Cloxacillin (Oxacillin) hydrolysing enzymes	31
	2e	Cephalosporinases inhibited by clavulanic acid	20
	2f	Carbapenem-hydrolysing enzyme inhibited by clavulanic acid	4
B	3	Metallo-enzymes that hydrolyse carbapenems and other β-lactams except monobactams. Not inhibited by clavulanic acid	24
D	4	Miscellaneous enzymes that do not fit into other groups	9

2.6.2 Types of ESBL's

2.6.2.1 TEM type

The Temorina *Escherichia coli* Mutant (TEM) type was first isolated in Greece from *Escherichia coli* and was named after the first patient's name 'Temoniera' and the organism it was isolated from (Bradford, 2001; Rawat, D. and Nair, 2010; Sah and Hemalatha, 2015). It belongs to class A ESBL's and confers resistance to Ampicillin, Penicillin, first generation Cephalosporins such as Cephalothin and Cephaloridine (Bradford, 2001; Rupp and Fey, 2003). The enzyme has now expanded its hydrolytic capabilities to Extended Spectrum Cephalosporins and Aztreonam due to mutation in the *blaTEM-1* structural gene (Stürenburg and Mack, 2003). TEM-1 and TEM-2 are the most commonly isolated derivatives and mostly found in *E.coli* and *K. pneumoniae* but they are also found in different genera of *Enterobacteriaceae* (Ghafourian *et al.*, 1995; Shaikh *et al.*, 2015).

There are currently over 200 TEM derivatives identified worldwide (http://www.lahey.or/studies/).

2.6.2.2 SHV type

The Sulfhydryl Variant (SHV) is the most prevalent of all the ESBL's specifically in clinical isolates (Ghafourian *et al.*, 1995; Jacoby and Han, 1996; Sah and Hemalatha, 2015). Its name is based on the assumption that its activity is related to the sulfhydryl substrate (Bradford, 2001). This type belongs to Class A ESBL's and isolated in a wide range of *Enterobacteriaceae* but mostly in *K. pneumoniae* strains (Ghafourian *et al.*, 1995; Bradford *et al.*, 1999). SHV was initially resistant to Penicillin and 1st generation Cephalosporins however, mutation within the *blaSHV-1* structural gene expanded its hydrolytic capabilities to extended spectrum Cephalosporins and Monobactams (Rupp and Fey, 2003; Shaikh *et al.*, 2015).

There are currently over 190 SHV derivatives reported worldwide (http://www.lahey.or/studies/).

2.6.2.3 CTXM type

The Cefotaximase- Munich (CTXM) type belongs to Class A ESBL's and was first isolated in 1989 in Germany but was then described by Tzouvelekis in 2000 (Tzouvelekis *et al.*, 2000). The name is derived

from their ability to hydrolyse Cefotaxime antibiotic but they are currently able to hydrolyse 2nd, 3rd and 4th generation Cephalosporins and Monobactams also (Bradford, 2001; Pitout *et al.*, 2005). They have been isolated from *Salmonella spp*, and *E. coli* as well as other *Enterobacteriaceae* (Bradford *et al.*, 2004). These enzymes originated from a horizontal gene transfer and subsequent mutation from the chromosomal AmpC β-lactamase of *Klyuvera ascorbate* (Humeniuk *et al.*, 2002). CTXM-15 is most widely described worldwide (Bush and Jacoby, 2010).

There are currently over 170 CTXM derivatives reported worldwide (http://www.lahey.or/studies/).

2.6.2 OXA type

The oxacillin (OXA) type belongs to Class D ESBL's and the name is derived or based on their ability to hydrolyse the antibiotic oxacillin (Paterson *et al.*, 2003; Valentin *et al.*, 2014). They were first reported in Turkey and mainly isolated from *P. aeruginosa* species (Shaikh *et al.*, 2015) although isolated in few other gram negative bacteria (Paterson *et al.*, 2003). These enzymes are characterized by their high levels of hydrolytic activities to oxacillin, cloxacillin and methicillin and confer resistance to ampicillin and cephalothin (Bradford *et al.*, 1999).

There are currently over 400 OXA derivatives reported worldwide (http://www.lahey.or/studies/).

2.6.3 Treatment options for ESBL infections

The plasmid carrying the ESBL encoding gene also carries genes encoding for resistance to aminoglycosides and trimethioprim/sulfamethoxazole thereby causing a serious limit to antibiotics for treatment of ESBL infections (Paterson and Bonomo, 2005). Carbapenems are the most effective β-lactams in the treatment of ESBLs as they are inactivated by the β-lactamases *in vitro* (Huang *et al.*, 2012). Carbapenems therefore, are the cornerstone and a treatment of choice against these serious infections and the development of resistance amongst ESBLs has been of great concern (Shah and Isaacs, 2003; Falagas and Karageorgopoulos, 2009; Coetzee and Brink, 2011; Weinstein and Logan, 2012; Govind *et al.*, 2013; Liao *et al.*, 2014; Woodford *et al.*, 2014; Ventola, 2015; Meletis, 2016). Use of β-lactam-β-lactamase inhibitors (BIBLIs) such as ticarcillin-clavulanate, piperacillin-tazobactam and

amoxicillin- clavulanate has been considered for treatment but are uncertain and *in-vitro* data and several other retrospective clinical studies prove that carbapenems are more efficient (Harris *et al*., 2015).

2.7 Carbapenem Antibiotics

Carbapenems are produced by substituting carbon for Sulphur at position 1 and an unsaturated bond between carbons 2 and 3 of the familiar Penicillin nucleus (Craig, 1997). Production of these antibiotics was inspired by Thienamycin antibiotic which is naturally produced by *Streptomyces cattleya* organisms found in the soil (Queenan and Bush, 2007). It was first reported in 1976 at the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) meeting that Thienamycin showed a broad-spectrum antibacterial and β-lactamase inhibitory activity and this attracted a lot of attention on this antibiotic. However, it was discovered that it is unstable in aqueous solution, sensitive to mild base hydrolysis (above pH 8.0) and also highly reactive to nucleophiles such as hydroxylamine, cysteine and its own primary amine (Papp-Wallace *et al.*, 2011). To increase the stability of Thienamycin derivates were developed, N-formimidoyl derivate was the first to be used for production of Imipenem. Therefore the thienamycin's hydroxyethyl side chain in Carbapenems is what differentiates them from conventional Penicillins and Cephalosporins (Papp-Wallace *et al.*, 2011) . They are tolerated well by humans with minimal toxicity, however seizures can be seen in high doses and require close monitoring (Baughman, 2009).

According to Shah and Isaacs, 2003 carbapenems are classified into 3 groups:

Group 1: broad-spectrum Carbapenems, with limited activity against non-fermentative Gram negative bacilli, that is suitable for community-acquired infections:

Group 2: broad-spectrum Carbapenems, with limited activity against non-fermentative Gram negative bacilli suitable for nosocomial infections; and

Group 3: Carbapenems with clinical activity against Methicillin Resistant *Staphylococci*.

2.7.1 Clinically prescribed Carbapenems

2.7.1.1 Imipenem

Imipenem was the first carbapenem to be developed in 1980's and was initially called MK0787. It was approved by the US Food and Drug Administration (FDA) in 1985 (Baughman, 2009). It proved to be stable against β-lactamase degradation with a high affinity for PBP's and has been the widely used carbapenem worldwide (Baughman, 2009). However, it was deactivated by dehydropeptidase 1 (DHP-1) found in human renal brush borders and therefore requires co-administration with cilastatin (Shah and Isaacs, 2003; Baughman, 2009; Papp-Wallace *et al.*, 2011). It's a broad spectrum antibiotic which is effective against aerobic and anaerobic pathogens and is rapidly bactericidal (Shah and Isaacs, 2003).

2.7.1.2 Ertapenem

Ertapenem was approved by the US Food and Drug Administration (FDA) in 2001 (Baughman, 2009). It proved to be more stable against DHP-1 than imipenem but also without anti-pseudomonal activity like imipenem (Perez and Van Duin, 2013). It is administered once daily due to its prolonged half-life and this made it more favourable for treating infections in community dwellers. It belongs to Group 1 of the carbapenems classification (Shah and Isaacs, 2003).

2.7.1.3 Meropenem

Meropenem was approved for use by the US Food and Drug Administration (FDA) in 1995 (Baughman, 2009). It has a 1-β-methyl group which renders it resistant to degradation by DHP-1 and therefore does not require co-administration with cilastin (Shah and Isaacs, 2003). When combined with clavulanic acid derivates meropenem is sensitive to multi-drug resistant *Mycobacterium tuberculosis* (MDRTB) an organism which chromosomally expresses β-lactamase and therefore not usually susceptible to β-lactams (Papp-Wallace *et al.*, 2011). As the only FDA approved beta-lactamase inhibitor clavulanic acid is used to inhibit BlaC irreversibly thereby rendering *Mycobacterium tuberculosis* susceptible to meropenem (Hugonnet *et al.*, 2010).

2.7.1.4 Doripenem

Doripenem was approved by the US Food and Drug Administration (FDA) in 2007 (Hilas, Ezzo and Jodlowski, 2008). It's a novel parenteral 1-β-methyl that is resistant to degradation by renal dehydropeptidase (Fritsche *et al*., 2005). It is also least susceptible to hydrolysis by carbapenemases (Papp-Wallace *et al.*, 2011). It is the most favourable of the carbapenems as it combined the superior *in-vitro* activities of imipenem against Gram positive cocci and that of meropenem against Gram negative pathogens (Fritsche *et al*., 2005).

2.8 Carbapenem Resistant *Enterobacteriaceae*

There has been a remarkable increased prevalence in infections caused by carbapenem resistant *Enterobacteriaceae* over the last decade worldwide, causing a great concern especially in health care settings (Adler *et al.*, 2011; Thirapanmethee, 2012; Nordmann and Poirel, 2014; Thaden *et al.*, 2014; Barbarini *et al.*, 2015). Carbapenem resistant *Enterobacteriaceae* are a group of Gram negative organisms that are resistant to the last resort carbapenem antibiotics as well as penicillins, cephalosporins, aminoglycosides and quinolones (Rupp and Fey, 2003; Tenover *et al.*, 2006; Zurawski, 2014). The Centre for Disease Control and Prevention has categorized these microorganisms as an urgent public health threat as they are resistant to almost all common antibiotics making it impossible for clinicians to treat them (Coetzee and Brink, 2011; Livermore *et al.*, 2011; Zurawski, 2014).

Resistance to carbapenems can be intrinsic in some species, as they naturally might possess enzymes that hydrolyse carbapenems. *Strenotrophomonas maltophilia* is an example of these bacteria as they possess metallo-β-lactamase (MBL) L1, so carbapenems are not recommended for treating such infections (Yigit *et al.*, 2001; Hawser *et al.*, 2012; Meletis, 2016). However, intrinsic resistance amongst clinically important microorganisms is not common.

2.8.1 Mechanism of resistance

The most common mechanisms of carbapenem resistance amongst Gram negative organisms can be categorized as follows:

2.8.1.1 Non-carbapenemase mediated carbapenem resistance in *Enterobacteriaceae*

Some species develop mutation or modification in the outer membrane porin specific for an antibiotic absorption into the cell or preventing them from reaching the penicillin binding proteins (Rupp and Fey, 2003; Tenover *et al.*, 2006; Zurawski, 2014) . OmpF and OmpC are the primary porins involved with antibiotic absorption in the *Enterobacteriaceae* family. Any changes or mutations to them will lead to resistance (Birgy *et al.*, 2012; Schultsz and Geerlings, 2012; Baroud *et al.*, 2013; Nordmann and Poirel, 2014). However, resistance due to this mechanism will only be conferred against carbapenems only as the porins are specific to carbapenems and not all β-lactams (Tenover *et al.*, 2006; Zurawski, 2014). Another form of resistance is active expulsion of carbapenems after entry into the periplasmic space via tripartite efflux pump system as was the case for ESBLs (Rupp and Fey, 2003; Gupta *et al.*, 2011; Birgy *et al.*, 2012; Meletis, 2016). The efflux pump system that expels carbapenems (especially meropenem) is common for penicillins, aminoglycosides, quinolones and cephalosporins, therefore over expression might lead to multi-drug resistance (Nordmann and Poirel, 2002; Baroud *et al.*, 2013; Meletis, 2016).

2.8.1.2 Carbapenemase-mediated Carbapenem resistance in *Enterobacteriaceae*

Production of carbapenemase enzymes i.e. β-lactamases capable of inactivating carbapenem antibiotics as well as other β-lactam antibiotics is the most common and most important cause of resistance to carbapenems (Yigit *et al.*, 2001; Baroud *et al.*, 2013; Meletis, 2016). These enzymes encode for genes which can be transferred horizontally by plasmids or transposons and also carry resistance determinants that confer resistance to penicillin, extended spectrum cephalosporins and aminoglycosides (Tenover *et al.*, 2006; Birgy *et al.*, 2012; Nordmann *et al.*, 2012; Schultsz and Geerlings, 2012; Zurawski, 2014). For this reason carbapenemase-mediated CRE are considered the most important clinically (Nordmann *et al.*, 2012).

Carbapenemase production is the mechanism used for classification of carbapenem resistant *Enterobacteriaceae* according to the Ambler Classification into class A, class B, class D and the rare chromosome-encoded cephalosporinases class C.

2.8.2 Classification of Carbapenemase Producing *Enterobacteriaceae* (CPE)

2.8.2.1 Class A

Plasmid-encoded enzymes are the most common β-lactamases from this class, which are:

• ***Klebsiella pneumoniae* carbapenemase (KPC)** was first reported in 1996 in New York, US and is currently the most important enzyme clinically (Gupta *et al.*, 2011; Mathers *et al.*, 2011; Falagas *et al.*, 2014). They are plasmid encoded, mediating resistance to extended spectrum cephalosporins and all β-lactams and are only inhibited partially by clavulanic acid, boronic acid and tazobactam (Tenover *et al.*, 2006; Meletis, 2016). KPC was initially isolated from *Klebsiella pneumoniae* but has since been isolated from *Escherichia coli, Citrobacter spp, Pseudomonas aeruginosa, Salmonella spp, Serratia marcescens, Proteus spp* etc.(Amjad *et al.*, 2011). In non-fermentative *Pseudomonas aeruginosa* KPC production causes high levels of carbapenem resistance (Bush *et al.*, 1987; Perez and Van Duin, 2013).

• **Guiana Extended-Spectrum β-lactamases (GES)** was first reported in Greece from a *Klebsiella pneumoniae* isolate in 2000 (Poirel *et al.*, 2004). The genes encoding for this enzyme have a broad spectrum hydrolysis including extended-spectrum cephalosporins and penicillins (Queenan and Bush, 2007). In addition to the initial *Klebsiella pneumoniae,* GES has also been isolated from *Pseudomonas aeruginosa* (Poirel *et al.*, 2001).

Others include the not so common chromosomally encoded enzymes, *Serratia marcescens* enzyme (SME), imipenem hydrolyzing β-lactamase (IMI), and no metalloenzyme carbapenemase (NMC-A) (Queenan and Bush, 2007; Nordmann and Poirel, 2014; Zurawski, 2014; Meletis, 2016).

2.8.2.2 Class B

Metallo-β-lactamases (MBL) are able to hydrolyse β-lactams with the exception of aztreonam. They contain zinc in their active centre which makes inhibition by clavulanic acid, boronic acid and tazobactam

impossible but only achieved *in vitro* using ethylenediaminetetraacetic acid (EDTA) (Guentzel, 1996; Walsh *et al.*, 2005; Meletis, 2016). MBLs were isolated from *Bacillus cereus, Aeromonas spp, Strenotrophomonas maltophilia* which are environmental and opportunistic organisms and their MBL genes were initially intrinsic and chromosomal (Nordmann and Poirel, 2002).

The two types of MBL most commonly identified in *Enterobacteriaceae* are:

• **Verona Integron-encoded MBLs (VIM)** was first reported in Verona, Italy in 1997, and they are mostly found in non-fermenting Gram negative organisms (Carattoli, 2009).

• **New Delhi metallo-β-lactamases (NDM-1)** was first identified from urinary *Klebsiella pneumoniae* isolate in Italy in 1997 from a patient who was initially hospitalized in New Delhi, India (Gupta *et al.*, 2011; Perez and Van Duin, 2013). All NDM-1 producing strains may express other unrelated resistance genes like ESBLs, carbapenemase, AmpC cephalosporinases as well as resistance to aminoglycosides, macrolides, sulfamethoxazole and macrolides (Schultsz and Geerlings, 2012).

Other isolated MBL include Sao Paulo metallo-β-lactamases (SPM), Seoul imipenemase metallo-β-lactamases (SIM) and German imipenemase metallo-β-lactamases (GIM) but they have not spread beyond their countries of origin (Queenan and Bush, 2007).

2.8.2.3 Class D

Class D MBL is an oxacillinase type carbapenemases which is not affected by clavulanic acid or EDTA inhibition but inhibited by sodium chloride (NaCl) (Walsh *et al.*, 2005). OXA-48 is the most prevalent of this class and isolates producing this enzyme do not usually exhibit high levels of resistance to carbapenems unless other mechanism of resistance are expressed (Nordmann and Poirel, 2002; Thomson, 2010).

2.8.3 Prevalence of Carbapenem Resistant *Enterobacteriaceae* in South Africa

Since the isolation of the first carbapenemase producing *Enterobacteriaceae* in South Africa in 2010 there has been reports of isolations of different types from all three classes. There also has been a report of resistance due to porin loss (Brink *et al.*, 2014). NDM-1 type might be endemic to South Africa as it had

been isolated from patients who had no history of travelling outside the country (Brink *et al.*, 2012; Rubin *et al.*, 2014).

NDM types have been reported as isolated from Durban (Lowman *et al.*, 2011; Rubin *et al.*, 2014), Gauteng (Brink *et al.*, 2012; Chibabhai and Perovic, 2014; De Jager *et al.*, 2015; Perovic *et al.*, 2016), and Cape Town (Malande *et al.*, 2016). GES type has been isolated in Cape Town (Malande *et al.*, 2016), and Gauteng (Perovic *et al.*, 2016). The first IMI-1 type isolated in the country has been reported in the Eastern Cape (Gqunta *et al.*, 2015). OXA-48 and its variants like OXA-181 have been reported in Gauteng (Chibabhai and Perovic, 2014; De Jager *et al.*, 2015), Cape Town (Jacobson *et al.*, 2015), and Eastern Cape (Singh-moodley *et al*., 2015). VIM type has been isolated in Gauteng (Brink *et al.*, 2014; Chibabhai and Perovic, 2014) and Eastern Cape (Singh-moodley *et al*., 2015; D. Annear, 2017). IMP type has been reported from Eastern Cape (Singh-moodley *et al*., 2015) and Gauteng (Chibabhai and Perovic, 2014). Singh-Moodley and Perovic, conducted a research from isolates collected all over the country in 2016 and reported one isolate that carried (a) combination(s) of different types.

