



Molecular epidemiology of Carbapenem-resistant gram-negative bacteria in a regional hospital in KwaZulu-Natal, South Africa

By

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Dedication

My dissertation is dedicated to my parents, supervisors, mentors, and friends for their constant support, encouragement, advice, and assistance. Thank you for always being there for me and encouraging me to strive for improvement every day.

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Abbreviations

<i>ACC</i>	<i>Hafnia alvei</i> -derived Ambler Class C-type ampC β -lactamase genes
AmpC	Ampicillin β -lactamases
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
<i>bla</i>	beta-lactamase
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BREC	Biomedical Research Ethics Committee
CHROMagar™	Chromogenic agar
<i>CIT</i>	<i>Citrobacter freundii</i> -derived ampC β -lactamase genes
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase-Producing <i>Enterobacterales</i>
CRE	Carbapenem-Resistant <i>Enterobacterales</i>
CSF	Cerebrospinal Fluid
<i>CTX-M</i>	Cefotaximase Beta-Lactamase
<i>DHA</i>	<i>Morganella morganii</i> -derived Dhahran Beta-Lactamase-type ampC β -lactamase genes
DNA	Deoxyribonucleic Acid
DPKISMH	Dr Pixley Ka Isaka Seme Memorial Hospital
<i>E. cloacae</i>	<i>Enterobacter cloacae</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>EBC</i>	<i>Enterobacter spp.</i> -derived ampC β -lactamase genes
eCIM	Ethylenediaminetetraacetic Acid (EDTA) modified Carbapenem Inactivation Method
EDTA	Ethylenediaminetetraacetic Acid
EQA	External Quality Assessment
ESBL	Extended-Spectrum β -Lactamase
FNA	Fine Needle Aspirates
<i>FOX</i>	FOX-type (cefotaxin-hydrolysing) ampC β -lactamase genes
GD	Glycine-aspartic acid
GERMS-SA	Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa
<i>GES</i>	Guiana-Extended-Spectrum
<i>GIM</i>	German Imipenemase
GLASS	Global Antimicrobial Resistance and Use Surveillance System
IALCH	Inkosi Albert Luthuli Central Hospital
ICU	Intensive Care Unit

<i>IMI</i>	Imipenem-hydrolysing β -lactamase
<i>IMP</i>	Imipenemase
IPC	Infection Prevention and Control
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
<i>KPC</i>	<i>Klebsiella pneumoniae</i> carbapenemase
KZN	KwaZulu-Natal
<i>M. morganii</i>	<i>Morganella morganii</i>
MBLs	Metallo- β -Lactamases
mCIM	modified Carbapenem Inactivation Method
MGMH	Mahatma Gandhi Memorial Hospital
MIC	Minimum Inhibitory Concentration
<i>MOX</i>	<i>Aeromonas spp.</i> -derived Moxalactam-hydrolysing-type ampC β -lactamase genes
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
<i>NDM</i>	New Delhi metallo- β -lactamase
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
NIH	National Institute of Health
<i>NMC</i>	Non-metalloenzyme Carbapenemase
non-CP-CRE	non-Carbapenemase-Producing-CRE
<i>OXA-48</i>	Oxacillinase-48
<i>OXA-48-like</i>	Oxacillinase-48-like
<i>P. mirabilis</i>	<i>Proteus mirabilis</i>
PBPs	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
<i>S. marcescens</i>	<i>Serratia marcescens</i>
SA	South Africa
<i>SHV</i>	Sulphydryl Variable Beta-Lactamase
<i>SIM-1</i>	Seoul Imipenemase
<i>SME</i>	<i>Serratia Marcescens</i> Enzyme
TBE	Tris-Borate EDTA
<i>TEM</i>	Temoniera Beta-Lactamase
tNGS	targeted Next-Generation Sequencing
<i>VIM</i>	Verona integron-encoded metallo- β -lactamase
WGS	Whole-Genome Sequencing
WHO	World Health Organisation

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Abstract

Introduction

Carbapenem-resistant *Enterobacterales* (CRE) are becoming a serious public health threat in South Africa. This is especially true in KwaZulu-Natal (KZN), where regional hospitals are important for patient referrals. However, CREs in these hospitals are not well understood in terms of molecular characterisation. This study examined the burden and genetic basis of carbapenem-resistant *Enterobacterales* in a new regional hospital compared with higher-level facilities in KZN.

Methods

We reviewed laboratory surveillance data from three public hospitals: Inkosi Albert Luthuli Central Hospital (quaternary), Mahatma Gandhi Memorial Hospital (regional), and the recently opened Dr Pixley Ka Isaka Seme Memorial Hospital (regional) between 2016 and 2024. We screened a total of 720 carbapenem-resistant *Enterobacterales* isolates. Phenotypic methods were used to confirm identity and carbapenem resistance of the isolates, and while Polymerase Chain Reaction (PCR) was used to screen isolates for carbapenemase, extended-spectrum β -lactamase (ESBL), and ampC β -lactamase genes.

Results

CRE accounted for 11.7% of all *Enterobacterales*, with an increasing trend observed across all hospitals. The newly established regional hospital showed the most rapid increase. The distribution of species varied by hospital level. *Klebsiella pneumoniae* was most common at the quaternary centre, while *Proteus mirabilis* was more frequent in regional hospitals. The main carbapenemase genes found were *bla*_{OXA-48-like} and *bla*_{NDM}. However, 15.4% of CRE did not harbour detectable carbapenemases, suggesting possible non-enzymatic resistance mechanisms. Notably, 81.5% of isolates carried ESBL and/or ampC genes, and 96.4% harboured multiple β -lactamases (carbapenemases, ESBLs, and/or ampCs), with only 3.6% lacking any screened β -lactamase genes.

Conclusion

The increasing prevalence of CRE and the presence of various resistance mechanisms in KZN highlight the urgent need for coordinated molecular surveillance, improved infection prevention and control, and synchronised antimicrobial stewardship across all hospital levels.

My dissertation consists of five chapters that investigate the burden and molecular characterisation of carbapenem-resistant *Enterobacterales* in KwaZulu-Natal, South Africa. Chapter 1 outlines the background, rationale, aim, and objectives of the study. It emphasises the clinical and public health importance of carbapenem resistance. Chapter 2 presents a detailed literature review. It covers the global, national, and provincial epidemiology of carbapenem-resistant *Enterobacterales*, focusing on molecular resistance mechanisms and emerging trends. Chapter 3 examines the prevalence of carbapenem-resistant *Enterobacterales* and the molecular characterisation of carbapenem-resistant *Enterobacterales* for the presence of carbapenemase genes in three hospitals, including a new regional hospital. Chapter 4 builds on these findings by exploring additional β -lactam resistance mechanisms, specifically extended-spectrum β -lactamase and ampC β -lactamase genes, to clarify complex resistance patterns. Chapter 5 synthesises the findings from all chapters, discusses their implications, and offers conclusions and recommendations for surveillance and infection prevention and control strategies

1. Chapter 1: Introduction

1.1 Background

Antimicrobial resistance (AMR) is one of the major public health challenges worldwide in the 21st century (1, 2). Multidrug-resistant gram-negative bacteria are bacteria that are resistant to one antibiotic in three or more antibiotic classes (3). The increasing presence of these organisms in health care settings has led to a decreased efficacy of many first-line antibiotics, especially in healthcare settings where infections tend to be severe and invasive (3). Among these pathogens, carbapenem-resistant *Enterobacteriales* (CRE) and other carbapenem-resistant gram-negative bacteria pose the greatest threat because they are resistant to carbapenems (4). Carbapenems have historically been seen as the last line of defence against serious infections (5). Carbapenems, such as imipenem, meropenem, and ertapenem, belong to the β -lactam class of antibiotics and are effective against many gram-negative organisms (4). The introduction of carbapenems positively changed the treatment course of severe infections caused by extended-spectrum β -lactamase (ESBL) producers (4). However, in the past two decades, the widespread use of carbapenems has led to the selection of resistant organisms (6). This is due to the acquisition of carbapenemase genes, reduced outer-membrane permeability, and increased efflux pump activity (5, 7).

1.2 Carbapenem-Resistant *Enterobacteriales* and Mechanisms of Resistance

Carbapenem-Resistant *Enterobacteriales* (CRE) are defined by bacteria (*Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloacae* (*E. cloacae*), *Proteus mirabilis* (*P. mirabilis*), *Serratia marcescens* (*S. marcescens*), *Morganella morganii* (*M. morganii*), etc) possessing resistance to at least one carbapenem antibiotic, usually through carbapenem-hydrolysing β -lactamases, also known as carbapenemases (4, 5). These enzymes are encoded by mobile genetic elements that can easily spread between bacterial species through horizontal gene transfer (8). Key carbapenemase families include *Klebsiella pneumoniae* carbapenemase (*KPC*), New Delhi metallo- β -lactamase (*NDM*), Verona integron-encoded metallo- β -lactamase (*VIM*), Imipenemase (*IMP*), and Oxacillinase-48 (*OXA-48*)-like variants (9, 10). The distribution of these genes varies by region (11). The *KPC* enzymes are the most common in the United States of America and Europe, while *NDM* and *OXA-48*-like variants are more prevalent in Asia, the Middle East, and Africa (12-14). These enzymes confer resistance to carbapenems and other β -lactam antibiotics, and are frequently carried on mobile genetic elements that also harbour resistance genes to aminoglycosides and fluoroquinolones (15, 16). Resistance can also develop through mutations in porins that lower antibiotic uptake or through increased efflux pump activity that reduces the drug concentration inside the bacteria (4, 17). These mechanisms often coexist with ESBL genes like Cefotaximase Beta-Lactamase (*CTX-M*), Sulfhydryl Variable Beta-Lactamase (*SHV*), and Temoniera Beta-Lactamase (*TEM*), increasing the level of resistance to β -lactam antibiotics (17, 18).

1.3 Global and African Epidemiology

The global spread of CRE has increased since 2010, with outbreaks reported in hospitals throughout Europe, Asia, and the Americas (19, 20). The WHO Global Antimicrobial Resistance Surveillance System (GLASS) report of 2024 states that the prevalence of CRE among *K. pneumoniae* bloodstream infections increased from 2.9% in 2017 to 6.7% in 2023 (21, 22). The mortality rate associated with CRE infections is estimated to be between 30% and 50%, mainly due to limited treatment options (5). The burden of CRE in Africa has often been underestimated due to limited molecular surveillance capacities (23). However, recent systematic reviews have shown the presence of NDM and OXA-48-like enzymes as the frequently detected carbapenemase genes (23, 24). A similar study noted the widespread presence of both genes across West Africa, along with an increasing detection of KPC and VIM variants (25).

1.4 South African Context

Molecular surveillance has greatly improved through national laboratory networks in South Africa (SA). A study that screened CRE isolates collected from 2016 to 2020 reported *bla_{NDM}* and *bla_{OXA-48}* variants as the most common genes (26). More recent data confirmed national findings from 2015 to 2021, showing that OXA-48-like enzymes were predominant, followed by NDM among 2100 confirmed CRE *Enterobacterales* isolates (12). A Gauteng study showed KPC-2, NDM-1, and OXA-181/232 variants (27). Hospital outbreaks in Johannesburg showed co-harboring of NDM-1 and OXA-181 genes in neonatal units (28). A study in Cape Town reported that OXA-48-like enzymes were the commonest among carbapenem-resistant *K. pneumoniae* isolates (29). Despite improvements in molecular surveillance, KwaZulu-Natal (KZN) has been identified as one of the provinces with higher hospital admission rates and antibiotic use compared to national trends (26, 30, 31). Furthermore, the limited national molecular surveillance makes KZN crucial for studying the spread of CRE. However, available data is only reported by tertiary hospitals or outbreak investigations, creating a gap in understanding CRE dynamics in regional hospitals, which initially manage patient referrals. (26, 30, 32, 33).

1.5 Co-harboring Resistance Genes and the Role of ESBLs

The combination of carbapenemase and ESBL mechanisms complicates management (26). Co-expression of *bla_{CTX-M-15}* with *bla_{OXA-48}* or *bla_{NDM-1}* has been frequently observed in SA (29). These combinations lead to high-level resistance, even to newer β -lactam/ β -lactamase inhibitor combinations (29). The interplay between ESBLs and carbapenemase genes globally enhances horizontal transfer through conjugative plasmids and increases the risk of treatment failure (26, 27, 29). Plasmid-mediated spread of carbapenemase and ESBL genes is well documented worldwide, including studies from Africa, with these genes frequently being carried on plasmids such as IncX3, IncL/M, IncF types (15, 27). These mobile genetic elements enable the rapid spread of resistance, leading to the emergence of dual or multiple resistance mechanisms within single isolates (15, 34). Environmental studies have shown that these genes persist in wastewater and effluent specimens, which

suggests a One Health aspect to CRE spread, linking clinical, environmental, and agricultural reservoirs (32, 34, 35).

1.6 Clinical Impact and Infection Prevention and Control

The clinical impact of CREs is significant; infections usually manifest as bloodstream, urinary tract, or respiratory infections with limited treatment options, such as colistin, tigecycline, and ceftazidime – avibactam (36). Delayed identification of CREs leads to poor outcomes (37). Recent research showed that previous carbapenem use and intensive care unit (ICU) admissions were major predictors of CRE infection (26). Another study showed that colonisation with NDM-producing *K. pneumoniae* in paediatric ICU patients often preceded bloodstream infections (38). Implementing effective infection prevention and control (IPC) programs—such as screening, cohort development, and environmental decontamination—is inconsistent across SA hospitals, especially in regional areas (39, 40). Therefore, early molecular detection and surveillance are crucial for reducing CRE transmission and improving antimicrobial stewardship efforts (26).

1.7 Problem Statement

The molecular epidemiology in newly established regional hospitals is not well defined, although CRE has been documented in South Africa since 2011 (41). There is a paucity of KZN data on CRE prevalence, resistance mechanisms, and gene distribution across hospital levels (26, 42). It is therefore challenging to implement effective IPC strategies or compare regional trends with national data (12, 43). Furthermore, the coexistence of ESBL and carbapenemase genes has not been studied in these hospitals, limiting the understanding of the resistance burden and its clinical effects (44, 45). It is crucial to determine whether regional hospitals act as reservoirs or pathways for the spread of CRE to implement provincial AMR containment efforts.

1.8 Rationale of the Study

Investigating the molecular epidemiology of CRE in regional hospitals offers insights into local transmission by identifying emerging high-risk strains. Our study will provide baseline molecular and phenotypic data from KZN, aligning with South Africa's National Antimicrobial Resistance Strategy Framework (2024–2030), which emphasises regional surveillance and genomic analysis of resistant pathogens. Our data will strengthen IPC in hospitals, guiding empirical treatments, and shaping national AMR policy.

1.9 Aims and Objectives

1.9.1 Aim

We aim to determine the prevalence and molecular characterisation of carbapenem-resistant gram-negative bacteria in a new regional hospital in KwaZulu-Natal (KZN), South Africa.

1.9.2 Objectives

- Determine the prevalence of carbapenem-resistant Enterobacterales (CRE) in a new regional hospital compared with other regional hospitals in KZN.
- Identify the main species of CRE in the new regional hospital in KZN.
- Characterise the antibiotic resistance profiles of CRE isolates using phenotypic data obtained from routine laboratory records from antibiotic susceptibility testing and genotypic techniques.
- Describe the genetic sequencing of the detected target β -lactam genes (*bla_{TEM}*, *bla_{SHV}*), ESBL genes (*bla_{CTX-M}*) and ampC genes (*bla_{ampC}*) to confirm the genes of interest

Chapter 1 has described the background, with the aim and objectives of our study. Chapter 2 is the literature review, which describes global, national, and provincial trends, carbapenem antibiotics, resistance mechanisms, and emerging patterns relevant to this study.

1.10 References

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2. Chapter 2: Literature Review

Chapter 1 provided an overview of carbapenem-resistant *Enterobacterales* and their importance as a growing public health issue, especially in healthcare facilities in KwaZulu-Natal, South Africa. It explained the study's reasons, goals, and objectives, highlighting gaps in local molecular surveillance and the need to improve our understanding of resistance mechanisms in newly opened hospitals. Chapter 2 continues from this foundation by examining the current literature on carbapenem-resistant *Enterobacterales*. It focuses on global, national, and provincial trends, carbapenem antibiotics, resistance mechanisms, and emerging patterns relevant to this study.

Carbapenem-Resistant *Enterobacterales*: A Narrative Review of Prevalence, Resistance Mechanisms, and Molecular Characterisation

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Abstract

Carbapenem-resistant *Enterobacterales* (CRE) have become a major global public health issue because of limited treatment options and increasing morbidity and mortality rates. This review summarises current evidence on the prevalence, epidemiology, resistance mechanisms, and molecular traits of CREs, with a focus on global trends and the situation in South Africa. The review brings together findings that explain how carbapenem resistance occurs, including carbapenemase production, the co-occurrence of extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases, and non-enzymatic mechanisms such as loss of porins and overexpression of efflux pumps. It pays particular attention to key carbapenemase genes, such as *bla_{KPC}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*, *bla_{OXA-48-like}*, and *bla_{GES}*, and their influence on resistance profiles. The review also covers laboratory detection methods, how these bacteria spread in healthcare settings, and the implications for clinical and public health. Evidence from South Africa shows rapid spread across hospitals, highlighting the need for better molecular diagnostics, improved infection prevention and control, and strong antimicrobial stewardship.

2.1 Introduction

Antibiotic resistance in gram-negative bacteria has become a global concern (1, 2). The increased prevalence of these bacteria has raised attention from the World Health Organisation (WHO), as this rise is largely due to the inappropriate use of antibiotics in treating bacterial infections (2-5). Recent research showed a direct correlation between the frequency of bacterial exposure to antibiotics and levels of resistance (2, 6). The WHO and the National Institute of Health (NIH) have recognised these resistance levels as a problem in the future of patient care and infection management (1, 2, 4, 5, 7).

Carbapenem-resistant *Enterobacteriales* (CRE) have become a global threat in healthcare (8, 9). These bacteria cause severe infection and possess resistance to antibiotics (10). Various mechanisms of resistance are implicated in carbapenem resistance, namely carbapenemase production, efflux pump overexpression, porin loss or mutation, penicillin-binding protein alteration, and biofilm formation (9, 11). Carbapenemase production is the ability of bacteria to produce or synthesise the carbapenemase enzyme (11, 12). Carbapenemase is a β -lactamase enzyme capable of hydrolysing the β -lactam ring present in all β -lactam antibiotics (13).

The production of the carbapenemase enzyme is coded for by a set of genes, namely the β -lactamase (*bla*) *Klebsiella pneumoniae* carbapenemase (*KPC*); *bla* New Delhi metallo- β -lactamase (*NDM*); *bla* Verona integron-encoded metallo- β -lactamase (*VIM*); *bla* Imipenemase (*IMP*); *bla* Oxacillinase-48-like (*OXA-48-like*) and *bla* Guiana-Extended-Spectrum (*GES*), amongst other genes (9, 12). There has been an increase in CRE infections in South Africa (SA) (14). These bacteria cause hospital-acquired infections, including ventilator-associated pneumonia, urinary tract infections, central line-associated bloodstream infections, intra-abdominal infections, and other healthcare-associated infections (10). The molecular epidemiology of CREs shows complex dynamics in KwaZulu-Natal (KZN) (15, 16). They are characterised by the co-circulation of multiple carbapenemase genes, including *bla_{NDM}* and *bla_{OXA-48-like}* variants (17, 18), along with frequent co-harboursing with other β -lactamase genes, such as ESBLs and *ampC* (18, 19), and with variation in resistance patterns across different hospital levels (18). These dynamics are influenced by patient referral networks among regional, tertiary, and quaternary hospitals, differences in antibiotic use, and variability in infection prevention and control (IPC) measures across hospitals (17-19).

Therefore, understanding the transmission patterns and genetics of these bacteria is critical for future implementation of effective infection prevention and control (IPC) practices. Our narrative review aims to explore the global, national and provincial trends of CREs, focusing on carbapenem antibiotics, resistance mechanisms, and emerging patterns of CREs. Thus, providing an overview of critical healthcare needs which require further understanding.

2.2 Carbapenems

Although penicillins are the standard antibiotic choice for routine infections, carbapenems are a class of broad-spectrum β -lactam antibiotics that are effective against a variety of bacterial species, including many multidrug-resistant gram-negative bacteria (20). Carbapenems possess three main features which contribute to stability against β -lactamases, i.e. a double bond between the second and third carbon atoms, a β -lactam ring fused to a five-membered ring, and a carbon atom instead of a sulphur atom (Figure 1)(20, 21).

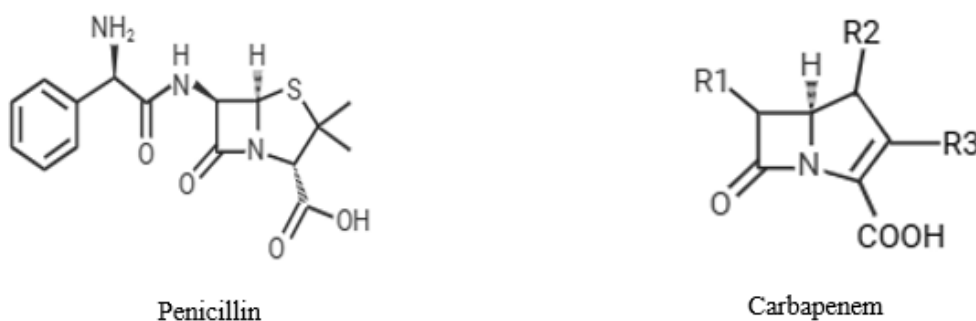


Figure 1: Comparison of the chemical structure of penicillin and carbapenem antibiotics (Created in <https://BioRender.com>)

These antibiotics cause bacterial cell death by inhibiting bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), which disrupts peptidoglycan cross-linking, which ultimately leads to cell lysis, causing cell death (21). The most used carbapenems in SA are imipenem, meropenem, and ertapenem (22). These antibiotics are reserved for the treatment of hospital-acquired infections, infections caused by ESBL-producing organisms, and multidrug-resistant gram-negative infections, septicemia, severe urinary tract infections and complicated intra-abdominal infections (Table 1) (8, 23).

Table 1: Common carbapenem antibiotics (adapted from Armstrong T, Fenn SJ, Hardie KR. J Med Microbiol. 2021;70(12):001462)(23)

Antibiotic	Uses
Imipenem	Severe infections, usually with cilastatin to prevent kidney metabolism.
Meropenem	Meningitis, intra-abdominal infections, and sepsis.
Ertapenem	Intra-abdominal and pelvic infections, UTIs.

While carbapenems are highly effective and crucial for the treatment of multidrug-resistant infections, the increased overuse and inappropriate use of carbapenem antibiotics in the healthcare system have unintentionally led to the development of carbapenem-resistant strains, especially among *Enterobacterales*, specifically the development of CRE. (24).

2.3 Carbapenem-resistant *Enterobacterales* (CRE)

Carbapenem-resistant *Enterobacterales* (CRE) are *Enterobacterales* that show resistance to at least one carbapenem antibiotic, which usually results from carbapenemase production or a combination of β -lactamase activity with porin loss and other resistance mechanisms (13). Carbapenem-resistant *Enterobacterales* (CRE) have four principal mechanisms of resistance, i.e. (a) carbapenemase production, (b) porin mutations and loss, (c) efflux pump overexpression, and (d) penicillin-binding protein (PBP) alteration (9).

(a) Carbapenemase Production

Carbapenemase production is the most common mechanism of resistance among CREs. Carbapenemases are β -lactamase enzymes with the ability to hydrolyse or degrade the β -lactam ring found in β -lactam antibiotics, e.g., carbapenems (25). The prevalent carbapenemase enzymes are the *Klebsiella pneumoniae* carbapenemase (*KPC*), New Delhi metallo- β -lactamase (*NDM*), Verona integron-encoded metallo- β -lactamase (*VIM*), Imipenemase (*IMP*), Oxacillinase-48 (*OXA-48*), and Guiana-Extended-Spectrum (*GES*) (25). These are coded for by a series of genes, namely the *bla_{KPC}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*, *bla_{OXA-48}* and *bla_{GES}* genes, respectively (12, 26). The enzymes are classified into three distinct classes based on the Ambler classification of β -lactamases as Class A, Class B, and Class D enzymes (27).