2.8.4 Treatment options for carbapenem resistant *Enterobacteriaceae* (CRE) infections

There are limited treatment options for carbapenem resistant *Enterobacteriaceae* as most of them are resistant to all or almost all β-lactams. They commonly carry genes encoding for resistance mechanisms against fluoroquinolones, and aminoglycosides (Coetzee and Brink, 2011; Meletis, 2016). This limited option has forced clinicians to opt for antibiotics which were previously phased out due to efficacy and or toxicity concerns such as fosfomycin, polymyxins and aminoglycosides (Coetzee and Brink, 2011; Schultsz and Geerlings, 2012; Perez and Van Duin, 2013; Van Duin *et al.*, 2013; Meletis, 2016). Additional to these is tigecycline which is a newer modified tetracycline (Schultsz and Geerlings, 2012).

According to Meletis (2016), depending on antimicrobial susceptibility results of a patient, clinicians have three therapy schemes to choose for treatment of CRE infections:

1. Monotherapy with either colistin, carbapenem, tigecycline or fosfomycin depending on which one is still active *in vitro.*
2. Combination therapy of any two active drugs between colistin, tigecycline and fosfomycin without including carbapenem.
3. Combination therapy with two or more of the active drugs, with at least one carbapenem which had MIC results of ≤4mg/L.

In a clinical trial conducted by Tzouvelekis and colleagues (2014), combination therapy which included carbapenem was more effective in treating CRE infections, as it resulted in lower mortality rate than the other two therapy schemes (Tzouvelekis *et al.*, 2014; Meletis, 2016).

Colistin (Polymyxin E) was discovered in 1949 but due to its toxicity (to the peripheral nervous system and renal system) and nephrotoxicity profile, it fell out of favour in the 1980's (Schultsz and Geerlings, 2012; Perez and Van Duin, 2013; Zurawski, 2014). Recent studies indicate fewer toxic effects due to use of lower doses, varied drug formulations and regular monitoring for nephrotoxic effects. It is recommended that urinalysis, creatinine and electrolyte measurements in serum and urine as well as neurological assessment be frequently monitored in patients receiving treatment with Colistin (Zurawski, 2014)

While tigecycline is a modified version of tetracycline, designed to overcome efflux pumps and ribosomal protection which are the two major mechanism of resistance against tetracycline (Schultsz and Geerlings, 2012; Van Duin *et al.*, 2013). Tigecycline binds the 30S ribosomal subunit with an affinity five time higher than Tetracycline, leading to bacterial protein synthesis inhibition (Van Duin *et al.*, 2013). The main side effects for this antibiotic are vomiting and nausea, but issues including extreme elevation of alkaline phosphatase (ALP) and pancreatitis have been reported (Perez and Van Duin, 2013). In 2010 the US Food and Drug administration issued a warning regarding the increase in mortality rates associated to the use of tigecycline (Perez and Van Duin, 2013; Campbell *et al.*, 2015).

Fosfomycin is a phosphoric acid derivate (cis-1,2- epoxy propyl phosphoric acid) initially extracted from a *Streptomyces* species culture in 1969 (Schultsz and Geerlings, 2012). Fosfomycin tromethamine (a soluble salt with improved bio-availability) is currently used and is active against a broad-spectrum of Gram negative and Gram positive micro-organisms (Van Duin *et al.*, 2013). It inhibits synthesis of bacterial cell wall by inactivating pyruvic transferase which is an enzyme involved in peptidoglycan synthesis (Perez and Van Duin, 2013). It is well tolerated (even by neonates and children, for several months of administration), has few side effects and safe to use during pregnancy (Lewis *et al.*, 2013). Its major disadvantages however, are that it is suitable for treatment of uncomplicated lower urinary tract infections only and its high cost compared to other antimicrobial agents is the other disadvantage (Schultsz and Geerlings, 2012; Lewis *et al.*, 2013; Perez and Van Duin, 2013).

However, several studies have recorded resistance of carbapenem resistant *Enterobacteriaceae* against the above mentioned antimicrobial agents (Coetzee and Brink, 2011; Livermore *et al.*, 2011; Schultsz and Geerlings, 2012; Perez and Van Duin, 2013; Zurawski, 2014).

2.9 Use of antibiotics in animal species and transmission of resistant organisms to humans

All organisms, including humans, animals, bacteria, plants etc., interact a lot as they share the same environment and resources (Valentin *et al.*, 2014). This interaction makes it possible for bacteria, including multi-drug resistant ones to transfer genetic traits from one species to the other. Most pathogens that cause infections in humans originate from animals and can be transmitted to humans in a variety of ways (Geser *et al*., 2012; Valentin *et al.*, 2014).

Antimicrobials are used on animal farms to either, treat diseases, control diseases, and prevent diseases or as a growth promoter. Amoxicillin, aminoglycosides, ceftiofur, penicillin, erythromycin, quinolones, gentamicin, novobiocin, tylosin, tilmicosin and tetracycline are extensively used on cattle farms. These antibiotics are mainly used for treatment and prevention of bovine pneumonia, diarrhoea, respiratory diseases and shipping fever which are common problems (Geser *et al.*, 2012; Economou and Gousia,

2015; Webb *et al.*, 2016). In cases of disease prevention (prophylaxis) animals with no clinical signs are given the antibiotics for several weeks in small amounts either in feed or water when a risk of infection exists. In some cases the whole flock is treated with high doses of antibiotics over a short period of time, despite the exhibition of clinical symptoms in few animals (metaphylaxis) (Economou and Gousia, 2015). Several studies have then reported CRE isolation in livestock (Wang and Quinn, 2010; Livermore *et al.*, 2011; Poirel *et al.*, 2012; Guerra *et al*., 2014) even though carbapenems are not approved for use in livestock production anywhere in the world (Webb *et al.*, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Sampling Information

3.1.1 Sample size determination

This study was conducted at the North West University, Mafikeng Campus North West Province, South Africa. Cattle faecal samples were randomly collected from both communal and commercial farms. The number of samples that were collected during the study was determined using the formula below:

$$\text{Sample (n)} = \frac{Z_{1-\alpha/2}^2 P (1-P)}{d^2}$$

$Z_{1-\alpha/2}$ = standard normal variable (at 5% type I error ($P< 0.05$) it is 0.05)

P = expected prevalence in population based on previous studies

d = absolute error or precision (which is 5%)

$$\text{Sample size (n)} = \frac{(1.96)^2(0.16)\ (1 - 0.16)}{(0.05)^2} = 206$$

For estimation of the prevalence of Carbapenem resistant ESBL-producing *Enterobacteriaceae*, the sample size for this study was determined using the prevalence of 16 % obtained from a previous study conducted by Villar *et al*., (2013) at the 95% confidence level and absolute error of 5% using the formula described earlier (Charan and Biswas, 2013). Accordingly, the minimum sample size for the study was 206 samples.

3.1.2 Sampling Sites

A total of 233 faecal samples were collected from both communal and commercial cattle in randomly selected farms around the North West Province. The number of samples collected from the different sampling points is shown in Table 3.1.

Table 3.1: Sample stations and the total number of samples collected during the study

Nature of the farm	**Sample station**	**No. of samples collected**
Communal	Lokaleng	35
	Ntontonyane	80
Commercial	Koster	53
	Rooigrond	65
Total		**233**

3.1.3 Collection of faecal samples from cattle

Animals were put in crush pens and minimally restrained to ensure all ethical and humane procedures were adhered to. Faecal samples were collected directly from the rectum of the animals using sterile arm-length gloves by trained Animal Health technicians and in order to avoid duplication of sampling, the cattle were locked in their respective handling pens. The samples were placed in sterile sample collection bottles, labelled properly and immediately transported on ice to the laboratory for analysis. Data on antibiotic type and treatment history were collected for the purpose of understanding antibiotic exposure histories of isolates from the study population. Sampling was done between July 2016 and August 2017.

3.2 Ethical Clearance

Ethical clearance for the study was obtained from the Mafikeng Animal Research Ethics Committee.

3.3 Control Strains

Commercially obtained bacterial strains of *Staphylococcus aureus* (ATCC 33592), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603 and 13883), *Salmonella enterica* (ATCC 14028 and 12325), *Shigella sonnei* (ATCC 9290 and 29930) and *Enterobacter aerogenes* (ATCC 13048) were used as control strains in all bacterial identification experiments during the study.

3.4 Sample Processing and Bacterial Isolation

Upon arrival in the laboratory, the samples were analysed for the presence of *Enterobacteriaceae* using MacConkey agar (Biolab, City, South Africa). However, when it was not possible to analyse samples immediately, the samples were stored at 4°C and analysed within 24 hours. Approximately 2 g of faecal sample was dissolved in 2% (w/v) sterile buffered peptone water (Biolab, Lawrenceville, Georgia, USA-South Africa) and mixed properly using a vortex mixer (Scientific Industries, Patent, US). Aliquots of 100 µL from each sample were spread-plated on solidified MacConkey agar supplemented with crystal violet and salt (Biolab, Lawrenceville, Georgia, USA) using a standard procedure (Sanders, 2012). The plates were incubated aerobically at 37°C for 24 hours.

3.4.1 Purification of Bacteria Isolates

After 24 hours of incubation, plates were observed for bacterial growth and colony morphology was used as the main criterion to preliminarily differentiate between isolates. Colonies that showed differences in shape, colour and margins were considered to belong to different species and were sub-cultured onto MacConkey agar with Crystal Violet, with salt (Biolab, Lawrenceville, Georgia, USA) in order to isolate

single pure colonies. All plates were incubated aerobically at 37 °C for 24 hours and one pure colony per plate was picked into sterile tryptic soya broth and further incubated aerobically at 37 °C for 24 hours. These cultures were used to prepare glycerol stocks that were stored at -80 °C in order to preserve the isolates for future use. Isolates were later subjected to preliminary and confirmatory bacteria identification tests that are specific for *Enterobacteriaceae*.

3.5 Bacterial Identification by Cellular Morphology

Gram-staining standard technique as described by Cruikshank *et al.*, (1975) was used to determine the cellular morphologies of the isolates. Bacteria smears were prepared, stained and observed using a compound microscope (Cruikshank *et al.,* 1975). All Gram-negative rod shaped bacteria were subjected to preliminary biochemical identification tests that are specific for *Enterobacteriaceae*.

3.6 Preliminary Identification of *Enterobacteriaceae* Isolates

3.6.1 Oxidase Test

Presumptive isolates were screened using the Test Oxidase™ reagent (PL.390) obtained from Mast Diagnostics (Neston, Wirral, U.K.) in accordance to the manufacturer's protocol. The oxidase test identifies the ability of test isolates to produce cytochrome c oxidase, an oxidizing enzyme which plays a vital role in the electron transport chain (Capaldi, 1990). Cytochrome c oxidase oxidizes tetramethyl-p-penylenediamine and converts it to indophenols resulting in a purple colour. Oxidase positive isolates produce a purple colour and vice versa. Organisms belonging to the family *Enterobacteriaceae* are oxidase negative and results were recorded accordingly.

3.6.2 Triple Sugar Iron (TSI) Test

Triple sugar iron (TSI) agar obtained from Biolab, (Biolab, Lawrenceville, Georgia, USA) was used to determine the ability of the isolates to metabolise substrates glucose, sucrose and lactose at

concentrations of 0.1%, 1.0% and 1.0%, respectively. In order to perform the test, a single pure isolate was stab-inoculated in the butt of the TSI medium contained in McCartney bottles and latter streaked on the slant in a *zig-zag* motion. The bottles were loosely closed and incubated aerobically at 37°C for 24 hours. After incubation, media were observed and the ability of the isolates to ferment carbohydrates and produce acid, gas, hydrogen sulfide and metabolize lactose or sucrose or both was assessed visually and results were interpreted as previously indicated (Forbes and Weissfeld, 1998). Table 3.2 provides detailed information of the parameters used in interpreting the results. Data in Table 3.3 outlines the major characteristics of bacterial species belonging to the family *Enterobacteriaceae* when subjected to the TSI test.

Table 3.2: Ingredients of the Triple Sugar Iron Agar and Reactions Interpretation

Agar ingredients	**Function**	**Interpretation**
Phenol red	pH indicator	Acids are produced during fermentation, therefore acidic environment turns the agar yellow. If no fermentation occurred the agar remains red indicating that the organism is an obligate aerobe
1 % Lactose and 1% Sucrose	Fermentation of either Lactose or Sucrose produces large amounts of acid	Production of large amount of acid results in a yellow colour in both slant and butt.
0.1% Glucose	Fermentation of only Glucose produces a small amount of acid (due to the small amount of Glucose in the agar)	Production of small amount of acid will only turn the butt yellow and the slant remains red. This indicates that the organism is only able to ferment Glucose and not Sucrose and Lactose.
Ferrous sulfate	Source of Iron and Sulphur	Organism reduces Sulfate to Hydrogen sulfide. Combination of Iron and Hydrogen sulfide forms a black compound called Ferrous sulfide at the butt of the tube.

Table 3.3: Characteristics of the *Enterobacteriaceae* Family Species on the Triple Sugar Iron Test

Organisms	Slant	Butt	Gas production	Hydrogen Sulphide production
Klebsiella spp	Yellow	Yellow	Positive	Negative
Escherichia spp	Yellow	Yellow	Positive	Negative
Enterobacter spp	Yellow	Yellow	Positive	Negative
Shigella spp	Red	Yellow	Negative	Negative
Salmonella spp	Red	Yellow	Positive	Positive
Proteus spp	Red	Yellow	Positive	Positive

3.6.3 Simons Citrate Test

The Simmons citrate agar (Biolab, Lawrenceville, Georgia, USA) was used to determine the ability of the isolates to utilize citrate as the sole source of carbon (MacFaddin, 2000). An organism that metabolizes citrate is able to convert ammonium salts into ammonia increasing the alkalinity of the agar. Bromothymol blue an indicator that is incorporated into the culture medium changes its colour from green to blue due to a shift in the pH to 7.6 and above. All plates were inoculated with the isolates and were incubated aerobically at 37°C for 24 hours. Data in Table 3.4 was used to presumptively identify the isolates.

Table 3.4: Expected results for members of the family *Enterobacteriaceae* based on the Simons Citrate agar

Growth	**Organisms species**
Citrate Positive (Deep blue colour)	*Klebsiella* spp*, Enterobacter* spp*, Citrobacter* spp*, Salmonella* Paratyphi*, Salmonella* Enteritidis*, Salmonella* Typhimurium
Citrate Negative (Green) or No growth	*Escherichia* spp*, Shigella* spp*, Salmonella* Typhi*, Salmonella* Paratyphi A

3.7 Phenotypic detection of carbapenem resistant *Enterobacteriaceae* (CRE)

3.7.1 Disk Diffusion Test to Detect Carbapenem Resistant Isolates

All isolates that satisfied the preliminary identification tests specific for *Enterobacteriaceae* were subjected to the disc diffusion test to detect CRE resistant isolates. The test was performed as recommended by the Clinical Laboratory Standards Institute (2007) using the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) on a plate of Mueller-Hinton Agar (Biolab, Lawrenceville, Georgia, USA). In order to perform the test, isolates were revived and bacterial suspensions were prepared using sterile distilled water. Aliquots of 100 µL from each suspension were spread-plated on Muller-Hinton agar and antibiotic discs obtained from Mast Diagnostics, South Africa that appear in Table 3.5 were placed on the inoculated agar plates at equidistant points. The plates were incubated aerobically at 37 °C for 24 hours. Antibiotic growth inhibition zone diameter data were measured in millimeters and standard reference

values that appear in Table 3.5 were used to determine if the isolates were susceptible, intermediate resistant or resistant to a particular antibiotic (Clinical Laboratory Standard Institute, 2007).

Table 3.5: Antibiotics that were used to screen for Carbapenem Resistant *Enterobacteriaceae* (CRE)

Antibiotic	**Abbrev**	**Disc conc**	**Sensitive (mm)**	**Intermediate (mm)**	**Resistant (mm)**
Imipenem	IPM	10µg	≥23	20-22	≤19
Ertapenem	ETP	10µg	≥22	19-21	≤18
Meropenem	MEM	10µg	≥23	20-22	≤19
Doripenem	DOR	10µg	≥23	20-22	≤19

3.7.2 Detection of Carbapenemase Production Using the Modified Hodge Test (MHT)

All isolates that were of intermediate resistance or sensitive to Imipenem were subjected to a Modified Hodge Test (Amjad *et al.*, 2011) which is a phenotypic assay that is used to detect the ability of test strains to produce carbapenemase. Isolates that produce the carbapenemase enzyme are resistant to carbapenem antibiotics. This test was performed and interpreted as described by Amjad *et al*. (2011). A 0.5 McFarland standard suspension of *Escherichia coli* (ATCC 25922) control strain was prepared using saline and a 1:10 dilution was later prepared from the solution. Aliquots of 100 µL of the suspension with the lowest dilution factor were spread-plated onto a Mueller-Hinton Agar (Biolab, Lawrenceville, Georgia, USA) in order to prepare a lawn and a 10 µg ertapenem antibiotic disc was placed at the centre of the plate. Test organisms and a positive control strain (*Klebsiella pneumoniae* ATCC 1705) were streaked from the edge of the disk to the edge of the plate in straight lines (Figure 3.1). All the plates were incubated aerobically at 37 °C for 24 hours. Positive isolates produce indentation due to the production of carbapenemase (Figure 3.1) and vice versa (Amjad *et al.,* 2011).