Class A and D β -lactamases require serine at the active sites, while class B, the metallo- β -lactamases (MBLs), require zinc for β -lactam hydrolysis (28). Class A serine β -lactamases include Non-metalloenzyme Carbapenemase (*NMC*)/Imipenem-hydrolysing β -lactamase (*IMI*), *Serratia Marcescens* Enzyme (*SME*), *GES*, and *KPC*, which require an active site containing the amino acid serine in order to hydrolyse the β -lactam (29). Class B metallo- β -lactamases hydrolyse carbapenems by the interaction of the zinc in the enzyme's active site with the β -lactam of carbapenems and other β -lactam drugs. Class B includes, namely, *NDM*, *VIM*, *IMP*, German imipenemase (*GIM*), and Seoul imipenemase (*SIM*) variants (30). Class D serine- β -lactamases, also known as the OXA β -lactamase, which is Oxacillinase β -lactamase, are the commonest plasmid-encoded β -lactamases with the ability to hydrolyse oxacillin and cloxacillin. There are currently 121 different variants of the OXA gene, 45 of which have the ability to hydrolyse carbapenem (25, 31-33).

Testing for these genes is done for three reasons: (i) the prevalence of genes as carbapenemase production, is a common and clinically significant resistance mechanism of CREs, (ii) transmission potential, as carbapenemase genes are often located on plasmids, which enables rapid horizontal gene transfer between bacteria leading to dissemination, (iii) clinical and diagnostic utility, e.g. molecular methods that allow for rapid and specific detection of carbapenemase genes (20, 34).

(b) Porin mutations and loss

Porin mutations and loss can contribute to antibiotic resistance (35). Porins are proteins that form channels in the bacterial outer membrane, allowing the passive diffusion of molecules, including antibiotics (36). A loss or mutation of these proteins may affect or prevent the entry of antibiotics (35).

(c) Efflux pump overexpression

Efflux pump overexpression contributes to carbapenem resistance in *Enterobacterales* by actively transporting antibiotics such as carbapenems out of the bacterium (36). Efflux pumps are membrane-bound transport proteins that reduce the intracellular concentration of antibiotics, preventing antibiotics from reaching their targets or achieving effective bactericidal levels (37).

(d) Penicillin-Binding Proteins

Penicillin-Binding Proteins (PBPs) are essential enzymes involved in the final stages of bacterial cell wall synthesis (38). Carbapenems exert a bactericidal effect by binding to these PBPs and inhibiting their activity (13). However, alterations or mutations in PBPs can reduce the affinity of carbapenems, thereby decreasing the antibiotic's ability to inhibit cell wall synthesis (39).

Biofilm formation, although not a primary mechanism of CRE resistance, contributes to antibiotic resistance, and occurs when bacteria are encased in a self-produced extracellular matrix, aiding the survival and resistance of bacteria by limiting antibiotic penetration, facilitating the exchange of resistance genes, and protecting bacteria from host immune responses (39-41).

The ESBLs are enzymes encoded by genes carried on plasmids belonging to the Cefotaximase Beta-Lactamase (*CTX-M*) family, while certain variants in the Temoniera Beta-Lactamase (*TEM*), and Sulfhydryl Variable Beta-Lactamase (*SHV*) families also exhibit ESBL properties (42). The ESBLs hydrolyse third-generation cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime, and are inhibited by β -lactamase inhibitors like clavulanic acid (43). The *CTX-M* family, especially *CTX-M-15*, has become the dominant ESBL genotype in *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*), which is widely reported across Africa and South Africa in the past decade (44). The ESBL producers increase the selective pressure for carbapenem use, which is the escalation therapy for serious ESBL infections, which contributes to the rise and spread of CRE (42).

The Ampicillin β -lactamases (AmpC) are an important resistance mechanism that is increasingly relevant in clinical settings, in addition to ESBLs (45). Plasmid-mediated AmpC enzymes, such as those encoded by *bla_{ampC}*, are not affected by clavulanic acid, which also provides resistance to cephamycins and third-generation cephalosporins (46). Recent surveillance has revealed a rise in the detection of plasmid-mediated Amp-C genes, such as *Citrobacter freundii*-derived ampC β -lactamase genes (*CIT*), *Morganella morganii*-derived Dhahran Beta-Lactamase-type ampC β -lactamase genes (*DHA*), FOX-type (cefoxitin-hydrolysing) ampC β -

lactamase genes (*FOX*), *Hafnia alvei*-derived Ambler Class C-type ampC β -lactamase genes (*ACC*), and *Enterobacter* spp.-derived ampC β -lactamase genes (*EBC*), *Aeromonas* spp.-derived Moxalactam-hydrolysing-type ampC β -lactamase genes (*MOX*) in *E. coli* and *K. pneumoniae* (45).

The *bla_{ampC}* gene and its variants, including DHA types, have been identified in modern *Enterobacterales* collections (45). AmpC β -lactamases are typically consistently expressed at low levels but is also induced in the presence of β -lactam antibiotics, leading to increased enzyme production and enhanced resistance during therapy (47). These contribute to resistance phenotypes and present diagnostic challenges (48). Standard ESBL tests overlook Amp-C activity, allowing it to spread unnoticed (49).

Plasmid-mediated Amp-C is less common than CTX-M, TEM, or SHV ESBLs, but it limits treatment options with β -lactams and may lead to unnecessary use of carbapenems (45, 48). Plasmids are crucial for spreading Amp-C and carbapenemase genes, which can lead to co-carriage and complex multidrug-resistant characteristics (50). These isolates are difficult to detect and treat when *bla_{ampC}* occurs alongside ESBLs or carbapenemases, which highlights a need for molecular diagnostics in combination with phenotypic testing (48, 51). These changing β -lactamase patterns directly influence the epidemiology of CREs observed in our region (48).

2.4 Epidemiology

The global spread of CREs has increased in the last decade and poses a risk for healthcare-related illness and death worldwide (9, 52). Recent global surveillance studies have shown significant differences in carbapenemase distributions (52, 53). A global study indicated that of 7,731 carbapenem-resistant *Escherichia coli*, NDM made up 52.2% of isolates, OXA-48/-like accounted for 30.1%, and KPC represented 14.7% (52). The NDM and OXA-48-like enzymes represented 43.1% and 42.9% of CRE isolates in Africa, which highlights geographic differences in enzyme prevalence with a changing distribution of carbapenemases by region (52). A recent meta-analysis published in 2024, which pooled global CRE prevalence, estimated the total prevalence at 43.1%, with variation by region (54). A global study showed the prevalence of carbapenem-resistant *Klebsiella pneumoniae* was estimated to be about 28.7% among clinical isolates, with regional variation, such as occurrence rates of 14.3% in North America and more than 66% in South Asia (55). KPC is still the most common in parts of the Americas and some European areas (9, 52). NDM is very common in South Asia and has spread globally (9, 56). OXA-48 and similar variants have become prevalent in North Africa, parts of Europe, and across Africa (57, 58). These changing patterns, highlighted by WHO/GLASS surveillance analyses and various genomic and epidemiologic reviews, show the need for coordinated national and international surveillance (52, 53). There is also a need for linking routine molecular characterisation with laboratory data to infection prevention and treatment strategies (9, 56).

Research in sub-Saharan Africa has indicated a growing presence of ESBL-producing *Enterobacterales* and a rapid increase in CRE detection in clinical specimens and surveillance isolates (52). Limited resources and varying laboratory capabilities make local monitoring essential for tracking regional trends (59). National consolidated surveillance by the Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa (GERMS-SA) / National Institute of Communicable Diseases (NICD) revealed a measurable and growing CRE burden (60-64). A recent analysis of GERMS-SA data from 2015 to 2021 identified 5,258 CRE bloodstream infection (BSI) events among 270,239 BSI records, which is about 1.9% of all BSI (64). It also reported a case-fatality rate of 36.1% among cases with outcome data, which shows significant mortality and the need for improved surveillance and stewardship (64). The 2023 GERMS-SA report summarises thousands of laboratory-confirmed cases in the country, confirming ongoing CRE detection and shifts in gene types that justify genomic surveillance at the provincial level (63). There has been a relative decrease in *bla*_{NDM} and a rise in *bla*_{OXA-48-like} variants among clinical bloodstream isolates (63, 65). The national data also reveal an increase in CRE bloodstream infections, highlighting the need for genomic surveillance at the provincial level (64, 66).

KwaZulu-Natal (KZN) has a high burden of CRE colonisation and invasive infections (60, 63). Provincial and hospital reports indicated that KZN has active CRE detection with documented clusters in referral hospitals (66). KwaZulu-Natal-based surveillance and hospital studies indicated an increase in CREs at regional, tertiary, and quaternary centres, which is consistent with referral pathway effects and variations in laboratory capacity among hospitals (64).

Regional hospital surveillance and reports from paediatric intensive care units (PICUs) have shown rising CRE carriage and outbreaks in provincial facilities, emphasising the need to triangulate NICD and GERMS-SA national data with local hospital surveillance to accurately assess the provincial burden (67). The network of hospitals in KZN may create pathways for transmission that might facilitate the spread of clones if IPC and monitoring are not effective. (67, 68).

It is important to understand the molecular and biochemical processes underlying CRE following the global rise of CRE and its presence in SA, which will improve testing and treatment.

2.5 Mechanisms of Resistance

Carbapenem resistance in *Enterobacteriales* is due to four overlapping mechanisms: (1) the production of carbapenemases, including serine β -lactamases like KPC and OXA-48-like, and metallo- β -lactamases such as NDM, VIM, and IMP; (2) the combined production of ESBL or Amp-C β -lactamase along with porin loss or changes that decrease antibiotic entry; (3) the upregulation of efflux pumps; and (4) target modification or other chromosomal mutations, including changes in penicillin-binding proteins (69). Carbapenemase production is the main mechanism because it occurs on plasmids that can be shared (11, 70). Recent reviews highlight that resistance traits involve several mechanisms, such as ESBL, omp porins distribution, and low-level carbapenemases (13, 42, 71). The complexity makes it difficult to detect phenotypes and interpret susceptibility (13, 72).

The understanding of carbapenem resistance development in *Enterobacteriales* is necessary to examine the genetic changes that drive these traits. The three key mechanisms are:

- a) Mutations that lower outer membrane permeability, especially in the *ompK35* and *ompK36* porin genes of *Klebsiella pneumoniae*.
- b) Structural changes in β -lactamase enzymes like *KPC* and *OXA-48-like* variants, which affect hydrolytic activity or inhibitor binding.
- c) The spread of metallo- β -lactamases, such as *NDM*, with various allelic variants leads to broad-spectrum resistance.

These examples in Table 2 show how changes in nucleotides or amino acids can alter antibiotic resistance profiles and clinical outcomes, highlighting the need to include molecular tracking in programs that monitor antimicrobial resistance (35, 72-75).

Table 2: Common mutation/alteration sites linked to carbapenem resistance, adapted from recent global genomic surveillance studies (2020-2025)

Mechanism Category	Gene/target	Typical mutation/alteration	Functional effect	References
Porin alterations (<i>K. pneumoniae</i>)	<i>ompK35</i>	Frameshift/truncation (e.g., nucleotide deletion causing premature stop)	Loss of or reduced porin leading to decreased carbapenem influx	(73)
Porin alterations (<i>K. pneumoniae</i>)	<i>ompK36</i>	Glycine-aspartic acid (GD) insertion at aa 134–135; IS element insertions in promoter	Alters channel size/selectivity, leading to reduced permeability	(73)
KPC enzyme	<i>bla_{KPC}</i> (Ω-loop residues 164–179)	Ω-loop substitutions (e.g., D179Y / D179N, L169P)	Altered inhibitor binding leading to ceftazidime–avibactam resistance, which may alter carbapenem susceptibility	(74)
OXA-48 family	<i>bla_{OXA-48-like}</i>	Point variations and different variants (OXA-181, OXA-232 etc.)	Causes variable hydrolytic profiles; also results in poorer detection by some phenotypic screens	(75)
NDM family	<i>bla_{NDM}</i>	Multiple allelic variants (NDM-1, NDM-5)	Metallo-β-lactamase activity (zinc-dependent), which causes broad β-lactam hydrolysis	(72)

Insertion sequence (IS), β-lactamase (*bla*); *Klebsiella pneumoniae* carbapenemase (*KPC*); New Delhi metallo-β-lactamase (*NDM*); *Oxacillinase*-48-like (*OXA-48-like*).

In addition to producing carbapenemase, non-carbapenemase mechanisms can also lead to carbapenem resistance in *Enterobacterales* (13). These include changes to porins, the activity of efflux pumps, and modifications of targets (76). As shown in Table 2, mutations in porin genes, especially *ompK35* and *ompK36* in *Klebsiella pneumoniae*, lower the permeability of the outer membrane and restrict the entry of antibiotics (73). Efflux pump systems like AcrAB-TolC further enhance resistance by actively expelling antibiotics from the bacterial cell (77, 78). While less commonly noted, changes in PBPs can also lessen antibiotic binding (79). These mechanisms often work together with β-lactamase production, leading to complex resistance traits (80, 81).

2.6 Laboratory Diagnosis

Characterising CRE in clinical settings requires precise laboratory detection and confirmation of resistance mechanisms based on international standards like the Clinical and Laboratory Standards Institute (CLSI) M100 (82). Laboratories in SA use CLSI M100 performance standards as the main reference for antimicrobial susceptibility testing (AST) (82). The current edition should be applied for breakpoints, quality control, and method recommendations (82). Many laboratories use ertapenem non-susceptibility or reduced susceptibility to any carbapenem as an initial CRE screen, which is followed by confirmatory phenotypic tests, such as the modified carbapenem inactivation method (mCIM), or molecular tests for carbapenemase genes (83). Recent CLSI updates highlighted the need for local validation of automated AST systems and caution against misclassification of organisms and carbapenemase types by routine platforms (82, 83).

Initial diagnostic steps include microscopy with gram staining to identify gram-negative bacilli, which is followed by culture on routine media like blood agar and MacConkey agar to evaluate colony shape, lactose fermentation, and purity (84). Selective media with carbapenems, such as MacConkey supplemented with meropenem or ertapenem, may be used to improve recovery of carbapenem-resistant strains (85).

Chromogenic media such as chromogenic agar (CHROMagar™) CRE (CHROMagar, Paris, France) or CHROMagar™ ESBL (CHROMagar, Paris, France) also aid in the early detection and preliminary differentiation of resistant *Enterobacterales* by producing distinct colony colours based on enzyme activity (86, 87). These media are recommended for screening rectal swabs and surveillance cultures in high-risk situations or during outbreaks to increase detection sensitivity and reduce turnaround time (86, 87). Culture is essential for phenotypic AST, archiving isolates for sequencing, and detecting mixed populations that molecular tests might miss (84) (Supplementary Tables, Table S1).

Species identification by MALDI-TOF (bioMérieux, Marcy l'Étoile, France) mass spectrometry provides rapid identification, usually within 1 to 2 hours from a colony (88). Laboratories without MALDI-TOF (bioMérieux, Marcy l'Étoile, France), use validated biochemical panels, like API 20E, which are still acceptable, although these are slower and less precise (89, 90). Laboratories may combine species identification (MALDI-TOF/API) (bioMérieux, Marcy l'Étoile, France) with AST workflows, which ensures bacteria identification, informing the interpretative criteria and targeted molecular testing (88, 89). Bacterial identification using methods like MALDI-TOF, API, or automated systems is a crucial first step in diagnosing CRE (91). It helps interpret antimicrobial susceptibility results correctly and directs further confirmatory testing for carbapenem resistance mechanisms (91).

Susceptibility testing can be done using reference broth microdilution, validated automated platforms such as VITEK®2 (bioMérieux, Marcy l'Étoile, France), or disk diffusion interpreted by CLSI criteria (Kirby-Bauer) (92, 93). These susceptibility testing methods are key to identifying CREs, as reduced susceptibility to carbapenems is the main characteristic that leads to further investigation for underlying resistance mechanisms

(94). Integrated platforms like VITEK®2 combine organism identification and AST. They support quick detection of resistance types and simplify the diagnostic workflow for CRE in clinical laboratories (95). Additionally, methods such as E-test (gradient diffusion) and synergy testing are also increasingly used, particularly for confirmatory purposes and the detection of specific resistance mechanisms (96). Methods like E-test and synergy testing help characterise resistance types and can also identify isolates that need confirmatory molecular testing for carbapenemase production (97). Each method has its limitations (92, 96, 98). Automated cards and some disk protocols can miss OXA-48-like producers or give inconsistent minimum inhibitory concentrations (MICs) for new β -lactam/ β -lactamase inhibitor combinations (65, 99, 100). Therefore, confirmatory testing with reference methods or targeted molecular testing for suspected carbapenemase producers is advisable (65, 101). Local quality control, involvement in external quality assessment (EQA) schemes, and cross-method verification, like comparing VITEK®2 (bioMérieux, Marcy l'Étoile, France) to broth microdilution and disk diffusion, enhance result reliability (102).

Phenotypic confirmatory assays have performed well in recent evaluations (103). The multicentre evaluations of the modified carbapenem inactivation method (mCIM) report sensitivities of 97% to 100%, and specificities between 95% and 99% for detecting carbapenemase-producing *Enterobacterales* (103). The use of mCIM and Ethylenediaminetetraacetic acid (EDTA) modified carbapenem inactivation method (eCIM) as reliable, low-cost confirmatory tests is suitable for routine use where molecular testing is limited (103, 104).

2.7 Molecular Diagnosis

Targeted molecular assays are essential for quickly identifying carbapenemase-producing *Enterobacterales* (CPE) (105, 106). Conventional polymerase chain reaction (PCR) amplifies specific gene fragments related to key carbapenemase genes, such as *bla_{KPC}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*, and *bla_{OXA-48-like}* (107, 108). The PCR uses gene-specific primers to enable rapid DNA amplification (108). Results are visualised using agarose gel electrophoresis or fluorescence-based systems (107). Conventional PCR is still commonly used in many laboratories because it is affordable, simple to execute, and works well with basic molecular tools (107). It enables the targeted detection of key carbapenemase genes with high accuracy when the right primers are used (107, 108). Real-time PCR improves detection efficiency by using fluorescent probes or intercalating dyes, which allows monitoring of gene amplification in real time (105). The Real-time PCR shortens turnaround time, lowers contamination risk and provides information on gene copy number (105). Multiplex Real-time PCR platforms and validated in-house assays are commonly used to detect multiple carbapenemase genes, which enable same-day confirmation from bacterial isolates or clinical specimens (106). These methods are useful for screening high-risk patients, detecting colonisation early, and guiding quick infection prevention and control actions (105, 106). These PCR-based tests of rectal swabs or enrichment broths have shown better sensitivity and specificity than culture alone for colonisation surveillance, significantly improving early detection and reducing transmission in healthcare settings (108, 109).

In addition to conventional and real-time PCR methods, several commercial molecular tests are available for the quick detection of CPE (110). These include platforms like the Xpert® Carba-R assay (Cepheid, California, USA), the BD MAX™ Check-Points CPO assay (Becton, Dickinson and Company, New Jersey, USA; Check-Points, Wageningen, The Netherlands), and the eazypex® SuperBug CRE system (AmplexDiagnostics GmbH, Gars-Bahnhof, Germany) (111-113). They can detect key carbapenemase genes, including *bla_{KPC}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*, and *bla_{OXA-48-like}*, directly from clinical samples or bacterial isolates (112). These tests offer rapid turnaround times, often within one hour (114). They have shown high sensitivity and specificity, making them useful tools for preventing infections, controlling outbreaks, and making clinical decisions in both high- and low-resource settings (115).

Sequencing technologies have changed molecular epidemiology and resistance surveillance following targeted PCR (68). Whole-genome sequencing (WGS) and targeted next-generation sequencing (tNGS) provide detailed data on bacterial genomes, which allows for thorough analysis of clonality, resistome composition, and plasmid structure (68, 116). Whole-genome sequencing (WGS) offers complete genomic resolution and reveals the presence of multiple resistance genes, such as *bla_{NDM}* and *bla_{OXA-48-like}*, within the same strain (117). It also identifies mobile genetic elements like IncX3 and IncL/M plasmids that enable horizontal gene transfer (117). However, tNGS focuses on selected resistance-related sites and is a quicker, more cost-effective option for surveillance (68). Sequencing technologies have transformed outbreak investigations and tracking of infections but use in routine diagnostics remains limited in many low- and middle-income countries (59). Barriers include high implementation costs, the need for advanced sequencing equipment, and specialised bioinformatics skills (59, 68). Hybrid diagnostic frameworks that combine centralised WGS with decentralised PCR screening are increasingly used in resource-limited healthcare systems (68). The clinical management of CRE infections has become increasingly difficult due to the complex nature of resistance mechanisms and the diagnostic challenges mentioned.

2.8 Antibiotic Treatments of CRE Infections

Treatment options for CRE infections in many public hospitals in South Africa are limited due to drug availability, concerns about toxicity, and local resistance patterns (118, 119). Polymyxins (colistin) and tigecycline were the main options for salvage therapy in the past (119). However, new evidence highlights drawbacks, such as nephrotoxicity, inadequate serum levels for bloodstream infections, and varying clinical success (119). Newer targeted combination drugs like ceftazidime-avibactam and cefiderocol are advances in therapy (120). Ceftazidime-avibactam pairs a third-generation cephalosporin with a non-β-lactam β-lactamase inhibitor (avibactam), effectively neutralising KPC and most OXA-48-like carbapenemases (120). However, it does not work against MBLs like NDM, VIM, and IMP (120). Cefiderocol is a new siderophore cephalosporin that uses iron-uptake pathways to enter bacterial cells (121).

It has shown strong activity against many carbapenemase producers, including many MBL-producing strains, although reports of emerging resistance during treatment exist (121, 122). Ceftazidime-avibactam and cefiderocol have demonstrated better clinical outcomes and lower toxicity compared to polymyxins but are expensive (123, 124). South African treatment guidelines now recommend mechanism-guided therapy, which involves choosing treatment based on the identified carbapenemase gene, with support from infectious disease consultation and combination therapy when suitable (120). However, colistin remains the most commonly used option in many hospitals, while access to ceftazidime-avibactam and cefiderocol is mostly limited to tertiary and quaternary centres (119, 125). South African hospitals have shown varying outcomes with colistin-based regimens and suggest improved survival when newer agents are available and used correctly (126, 127).

A national surveillance analysis of 2,919 patients with CRE bloodstream infections in SA from 2015 to 2021 reported a case-fatality rate of 36.1%, corresponding to 1,055 deaths (64). These results indicate the clinical impact and high mortality rates associated with CRE infections (64). The study highlights the limited treatment options available in SA hospitals and the continued reliance on older, toxic drugs like colistin and tigecycline (64). The newer targeted therapies, such as ceftazidime-avibactam and cefiderocol, have shown better safety and efficacy in other countries, but availability in the SA public sector is limited, especially outside tertiary centres (119, 123, 126). These observations require the need to improve access to mechanism-guided therapy while enhancing antimicrobial stewardship and IPC measures to prevent further spread and improve patient outcomes (59, 120).

Strong IPC practices are crucial to reduce CRE spread in addition to antibiotics (128). Key strategies include early identification and isolation or grouping of colonised patients, strict hand hygiene, contact precautions, environmental cleaning with sporicidal agents, and routine screening of high-risk wards (129). Molecular surveillance and targeted therapy are essential for controlling the ongoing spread of CRE in SA healthcare facilities by combining IPC with antimicrobial stewardship (64, 68, 120).

2.9 Conclusion

The increasing occurrence of CRE infections in KZN is a threat to the healthcare system, which highlights the need for a better understanding of CREs in KZN. There is a need for more effective monitoring of the prevalence and detection of CRE genes to facilitate better antimicrobial stewardship in the healthcare system of the province.