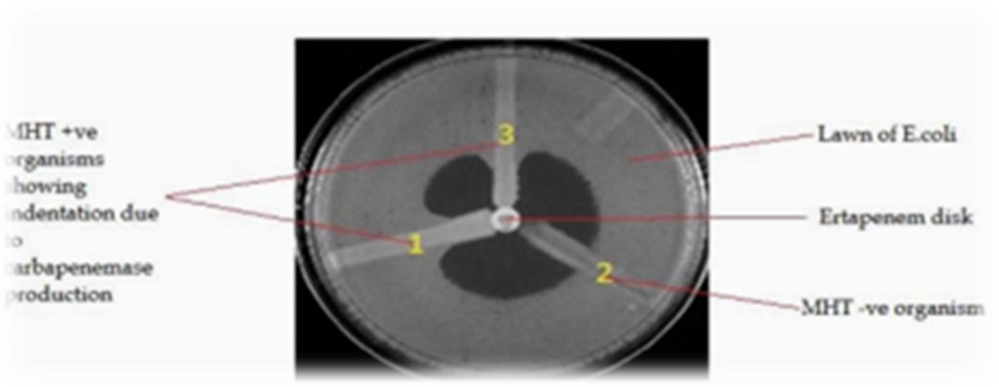


Figure 3.1: Modified Hodge Test demonstrating positive and negative organisms (Amjad *et al.,* 2011)

3.7.3 Culture on Brilliance™ Carbapenem Resistant *Enterobacteriaceae* (CRE) Agar

All the isolates that were either resistant or intermediately resistant to at least one carbapenem antibiotic were sub-cultured onto Brilliance™ CRE agar (Thermo-fisher Scientific, USA). The Brilliance™ CRE agar is a chromogenic medium that is used for presumptive detection of carbapenem resistance among *Enterobacteriaceae*. A 0.5 McFarland turbidity standard was prepared from fresh pure colonies and inoculated onto commercially obtained Brilliance™ CRE agar plates that were incubated aerobically at 37 °C for 24 hours. All plates were read according to manufacturer's instructions using standard guidelines that appear in Table 3.6.

Table 3.6: Morphological characteristics of *Enterobacteriaceae* on Brilliance™ CRE agar

Color produced	**Organisms species**
Pink	*Escherichia coli*
Blue	*Klebsiella, Enterobacter, Serratia* and *Citrobacter* (KESC)
Tan with Brown halo	*Proteus, Morganella* and *Providencia* (PMP)
White or Colorless	Resistant non-*Enterobacteriaceae*

3.8 Phenotypic Detection of Extended Spectrum Beta-Lactamase (ESBL) Producing Isolates

3.8.1 Culture on Brilliance Extended Spectrum Beta-Lactamase Agar

All presumptive isolates that were either intermediately resistant or resistant to carbapenem antibiotics were sub-cultured on Brilliance ESBL agar obtained from Biolab, (Biolab, Lawrenceville, Georgia, USA) in order to determine their ability to produce extended spectrum beta-lactamases. Brilliance ESBL agar is a chromogenic medium that is used for detecting ESBL producing organisms while inhibiting growth of other bacteria including those carrying *Amp*C type resistance. A 0.5 McFarland turbidity standard was prepared from fresh pure colonies and inoculated onto solidified agar plates and incubated aerobically at 37 °C for 24 hours. Plates were read according to the manufacturer's instructions as demonstrated in Table 3.7.

Table 3.7: Morphological characteristics of *Enterobacteriaceae* on Brilliance ESBL agar

Color produced	**Organisms species**
Blue or Pink	*Escherichia coli*
Green	*Klebsiella, Enterobacter, Serratia* and *Citrobacter* (KESC)
Tan with Brown halo	*Proteus, Morganella* and *Providencia* (PMP)
Colorless	*Salmonella, Acinetobacter* and others

3.8.2 Antimicrobial Resistance Profiles of the Isolates

All the isolates that possessed both CRE and ESBL characteristics by phenotypic agar screening assays were subjected to the Kirby-Bauer agar disc diffusion antimicrobial susceptibility test (Bauer *et al.*, 1966) against a panel of 13 different antimicrobial agents obtained from Mast Diagnostics, (Mast Diagnostics, Bootle, United Kingdom) (Table 3.8). The test was performed as recommended by the Clinical Laboratory Standards Institute (CLSI, 2007). In order to perform the test all isolates were revived by culturing on MacConkey agar and plates were incubated at 37 °C for 24 hours. A 0.5 McFarland turbidity bacteria suspension was prepared using fresh pure colonies from MacConkey agar and aliquots of 100 µL were spread-plated onto Mueller-Hinton Agar (Biolab, Lawrenceville, Georgia, USA) plates. The selected

antibiotic discs were placed on inoculated plates at equal distances and plates were incubated aerobically at 37 °C for 24 hours. Growth inhibition zone diameter data were determined in millimeters and results were used to classify isolates as susceptible, intermediate resistant or resistant to an antibiotic based on standard reference values (Clinical Laboratory Standard Institute, 2007).

Table 3.8: Details of the different antibiotics used in the study for antibiotic susceptibility test of *Enterobacteriaceae*

Antibiotic	**Abbrev**	**Disc concentration**	**Type of Antibiotic**	**Sensitive (mm)**	**Intermediate (mm)**	**Resistant (mm)**
Piperacillin	PRL	30µg	Broad-spectrum penicillin	≥21	18-20	≤17
Ticarcillin	TC	75µg	Extended-spectrum penicillin	≥20	15-19	≤14
Amoxicillin	A	25µg	Aminobenzyl-penicillin	≥17	14-16	≤13
Amoxicillin-clavulanate	AMC	30µg	Aminobenzyl-penicillin with β-lactamase inhibitor	≥18	14-17	≤13
Cephalothin	KF	30µg	1st generation Cephalosporin	≥18	15-17	≤14
Cefoxitin	FOX	30µg	2nd generation Cephalosporin	≥18	15-17	≤14
Cefuroxime	CXM	30µg	2nd generation Cephalosporin	≥18	15-17	≤14
Cefotaxime	CTX	30µg	3rd generation Cephalosporin	≥26	23-25	≤22
Ceftazidime	CAZ	30µg	3rd generation Cephalosporin	≥21	18-20	≤17
Ceftiofur	EFT	30µg	3rd generation Cephalosporin for veterinary use	≥21	18-20	≤17
Cefepime	CPM	30µg	4th generation Cephalosporin	≥25	19-24	≤18
Aztreonam	ATM	30µg	Monobactam	≥21	18-20	≤17
Ciprofloxacin	CIP	5µg	Quinolones Group II	≥21	16-20	≤15

3.9 Molecular Characterisation of *Enterobacteriaceae*

3.9.1 DNA Extraction

In order to avoid any bias, all the 151 presumptive isolates that possessed ESBL and CPE phenotypic traits on chromogenic agar and were resistant or intermediately resistant to at least one carbapenem were subjected to genotypic characterisation assays using PCR analysis. The isolates were revived by sub-culturing on Nutrient agar (Biolab, Lawrenceville, Georgia, USA) and plates were incubated aerobically at 37 °C for 24 hours. Fresh pure colonies were inoculated into 5 mL Nutrient broth (Biolab, Lawrenceville, Georgia, USA) and incubated aerobically at 37 °C for 24 hours. Bacterial chromosomal DNA was extracted using the Zymo Research Genomic DNA™ –Tissue MiniPrep Kit (Zymo, Irvine, California, USA) following the manufacturer's instructions. The extracted DNA (100 µL) from isolates was stored at -80°C and used for PCR amplifications.

3.9.2 Determination of the quality and quantity of DNA extracted

The quality and quantity of genomic DNA extracted from the isolates was determined using a WPA Lightwave UV/visible spectrophotometer (WPA, UK) by measuring the optical density at 260 nm and 280 nm (Sambrook *et al.*, 1989). The quantity of DNA was assessed by conventional 1 % (w/v) gel electrophoresis.

3.9.3 Amplification of 16S rRNA gene fragments

Bacterial 16S rRNA gene fragments were amplified using bacterial universal primers from the genomic DNA extracted from all the 151 isolates. PCR reactions were prepared as standard 25 µL reaction mixtures comprising 12.5 µL of ready to load 2X DreamTaq Green Master Mix, 0.25 µL of each oligonucleotide primer, 11 µL RNase-DNase free water and 1 µL template DNA. The oligonucleotide primer sequences as well as the cycling conditions that appear in Table 3.9 were used to amplify the bacterial 16S rRNA gene fragments (Korzeniewska and Harnisz, 2013). PCR amplifications were

performed using C1000 Touch™ Thermal cycler (Bio-Rad, Hercules, California, USA). All PCR reagents used were Fermentas, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR products were stored at 4 °C and later resolved by agarose electrophoresis.

Table 3.9: Oligonucleotide primers used for 16S rRNA amplification and PCR conditions

Primers	**Sequence (5'-3')**	**Targeted gene**	**Amplicon size (bp)**	**PCR condition and cycles**	**References**
27F	AGAGTTTGATCATGGCTCAG	*16S rRNA*	1420	Initial denaturing at 95°C for 5 min and 30 cycles of: Denaturing at 94°C for 1 min Annealing at 55°C for 1 min Extension at 72°C for 1 min Final Extension 72°C for 10 min	(Korzeniewska and Harnisz, 2013)
1492R	GGTACCTTGTTACGACTT				

3.9.4 Genus Specific PCR to Confirm Identities of *Enterobacteriaceae*

Genus specific PCR analyses designed to amplify the *uidA*, *ntrA*, *tuf*, *invA* and *shg* gene fragments that are specific for *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Salmonella* species and *Shigella* species respectively (Salehi *et al*., 2005; Anbazhagan *et al*., 2011; Kupradit *et al*., 2013) were performed on all 151 isolates. Amplifications were performed using primer sequences and cycling conditions outlined in Table 3.10. PCR cycling was performed using the C1000 Touch™ Thermal cycler (Bio-Rad, Hercules, California, California, USA) and PCR reactions were standard 25 µL volumes comprising 12.5 µL of 2X DreamTag Green Master Mix, 11 µL RNase-DNAse free distilled water, 0.25 µL of each oligonucleotide primer, and 1 µL of template DNA. All PCR reagents used for the PCR were from Thermo-Fischer, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR products were held at 4 °C and later separated by agarose gel electrophoresis.

3.10 Molecular Detection of the ESBLs Encoding Genes in *Enterobacteriaceae*

3.10.1 Detection of Bla_{CTX-M}, Bla_{OXA}, Bla_{SHV}, and Bla_{TEM} genes

All the 151 isolates were subjected to a gene specific multiplex PCR assay in order to screen for the presence of ESBL genes bla_{CTX-M}, bla_{OXA}, bla_{SHV} and bla_{TEM} using oligonucleotide primers (Al-Mayahie, 2013) that appear in Table 3.11. Amplifications were performed with C1000 Touch™ Thermal cycler (Bio-Rad, Hercules, California, USA) using PCR cycling conditions that are shown in Table 3.11. PCR reactions were standard 25 µL volumes comprising12.5 µL of 2X DreamTag Green Master Mix, 9.5 µL RNase free distilled water, 0.25 µL of each primer and 1 µL of template DNA. All PCR reagents used in the analysis were from ThermoFischer, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR products were held at 4 °C and later resolved by agarose gel electrophoresis.

3.11 Molecular Detection of Carbapenem Resistant *Enterobacteriaceae*

3.11.1 Detection of Carbapenemase Resistance Genes in isolates using Multiplex PCR

In order to avoid bias, all the 151 isolates that were screened for the presence of ESBL genes were also subjected to a multiplex PCR designed to screen for the presence of carbapenemase resistance genes (*bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{OXA-23}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$) (Gheorghe *et al.*, 2014). Multiplex PCR was performed using oligonucleotide primer sequences that appear in Table 3.12. The six primers were divided into two groups based on their amplicon sizes. In Group 1 were *bla*$_{KPC,}$ *bla*$_{NDM}$ and *bla*$_{OXA-23}$ and Group 2 had *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$.

Table 3.10: Oligonucleotide primers used for genus specific PCR amplification and amplification conditions

Primer	Primer sequence (5'-3')	Target gene	Amplicons size (bp)	PCR condition and cycles	References
ntrA-F	CATCTCGATCTGCTGGCCAA	*ntrA*	90bp	**Initial denaturing** at 95°C for 10 min 35 cycles of: **Denaturing** at 95°C for 45seconds **Annealing** at 52°C for 45 seconds **Extension** at 72°C for 1 min 30 seconds **Final Extension** 72°C for 10 min	(Anbazhagan *et al*., 2011)
ntrA-R	GCGCGGATCCAGCGATTGGA				
uidA-F	CTGGTATCAGCGCGAAGTC	*uidA*	556bp	**Initial denaturing** at 95°C for 10 min 35 cycles of: **Denaturing** at 95°C for 45 seconds **Annealing** at 52°C for 45 seconds **Extension** at 72°C for 1 min 30 seconds **Final Extension** 72°C for 10 min	(Anbazhagan *et al*., 2011)
uidA-R	AGCGGGTAGATATCACACTC				
Tuf-F	TCTACTTCACACGTAG	*tuf*	240bp	**Initial denaturing** at 95°C for 10 min 35 cycles of: **Denaturing** at 95°C for 45 seconds **Annealing** at 58°C for 45 seconds **Extension** at 72°C for 1 min 30 seconds **Final Extension** 72°C for 10 min	(Anbazhagan*et al*., 2010)
Tuf-R	TTCTAACAGCTCTTCA				
invA-F	GTGAAATTATCGCCACGTGGCAA	*invA*	284bp	**Initial denaturing** at 94°C for 60 seconds 35 cycles of: **Denaturing** at 94°C for 60 seconds **Annealing** at 64°C for 30 seconds **Extension** at 72°C for 30 seconds **Final Extension** 72°C for 7 min	(Salehi *et al*., 2005)
invA-R	TCATCGCACCGTCAAAGGAACC				

Table 3.11: Oligonucleotide primers used for the detection of ESBL genes and multiplex-PCR amplification conditions

Primer	Primer sequence (5'-3')	Target gene	Amplicons size (bp)	PCR condition and cycles	References
blaTEMF	AAACGCTGGTGAAAGTA	*bla*$_{TEM}$	822	**Initial denaturing** at 94°C for 5 min 35 cycles of: **Denaturing** at 94°C for 30 seconds **Annealing** at 45°C for 1 min **Extension** at 72°C for 1 min **Final Extension** 72°C for 10 min	(Al-Mayahie, 2013)
blaTEMR	AGCGATCTGTCTAT				
blaSHVF	ATGCGTTATATTCGCCTGTG	*bla*$_{SHV}$	753		
blaSHVR	TGCTTTGTTATTCGGGCCAA				
blaCTX-MF	CGCTTTGCGATGTGCAG	*bla*$_{CTX-M}$	550		
blaCTX-MR	ACCGCGATATCGTTGGT				
blaOXAF	ATATCTCTACTGTTGCATCTCC	*bla*$_{OXA}$	619		
blaOXAR	AAACCCTTCAAACCATCC				

Table 3.12: Oligonucleotide primers used for the detection of Carbapenemase resistance genes and multiplex-PCR amplification conditions

Primer	Primer sequence (5'-3')	Target gene	Amplicons size (bp)	PCR condition and cycles	References
KPC-Fm	CGTCTAGTTCTGCTGTCTTG	*bla*$_{KPC}$	798	**Initial denaturing** at 94°C for 10 min 36 cycles of: **Denaturing** at 94°C for 30 seconds **Annealing** at 52°C for 40 seconds **Extension** at 72°C for 50 seconds **Final Extension** 72°C for 5 minutes	(Poirel *et al.*, 2011; Gheorghe *et al.*, 2014)
KPC-Rm	CTTGTCATCCTTGTTAGGCG				
NDM-F	GGTTTGGCGATCTGGTTTTC	*bla*$_{NDM}$	621		
NDM-R	CGGAATGGCTCATCACGATC				
OXA-23-F	ATGAGTTATCTATTTTTGTC	*bla*$_{OXA-23}$	501		
OXA-23-R	TGTCAAGCTCTTAAATAATA				
GES-C	GTTTTGCAATGTGCTCAACG	*bla*$_{GES}$	371		
GES-D	TGCCATAGCAATAGGCGTAG				
VIM-F	GATGGTGTTTGGTCGCATA	*bla*$_{VIM}$	390		
VIM-R	CGAATGCGCAGCACCAG				
OXA-48-F	TTCGGCCACGGAGCAAATCAG	*bla*$_{OXA-48}$	438		
OXA-48-R	GATGTGGGCATATCCATATTCATC GCA				

3.12 Agarose Gel Electrophoresis and Visualization

PCR products were separated by electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide (0.1µg/mL) (Sambrook *et al.*, 1989). Depending on the size of the amplicons, a 1Kb or 100 bp DNA molecular weight marker (Fermentas, USA) was included in each gel. Electrophoresis was conducted in a horizontal Pharmacia biotech equipment system (Model Hoefer HE 99X, Amersham Pharmacia biotech, Sweden) for 1 hour at 80V using 1X (v/v) TAE buffer. A ChemiDoc Imaging System (Bio-Rad ChemiDoc™ MP Imaging System, Hercules, California, USA) was used to capture the image.

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 Identification of *Enterobacteriaceae* Isolates

4.1.1 Colony and Cellular Morphology

A total of 233 faecal samples were collected from the different cattle farms located in North West Province of South Africa. The samples were assessed for characters of *Enterobacteriaceae* by culturing on MacConkey agar supplemented with Crystal Violet and salt. Isolates were selected based on colony morphology depending on their colony colour, size, margin, and shape. *Enterobacteriaceae* are known to exhibit distinguishable colonies on MacConkey agar supplemented with Crystal Violet and salt (Health Protection Agency, 2013). Lactose fermenting organisms such as *Escherichia coli* produce pink colour, while non-lactose fermenters such as *Proteus*, *Shigella* etc. will grow on the media but produce colourless colonies. A total of 314 presumptive isolates were selected from the 233 samples based on the aforementioned characteristics. Gram staining revealed that all the 314 presumptive isolates were Gram negative non-spore forming straight rods, with different sizes. These isolates were subjected to other biochemical tests specific for *Enterobacteriaceae*.

4.1.2 Preliminary Biochemical Tests

All the 314 presumptive isolates were subjected to biochemical identification tests specific for *Enterobacteriaceae*. Out of the 314 presumptive isolates, 34 isolates were positive for the spot oxidase test and were excluded from the study while the remaining 280 presumptive isolates were oxidase negative (Table 4.1). The 280 isolates also satisfied the specific characteristics for *Enterobacteriaceae* based on the Triple Sugar Iron agar and Simmons Citrate agar tests (Table 4.1) and were subjected to further biochemical identification tests.