Chapter 2 has reviewed the existing literature on CREs. It outlines their global emergence, molecular resistance mechanisms, and epidemiology in South Africa and KwaZulu-Natal. The review identified significant gaps in molecular surveillance data from regional hospitals and stressed the importance of understanding local resistance patterns. Chapter 3 addresses these gaps by investigating the prevalence, species distribution,

antimicrobial resistance profiles of CREs and carbapenemase gene presence in multidrug-resistant *Enterobacterales* isolates in three KwaZulu-Natal hospitals, including a new regional hospital

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Supplementary Tables

Table S1: Types of Agars adapted from recent studies

Media	Constitution	References
Blood Agar (BA)	• Tryptic soy agar or nutrient agar base. • 5-10% defibrinated sheep blood.	(130)
MacConkey Agar (MAC)	• Peptone and proteose peptone. • Lactose. • Bile salts & crystal. • Natural red.	(131)
MacConkey + Meropenem/Ertapenem	• MacConkey Agar base (listed above). • Carbapenem antibiotics (meropenem or ertapenem), at 1-2µg/ml.	(132)
CHROMagar™ CRE	• Peptones and an agar base. • Chromogenic substrates linked to specific bacterial enzymes like β-glucosidase or β-galactosidase. • carbapenem supplements.	(133)
CHROMagar™ ESBL	• Peptones and an agar base. Chromogenic substrates linked to specific bacterial enzymes like β-glucosidase or β-galactosidase. • Third-generation cephalosporins.	(134)

3. Chapter 3

The literature reviewed in Chapter 2 indicates that carbapenem-resistant *Enterobacterales* continue to proliferate both globally and within South Africa, with evolving molecular resistance patterns and a growing clinical impact. However, limited data are available from regional hospitals, particularly those that are newly established. Chapter 3 aims to investigate the prevalence of Carbapenem-resistant *Enterobacterales* and molecularly characterise local Carbapenem-resistant *Enterobacterales* isolates to determine the presence of carbapenemase genes, in hospitals with different referral roles, providing essential baseline data to understand the dynamics of carbapenem resistance in KwaZulu-Natal.

The Molecular Characterisation of Carbapenem-Resistant *Enterobacterales* in a New Regional Hospital in KwaZulu-Natal, South Africa

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Abstract

Introduction

Carbapenem-resistant *Enterobacterales* (CRE) are a growing public health concern in hospitals facing high burdens. Data from newly established hospitals in South Africa are still limited.

Methods

We reviewed laboratory records from three KwaZulu-Natal (KZN) hospitals (Dr Pixley Ka Isaka Seme Memorial Hospital (DPKISMH), Inkosi Albert Luthuli Central Hospital (IALCH) and Mahatma Gandhi Memorial Hospital (MGMH)) to determine the annual prevalence, bacterial species distribution and antibiotic resistance levels of CREs from 2016 to 2024. A subset of 720 stored carbapenem-resistant *Enterobacterales* isolates underwent multiplex Polymerase chain reaction (PCR) to detect key carbapenemase genes.

Results

CRE prevalence increased in all hospitals, including a rapid rise at a newly opened DPKISMH. We noted diverse species distributions, with *Klebsiella pneumoniae* prevalent at IALCH and *Proteus mirabilis* at regional sites. The main carbapenemases were *bla_{NDM}* and *bla_{OXA-48-like}*, with a clear shift from NDM to OXA-48-like at IALCH. Dual and multiple carbapenemase gene carriage was common, while 15.4% of isolates did not show detectable carbapenemase genes.

Discussion

The rise in CRE prevalence and changing carbapenemase dominance indicate ongoing selection pressure and the spread of resistance across referral hospitals. The rapid development of complex resistance in a new hospital points to inter-facility transmission rather than isolated cases. The increasing prevalence of OXA-48-like enzymes raises diagnostic issues, while carbapenemase-negative CRE suggests other resistance mechanisms are present.

Conclusion

Both the prevalence and genetic complexity of CRE are on the rise in KZN. This reinforces the necessity for ongoing molecular surveillance and improved infection control measures.

3.1 Introduction

The World Health Organisation (WHO) highlights the rising global burden of drug-resistant bacterial infections (1-3). Carbapenems are typically the last-line antibiotics for severe infections caused by gram-negative bacteria. However, carbapenem-resistant *Enterobacterales* (CRE) such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Enterobacter cloacae* (*E. cloacae*), *Proteus mirabilis* (*P. mirabilis*), *Serratia marcescens* (*S. marcescens*), *Morganella morganii* (*M. morganii*), etc, have become a clinical challenge (4). Resistance to carbapenems is mainly caused by carbapenemase enzymes like *Klebsiella pneumoniae* carbapenemase (*KPC*), New Delhi metallo- β -lactamase (*NDM*), Verona integron-encoded metallo- β -lactamase (*VIM*), Imipenemase (*IMP*), Oxacillinase-48-like (*OXA-48-like*) and Guiana-Extended-Spectrum (*GES*) (4).

Recent surveillance shows a rapid emergence of CREs in South Africa (5). National surveillance reported a rising incidence of carbapenem resistance in *Enterobacterales* (6) which detected NDM and OXA-48-like enzymes as the most frequent. KwaZulu-Natal (KZN) in South Africa has an estimated 12.3 million residents (7), which is the largest province with a demographic diversity, having a distinct urban and rural population (7). The KZN population trends have an impact on healthcare use and infectious diseases, leading to a heavier burden of communicable diseases compared to other provinces (8). KZN healthcare includes quaternary, regional and district hospitals (9). It appears that laboratories do not routinely test for CRE resistance mechanisms (10).

We aimed to determine the prevalence and molecular characterisation of CREs at the newly opened Dr Pixley Ka Isaka Seme Memorial Hospital (DPKISMH) (2022–2024) compared with two other established referral hospitals (Inkosi Albert Luthuli Central Hospital (IALCH) and Mahatma Gandhi Memorial Hospital (MGMH) (2016–2024). We further aimed to identify the dominant CRE and characterise CRE profiles using phenotypic and genotypic techniques in DPKISMH.

3.2 Methods

3.2.1 Laboratory Database

We reviewed laboratory records from three public hospitals in KZN: IALCH (quaternary) and MGMH (regional) from 2016 to 2024, and DPKISMH (regional) from 2022 to 2024. All laboratory records were retrieved from the National Health Laboratory Service (NHLS) TrackCareLab database to evaluate annual prevalence trends. All gram-negative *Enterobacterales*, resistant to at least one carbapenem antibiotic (ertapenem, meropenem, or imipenem) were included, which were mainly from: blood, urine, respiratory specimens, swabs, and fluids. All non-*Enterobacterales* were excluded. Specimens from the same patient, with the same bacteria, and same specimen type collected within 14 days were considered duplicates and excluded, while specimens outside of the 14-day period were considered non-duplicates and included. Prevalence data

were calculated using available laboratory records and were not adjusted for hospital admission volumes, as this data was not available.

3.2.2 Laboratory Methods

Phenotypic Identification

Seven hundred and seventy (770) stored isolates classified as carbapenem-resistant were retrieved and revived from cryobeads on 5% sheep blood agar and incubated at 37°C for 48 hours before further analysis. Isolates chosen for molecular analysis were based on what was available in stored laboratory collections at the hospitals. Only isolates that were successfully regrown were included in the molecular part of the study. Therefore, the molecular subset shows isolate availability and regrowth success instead of a proportional representation of all species found in the complete dataset. Isolates that did not revive after two attempts of subculturing were excluded. Therefore, a total of 720 isolates with successful regrowth from IALCH (n = 587), MGMH (n = 48), and DPKISMH (n = 85) were included in the analysis. Identification of bacteria and antimicrobial susceptibility testing (AST) were done previously as part of routine diagnostic laboratory procedures using the Vitek®2 automated system (bioMérieux, Marcy l'Étoile, France), and AST was interpreted according to Clinical and Laboratory Standards Institute (CLSI) M100-34th edition (2024) guidelines. Phenotypic variables, including bacterial identity and complete AST profiles, were collected and analysed by hospital and by year.

Genotypic Identification

DNA Extraction

Deoxyribonucleic Acid (DNA) was extracted from all isolates using the QuickExtract™ rapid protocol following the manufacturer's instructions (Lucigen®, Wisconsin, USA). Briefly, 100 µL of QuickExtract™ DNA Extraction Solution was added to 100µL of specimen, followed by heating at 65°C for 5 minutes and then at 98°C for 5 minutes. The lysate was centrifuged at 12,000rpm for 10 minutes, and the resulting supernatant was stored at -20°C until needed. DNA concentrations were measured by NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. ND-8000-GL) to ensure enough nucleic acid for PCR input, and specimens with an A260/A280 ratio of ≥ 1.8 were considered sufficiently pure.

Polymerase Chain Reaction (PCR)

A Multiplex PCR assay was optimised to detect Class A, B and D carbapenemase genes in all isolates (*bla_{KPC}*, *bla_{IMP}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{OXA-48-like}*, and *bla_{GES}*) (11). Negative and Positive controls for carbapenemase detection: *bla_{KPC}* (American Type Culture Collection (ATCC) BAA-1705 KLEPN 05), *bla_{IMP}* (NCTC 13476 ESCCO 28), *bla_{NDM}* (ATCC BAA-2146 KLEPN 10), *bla_{VIM}* (National Collection of Type Cultures (NCTC) 13439 KLEPN 12), *bla_{OXA-48}* (NCTC 13442 KLEPN 11), and *bla_{GES}* (Clinical Isolate KLEPN 14/KG914(12)) were included. The PCR was done on the Bio-Rad T100 Thermal Cycler (LASEC, Cape Town, South Africa,

catalogue no. 1861096). Each PCR Master mix consisted of 10.0µl of DreamTaq HS Green Master Mix (2×) (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. K9022), 0.2µl of Forward primer (10 µM) for each target gene, 0.2µl of Reverse primer (10 µM) for each target gene, 3.2µl of Nuclease-free water and 2µl Template DNA (20ng/µl)(11). PCR cycling conditions included an initial activation at 94°C for 3 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 60 seconds. A final extension step was done at 72°C for 3 minutes, after which specimens were held at 4°C (11). Previously published primer sequences, Master mix reaction mixes, and references are listed in Supplementary Tables S1 & S2 (11).

Gel Electrophoresis

All PCR amplicons were analysed on 1.5% agarose gels (TopVision Agarose Tablets, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. R2802) prepared in 1× Tris-Borate Ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (TBE (10X), RNase-free, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. AM9865). Gels were run at 80V for 2 hours to allow for clear separation of multiplexed targets of different lengths. A 100-base pair (bp) DNA ladder (GeneRuler Ready-to-Use DNA Ladders, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. SM0323) served as the molecular size marker. DNA bands were visualised using DNA Gel Loading Dye (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. R0611) mixed with GelRED (GelRed® Nucleic Acid Stain 10000X Water, Sigma Aldrich, Missouri, USA, catalogue no. SCT123) under UV light using a BIO RAD GelDoc Go imaging system (LASEC, Cape Town, South Africa, catalogue no. 12009077). Amplicon sizes were interpreted based on expected fragment lengths listed in Supplementary Table S1 (11, 13, 14).

3.2.3 Statistical Analysis

The CRE prevalence was calculated annually for each hospital. Trends from 2016 to 2024 were examined using a two-way repeated-measures ANOVA, with hospital as the column factor and year as the row factor. The CRE distribution and antibiotic resistance profiles were compared among hospitals using Chi-square (cell count ≥ 5) or Fisher's exact tests (cell count ≤ 5), based on the suitability of the data. The co-occurrence of carbapenemase genes was summarised using descriptive frequencies and percentages. The Chi-square or Fisher's exact tests were used to assess differences in the distribution of single, dual, and multiple gene combinations across the hospitals. Statistical analyses were conducted using GraphPad Prism (version 10.6.1 (892) for Windows; GraphPad Software, Massachusetts, USA, www.graphpad.com), with p-values less than 0.05 considered statistically significant. Comparative analyses were conducted using the data between 2016 to 2024 from each hospital. Since DPKISMH is a newly opened hospital, only data from 2022 onward was available. However, the main goal of the study was to assess overall trends and distribution across the entire study period."