Table 4.1 Biochemical Tests Results of the Presumptive Isolates

Test	**Results**			**Total**
Oxidase	Negative		280	314
	Positive		34	
Simmons Citrate	Blue Pigmentation		267	280
	No colour		13	
Triple Sugar Iron	Yellow	Butt	38	280
		Slant	236	
		No reaction	6	
	Gas Production		218	280
	No Gas Production		62	

4.2 Proportion of *Enterobacteriaceae* that possessed Carbapenem Resistance phenotypes

4.2.1 Proportion of isolates with resistance to Carbapenem antibiotics

All the 280 presumptive isolates that satisfied the preliminary identification tests for *Enterobacteriaceae* were screened for resistance against the following carbapenem antibiotics ertapenem, imipenem, meropenem and doripenem. All isolates that were either resistant or intermediately resistant against one or more of these antibiotics were treated as carbapenem resistant *Enterobacteriaceae* as previously defined (Centre for Disease Control and Prevention, 2015). Detailed data of results obtained are shown in Figure 4.1 As indicated in Figure 4.1, a total of 189 isolates were either resistant or intermediate resistant to one or more than one of the carbapenem antibiotics tested. In the current study 91 isolates were susceptible to all the Carbapenems tested, the proportion of isolates that were resistant to imipenem (42%) was larger than those resistant to ertapenem (35%), doripenem (30%) and meropenem (28%). A large proportions of the isolates showed intermediate resistance to meropenem (41%), and most isolates were susceptible to doripenem (36%) followed by ertapenem (34%) (Figure 4.1). The distribution of carbapenem resistance amongst the 189 isolates according to sample location is detailed in Table 4.2 below.

Table 4.2: Distribution of carbapenem resistant isolates according to collection location.

Type of farm	Collection Location	Samples collected	Presumptive isolates	Carbapenem resistant isolates
Communal Farms	**Lokaleng**	35	28	8
	Ntontonyane	80	92	52
Commercial Farms	**Koster**	53	58	45
	Rooigrond	65	102	84
Total		**233**	**280**	**189**

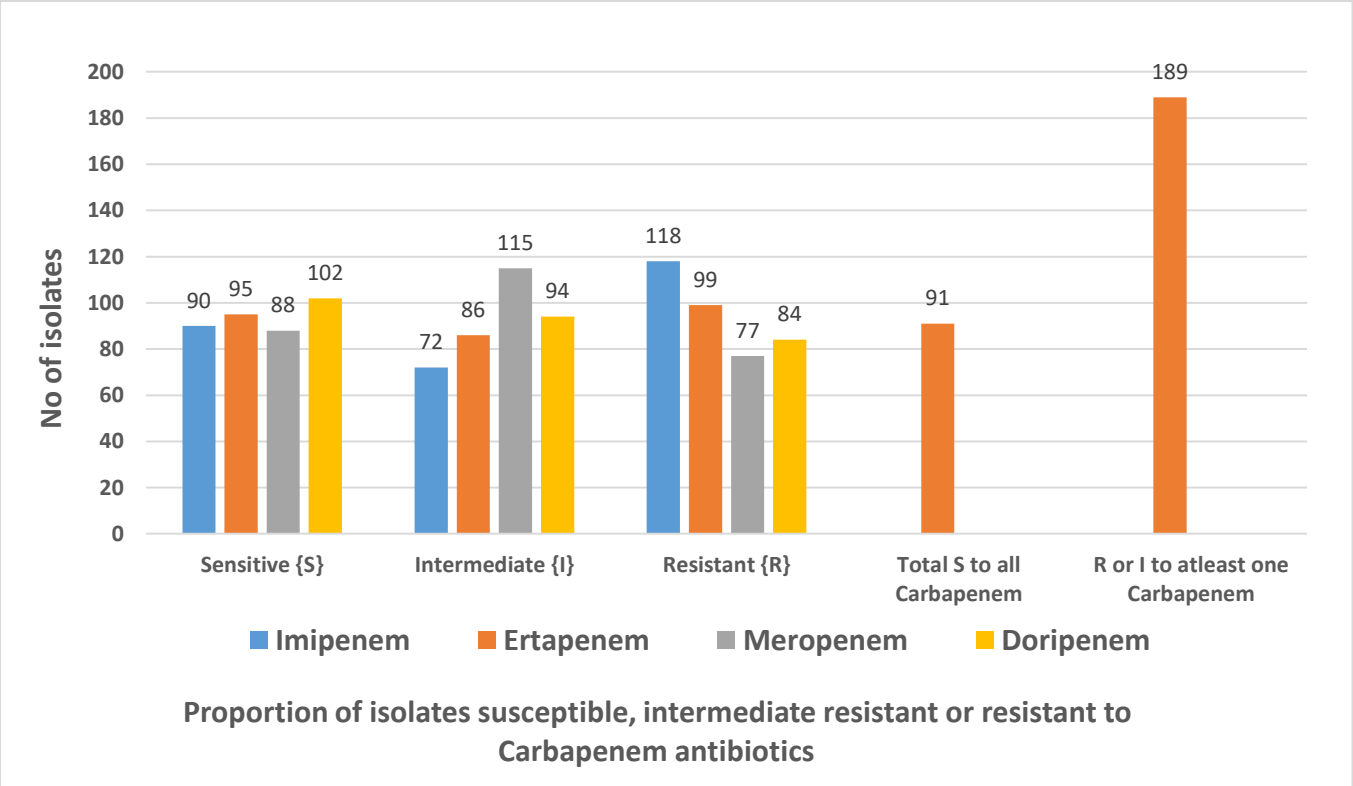


Figure 4.1: Susceptibility test results for different Carbapenem antibiotics

4.2.2 Detection of Carbapenemase producing phenotypes among *Enterobacteriaceae* using the Modified Hodge test

All the isolates that were either susceptible or intermediately resistant to imipenem were screened for the production of carbapenemase using the Modified Hodge test. A total of 162 isolates were subjected to this phenotypic assay and only 31 presumptive isolates were positive for the test. Among the 31 isolates

that were positive for carbapenemase production based on the Modified Hodge test, 16 were susceptible while 15 were intermediately resistant to imipenem based on the disc diffusion test. Detailed results are shown in Table 4.3

Table 4.3: Modified Hodge Test results for isolates susceptible or intermediate resistant to Imipenem

Modified Hodge Test		
Proportion of isolates subjected to the test		**Proportion of isolates positive for the test**
Intermediate isolates	72	15
Sensitive isolates	90	16
Total MHT positive isolates		**31**

4.2.3 Brilliance™ Carbapenem Resistant *Enterobacteriaceae* Agar

In order to avoid bias, all 280 presumptive isolates were plated onto Brilliance™ CRE agar to test for their resistance to the carbapenem antibiotic phenotypically. From these isolates, a total of194 isolates grew on the medium comprising an addition of 5 isolates to the 189 isolates that were initially resistant to carbapenems based on the disc diffusion test. Isolates produced colours that ranged from pale pink and blue indicative of carbapenem-resistant *E. coli* and carbapenem-resistant *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) respectively (Table 4.4 and Figure 4.2). As shown in Table 4.4, a large proportion (53.1%) of the isolates were carbapenem-resistant KESC.

Table 4.4: Colour Production as Observed on the Brilliance™ CRE Agar

Colour on Agar	**Positive Isolates**	**Total**
Pale pink	68	194
Blue	103	
Tan with Brown Halo	7	
White and Colourless	16	
No growth	86	86

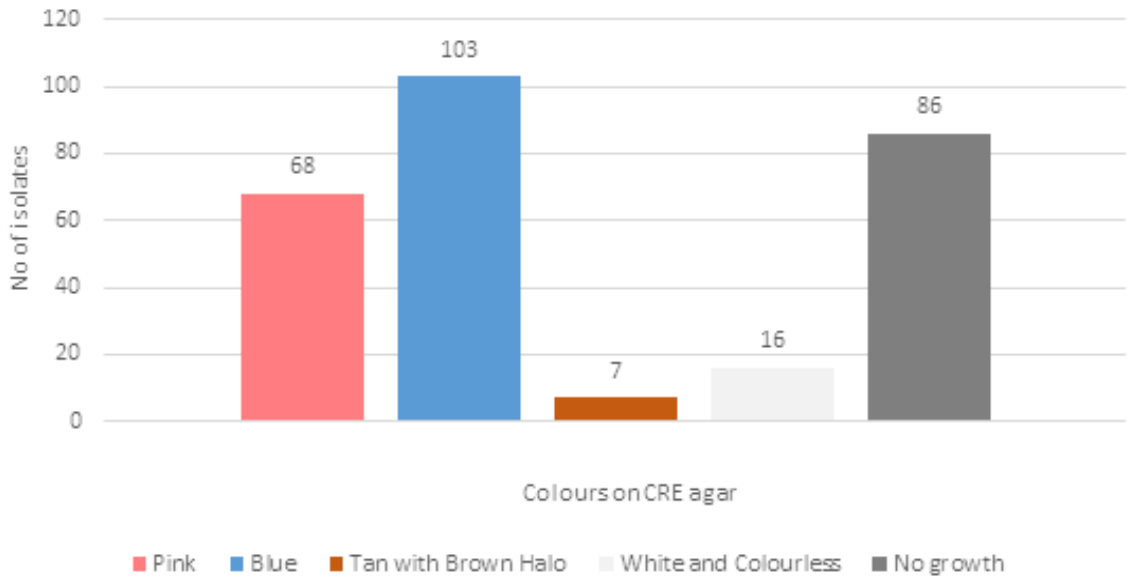


Figure 4.2: The presence of Carbapenem resistant Enterobacteriaceae as observed on the Brilliance™ CRE agar

4.3 Proportion of *Enterobacteriaceae* with ESBL and Carbapenem producing traits

4.3.1 Brilliance™ ESBL Agar

The ability of isolates to produce extended spectrum beta-lactamase (ESBL) was determined on all the 194 isolates in order to avoid any bias. All the 194 isolates that grew on the Brilliance™ CRE Agar were subjected to culture onto Brilliance ESBL agar plate to test for their ability to produce beta-lactamase enzyme. Results indicated that a total of 151 isolates were positive for ESBL production phenotypically and detailed results are shown in Table 4.5 and Figure 4.3. A large proportion (54.9%) of the ESBL-producing isolates produced green colonies indicative of ESBL-producing *Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* (KESC) (Table 4.5). In addition, 51 (33.8%) isolates produced blue coloured colonies and were presumptively identified as *E. coli*. A small proportion (3.3%) of the isolates produced tan colonies with brown halos due to the fact that they do not utilise either chromogen, but are able to deaminase tryptophan thus indicative of either *Proteus* or *Morganella* and *Providencia*.

Table 4.5: Different colour morphology of bacteria observed on the Brilliance™ ESBL Agar

Colour on Agar	No of positive isolates	Total
Blue	51	151
Green	83	
Tan with Brown Halo	5	
Colourless	12	
No growth (excluded)	43	43

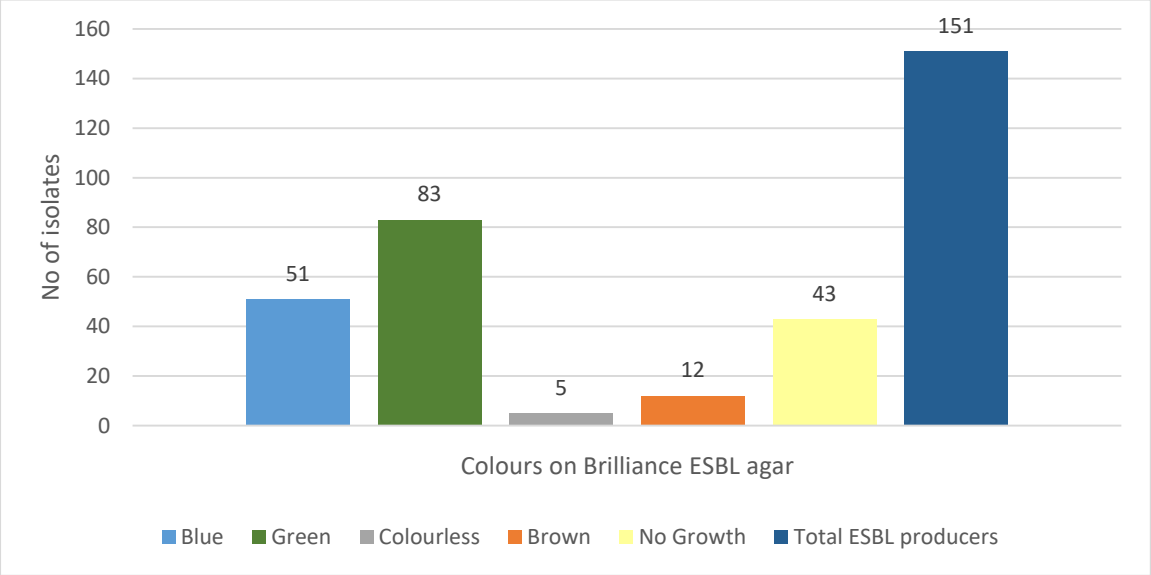


Figure 4.3: The persistence of Carbapenem resistant *Enterobacteriaceae* isolates on Brilliance ESBL agar.

4.3.2 Antimicrobial Resistance Profile

The 151 isolates that were carbapenem resistant and also positive for ESBL production were taken through the disk diffusion test with the use of the 13 antibiotics listed in Table 3.8. The results for antibiotic susceptibility test revealed that the isolates were mostly resistant to cefotaxime, aztreonam and cefotaxime. In addition, large proportions (39.1% to 43.7%) of these isolates were also resistant to ceftiofur, amoxicillin, piperacillin, ticarcillin, cephalothin, ceftazidime and cefoxitin (Table 4.6 and Figure 4.4.). On the contrary, most were susceptible to ciprofloxacin (12.6%) and amoxicillin-clavulanate (9.3%).

Table 4.6: Disk diffusion test results for the different antibiotics types

	Percentage antibiotic resistance												
	CXM	**CPM**	**ATM**	**EFT**	**A**	**PRL**	**TC**	**KF**	**CTX**	**CAZ**	**FOX**	**CIP**	**AMC**
Intermediate	N= 20 %=13.2	N=52 %=34.4	N=34 %=22.5	N=36 %=23.8	N=35 %=23.2	N=39 %=25.8	N=43 %=28.5	N=20 %=13.2	N=22 %=14.6	N=31 %=20.5	N=23 %=15.2	N=38 %=25.2	N=8 %=5.3
Resistant	N=72 %=47.7	N=30 %=19.9	N=82 %=54.3	N=60 %=39.7	N=60 %=39.7	N=66 %=43.7	N=59 %=39.1	N=62 %=41.1	N=90 %=59.6	N=62 %=41.1	N=64 %=42.4	N=19 %=12.6	N=14 %=9.3
Susceptible	N=59 %=39.1	N=69 %=45.7	N=35 %=23.2	N=55 %=36.4	N=56 %=37.1	N=46 %30.5	N=49 %=32.5	N=69 %=45.7	N=39 %=25.9	N=58 %=38.4	N=64 %=42.4	N=94 %=62.3	N=129 %=85.4
Total	**151**												

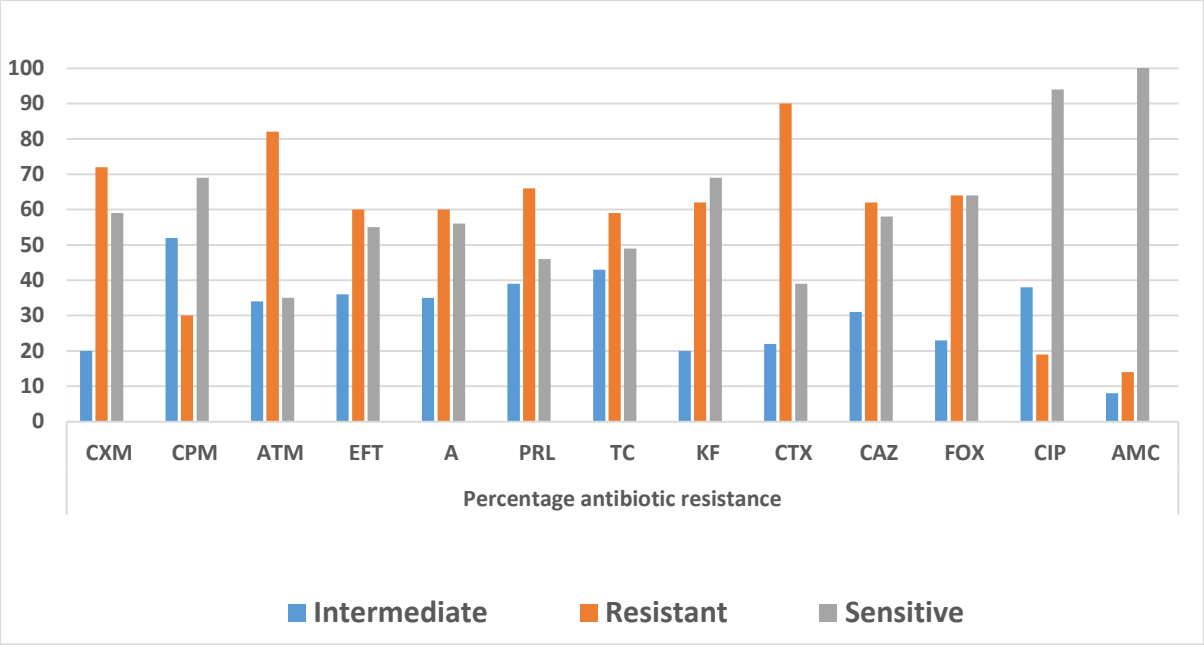


Figure 4.4: Percentage antibiotics resistance of isolates tested with Disk Diffusion test

4.4 Molecular Characterization of ESBL producing Carbapenem Resistant *Enterobacteriaceae*

4.4.1 16S rRNA Gene PCR analysis

Using bacterial universal primers, the DNA from all the 151 isolates which showed carbapenem resistance and ESBL producing traits was used to carry out the 16S rRNA gene PCR. The PCR results indicated that the desired 1420 bp gene fragments were successfully amplified from all 151 the isolates. Figure 4.5 indicates a 1% (w/v) agarose gel image of the bacterial 16S rRNA gene fragments.