3.2.4 Ethical Approval

This study received approval from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00007433/2024)

3.3 Results

3.3.1 Results of the Laboratory Database

Carbapenem-Resistant *Enterobacterales* (CRE) Prevalence

The prevalence data from three public hospitals (IALCH, MGMH & DPKISMH) in KZN were analysed from laboratory records. Sixty-six thousand and ninety-three (66093) gram-negative bacteria were detected: *Enterobacterales* 62.1% (41032/66093), and the remaining 37.9% (25061/66093) were non-*Enterobacterales*. CREs accounted for 11.7% (4820/41032) of all *Enterobacterales*: 13.1% (3752/28611) at IALCH, 8.1% (825/10217) at MGMH and 11% (243/2204) at DPKISMH. An increase in CRE prevalence was observed. An increasing trend in CRE was observed in IALCH from 3.3% (182/5530) in 2016 to 12.4% (609/4931) in 2024 ($p < 0.0001$). At MGMH, a fluctuating CRE prevalence was observed, from 2.8% (53/1888) in 2016 to 8.3% (91/1108) in 2024 ($p < 0.0001$). An increasing trend in CRE prevalence was observed at DPKISMH of 4.7% (12/255) in 2022 to 7.8% (113/1450) in 2024 ($p = 0.2894$) (Figure 1).

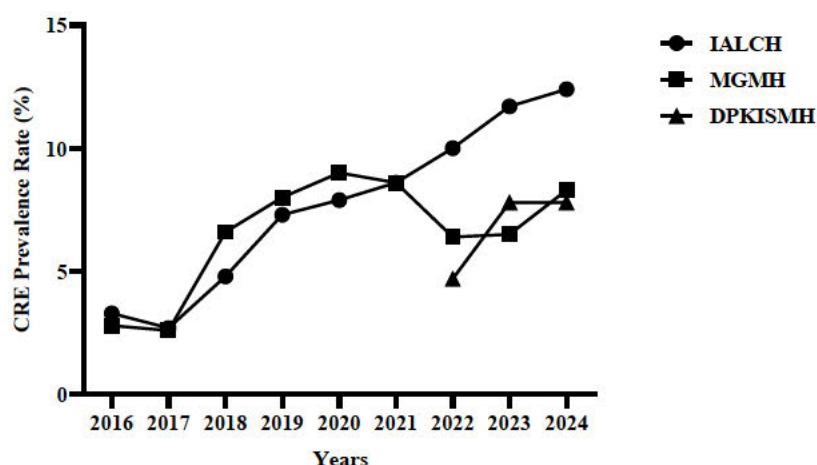


Figure 1: Prevalence of CREs (2016 to 2024) (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

The CRE prevalence at IALCH and MGMH from 2016 to 2024 showed no significant difference ($p = 0.2280$). However, the analysis from 2022 to 2024 showed a significant difference between IALCH and MGMH ($p = 0.0050$) and between IALCH and DPKISMH ($p = 0.0039$). No significant difference was observed between the prevalence at MGMH and DPKISMH ($p = 0.8819$) (Figure 2).

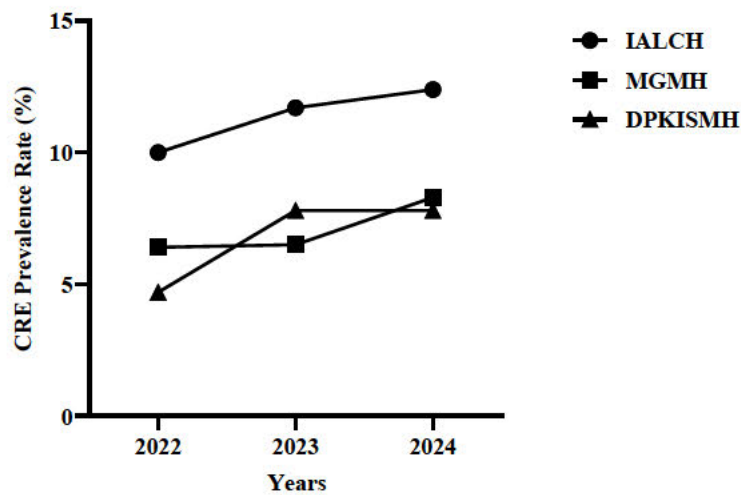


Figure 2: Prevalence of CREs (2022 to 2024) (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

Carbapenem-Resistant Enterobacterales (CRE) Distribution

The CRE distribution patterns were analysed to determine any differences in the hospitals. A significant difference was observed in the distribution of CREs (IALCH v/s MGMH $p < 0.0001$, MGMH v/s DPKISMH $p < 0.0001$, IALCH v/s DPKISMH $p < 0.0001$), indicating that each hospital had a distinct CRE pattern. At IALCH, the most prevalent bacterium was *K. pneumoniae* 78.2% (2934/3752), followed by *P. mirabilis* 7.1% (268/3752) and *E. cloacae* 4.6% (172/3752). However, *P. mirabilis* was the most prevalent at MGMH, 44.7% (369/825), and DPKISMH, 32.1% (78/243), followed by *K. pneumoniae*, at 31% (256/825) and 22.6% (55/243), respectively (Figure 3). These bacteria were detected in multiple sources (Tables S3–S5), especially in blood cultures in IALCH (1115/3752 (29.7%)), but in pus at MGMH (384/825 (46.5%)) and DPKISMH (107/243 (44%)).

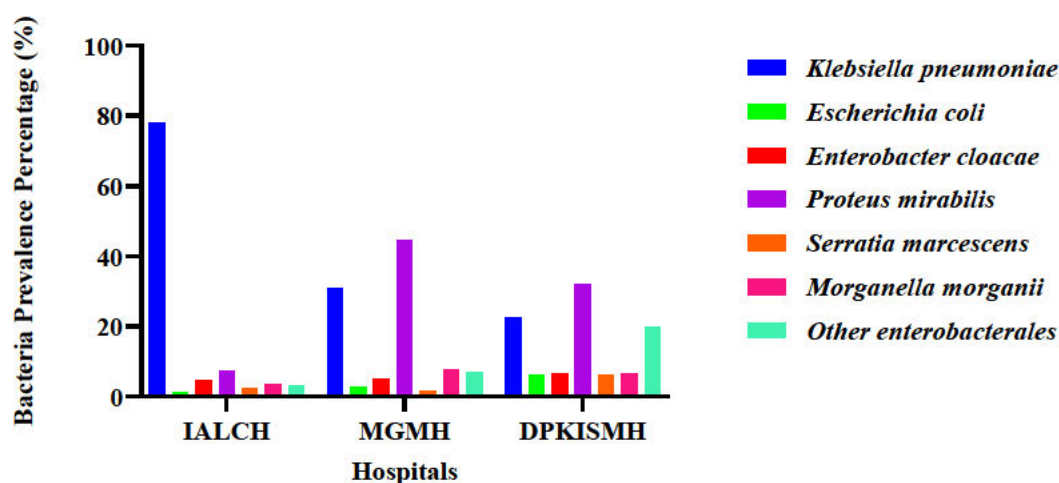


Figure 3: Prevalence of CRE bacterium per hospital (2016 to 2024) (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

Antimicrobial Susceptibility

Phenotypic AST was done using the Vitek®2 automated system (bioMérieux, Marcy l'Étoile, France) to compare antibiotic resistance among CREs and showed significant differences in antibiotic resistance among CREs across hospitals (Figure 4). At IALCH, resistance to meropenem was 81.1% (3043/3752), imipenem was 82.5% (3097/3752), and ertapenem was 41.8% (1567/3752). At MGMH, resistance to meropenem was 42.8% (353/825), imipenem was 89.9% (742/825), and ertapenem was 1.3% (11/825). At DPKISMH, meropenem resistance was 41.6% (101/243), imipenem was 80.7% (196/243), and ertapenem was 0.4% (1/243). Meropenem resistance varied between hospitals (IALCH v/s MGMH $p < 0.0001$, MGMH v/s DPKISMH $p = 0.0012$, IALCH v/s DPKISMH $p < 0.0001$), while imipenem resistance was significantly different at IALCH v/s MGMH ($p < 0.0001$) and MGMH v/s DPKISMH ($p < 0.0001$) but not at IALCH v/s DPKISMH ($p = 0.4545$). Although ertapenem resistance was low at MGMH and DPKISMH, a significant difference was noted at IALCH v/s MGMH ($p < 0.0001$) and IALCH v/s DPKISMH ($p < 0.0001$), while for MGMH v/s DPKISMH ($p = 0.3167$) was not significant.

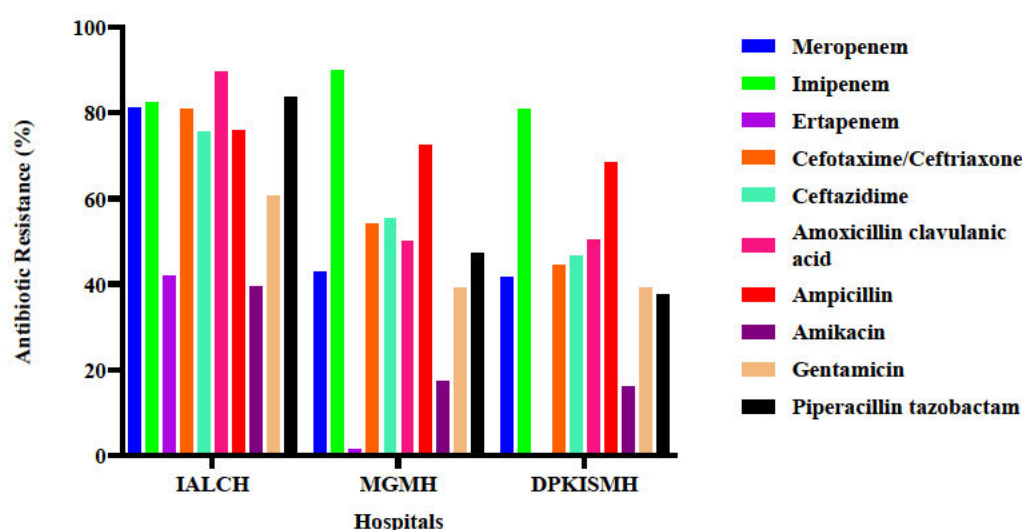


Figure 4: Antibiotic resistance among CREs per hospital (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

Resistance to cefotaxime/ceftriaxone was seen at IALCH 80.7% (3028/3752), 54.2% (447/825) at MGMH, and 44.4% (108/243) at DPKISMH and did not vary significantly at IALCH v/s MGMH ($p < 0.0001$), MGMH v/s DPKISMH ($p = 0.0076$), IALCH v/s DPKISMH ($p < 0.0001$). While resistance to ceftazidime was 75.5% (2833/3752) at IALCH, 55.2% (455/825) at MGMH, and 46.5% (113/243) at DPKISMH and did not differ significantly at IALCH v/s MGMH ($p < 0.0001$), MGMH v/s DPKISMH ($p = 0.0176$), IALCH v/s DPKISMH ($p < 0.0001$). Resistance to amoxicillin-clavulanic acid was highest at IALCH at 89.6% (3362/3752), followed by MGMH at 50.1% (413/825) and DPKISMH at 50.2% (122/243) and varied significantly at IALCH v/s MGMH ($p < 0.0001$) and IALCH v/s DPKISMH ($p < 0.0001$) but not at MGMH v/s DPKISMH ($p = 0.9683$). Ampicillin/amoxicillin resistance: IALCH 75.8% (2843/3752), compared to 72.4% (597/825) at MGMH and 68.3% (166/243) at DPKISMH, also showed significant variation at IALCH v/s MGMH ($p = 0.0402$), and IALCH v/s DPKISMH ($p = 0.0090$) but not at MGMH v/s DPKISMH ($p = 0.2192$). Differences were observed

for amikacin resistance: 39.4% (1480/3752) at IALCH, 17.3% (143/825) at MGMH, and 16.0% (39/243) at DPKISMH, showing significant differences at IALCH v/s MGMH ($p < 0.0001$) and IALCH v/s DPKISMH ($p < 0.0001$) but not at MGMH v/s DPKISMH ($p = 0.6399$). Similar variability was found for gentamicin: IALCH reached 60.7% (2277/3752), while MGMH reported 39.2% (323/825), and DPKISMH reported 39.1% (95/243), showing significant differences at IALCH v/s MGMH ($p < 0.0001$), IALCH v/s DPKISMH ($p < 0.0001$) but not at MGMH v/s DPKISMH ($p = 0.9873$). Piperacillin-tazobactam resistance showed variation: IALCH reported 83.6% (3138/3752), while MGMH reported 47.3% (390/825), and DPKISMH only 37.4% (91/243) (IALCH v/s MGMH $p < 0.0001$, MGMH v/s DPKISMH $p = 0.0068$, IALCH v/s DPKISMH $p < 0.0001$). Resistance patterns among CREs differed significantly between IALCH v/s MGMH and IALCH v/s DPKISMH, but not between MGMH v/s DPKISMH, for multiple antibiotics (Figure 4). IALCH consistently showed higher resistance levels, followed by MGMH and then DPKISMH.