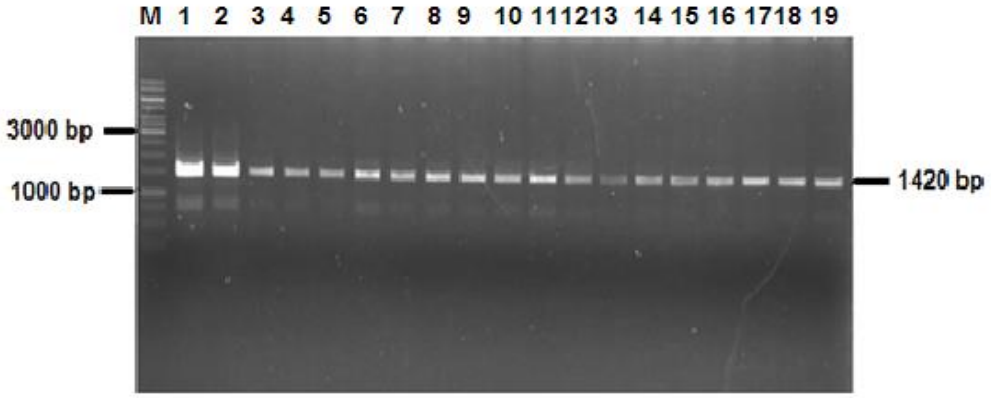


Figure 4.5: Agarose gel (1% w/v) image showing universal 16S rRNA gene fragments (1420 bp) amplified from the selected isolates and control strains. M= 1 kilo base pairs O'GeneRuler, Lane 1= *K. pneumoniae* (ATCC 13883) control strain, Lane 2= *E. coli* (ATCC 25922) control strain, Lane 3 - 19 = gene fragments of the tested isolates.

4.4.3 Genus Specific PCR

4.4.3.1 Amplification of *uidA* gene

All 151 presumptive isolates that were positive for the 16S rRNA gene fragments were screened for the *uidA* gene fragment that is specific for *Escherichia coli*. In the present study, a total of 52 (33.77%) isolates were positive for the *uidA* 556 bp gene fragments. Figure 4.6 shows a 1% (w/v) agarose gel image of the *E. coli uidA* gene fragments.

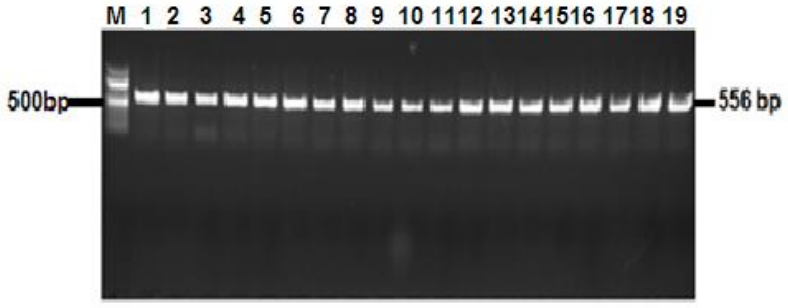


Figure 4.6: Agarose gel (1% w/v) image showing PCR products for *uidA* (556bp) gene fragments. Lane M = 100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1= *uidA* gene fragments amplified from *E. coli* (ATCC 25922) and lanes 2-19= *uidA* gene fragments amplified from *E. coli* isolates.

4.4.3.2 Amplification of *ntrA Klebsiella pneumoniae* gene fragments

A total of 99 isolates were processed for the *ntrA* gene PCR that is specific for *Klebsiella pneumoniae* species. Results indicated that the 90 bp *ntrA* gene fragments were successfully amplified in 66 (66.7%) of the isolates and Figure 4.7 shows a 1% (w/v) agarose gel image of the *Klebsiella pneumoniae ntrA* gene fragments.

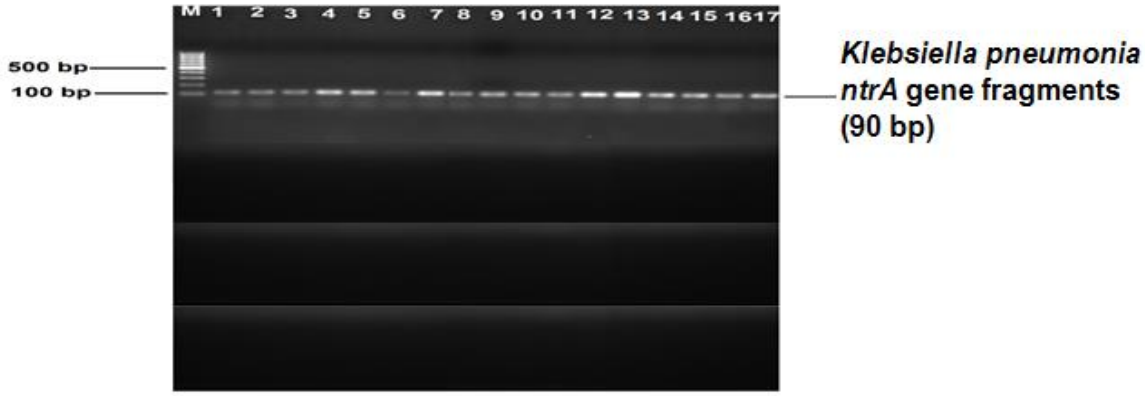


Figure 4.7: Agarose gel (1% w/v) image showing PCR products for *ntrA* (90bp) gene fragments amplified. Lane M = 100 base pairs ThermoFischer O'GeneRuler DNA Ladder, Lane 1= *ntrA* gene fragments amplified from *K. pneumoniae* (ATCC 13883) and lanes 2-17 = *ntrA* gene fragments amplified from isolates.

4.4.3.3 Proportion of isolates positive for the *tuf* gene

The remaining 33 isolates were processed for the *tuf* gene PCR analysis that is specific for *Proteus mirabilis* and 2 (1.3%) isolates possessed the desired 240 bp gene fragment (Figure 4.8).

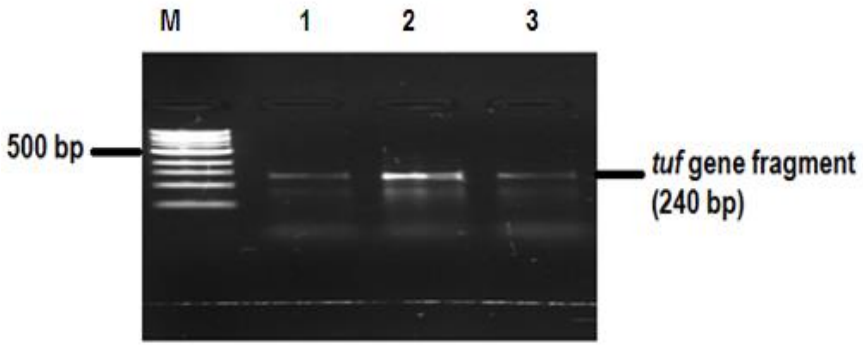


Figure 4.8: Agarose gel (1% w/v) image showing PCR amplification for *tuf* (240bp) gene fragments of the tested isolates. Lane M =100 base pairs ThermoFischer O'GeneRuler DNA Ladder, Lane 1 = *tuf* gene fragments amplified from *P. mirabilis* (ATCC 29930) and Lanes 2 – 3 = *tuf* gene fragments amplified from isolates.

4.4.3.4 Amplification of *invA* gene

A total of 31 isolates were subjected to the *invA* gene PCR analysis that is specific for *Salmonella* species. Results indicated that 7 (4.6%) possessed this gene sequence and generated the desired 284 bp amplicons. Figure 4.9 indicates a 1% (w/v) agarose gel of the gene *invA* fragments amplified during the study.

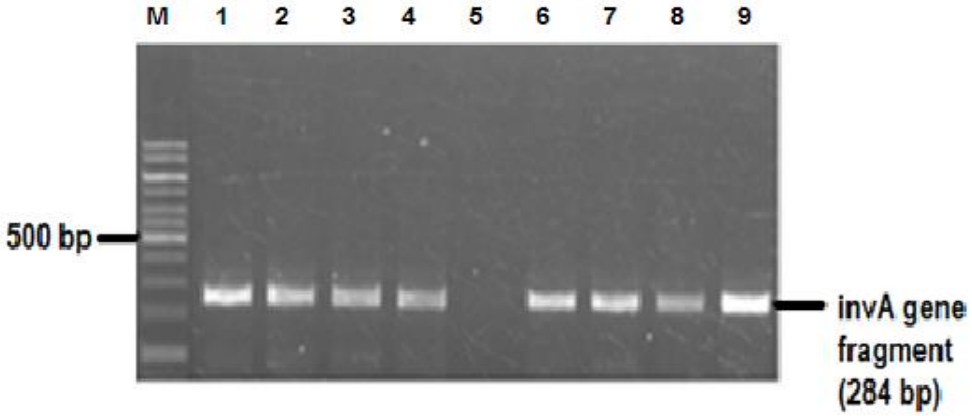


Figure 4.9: Agarose gel (1% w/v) image showing PCR products for *invA* (284bp) gene fragments amplified from isolates. Lane M =100 base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1= *invA* gene fragments amplified from *S. enterica* (ATCC 12325) and lanes 2-4 and 6-9= *invA* gene fragments amplified from *Salmonella* isolates. Lane 6= Negative control reaction.

4.4.4 Detection of CRE resistant genes in isolates using Multiplex PCR analysis

In this study, a total of 24 isolates were not fully identified by PCR as *Enterobacteriaceae* but were also included in the multiplex PCR assay designed to screen for CRE resistant genes. Thus all the 151 isolates where the CRE genes was as presented in Table 4.7. The PCR assay was performed in two groups of three primers each (*bla*$_{KPC,}$ *bla*$_{NDM}$, *bla*$_{OXA-23}$, and *bla*$_{VIM,}$ *bla*$_{OXA-23}$ and *bla*$_{GES}$) based on the expected amplicon sizes of the target genes. A total of 96 isolates harboured either one or more of the *bla*$_{KPC,}$ *bla*$_{NDM}$ and *bla*$_{OXA-23}$ gene sequences. Results revealed that the *bla*$_{KPC}$ (798 bp) was frequently detected (54; 33.8%) in the isolates when compared to the *bla*$_{NDM}$ (621 bp) gene (31; 20.5%) and the *bla*$_{OXA-23}$ (501 bp) (5; 3.3%) gene. An analysis of isolates for associations of the different resistance genes revealed that six (6) isolates had multiple resistant genes comprising four that were positive for the *bla*$_{KPC}$ and *bla*$_{OXA-23,}$ genes while two possessed the bla$_{KPC}$ and *bla*$_{NDM}$ genes. Figure 4.10 is a (1% w/v) agarose gel image of the *bla*$_{KPC,}$ *bla*$_{NDM}$ and *bla*$_{OXA-23}$ CRE genes amplified from the isolates.

In order to avoid bias, all the 151 isolates were also subjected to the *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$ gene PCR analysis and results indicated that a total of 55 (36.4%) isolates were positive for these gene sequences. Detailed analysis revealed that the *bla*$_{GES}$ gene segment was dominant 27 (17.9 %) in the isolates when compared to the *bla*$_{oxa-48}$ 16 (10.6 %) and *bla*$_{VIM}$ 10 (6.6 %) genes thus producing the desired amplicon sizes of 371 bp, 438 bp and 390 bp respectively (Figure 4.11). In addition, only a very small proportion (2; 1.3%) of the isolates possessed both the *bla*$_{GES}$ and *bla*$_{OXA-48}$. Figure 4.11 indicates a (1% w/v) agarose gel image of the *bla*$_{VIM}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$ CRE genes amplified from the isolates.

Table 4.7: Proportion of isolates that carry the different CRE resistant gene determinants

	CRE genes									
	***bla*$_{KPC}$**	***bla*$_{NDM}$**	***bla*$_{OXA-23}$**	***bla*$_{VIM}$**	***bla*$_{OXA-48}$**	***bla*$_{GES}$**	***bla*$_{KPC}$ + *bla*$_{OXA-23}$**	***bla*$_{KPC}$ + *bla*$_{NDM}$**	***bla*$_{GES}$ + *bla*$_{OXA-48}$**	**Total**
E. coli	18	17	2	1	7	5	0	2	0	**52**
K. pneumoniae	28	8	1	2	4	18	4	0	1	**66**
Proteus mirabilis	0	0	1	0	1	0	0	0	0	**2**
Salmonella spp	2	3	1	0	1	0	0	0	0	**7**
Non-genus specified										
KESC group	3	2	0	5	3	2	0	0	1	**16**
PMP group	2	1	0	0	0	0	0	0	0	**3**
SAO group	1	0	0	2	0	2	0	0	0	**5**
Total	**54**	**31**	**5**	**10**	**16**	**27**	**4**	**2**	**2**	**151**

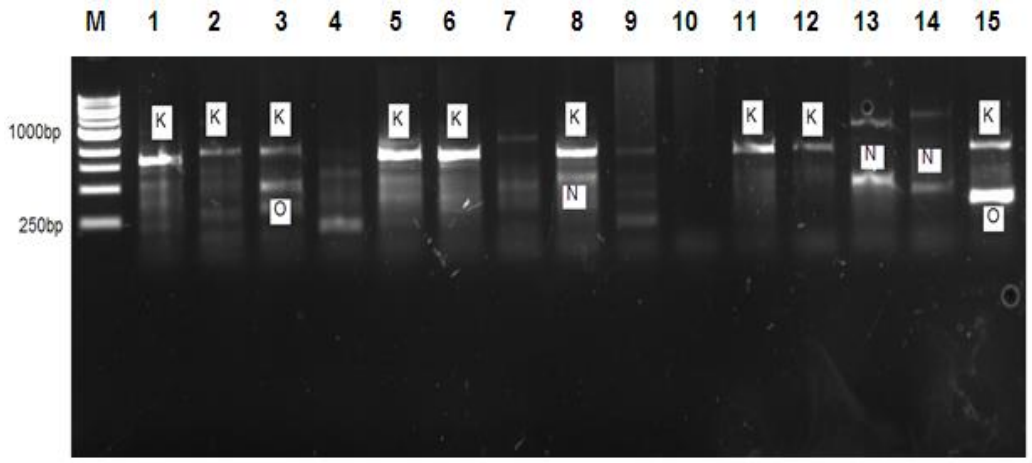


Figure 4.10: Agarose gel (1% w/v) image showing multiplex PCR for CRE genes, KPC (798 bp), NDM (621 bp), OXA-23 (501 bp). Lane M=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1,2,5,6,11 and 12= KPC gene fragments, Lane 3 and 15= KPC and OXA gene fragments, Lane 8= KPC and NDM gene fragments, and Lane 13 and 14= NDM gene fragment. Lane 4, 7, 9 and 10= No gene fragments amplified.

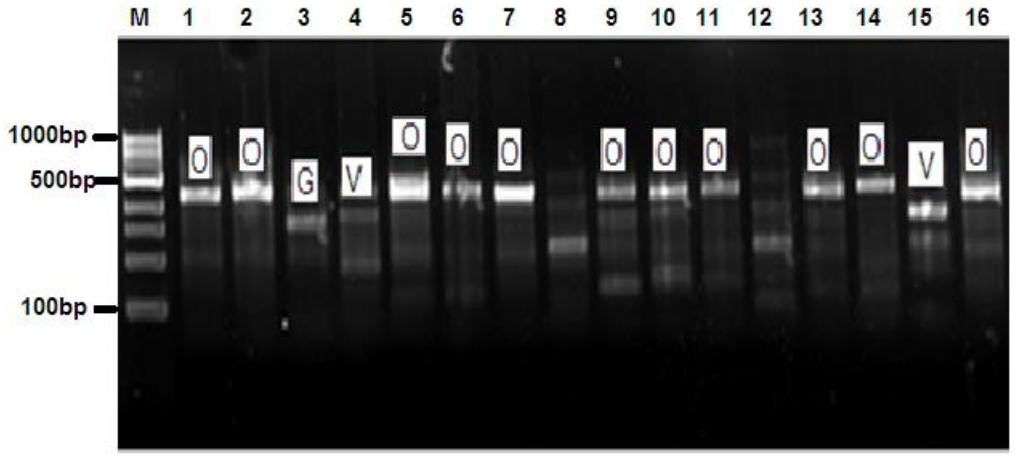


Figure 4.11: Agarose gel (1% w/v) image showing multiplex PCR for CRE genes, OXA-48 (438bp), VIM (390bp), and GES (371bp). Lane M=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lane 1,2, 5-7, 9-11, 13-14 and 16=OXA-48 gene fragments, Lane 3 = GES gene fragment, Lane 4 and 15= VIM gene fragment, Lane 8 and 12= No gene fragments amplified.

4.4.5 Proportion of isolates with ESBL genes

All 151 isolates were screened for ESBL encoding genes (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTXM}$, and *bla*$_{OXA}$) by multiplex PCR and all the 151 isolates were positive for at least one of the target genes as illustrated in Table 4.8. Amplifications of the ESBL genes by PCR produced the desired amplicon sizes of 753 bp, 822 bp, 550 bp and 619 bp for the *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTXM}$, and *bla*$_{OXA}$ genes respectively. Of 151 isolates, a large proportion (132; 87.4%) of the isolates had a single ESBL gene while only a small proportion (19; 12.6%) possessed more than one of the ESBL genes. An analysis of the isolates for associations of the different genes revealed that the *bla*$_{SHV}$ gene was dominant among the isolates and was detected in 50 isolates while the proportion of isolates that harboured the *bla*$_{TEM}$ gene was higher (34; 22.5%) than those with the *bla*$_{CTXM}$ (31; 20.5%) and the *bla*$_{OXA}$ (17; 11.3%) gene. In addition, 11 and 8 isolates possessed a combination of the *bla*$_{OXA}$ and *bla*$_{CTXM}$ as well as the *bla*$_{SHV}$ and *bla*$_{TEM}$ gene sequences respectively.