3.3.2 Results of the Laboratory Methods

Carbapenemase genes

To understand the genetic mechanisms responsible for the phenotypic carbapenem resistance in the hospitals, multiplex PCR was used to further investigate key carbapenemase-encoding genes. The Vitek®2 automated system (bioMérieux, Marcy l'Étoile, France) identified: *K. pneumoniae*, *E. coli*, *E. cloacae*, *P. mirabilis*, *S. marcescens*, *M. morganii* and other *Enterobacterales* (Figure S2), including the antibiotic resistance (Figure S3) of all 720 stored isolates. Among these isolates from 2016 to 2024, the *bla_{OXA-48-like}* 42.5% (n=306/720) gene was the most prevalent, followed by *bla_{NDM}* 40.1% (n=289/720) and *bla_{KPC}* 11.5% (n=83/720). The *bla_{VIM}*, *bla_{IMP}* and *bla_{GES}* were detected at low frequencies (<6% per gene).

Gene distribution patterns varied across facilities: at IALCH, *bla_{NDM}* 43.6% (256/587) was predominant, at MGMH, *bla_{NDM}* and *bla_{OXA-48-like}* were equally detected at 39.6% (19/48) respectively. At DPKISMH, *bla_{OXA-48-like}* 51.8% (44/85) was most prevalent (Figure 5). Gene distribution revealed significant differences for *bla_{GES}* at IALCH v/s MGMH ($p < 0.0001$) and IALCH v/s DPKISMH ($p = 0.0008$) but not at MGMH v/s DPKISMH ($p = 0.4274$), *bla_{IMP}* at IALCH v/s MGMH ($p < 0.0334$), and IALCH v/s DPKISMH ($p = 0.0111$) but not at MGMH v/s DPKISMH ($p > 0.9999$) and *bla_{NDM}* at MGMH v/s DPKISMH ($p = 0.0030$) IALCH v/s DPKISMH ($p < 0.0001$) but not at IALCH v/s MGMH ($p = 0.5881$) suggesting an uneven spread. Conversely, there were no significant differences for *bla_{KPC}* (IALCH v/s MGMH $p = 0.4855$, MGMH v/s DPKISMH $p = 0.6401$, IALCH v/s DPKISMH $p = 0.8873$), *bla_{OXA-48-like}* (IALCH v/s MGMH $p = 0.8062$, MGMH v/s DPKISMH $p = 0.1766$, IALCH v/s DPKISMH $p = 0.0709$), *bla_{VIM}* (IALCH v/s MGMH $p = 0.3863$, MGMH v/s DPKISMH $p > 0.9999$, IALCH v/s DPKISMH $p = 0.1532$). However, isolates without detectable carbapenemase genes showed a significant difference at IALCH v/s DPKISMH ($p = 0.0156$) and MGMH v/s DPKISMH ($p = 0.0460$) but not at IALCH v/s MGMH ($p = 0.4377$).

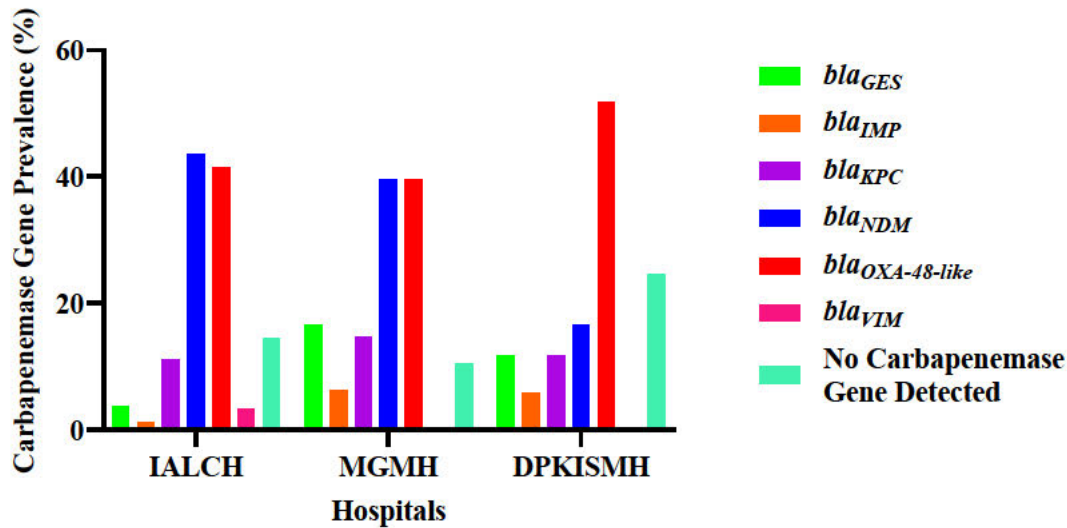


Figure 5: Carbapenemase gene distribution per hospital (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

Temporal trends of *bla*_{NDM} and *bla*_{OXA-48-like} at IALCH (2016-2023)

Temporal trends in carbapenemase gene distribution were examined to see if the leading carbapenemase type at IALCH changed during the study period. A change in carbapenemase gene patterns was observed (Figure 6) from 2016 to 2023. Isolates with *bla*_{NDM} showed a significant steady decrease from 70.0% in 2016 to 14.3% in 2023. In contrast, *bla*_{OXA-48-like} increased significantly during the same time, increasing from 0% in 2016 to 75.0% in 2023. The opposing trend indicates a shift in the main types of carbapenemases present in the facility over eight years. The sharp rise in OXA-48-like from 2020 onward corresponds with a decline in NDM.

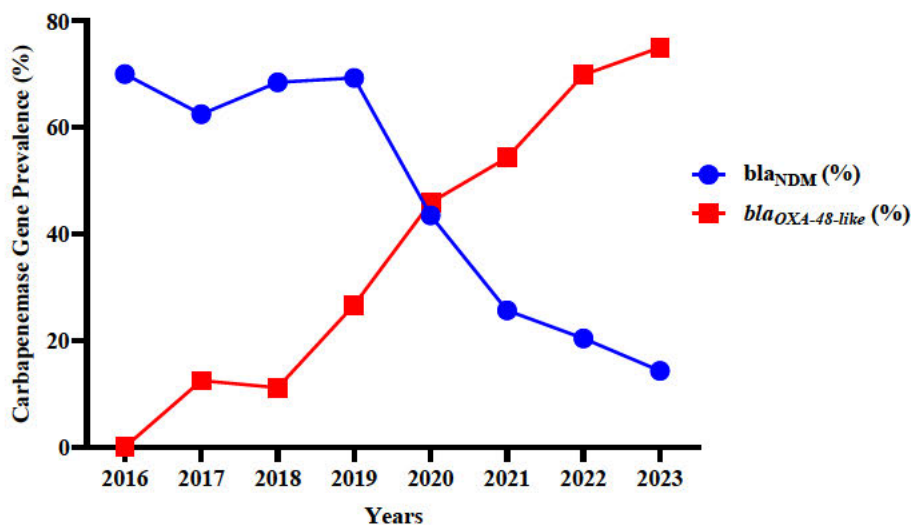


Figure 6: Temporal trend of *bla*_{NDM} and *bla*_{OXA-48-like} genes observed among isolates from IALCH (2016-2023) (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

Co-harboursing of carbapenemase genes

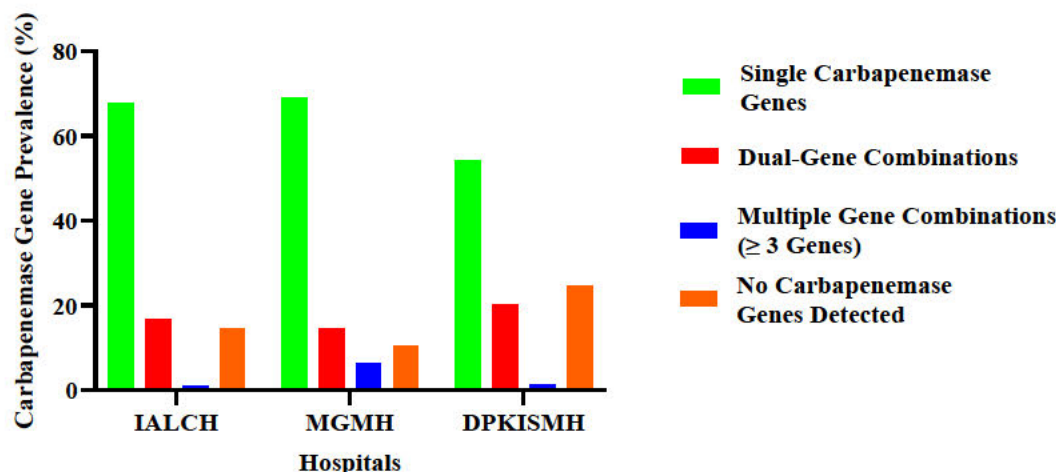


Figure 7: CRE Co-harboursing gene combination distribution across all hospitals (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

We assessed the co-harboursing of carbapenemase genes to determine if isolates had more than one resistant gene present (Figure 7, Table S6). Most isolates carried a single carbapenemase gene (66.3%), predominantly *bla*_{OXA-48-like} 34.2% (246/720) and *bla*_{NDM} 24.2% (174/720). Co-harboursing of two carbapenemase genes was observed in 16.9% of isolates, with the most frequent combinations being *bla*_{OXA-48-like} + *bla*_{NDM} (6.3%, 45/720) and *bla*_{NDM} + *bla*_{KPC} (7.5%, 54/720). Triple-gene carriage, such as *bla*_{IMP} + *bla*_{KPC} + *bla*_{OXA-48-like} (4/720), *bla*_{KPC} + *bla*_{NDM} + *bla*_{OXA-48-like} (3/720) and *bla*_{KPC} + *bla*_{OXA-48-like} + *bla*_{VTM} (1/720) was observed in 1.4% of isolates. 15.4% (111/720) of isolates tested negative for all screened carbapenemase genes.

There were no significant differences among hospitals for isolates carrying single carbapenemase genes at IALCH v/s MGMH ($p = 0.8925$) and MGMH v/s DPKISMH ($p = 0.0989$), but there was at IALCH v/s DPKISMH ($p = 0.0128$). No significant differences among hospitals were observed for isolates with dual-gene combinations (IALCH v/s MGMH $p = 0.7050$, MGMH v/s DPKISMH $p = 0.4353$, IALCH v/s DPKISMH $p = 0.4496$). Isolates with multiple (≥ 3) carbapenemase genes, however, showed a difference at IALCH v/s MGMH ($p = 0.0247$) but not at MGMH v/s DPKISMH ($p = 0.1336$) and IALCH v/s DPKISMH ($p > 0.9999$). Isolates with no detectable carbapenemase genes showed no significant variation at IALCH v/s MGMH ($p = 0.4377$) but did MGMH v/s DPKISMH ($p = 0.0460$) and IALCH v/s DPKISMH ($p = 0.0156$).

Hospital-specific co-harboursing of genes varied, with IALCH showing predominance of *bla*_{NDM} + *bla*_{KPC}, while MGMH had a heterogeneous profile of *bla*_{NDM} and *bla*_{OXA-48-like} with several low-frequency combinations. DPKISMH had a predominance of dual carriage of *bla*_{NDM} + *bla*_{OXA-48-like}. Notably, 15.4% of isolates tested negative for all screened carbapenemase genes, suggesting the presence of alternative resistance mechanisms.

3.4 Discussion

Carbapenem-resistant *Enterobacterales* (CRE) are a significant cause of infections in healthcare settings, limiting treatment options, resulting in high mortality rates (15). The CRE spread in hospitals is a public health issue, globally and in South Africa (15-18). We aimed to determine the prevalence, resistance profiles, and distribution of carbapenemase genes in carbapenem-resistant *Enterobacterales* in three hospitals in KZN. The CRE prevalence increased in all three hospitals. Our work offers the first description of CRE prevalence and molecular findings at DPKISMH. Additionally, we showed how rapidly a new hospital can develop CRE profiles similar to well-established hospitals (IALCH & MGMH).