Table 4.8: Proportion of isolates that harbour the different ESBL resistant gene determinants

	ESBL genes						Total
	***bla*$_{SHV}$**	***bla*$_{TEM}$**	***bla*$_{CTXM}$**	***bla*$_{OXA}$**	***bla*$_{OXA}$ + *bla*$_{CTXM}$**	***bla*$_{SHV}$ + *bla*$_{TEM}$**	
E. coli	12	14	18	1	2	5	**52**
K. pneumoniae	30	11	6	9	8	2	**66**
Proteus mirabilis	0	1	0	1	0	0	**2**
Salmonella spp	0	2	4	0	1	0	**7**
Non-genus specified	8	6	3	6	0	1	**24**
Total	**50**	**34**	**31**	**17**	**11**	**8**	**151**

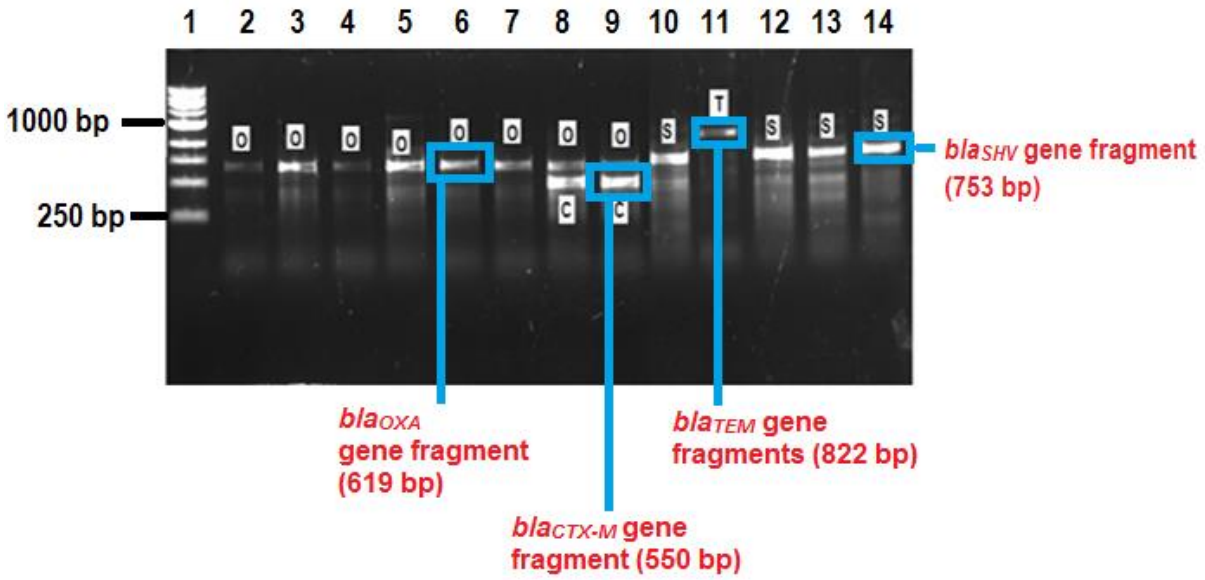


Figure 4.12: Agarose gel (1% w/v) image showing multiplex PCR for ESBL genes OXA (619bp), CTX-M (550 bp), SHV (822 bp) and TEM (753 bp). Lane 1=1 kilo base pairs Fermentas O'GeneRuler DNA Ladder, Lanes 2-7 = *bla*$_{OXA}$ gene from isolates, Lane 8-9= *bla*$_{CTX-M}$ gene fragments, Lane 10, 12-14= *bla*$_{SHV}$ gene fragments, and Lane 11 = *bla*$_{TEM}$ gene fragment from isolates.

CHAPTER 5

DISCUSSIONS AND INTERPRETATION

Resistance of *Enterobacteriaceae* to commonly prescribed antibiotics is currently a major issue of global public health concern due to its impact on therapeutic processes in both human and animal medicine (Carlet *et al.*, 2012; Chang *et al.*, 2015; Frieri, Kumar *et al*., 2017). The detection of multiple antibiotic resistance phenotypes as well as genotypes is currently on the increase among bacterial pathogens especially those belonging to the family *Enterobacteriaceae* (Zurfluh *et al.*, 2013; Singh-Moodley and Perovic, 2016b; Harada *et al.*, 2017). In addition, a number of these species have displayed resistance to beta-lactam antibiotics that were used as treatment options for multiple antibiotic resistant bacteria mainly through the production of beta-lactamases resulting in a very high prevalence of ESBL-producing *Enterobacteriaceae* (Bradford, 2001; Adler *et al.*, 2011; Thaden *et al.*, 2014; Barbarini *et al.*, 2015).

In order to counteract the negative therapeutic impacts of ESBL-producing *Enterobacteriaceae* carbapenems were approved as suitable and reliable agents for the treatment of infections caused by multi-drug resistant ESBL-producing *Enterobacteriaceae* (Hilas *et al.*, 2008; Baughman, 2009; Papp-Wallace *et al.*, 2011). However, studies conducted in recent years have revealed the presence of carbapenem resistant *Enterobacteriaceae* that may also harbour ESBL resistant traits (Yigit *et al.*, 2001; Gupta *et al.*, 2011; Baroud *et al.*, 2013; Perez and Van Duin, 2013; Zurawski, 2014; Meletis, 2016) thus significantly limiting treatment options for their associated infections. The situation is worsened by the very slow pace in the development of new antibiotics by pharmaceutical companies despite the very high morbidity and mortality in humans caused by antibiotic resistant bacteria (Tacconelli *et al.*, 2017). With this in mind, knowledge of the occurrence and antibiotic resistance profiles of bacterial species in a given

area is of paramount importance. In addition, the generation of data that indicates the coexistence of both carbapenem and extended spectrum beta-lactam resistant gene determinants in *Enterobacteriaceae* may also be of great epidemiological importance.

Fosfomycin, polymyxins and aminoglycosides were previously used to treat infections caused by carbapenemase-producing *Enterobacteriaceae* (Coetzee and Brink, 2011; Schultsz and Geerlings, 2012; Perez and Van Duin, 2013; Meletis, 2016). More recently, it has also been reported that isolates belonging to the family *Enterobacteriaceae* that were capable of producing carbapenemases also harboured genes encoding for resistance against fluoroquinolones and aminoglycosides (Coetzee and Brink, 2011; Meletis, 2016), thereby limiting treatment options for infections caused by these organisms. To salvage this situation, a combination therapy that includes at least a carbapenem has proven to be very effective in lowering mortality rates in humans (Tzouvelekis *et al.*, 2014; Meletis, 2016). This therefore means that the constant detection and frequent spread of CRE particularly those that also possess ESBL resistance genes may provide severe public health challenges for the future which indicates the need to limit development as well as dissemination of these multi-drug resistant strains.

The natural hosts of *Enterobacteriaceae* are ruminant animals particularly cattle and thus the consumption of undercooked contaminated meat from animals is known to be one of the major sources of transmission and infections in humans. Several diseases caused by *Enterobacteriaceae* originate from food producing animals (Van Reeth, 2007; Abente *et al.*, 2017; Bourret *et al.*, 2017) and this does not only affect the agricultural sector but poses severe financial constrains to the economy of any country (Torry, 2015). In 2015, the consumption of poultry, beef and pork in South African was at 2.9 million tons per annum of which 2.4 million tons were from local production facilities while the rest was imported from Brazil, Netherlands, United Kingdom and Germany (Torry, 2015). This resulted in an average spending on meat products of R165 billion which is one-third of the total food expenditure in South Africa per annum

(Torry, 2015). Given that the demand for meat and its associated products is high in South Africa, this provides opportunities for possible cross contamination between faeces and carcasses during the production process increasing the risk of food-borne infections in humans. Moreover, the health impacts of these pathogens are even higher especially when they harbour antibiotic resistant determinants (Schultsz and Geerlings, 2012; Meletis, 2016; Webb *et al.*, 2016). Given that multi-drug resistant *Enterobacteriaceae* have been detected even in countries like Brazil (Paterson *et al.*, 2003; Carmeli *et al.*, 2010; Karaiskos and Giamarellou, 2014), the Netherlands (Bos *et al.*, 2015), UK (Ventola, 2015; Hijazi *et al.*, 2016) , and Germany (Karaiskos and Giamarellou, 2014; Webb *et al.*, 2016) that have more advanced public health facilities, the importance of assessing the occurrence of these pathogens in developing countries like South Africa cannot be over-emphasized.

The primary aim of this study was to isolate and confirm the identities of *Enterobacteriaceae* using both preliminary identification tests and PCR assays. In the current study, a total of 314 presumptive isolates based on differences in colonial morphology were screened for presumptive identification characters of *Enterobacteriaceae*. A large proportion (280) of these isolates were oxidase negative also satisfied the specific characteristics for *Enterobacteriaceae* based on the Triple Sugar Iron agar and Simmons Citrate agar tests. Despite their lack of high sensitivity and specificity, preliminary biochemical tests play a crucial role for presumptive identification of bacterial isolates due to the fact that they provide a valuable direction on the identification scheme that may be used especially in facilities that do not have specialised equipment as well as expertise (Szabó *et al.*, 2005). The TSI and SCA have also been used for identifying *Enterobacteriaceae* to species level based on the production of blue coloured pigmentation. Citrate production is considered a characteristic of organisms belonging to the genus *Klebsiella, Enterobacter, Citrobacter* and *Salmonella* and therefore used to distinguish these organisms from citrate negative organisms in the family. This therefore explains the importance of supplementing these tests with more reliable and highly specific molecular identification tests. Against this background, effective identification

and surveillance of foodborne pathogens including those belonging to the family *Enterobacteriaceae* can be achieved through a combination of the conventional and PCR-based techniques (Loncarevic *et al.*, 2008; Aurora *et al*., 2009; Adzitey and Corry, 2011). A large proportion (39.07%) of the isolates were positively identified as *E. coli*, and *Shigella* species based on the presence of specific housekeeping genes. Given that PCR-based techniques are rapid, less laborious, and more sensitive, specific and efficient compared to the conventional preliminary identification tests (Magistrado *et al*., 2001; Keramas *et al.*, 2004) the detection of large proportions of *Enterobacteriaceae* in food-producing animals may provide opportunities for microbial cross-contamination to meat products if proper farm management techniques as well as standard operational procedures and proper hygiene are not practiced.

Another objective of the study was to assess the potential of isolates to produce enzymes that code for extended spectrum beta-lactamases and carbapenemase as well as the proportion of isolates that harbour genes encoding for ESBL and carbapenem resistance. This is motivated by the recent increase in the detection of both carbapenem and ESBL resistant *Enterobacteriaceae* among environmental and hospital settings in many countries worldwide and in South Africa in particular (Lowman *et al.*, 2014b; Gqunta *et al.*, 2015; Malande *et al.*, 2016; Perovic *et al.*, 2016; D. Annear, 2017). The development and spread of antibiotic resistant determinants from animals to human has been strongly linked to the misuse of antimicrobial agents in veterinary medicine and agriculture in general (Ateba and Bezuidenhout, 2008), coupled with the fact that the different classes of antibiotics routinely used for treatment of human infections are also used in animals either for therapy or for growth promotion (Wegener *et al*., 1999). Several studies have shown that there is a close association between the amounts of antibiotics used and the levels of resistance observed among bacteria isolates (Seiffert *et al.*, 2013; Guerra *et al*., 2014; Economou and Gousia, 2015; Webb *et al.*, 2016). In addition, resistant bacteria have frequently been detected in food products especially chicken, beef meat and pork (Ateba and Mbewe, 2011) and the isolates from these food products have been linked to isolates from patients in hospitals (Ateba and

Mbewe, 2013, 2014). Bacterial contaminants in food products may pose severe health challenges to consumers especially if they harbour multiple virulence gene determinants. This therefore indicates that antibiotic resistance in zoonotic bacteria constitutes a public health hazard that primarily results from increased risk of treatment failures (Da Costa *et al*., 2013) Interestingly, despite the fact that carbapenems are not approved for use in veterinary cases worldwide (Webb *et al.*, 2016) when compared to other antibiotics such as quinolones, tetracyclines, aminoglycosides, and aminobenzyl-penicillins (Economou and Gousia, 2015; Webb *et al.*, 2016) there is a steady increase in the detection of carbapenem resistant bacteria. Although, health care facilities are hot spots for resistant bacteria and are highly implicated in the spread of resistant strains (Tacconelli *et al.*, 2013), the trading of meat, fruits, vegetables, seeds, grain, and animals, the list of goods that are being imported and exported to and from different countries all over the world is extensive (Grami *et al.*, 2016).

There is minimum research available which characterizes carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae* isolated from animals and meat products (Tramuta *et al.*, 2011; Poirel *et al.*, 2012; Xu *et al.*, 2015; Harada *et al.*, 2017). This, despite strong evidence suggesting that animals (companion and food producing) play a crucial role in transfer of multi-drug resistant *Enterobacteriaceae* bacteria to humans (Da Costa *et al*., 2013; De Jager *et al.*, 2015). This amplifies the need for investigation and characterization of carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae.* In South Africa there is more information on clinical cases than veterinary, making it difficult to compare findings for animal isolates. The National Institute for Communicable Diseases (NICD) recorded an increase in carbapenem producing *Enterobacteriaceae* in South Africa for 2017 amongst clinical isolates (NICD, 2018).

With the use of the guidelines as recommended by the Clinical Laboratory Standards Institute (Wayne, 2007) the isolates were successfully identified and characterised using molecular techniques. According to Paterson and Bonomo (2005), molecular techniques are the referral standard for the identification, differentiation and characterization of carbapenem resistant *Enterobacteriaceae.*

There has been a dramatic increase in carbapenem resistance amongst *Enterobacteriaceae* worldwide and notably in South Africa (Thaden *et al.*, 2014; Barbarini *et al.*, 2015; Malande *et al.*, 2016; Perovic *et al.*, 2016). A Disk Diffusion test for four (ertapenem, meropenem, doripenem and imipenem) clinically prescribed carbapenems was performed as an inclusion criterion for all resistant isolates. An isolate has to be resistant or intermediate to at least one carbapenem to be considered as carbapenem resistant (Baran and Aksu, 2016). Disk Diffusion test, like the other biochemical test is also unreliable as a number of factors, including human error, play a crucial role in scoring the results. In the present study the highest level of resistance of 68.57% was observed against meropenem followed by imipenem (67.86%) and ertapenem (66.07%) while the least resistance was observed for doripenem (63.57%). Of the 280 isolates tested a total of 189 (67.5%) were resistant to at least one of the carbapenem antibiotics highlighting the high levels on CRE from animal isolates. The Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar was also used to identify isolates that were carbapenem resistant and was compared with the disk diffusion tests results. Although the agar does not give an indication of which carbapenem the isolates are resistant to it however identifies the organism species of the isolate based on the growth colour of the inoculum. The Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar is a good medium for detecting carbapenemase-producing *Enterobacteriaceae* due to its high sensitivity and specificity (Gazin *et al.*, 2012; Bracco *et al.*, 2013; Simner *et al.*, 2015; Osei Sekyere, 2016). Bracco *et al.*, (2013) noted that even isolates with very low carbapenem minimum inhibitory concentration managed to grow on the Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar in their study. It was no surprise that in the current study a total of five presumptive isolates that were initially identified as carbapenem sensitive in the Disk Diffusion test grew on this medium and were thereafter treated as carbapenem resistant. A total of 194 (69.29%) isolates grew on the Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar. Of these 53.10% belonged to the *Klebsiella, Enterobacter, Serratia and Citrobacter* (KESC) group, 35.05% were *Escherichia coli*, 8.25% were in the group of non-*Enterobacteriaceae* species, while 3.61% were classified under the *Proteus, Morganella* and *Providencia* (PMP) group. Several studies including the

NICD report for 2017 acknowledge that species from the KESC group are the most isolated in carbapenem resistant *Enterobacteriaceae* clinical cases, followed by *Escherichia coli* (Coetzee and Brink, 2011; Baran and Aksu, 2016; Lee *et al.*, 2016; Enyinnaya *et al.*, 2017; NICD, 2018). The five presumptive isolates that were initially identified as carbapenem sensitive in the disk diffusion test were then treated as carbapenem resistant after they grew on the Brilliance ™ carbapenem resistant *Enterobacteriaceae* agar. This proved the unreliability of phenotypic tests and the need for confirmatory tests and molecular tests in diagnostic cases for more specificity and reliability. *Enterobacteriaceae*'s ability to produce an enzyme carbapenemase is one of the most common and clinically important mechanism of carbapenem resistance. The Modified Hodge test is the test of choice for phenotypically determining production of carbapenemase amongst *Enterobacteriaceae* (Amjad *et al.*, 2011) and in one study was proved to have 94% sensitivity and 100% specificity (Townell *et al.*, 2014). This test can either be performed with meropenem or ertapenem, however Baran and Aksu (2016), proved an insignificant sensitivity difference of only 0.6% between the two antibiotics. The modified Hodge test was performed on 162 isolates which were either susceptible or intermediately resistant to imipenem to test for their ability to produce carbapenemase enzyme. Only 31 (19.14%) isolates were positive for carbapenemase production in the current study of which 9.26% (15) were form the intermediate group and 9.88% (16) were form the susceptible group. Isolates can be susceptible to carbapenems while harbouring carbapenemase enzymes which are capable of causing resistance to the antibiotics leading to treatment failure (Amjad *et al.*, 2011). The organism's ability to produce carbapenemase enzyme has been reported as the most common mechanism of resistance to carbapenem antibiotics (Yigit *et al.*, 2001; Baroud *et al.*, 2013; Meletis, 2016).

Production of extended spectrum beta-lactamase by the carbapenem resistant isolates is an important inclusion criterion for this study, as the aim was to characterise isolates that are extended spectrum beta-lactamase producers and also resistant to carbapenems. Brilliance ™ extended spectrum beta-lactamase agar is a chromogenic medium of choice with sensitivity of 94.9% and specificity of 95.7% according to

Gazin *et al.*, (2012). In identifying extended spectrum beta-lactamase producing *Enterobacteriaceae*, in the current study 194 isolates were inoculated on ESBL Agar medium and 151 (77.84%) were found to be positive for extended spectrum beta-lactamase production. Of these 54.97% belonged to *Klebsiella, Enterobacter, Serratia and Citrobacter* (KESC) group, 33.77% *Escherichia coli*, 7.95% *Salmonella, Acinobacter* and others group and 3.31% *Proteus, Morganella* and *Providencia* (PMP) group. The current study's findings agree with several studies that have identified *Klebsiella pneumoniae* and *Escherichia coli* as the most prevalently isolated species in the *Enterobacteriaceae* family in cases of extended spectrum beta-lactamase producing clinical infections (Bradford *et al.*, 1999, 2004; Monstein *et al.*, 2007; Shaikh *et al.*, 2015; Tseng *et al.*, 2015; NICD, 2018).

The isolates were also screened against 13 antibiotics which belonged to different classes. This was done to determine isolates which were multi drug resistant, a situation defined as resistance to three or more antibiotics belonging to different classes (Wayne, 2007; Baran and Aksu, 2016). A total of 94.04% isolates were multi-drug resistant and results for individual collection site are illustrated in Appendix 5. The highest level of resistance was for CTX (59.60%), ATM (54.30%), CXM (47.68%), PRL (43.71%), FOX (42.38%), KF (41.06%), CAZ (41.06%), EFT (39.74%), A (39.74%), TC (39.07%), CPM (19.87%), CIP (12.58%), and lastly AMC (9.27%). The highest level of resistance was from a 3rd generation cephalosporin in the current study. This group of antibiotics are widely used in treatment and prevention of serious Gram negative bacterial infections (Mir *et al.*, 2016). The least resistance was observed in amoxicillin-clavulanate. Clavulanate is an inhibitor which protects amoxicillin from being destroyed by the β-lactamase enzyme thereby avoiding destruction of the β-lactam ring (Carceles *et al*., 1995). Mir *et al*. (2016), discovered that resistance to these antibiotics is not necessarily due to use for veterinary medicine but might be introduced through exposure from the soil, plants and the environment. The use of antibiotics in veterinary medicine however has been documented as one of the major causes of multi-drug resistance in food producing animals (Geser *et al*., 2012; Economou and Gousia, 2015; Webb *et al.*, 2016; Lhermie *et al*., 2018). The high level of resistance to this diverse group of clinically prescribed

antibiotics is of great concern as it reveals the difficulties faced by clinicians in prescribing safe and reliable antibiotics in clinical cases.

Molecular techniques are more reliable, accurate and fast; therefore in the present study all the 151 isolates were identified using species specific genes by PCR amplification. *Escherichia coli* was screened with *uidA* gene, *Klebsiella pneumoniae* with *ntrA* gene, *Proteus mirabilis* with *tuf* gene and *Salmonella* species with *invA* gene. The *E.coli uidA* (β-glucuronidase) gene encodes for β-D-glucuronidase, which is an acid hydrolase that catalyses the cleavage of a wide range of β-glucuronides (Horakova *et al*., 2008). This gene sequence is unique to *E. coli* species, although it can be associated with *Shigella* species due to their close relation (Godambe *et al*., 2017) . The *ntrA* (nitrogen-regulation) gene is a species specific identification gene involved in controlling the *nif* gene expression in *K. pneumoniae* bacteria. It is one of the five regulatory genes located outside the *nif* gene cluster and it regulates the operons needed for the assimilation of poor nitrogen source (Dixon., 1986). The *tuf* (protein elongation factor Tu) gene is a *Proteus mirabilis* species identification gene encoding for elongation factor Tu which, during protein synthesis, carries aminoacyl-tRNA to the programmed ribosomes (Isabel *et al.*, 2008). The *invA* gene is a genus specific primer for confirmation of isolates as *Salmonella* species (Salehi *et al*., 2005). The invA gene encodes for bacterial putative inner membrane protein, necessary for the host's epithelial cells invasion (Darwin and Miller, 1999). The primers used in this study have been used elsewhere (Salehi *et al*., 2005; Anbazhagan *et al*., 2010; Anbazhagan *et al.*, 2011; Kupradit *et al*. , 2013) and proved to be specific to the targeted species. Of the 151 isolates, 66 (43.70%) isolates were identified as *Klebsiella pneumoniae*, 52 (34.43%) as *Escherichia coli*, 7 (4.63%) as *Salmonella* species and 2 (1.32%) as *Proteus mirabilis*. These findings were different from most studies which isolated *E. coli* as the most common species (Corbella *et al.*, 2000; Villar *et al*., 2013; Guerra *et al*., 2014; Monaco *et al.*, 2014; Tekiner and Özpınar, 2016). However, according to a study conducted in Nigeria on healthy pigs *K. pneumoniae* were the most prevalent species, followed by *Salmonella* species then *E. coli* (Ugwu *et al.*, 2015). The NICD

report for 2017 states that amongst carbapenem resistant clinical isolates for the year, *K. pneumoniae* accounted for 77.23%, *E. coli* 3.48%, *Proteus mirabilis* 0.32% and *Salmonella* species were at 0.08% (NICD, 2018). Although the percentages are different the current study's findings correspond to those of the NICD in that *K pneumoniae* was the most prevalent followed by *E.coli*. Upon comparing the result of 151 isolates screened on the Brilliance ™ ESBL agar and molecular identification, *Escherichia coli* was correctly identified as 33.77% on both tests. Of these isolates 79.52% those which belonged to the *Klebsiella, Enterobacter, Serratia and Citrobacter* (KESC) group were identified as *Klebsiella pneumoniae*. 58.33% of the isolates from the *Salmonella, Acinobacter* and others group were identified as *Salmonella* species. 40% of the isolates from the *Proteus, Morganella* and *Providencia* (PMP) group were identified as *Proteus mirabilis*.

Multiplex PCR (mPCR) is universally used for rapid detection of various pathogenic bacteria both from environmental and food products, with high specificity and sensitivity (Anbazhagan *et al.*, 2011). Therefore in the current study mPCR was used for the detection of both ESBL and CRE genes. The four ESBL genes targeted in this study were $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{SHV} and bla_{TEM} which encode the four most common ESBL types currently isolated worldwide (Bush and Jacoby, 2010; Sah and Hemalatha, 2015; Shaikh *et al.*, 2015). According to several studies on animals and animal products $bla_{CTX\text{-}M}$ was found to be the most dominant ESBL gene (Guerra *et al.*, 2014; García-Cobos *et al.*, 2015; Argudín *et al.*, 2017). However, Tekiner and Özpınar (2016) identified bla_{TEM} as the most prevalent gene isolated from foods of animal origin. Contrary to all the studies above, in the present study bla_{SHV} was found to be the most prevalent amongst the isolates. The ESBL gene detected most frequently in this study was bla_{SHV} followed by bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{OXA} in 33.11%, 22.52%, 20.53% and 11.26% isolates, respectively. Nineteen isolates showed multiple genes of which 11 (7.28%) isolates harboured bla_{OXA} and $bla_{CTX\text{-}M}$ while 8 (5.30%) isolates carried bla_{SHV} and bla_{TEM}. The 6 CRE genes targeted in this study are amongst the most commonly or routinely isolated worldwide (Poirel *et al.*, 2001; Johnson and Woodford, 2013; Ouertani *et al.*, 2016; Rodrigues *et al.*, 2016) and also in South Africa especially in clinical cases (Coetzee

and Brink, 2011; Lowman *et al.*, 2011; Brink *et al.*, 2012, 2013; Chibabhai and Perovic, 2014; Malande *et al.*, 2016). According to the NICD, in clinical isolates the most prevalent CRE gene was bla_{OXA-48} and variants, followed by $bla_{NDM,}$ $bla_{VIM,}$ bla_{KPC} and bla_{GES} at 77.71%, 20.24%, 1.11%, 0.63% and 0.32% occurrence rate, respectively (NICD, 2018). The most isolated CRE gene in the current study was bla_{KPC}, bla_{NDM}, bla_{GES}, bla_{OXA-48}, bla_{VIM} and bla_{OXA-23} at 35.76%, 20.53%, 17.88%, 10.60%, 6.62% and 3.31%, respectively. Interestingly eight (8) of the 151 isolates were found carrying multiple genes, four (4) (2.65%) isolates had bla_{KPC} and bla_{OXA-23} genes, 2 (1.32%) isolates harboured bla_{KPC} and bla_{NDM} while the 2 (1.32%) isolates harboured bla_{GES} and bla_{OXA-48} genes.

In the present study all the 66 isolates identified by molecular technique (*ntrA* gene) as *Klebsiella pneumoniae*, harboured all CRE genes and ESBL genes. For CRE genes, bla_{KPC} was detected in 42.42% of the *K. pneumoniae* isolates, bla_{GES} detected in 27.27% isolates, bla_{NDM} detected in 12.12% isolates, while bla_{VIM} detected in 3.03% isolates, bla_{OXA-48} detected in 6.06% isolates and bla_{OXA-23} detected in 1.52% of isolates. Five isolates harboured multiple genes in combination of bla_{KPC} with bla_{OXA-23} and bla_{OXA-48} with bla_{GES} in 6.06% and 1.52% isolates, respectively. According to the NICD bla_{OXA-48} and variants were the most common amongst *K. pneumoniae* isolates, followed by bla_{NDM}, bla_{VIM}, bla_{KPC} and lastly bla_{GES} (NICD, 2018). For ESBL genes bla_{SHV} was the mostly detected in 45.45% isolates followed by bla_{OXA} in 13.64% isolates, bla_{TEM} in 16.67% isolates, and bla_{CTX-M} was the least detected in 9.09% isolates. A total of 10 isolates harboured multiple genes in combination of bla_{OXA} with bla_{CTX-M} and bla_{SHV} with bla_{TEM} in 12.12% and 3.03% isolates, respectively.

The 52 isolates identified as *E. coli* also harboured all the CRE and ESBL genes. For CRE genes, bla_{KPC} was the mostly detected in 34.62% isolates followed by bla_{NDM} in 32.69% isolates, bla_{OXA-48} in 13.46% isolates, bla_{GES} in 9.62% isolates, bla_{OXA-23} in 3.85% isolates and bla_{VIM} was least detected in 1.92% of isolates. Two (3.85%) of the *E.coli* isolates had multiple genes (bla_{KPC} with bla_{NDM}). According to NICD report for 2017 bla_{OXA-48} and variants were the most prevalent amongst *E coli* species, followed by bla_{NDM} then bla_{GES} (NICD, 2018). For the ESBL genes detected bla_{CTX-M} was detected the most in 34.62%

isolates, bla_{TEM} in 26.92% isolates, bla_{SHV} in 23.08% isolates, and bla_{OXA} in 1.92% isolates. Seven (13.46%) of these *E. coli* isolates had multiple gene in two combinations bla_{SHV} and $bla_{TEM/}$ bla_{OXA} and bla_{CTX-M} in 9.62% and 3.85% isolates, respectively.

Out of 2 isolates identified as *Proteus mirabilis* 50% of the isolates harboured bla_{OXA-48} and 50% harboured bla_{OXA-23} CRE genes. Also, they have 50% prevalence rate each for bla_{TEM} and bla_{OXA} ESBL genes. Out of 7 isolates identified as *Salmonella* species, 57.14% isolates harboured bla_{CTX-M} and 28.57% isolates harboured blaTEM genes. Two isolates contained multiple genes of which 50% harboured a combination of bla_{OXA} and bla_{CTX-M} while the other 50% had bla_{SHV} and bla_{TEM} genes. Comparing the above with NICD results for *Proteus* species bla_{OXA-48} and variants were the most dominant followed by $bla_{NDM,}$ while bla_{NDM} was the only gene isolated for *Salmonella* species (NICD, 2018).

In a study conducted from food of animal origin the most common gene co-existence was found to be bla_{TEM} and bla_{CTX-M} followed by bla_{SHV} and bla_{TEM}, the study also reported three genes $bla_{SHV,}$ bla_{TEM} and bla_{CTX-M} in co-existence in some samples (Tekiner and Özpınar, 2016). In the present study a total of 19 isolates harboured multiple ESBL genes of which 10 (52.63%) isolates were *K pneumoniae*, 7 (36.84%) isolates were *E coli* and 2 (10.53%) isolates were *Salmonella* species. Of these 19 isolates, 11 (57.89%) harboured a combination of bla_{OXA} with bla_{CTX-M} and 8 (42.11%) of the isolates harboured a combination of bla_{SHV} with bla_{TEM}. The NICD reported bla_{OXA-48} and variants and bla_{NDM} combination as the most commonly detected, followed by bla_{OXA-48} and variants and bla_{VIM}, then bla_{OXA-48} and variants and bla_{GES}, lastly bla_{NDM} and bla_{GES} and also bla_{NDM} and bla_{KPC} equally (NICD, 2018). A total of 7 isolates harboured multiple CRE genes in the current study, where *K pneumoniae* had 5 (71.43%) and *E coli* had 2 (28.57%) of the total isolates. A combination of bla_{OXA-23} and bla_{KPC} had a highest number of isolates i.e. 4 (57.14%), followed by bla_{KPC} and bla_{NDM} for 2 (28.57%) isolates, finally bla_{OXA-48} and bla_{GES} for 1 (14.29%) isolate. Three co-existence genes were reported in clinical isolates bla_{OXA-48} and variants with bla_{VIM} and bla_{NDM} the other was bla_{OXA-48} and variants with bla_{GES} and bla_{NDM}, unfortunately no three co-existing genes were detected in the current study.

Of the 28 presumptive isolates isolated from the samples collected in Lokaleng, only 8 (28.57%) were found to be resistant to carbapenem antibiotics. Five (5) (62.50%) of the eight (8) isolates were found to be multi-drug resistant carrying both ESBL and CRE genes, contributing 3.31% of the total 151 final isolates from the study. From Ntontonyane 52 (56.52%) of the 92 presumptive isolates were resistant to at least one carbapenem antibiotic. Fifty (50) (96.15%) of those 52 isolates were found to be carrying both ESBL and CRE genes, contributing 33.11% of the final isolates. Some 45 (77.59%) of the 92 presumptive isolates from Koster were resistant to at least on carbapenem antibiotic. Of these 34 (75.56%) were included in the final isolates which were multi-drug resistant, ESBL and CRE gene carriers, contributing 22.52% of the 151 isolates. At Rooigrond 84 (82.35%) of the 102 presumptive isolates were carbapenem resistant. 62 (73.81%) of them contributed 41.06% of the final 151 multi-drug resistant isolates carrying both ESBL and CRE genes.

In the current study *K.pneumoniae*, *E.coli*, *P.mirabilis* and *Salmonella* spp are the four most isolated organisms. These organisms are known to cause diseases in both animals and humans hence the presence of multi drug resistant strains amongst these organisms is of great concern. Diseases caused by these organisms include but are not limited to salmonellosis, urinary tract infections, diarrhoea, respiratory infections etc. (Nordmann *et al*., 2012; Korzeniewska and Harnisz, 2013; Hrabák *et al*., 2014). *Salmonella* Enterica has assumed epidemiological importance as it has caused several outbreaks through consumption of contaminated food and water (Da Silva *et al*., 2013). Diarrhoea and respiratory infections are usually the most contagious and common diseases in both animal and human cases (Bengtsson and Greko, 2014). It is also important to note that infections due to multi drug resistant strains causes a high mortality in both humans and animals (Da Silva *et al*., 2013).

CHAPTER 6

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusion

The number of clinical case infections caused by *Enterobacteriaceae* has increased in the recent years. Cross contamination between animals, food products and humans is one of the major health concerns. This cross transmission of disease causing organisms from one species to another leads to complicated/severe disease in the other species. However, there is a limited number of studies that characterize resistant *Enterobacteriaceae* from animals and animal products, as most research focuses on human clinical cases (Poirel *et al.*, 2012; Xu *et al.*, 2015; Harada *et al.*, 2017). This study aimed to characterize extended spectrum beta-lactamase producing carbapenem resistant *Enterobacteriaceae* from faecal matter collected from cattle in both commercial and communal farms in the North West Province, South Africa. This will help in understanding the persistence of most common bacterial species resistant to common antibiotics as well as the resistant genes carried by those bacteria. The North West Province is known as South Africa's 'Texas' due to the extensive cattle farming in the province.

The data in this thesis proves that cattle are carriers of bacteria which harbour multi drug resistant genes. Cattle from both communal and commercial farms in this study carry multidrug resistant bacteria with few options as treatment of choice should they cause an infection. These multidrug resistant bacteria have a potential of causing serious infections in humans through cross contamination or consumption of contaminated meat and meat products (Torry, 2015). In this study 48.09% of the initial presumptive isolates were found to be carbapenem resistant and also producers of extended spectrum beta-lactamase enzymes. The limited treatment options for multidrug resistant carbapenem resistant

Enterobacteriaceae makes treatment of infections caused by these bacteria a nightmare in clinical cases (Brink *et al.*, 2014; Satlin *et al*., 2014; Sampaio and Gales, 2016). In the present study a high percentage (94.04%) of the isolates which were carbapenem resistant and also producers of extended spectrum beta-lactamase enzymes were found to be multidrug resistant. A small percentage (2.58%) of the isolates were found to be sensitive to all carbapenems but grew on Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar. With molecular techniques, these isolates were later found to be carrying both extended spectrum beta-lactamase and carbapenem resistance genes. This might have been due to human error proving the unreliability of phenotypic tests or in agreement with finding noted in another study (Bracco *et al.*, 2013).

In this study *K. pneumoniae* was identified as the most prevalent organism isolated in cattle faeces followed by *E. coli*, *Salmonella* spp., and *Proteus mirabilis* respectively. Upon characterizing extended spectrum beta-lactamase genes bla_{SHV} was identified as the most common gene, followed by bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{OXA} respectively. In *K. pneumoniae* isolates bla_{SHV} was the most common gene and $bla_{CTX\text{-}M}$ was the most regularly detected in *E coli* and *Salmonella* spp. For carbapenem resistance genes characterization, bla_{KPC} was most common gene followed by bla_{NDM}, bla_{GES}, $bla_{OXA\text{-}48}$, bla_{VIM} and $bla_{OXA\text{-}23}$ genes, respectively. For *K. pneumoniae* and *E. coli* isolates bla_{KPC} was the most common gene and $bla_{CTX\text{-}M}$ was the most common gene in *Salmonella* species. Interestingly, multi-gene carrying isolates were identified for both extended spectrum beta-lactamase genes and carbapenem resistance genes. Most of these unique isolates were *K. pneumoniae* (52.63%), *E. coli* (36.84%), and *Salmonella* spp (10.53%). The NICD has isolated these rare isolates and some even had three different genes in them (NICD, 2018), but these were only isolated from human clinical samples. The results of this study prove that animals also harbour these multi-gene carrying bacterial isolates.

Even though most samples were collected from Ntontonyane (80 samples) Rooigrond had the highest number of presumptive isolates (102 isolates). Of all the collection sites Ntontonyane had the highest number of carbapenem resistant isolates that are multidrug resistant, harbouring both ESBL and CRE genes at 96.15% followed by Koster at 75.56%, Rooigrond at 73.81% and finally Lokaleng at 62.50%. All these areas showed a concerning high percentage of carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae* proving presence of these resistant bacteria amongst animals. In the current study communal farms had an overall high percentage of carbapenem resistant extended spectrum beta-lactamase producing *Enterobacteriaceae* (62.50+96.15/2= **79.33%**) than commercial farms (75.56+73.81/2= **74.69%**).

6.2 Limitations of the study

Although the four farms from where the samples were collected are representative of both commercial and communal farms, these farms represent a small fraction of the cattle farms in the North West province, South Africa. They however do give an overview of the presence of multidrug resistant *Enterobacteriaceae* on cattle farms and the extent of the problem. However, a more intense research with a larger variety of samples collected from all farms in the province is required. This would give a broader picture of the problem facing the country especially if done in other provinces as well. Also, in the present study the focus was on carbapenemase-mediated carbapenem resistance in *Enterobacteriaceae*. Non-carbapenemase-mediated carbapenem resistance in *Enterobacteriaceae* has either developed due to mutation or modification in the outer membrane porin specific for antibiotic absorption. This type of resistance also plays a crucial role in resistance and requires to be investigated. The sample size in this study was calculated using a prevalence of 16% which was obtained from a study conducted in Argentina by Villar *et al*., 2013. This is due to scarcity of national data on carbapenem resistant ESBL producing *Enterobacteriaceae* isolated from animals, as most studies focus on isolates from humans.

6.3 Recommendations

A more intense study on carbapenem resistant ESBL producing *Enterobacteriaceae* which includes a history of antibiotics use in the farms would be beneficiary. This would provide with a knowledge of the veterinary prescribed antibiotics that can lead to carbapenem resistance in animals as these antibiotics are not recommended for use in animals. Controlling and reducing multi drug resistance in animals would be beneficiary to human health care sector as these organisms cause severe, difficult to treat diseases.

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APPENDICES

APPENDIX 1: Culture Media and Gels used in this study.

1.1 Buffered Peptone Water	g/L
Peptone	10.0
Sodium Chloride	5.0
Di-sodium Hydrogen Phosphate	3.5
Potassium Dihydrogen Phosphate	1.5

Twenty grams (20 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. Buffered peptone water (Biolab, Lawrenceville, Georgia, USA) was used as a pre-enrichment medium for isolation of *Enterobacteriaceae*.

1.2 Nutrient Broth	g/L
Meat extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	8.0

Sixteen grams (16 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. Nutrient broth (Biolab, Lawrenceville, Georgia, USA) was used as pre-enrichment medium for overnight cultures during DNA extraction.

1.3 MacConkey Agar (with crystal violet and salt)	g/L
Peptone	20.0
Lactose	10.0
Bile Salts No 3	1.5
Sodium Chloride	5.0
Neutral Red	0.03
Crystal Violet	1.001
Agar	13.5

Fifty grams (50 g) of the above components were dissolved in 1L of distilled water. The media was autoclaved at 121 °C for 15 minutes. MacConkey agar (Biolab, Lawrenceville, Georgia, USA) was used as a differential medium for isolation of *Enterobacteriaceae*.

1.4 Triple Sugar Iron (TSI)	g/L
Meat extract	3.0
Yeast extract	3.0
Tryptone	18.0
Meat peptone	2.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferric Ammonium Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024

Agar	10.5

Sixty three grams (63 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. TSI agar (Biolab, Lawrenceville, Georgia, USA) was used as a medium for preliminary identification of *E. coli* based on its biochemical profile.

1.5 Simmons Citrate Agar	g/L
Ammonium Dihydrogen Phosphate	1
Dipotassium Phosphate	1
Sodium chloride	5
Sodium citrate	2
Magnesium Sulphate	0.2
Bromothymol Blue	0.08
Agar	15

Twenty two grams (22 g) of the above components were dissolved in 1L of distilled water. The medium was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. Simons Citrate agar (Biolab, Lawrenceville, Georgia, USA) was used as a medium for preliminary identification of *E. coli* based on its biochemical profile.

1.6 Mueller-Hinton Agar	g/L
Meat Infusion	5.0
Casein Hydrolysate	17.5
Soluble Starch	1.5
Agar	14.0

Thirty eight grams (38 g) of the above components were dissolved in 1L of distilled water. The medium was distributed into McCartney bottles and autoclaved at 121 °C for 15 minutes. The media was poured into plates and used to determine the antimicrobial susceptibility profiles of the isolates.

1.7 Nutrient Agar

Peptone

Beef extract

Agar

NaCl

Suspended 28 g of nutrient agar powder in 1 L of distilled water. Heated the mixture while stirring to fully dissolve all nutrient agar powder. Autoclaved the dissolved mixture at 121 °C for 15 minutes. Once the nutrient agar has been autoclaved, it was allowed to cool and poured out into petri dishes and allowed to solidify.

1.8 Brilliance ™ Carbapenem Resistant *Enterobacteriaceae* agar

Peptone	15.0
Carbohydrates	2.0
Titanium oxide	5.1
Chromogenic mix	1.0
Antibiotic mix	19mL
Agar	15.0

Prepared a 0.5 McFarland turbidity, of colony and sterile distilled water as per manufacturer's instructions. The medium was allowed to warm to room temperature and inoculated it with the mixture using a sterile cotton swab. Incubated overnight at 37 ºC and scored the results according to the colour of the growth on the medium. Negative plates were re-incubated for an additional 24 hours.

1.9 Brilliance ™ Extended Spectrum Beta-Lactamase agar

Reagent	**g**
Peptone	12.0
NaCl	5.0
Phosphate buffers	4.0
Chromogenic mix	4.0
Antibiotic mix	0.28
Agar	15.0

We prepared a 0.5 McFarland turbidity, of colony and sterile distilled water as per manufacturer's instructions. Allowed the medium to warm to room temperature and inoculated it with the mixture using a sterile cotton swab. Incubated overnight at 37 ºC and score the results according to colour of the growth on the medium. Negative plates were re-incubated for an additional 24 hours.

1.10 2% Agarose gel

Agarose powder	2g
TAE buffer	100mL
Ethidium bromide	0.1µL

Two (2) grams of agarose powder were added to a 500mL Duran bottle and added the TAE buffer. The contents were swiftly mixed and microwaved until the contents had dissolved leaving a clear liquid. Allowed the mixture was allowed to cool and 1µL of EtBr was added and mixed well. Once the content have cooled down it was carefully poured into electrophoresis trays with a comb.

1.11 1.5% Agarose gel

Agarose powder	1.5 g
TAE buffer	100mL
Ethidium bromide	0.1µL

Added the agarose powder to a 500mL Duran bottle and added TAE buffer. Swiftly mixed the contents and microwaved it until the contents have dissolved leaving a clear liquid. Allowed the mixture to cool down and added 1µL of EtBr and mixed well. Once the content have cooled down it was carefully poured into electrophoresis trays with a comb.

APPENDIX 2: Chemicals used in this study

2.1 50X TAE Buffer

Thermo Scientific 50X TAE Electrophoresis Buffer (40mMTris, 20mM Acetic Acid and 1mM EDTA) stock solution was supplied by Thermo Scientific, Johannesburg, South Africa. A 1X TAE buffer working solution was prepared and used for resolving either DNA or amplified PCR products by agarose gel electrophoresis.

2.2 Ethanol (70%)

Absolute ethanol (99% v/v) was supplied by Merck, Diagnostics, South Africa. A 70% (v/v) working solution was prepared by aliquoting 700 ml of absolute ethanol into a 1L Duran bottle and added 300 ml of distilled water. The solution was stored at room temperature and used for sterilising the work area.

2.3 PCR Master Mix (2X DreamTag Green)

Thermo Scientific (Catalogue #K1081 200 rxns) 2XDreamTag Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer) was used for PCR amplification of target genes. This enzyme was produced by ThermoFischer, USA and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. The Master Mix was stored at -20 °C when not in use.

2.4 Oligonucleotide primers

Primer sets used in this study to amplify various genes encoding ESBLs and *E. coli* housekeeping genes were synthesised and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. Each primer was supplied in a lyophilised state and with a synthesis report on how to prepare a

100 µM stock solution. The primer sets (Forward and Reverse) were stored in separate tubes at -4°C for future use. The working solution was prepared by aliquoting the required volume of forward and reverse primer set from the stock solution into a 1.5µl sterile Eppendorf tube.

2.5 100bp and 1Kb DNA Molecular Weight Markers

The standard DNA markers, O'GeneRuler 1 Kilo base pairs and Gene Ruler 100 base pairs ranging from 250-10000 bp to 100-1000 bp fragments respectively were supplied by Thermo Scientific Company and used to determine the relative sizes of all amplicons after agarose gel electrophoresis.

2.6 Glycerol

Absolute glycerol was supplied by Merck, Diagnostics, South Africa. A 70% (v/v) working solution was prepared by aliquoting 700 ml of absolute glycerol into 1L Duran bottle and added 300 ml of sterile distilled water. The solution was stored at room temperature and aliquoted into Eppendorf tubes for colony storage at -70 °C.

2.7UltraPure™RNase-DNase free distilled Water

UltraPure™ DNase/RNase-Free Distilled Water is designed for use in all molecular biology applications, it is synthesised and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa.

APPENDIX 3: Stains and Kits used in this study

3.1 Gram Staining

We mixed a single colony with a drop of sterile distilled water on a clean slide. This smear was heat-fixed by passing it over a flame, several times until it was dry. The slide was placed on the staining tray and followed the following staining procedure:

- Flooded the fixed smear with crystal violet solution for 1 minute.
- Rinsed off the crystal violet with tap water.
- Flooded the smear with iodine solution for 1 minute.
- Rinsed off the iodine solution with tap water.
- Flooded the smear with decolourizer 3 seconds.
- Rinsed off the decolourizer with tap water.
- Flooded the smear with safranin for 30 seconds.
- Rinsed off the safranin with tap water.
- Air dried the smear and place in an upright position.

Microscopically examine the slide for bacterial organisms under oil using 100X objective.

3.2 Zymo Research Genomic DNA ™ Tissue MiniPrep Kit

Protocol for biological liquids and cell suspensions:

- A colony was suspended into Nutrient broth and incubated overnight at 37 °C
- The mixture was centrifuged for 10 minutes, the supernatant was discarded
- Some 100µL of water were added
- About 95µL of 2X digestion buffer and 5µL Proteinase K were added
- The mixture was vortexed and incubated at 55 °C for 20 minutes in a water bath
- Some 700µL of Genomic Lysis Buffer was added and vortexed
- The mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10 000 x *g* for a minute.

- Some 200µL of DNA Pre-Wash buffer was added to the spin column in a new collection tube, centrifuged at 10 000 x *g* for a minute.
- About 400µL of g-DNA Wash buffer was added to the spin column then centrifuged at 10 000 x *g* for a minute.
- The spin column was transferred to a clean micro centrifuge tube
- About 50µL DNA elution buffer was added to the spin column and incubated for 5 minutes at room temperature.
- Centrifuged for 30 seconds to elute the DNA
- Stored the DNA at -70°C for future use.

3.3Ethidium Bromide

A stock solution of 10mg/ml was prepared in a 5 ml Duran bottle by dissolving the powder in sterile distilled water and the solution protected from light by wrapping the bottle with a masking tape and stored at 4 °C. A final volume of 0.1 µl was used for visualising DNA and PCR products in electrophoresis gel.

APPENDIX 4: Antibiotics discs used in this study

Antibiotic	Type of Antibiotic	Abbrev	Disc concentration
Piperacillin	Broad-spectrum penicillin	PRL	30µg
Ticarcillin	Extended-spectrum penicillin	TC	75µg
Amoxicillin	Aminobenzyl-penicillin	A	25µg
Amoxicillin-clavulanate	Aminobenzyl-penicillin with β-lactamase inhibitor	AMC	30µg
Cephalothin	1st generation Cephalosporin	KF	30µg
Cefoxitin	2nd generation Cephalosporin	FOX	30µg
Cefuroxime	2nd generation Cephalosporin	CXM	30µg
Cefotaxime	3rd generation Cephalosporin	CTX	30µg
Ceftazidime	3rd generation Cephalosporin	CAZ	30µg
Ceftiofur	3rd generation Cephalosporin for veterinary use	EFT	30µg
Cefepime	4th generation Cephalosporin	CPM	30µg
Aztreonam	Monobactam	ATM	30µg
Ciprofloxacin	Quinolones Group II	CIP	5µg
Imipenem	Carbapenem	IPM	10µg
Ertapenem	Carbapenem	ETP	10µg
Meropenem	Carbapenem	MEM	10µg
Doripenem	Carbapenem	DOR	10µg

APPENDIX 5: Antibiogram Data of all Isolates Tested with the 13 Antimicrobial Agents and Areas of Collection

Ntontonyane	CXM	CPM	ATM	EFT	A	PRL	TC	KF	CTX	CAZ	FOX	CIP	AMC
1B	R	I	R	R	I	R	S	S	I	I	I	S	S
3A	I	S	I	I	R	R	R	R	I	R	S	S	S
3B	S	I	R	I	S	I	I	R	S	S	S	S	S
4A	S	I	S	R	R	R	R	R	S	R	I	S	I
4B	I	S	R	R	I	R	I	S	S	I	S	I	R
5A	I	S	I	R	S	R	R	S	I	S	R	S	S
5B	S	I	S	S	I	R	I	S	R	R	S	S	S
7A	R	R	R	S	S	R	R	S	R	S	R	S	S
8A	R	I	S	R	R	R	I	R	S	R	S	R	S
8B	R	S	R	R	R	I	S	S	R	S	S	R	R
9A	S	S	S	I	R	R	R	R	S	R	S	S	I
9B	S	S	R	R	R	R	S	S	S	S	S	I	S
10	S	I	S	R	R	I	S	R	S	R	S	S	S
11A	I	I	I	R	R	S	S	R	R	S	S	I	S
11B	R	S	R	R	R	S	R	R	R	I	S	S	S
12A	R	S	I	R	R	I	R	I	I	S	R	S	S
12B	I	I	S	R	R	S	S	S	R	I	R	S	S
13B	S	S	I	R	S	I	R	R	I	S	R	S	S
14B	R	S	R	R	R	I	I	R	S	R	S	I	S
15A	S	S	R	S	I	I	S	R	S	R	R	S	S
16A	I	I	I	R	S	R	R	R	R	R	S	S	S
16B	R	S	R	I	I	R	R	I	R	I	S	I	S
18A	S	S	R	R	S	S	R	R	R	R	S	I	S
18C	R	S	I	R	R	I	I	I	R	I	R	R	S
19A	S	I	I	I	I	S	R	I	I	S	S	R	S
21	R	R	R	R	R	I	R	R	R	S	S	I	S
23A	I	I	S	I	I	S	I	S	S	R	S	I	S
24A	S	S	I	I	R	I	S	R	R	R	R	I	S
26A	I	I	I	R	S	S	I	I	I	R	R	I	R
26B	S	I	S	I	R	R	I	R	R	R	R	I	S
28	R	S	S	S	S	S	I	R	R	R	S	S	S
37B	I	S	S	R	R	R	S	S	S	S	R	S	S
39B	R	S	I	R	R	S	S	R	R	R	R	S	S

Koster	CXM	CPM	ATM	EFT	A	PRL	TC	KF	CTX	CAZ	FOX	CIP	AMC
1	S	S	S	S	S	R	S	I	R	R	I	S	S
2A	S	R	R	R	I	I	S	R	I	R	R	I	S
2B	S	S	S	S	S	R	I	S	I	R	S	I	S
4A	R	S	I	R	R	S	S	R	R	R	R	I	S
4B	R	S	R	I	I	R	I	I	S	I	I	R	S
5B	R	I	R	S	S	I	S	S	S	R	S	R	S
6A	R	I	R	R	S	S	R	I	I	I	I	S	S
6B	R	S	R	S	I	R	R	S	R	S	S	I	S
7A	S	S	I	R	I	S	S	I	R	R	R	S	S
7B	R	S	R	R	R	R	I	R	S	I	R	I	S
8B	S	I	R	I	I	S	R	R	S	S	R	S	S
9	S	S	I	I	R	R	I	R	S	S	R	I	S
13A	S	R	R	I	I	S	S	R	R	R	R	S	S
13B	R	S	R	I	I	I	I	R	R	R	R	R	S
13C	R	S	R	R	S	S	I	R	S	S	R	S	S
14	R	S	R	R	S	I	R	R	S	R	R	I	S
15	S	I	R	S	I	S	R	R	R	I	R	S	S
16	R	I	R	I	S	I	R	R	S	I	R	S	I
17A	I	S	S	S	R	S	S	R	I	R	S	I	S
17B	I	S	R	I	R	R	S	S	S	R	R	S	S
17C	R	S	R	S	S	R	R	I	I	R	I	S	S
19B	S	S	I	I	I	R	R	S	S	I	S	S	S
19C	S	S	R	S	S	I	I	S	R	R	I	R	S
20A	R	I	R	S	S	R	R	R	R	R	S	I	S
20C	S	S	R	I	I	I	R	R	R	R	R	S	S
21A	R	I	R	S	S	S	R	S	R	S	I	S	S
22	R	I	S	I	R	S	R	S	R	R	R	S	S
25	I	S	S	R	R	R	R	R	S	I	I	S	S
26C	S	R	R	S	I	R	R	R	R	I	R	I	S
27	R	S	R	S	S	R	R	S	R	I	S	S	S
28A	R	S	R	S	R	R	R	I	S	S	R	S	S
28B	R	S	R	S	R	R	S	R	R	S	I	I	S
28C	R	S	R	R	S	S	R	S	R	S	R	S	S
29A	I	I	R	S	S	R	I	S	R	S	R	S	S
29B	S	S	R	R	I	I	R	R	R	I	S	S	S
29C	R	R	I	S	S	I	R	S	R	S	I	S	S
30A	R	S	S	R	R	S	R	S	R	S	S	S	S
30C	S	I	I	R	R	R	S	S	R	S	S	I	R
32A	S	S	S	S	S	R	S	S	S	R	R	R	S
32B	I	S	R	S	R	I	I	S	R	I	R	R	S
36A	S	S	R	I	I	I	R	S	R	S	S	I	R
36B	R	I	R	I	R	I	I	S	R	S	S	S	R
37A	R	I	R	I	I	I	I	I	I	S	R	R	S
38	S	I	R	S	R	R	I	R	R	I	R	I	R
39	S	I	R	R	S	R	R	S	S	S	S	I	R
40A	R	S	R	S	I	S	R	R	R	S	I	S	S
41B	R	I	R	I	R	I	S	R	S	I	S	S	R