The CRE prevalence increased in all three hospitals. Our results suggest that CRE was quickly introduced into DPKISMH, likely due to patient transfers or environmental sources, which may be consistent with possible spread between hospitals. Global reports have shown community-level CRE carriage in India (18) and China (17). African studies have shown a similar pattern in Nigeria (19), Kenya (20) and other countries in Africa (21). Studies in South Africa showed a rising CRE prevalence, particularly in hospitals with high antibiotic use and complex patient care (8, 22). The Group of Enteric, Respiratory, and Meningeal disease Surveillance in South Africa (GERMS-SA) confirms widespread CRE occurrence (15). Our findings suggest that CRE circulation in KZN likely involves community and hospital sources. The rise at DPKISMH appears to be more rapid compared to global studies (17-19), which may be due to referrals to high-burden hospitals, such as MGMH and IALCH. The GERMS-SA report supports that CRE transmission in KZN includes inter-hospital and community spread rather than isolated hospital outbreaks (15).

Klebsiella pneumoniae was the dominant CRE at IALCH, while *P. mirabilis* was the most common at MGMH and DPKISMH. IALCH also had a higher rate of blood culture isolates, while MGMH and DPKISMH recorded more wound and pus samples. Global findings confirm *K. pneumoniae* as the main CRE organism in hospital settings, especially in ICUs and high-care units, where invasive devices increase infection (17, 18). African studies in Nigeria (19) and Kenya (20) show a similar pattern. Hospital studies in South Africa also identify *K. pneumoniae* as the leading CRE, particularly in bloodstream infections among critically ill patients (8, 22). The CRE distribution of *K. pneumoniae* at the quaternary hospital observed in our study aligns with research regarding the role of this bacterium in serious hospital-related CRE infections (8, 17-19, 22). The higher prevalence of *P. mirabilis* in regional hospitals contrasts with the quaternary hospital, which may be due to a higher burden of soft-tissue, wound, and community-related infections at the regional hospitals. These variations highlight the impact of specimen type, patient demographics, and service profiles on CRE distribution, emphasising the need for hospital-specific surveillance.

We found significant differences between hospitals with respect to third-generation cephalosporins, β -lactam/ β -lactamase inhibitor combinations, aminopenicillins, aminoglycosides, and piperacillin-tazobactam resistance by CREs. The relatively low resistance to ertapenem may be due to the presence of isolates that are resistant to just one carbapenem, thus leading to the inclusion of isolates with specific resistance profiles. IALCH exhibited high resistance across all antibiotic classes, indicating stronger selection pressure and increasing CREs at a quaternary level. However, these results were obtained phenotypically (Vitek[®]2 automated system (bioMérieux, Marcy l'Étoile, France), during routine laboratory processes at the various hospitals, and the assumption is that these results were verified by the respective laboratories during that time.

African surveillance reports tertiary hospitals having higher rates of multidrug-resistant *Enterobacterales*, resulting from increased antibiotic use, complex cases, and longer hospital stays (15, 16, 22). Our results reflect national and global data (23-25). Referral hospitals bear the highest burden of antibiotic resistance, while regional and newly established hospitals often show intermediate or lower levels of resistance (4, 26). The comparison highlights how hospital type, antibiotic use, and the local prevalence of bacteria influence trends in resistance, emphasising the need for tailored strategies in antibiotic stewardship and infection prevention and control (IPC) (27, 28).

Molecular screening detected all six carbapenemase genes in all three hospitals. The primers used in our study were selected based on previously published sequences for the carbapenemase genes *bla_{KPC}*, *bla_{IMP}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{OXA-48-like}*, and *bla_{GES}*, and were designed to detect the most common variants of the target carbapenemase genes, but may not detect all genotypes. The most common genes were *bla_{OXA-48-like}* and *bla_{NDM}*. The prevalence varied by hospital, with IALCH showing NDM predominance, MGMH showing equal NDM and OXA-48-like prevalence and DPKISMH showing OXA-48-like predominance. South African studies have highlighted the dominance of OXA-48-like and NDM mechanisms, but proportions differ between provinces (8, 24, 29). Global and African studies show regional differences, with OXA-48-like being more common in some areas, while NDM is prevalent in others (21, 25, 30, 31).

The similarities between our results and those reported in other studies (8, 21, 24, 25, 29-31), suggesting various carbapenem resistance mechanisms are circulating in the region. The higher prevalence of OXA-48-like at DPKISMH might signal unrecognised transmission due to lower carbapenem minimum inhibitory concentration (MIC). In contrast, the higher NDM prevalence at IALCH reflects the severity of infections and the complexity of patients at a quaternary centre. The differences likely arise from variations in plasmid circulation, antibiotic exposure, and referral patterns.

Our study revealed that NDM and OXA-48-like enzymes were the main carbapenemases in these hospitals in KZN, each presenting unique clinical challenges. NDM enzymes provide high-level resistance to nearly all β -lactams and resist most β -lactamase inhibitors currently available (8, 24). The OXA-48-like enzymes often

have lower carbapenem MICs to evade phenotypic detection and spread silently (32). Studies reported similar issues, with NDM being associated with limited treatment options, whereas OXA-48-like enzymes are linked to significant diagnostic challenges (8, 24, 29). Our findings align with global observations emphasising that antibiotic choice must have an understanding of resistance mechanisms (23, 25, 33). The differences between hospitals may likely reflect variations in antibiotic prescribing, case severity, and the circulation of plasmid-carrying resistance genes (34-36). These differences highlight the need for rapid molecular testing and targeted antibiotic treatment.

Our study showed a significant change in the distribution of carbapenemase genes between 2016 and 2023 at IALCH; *bla*_{NDM} decreased while *bla*_{OXA-48-like} increased. The opposing trends indicate a shift in the main carbapenemase-producing lineages. These findings align with South African studies showing a rise in OXA-48-like carbapenemases among *Enterobacterales* isolates. OXA-48 and its variants are often found as one of the main types of carbapenemases in national and regional data (37, 38). The observed shift coincides with the COVID-19 pandemic period, during which changes in hospital practices, increased antibiotic use, prolonged hospital stays and patient management have been reported to have influenced antimicrobial resistance patterns and possibly facilitated the emergence or selection of specific carbapenemase-producing strains (39, 40). While these factors may have contributed to the observed trends, the impact of the COVID-19 pandemic was not assessed in this study.

Several hospital outbreaks and molecular studies in Africa have documented the emergence and spread of OXA-48, OXA-181, and OXA-232 lineages (21, 23, 32, 41). Outbreaks involving NDM and OXA-48 variants have also been reported, highlighting local differences and occasional replacement or co-circulation (42). The observed replacement-like dynamics, with *bla*_{OXA-48-like} increasing as *bla*_{NDM} declines, may reflect one or more interacting mechanisms (43). These could include the introduction and growth of OXA-48-carrying strains, the horizontal transfer of *bla*_{OXA-48-like} bearing plasmids that spread easily in the environment (32), or changing selective pressures (23), such as antibiotic prescribing habits, that benefit OXA-48 producers. OXA-48-like enzymes can be challenging to detect phenotypically and are often associated with mobile plasmids (44), which facilitate growth (32).

We found that 16.9% of isolates carried two or more carbapenemase genes. The most common combinations across the three hospitals included *bla*_{OXA-48-like} + *bla*_{NDM} and *bla*_{NDM} + *bla*_{KPC}. The detection of multiple carbapenemase genes within single isolates highlights the increasing genetic complexity of CRE populations in our setting, suggesting resistance is actively acquired rather than just through clonal expansion. Globally, recent studies show that *Enterobacterales* are increasingly reported to carry dual and multiple carbapenemase genes, particularly combinations involving *bla*_{NDM} and *bla*_{OXA-48-like} enzymes (25, 30). Large genomic analyses reveal frequent co-occurrence of *bla*_{NDM-1} with *bla*_{OXA-48-like} carbapenemases across various sequence types and ecological niches, with some studies reporting isolates producing both enzymes simultaneously (34, 45).

African studies indicate a rising detection of carbapenemase co-harboured, with systematic reviews noting the presence of NDM + OXA-48-like and NDM + KPC combinations in multiple regions, reflecting widespread plasmid-mediated gene exchange(21, 31). In South African studies and national surveillance reports have found an increasing number of *Enterobacterales* isolates carrying multiple carbapenemase genes, often involving *bla*_{NDM} in combination with *bla*_{OXA-48-like} enzymes, in high-burden referral hospitals (15, 24). Our findings align with these recent reports, reinforcing the idea that carrying multiple carbapenemase genes is becoming a significant aspect of current CRE epidemiology. The proportion of dual-gene isolates observed in our study likely reflects ongoing horizontal gene transfer and the movement of mobile plasmids carrying multiple resistance genes. These processes are supported by antibiotic selection pressure and high patient turnover in referral hospital settings, which complicate IPC and limit treatment options (25, 34). The similarity between our data and recent genomic studies highlights the need for effective molecular diagnostics in surveillance and routine hospital practice.

Our study showed that 15.4% of the isolates were carbapenemase-negative CRE, showing that a share of resistance cannot be explained by known carbapenemase mechanisms used in this study. Similar findings have emerged globally, with multiple studies indicating that a notable portion of CRE exhibits carbapenem resistance through other mechanisms (25, 45). Data from Africa also show non-carbapenemase-producing CRE, with systematic reviews highlighting lesser-known resistance mechanisms across the continent (21, 31). South African studies have documented carbapenem-resistant isolates without identifiable carbapenemase genes, supporting the role of non-enzymatic resistance pathways (15, 24). Together, our findings align with recent global and regional literature and highlight the need for thorough molecular studies to fully understand carbapenem resistance beyond just detecting carbapenemases.

3.5 Strengths and Limitations

Our study has several strengths. It is one of the few multi-hospital analyses of carbapenem-resistant *Enterobacterales* in KwaZulu-Natal. It includes a newly established regional hospital, which allows for comparison across different levels of care. The number of isolates and the extended time frame enabled us to assess trends. We combined phenotypic susceptibility testing with molecular detection of key carbapenemase genes, which provided insight into resistance patterns and mechanisms. Identifying co-harboured carbapenemase genes and the shift from NDM to OXA-48-like at IALCH improves surveillance. However, certain limitations must be recognised. We relied on available records, which may have missing data and selection bias. We screened only selected carbapenemase genes; therefore, additional resistance mechanisms such as porin loss, efflux, and untested carbapenemase genes were not screened. We also lacked data on prescribing and antibiotic consumption. Our data was not stratified by sample type or group. We did not screen samples for community carriage and introduction. Our results were not normalised to admission numbers, which would have influenced detection rates. The role of Covid-19 on changing enzyme prevalence in 2019-2021 was not studied. Due to the fact that isolates from IALCH and MGMH were collected over a

longer period of time as compared to isolates collected from DPKISMH, which only opened in 2022, a full comparison was not possible, although future surveillance over a longer period of time would help strengthen the comparison

3.6 Conclusion

Our study explored the molecular characterisation of CREs in three hospitals in KZN, including the first description from a new regional hospital. The prevalence of CRE increased significantly at all sites, showing the rapid development of resistance, even in a new institution. *Klebsiella pneumoniae* was the main CRE, although the patterns varied by hospital. Molecular screening identified *bla_{NDM}* and *bla_{OXA-48-like}* genes as the main carbapenemases, with a shift from NDM to OXA-48-like over eight years at IALCH. Our findings highlight the need for regular molecular diagnostics, active antibiotic treatment, and specific IPC measures to prevent the spread of CREs in KZN hospitals.

3.7 Future Work

Further work should be done to determine additional resistance mechanisms causing carbapenemase-negative CRE. Community circulation and subclinical colonisation of CREs requires further investigation. Clinical data such as patient outcomes, prior antibiotic exposure, and comorbidities need to be further clarified. The clonal spread of CREs between hospitals needs to be assessed. Comparison of isolates from various clinical specimen types to better understand how resistance genes are distributed across infection and colonisation sites, may offer insight into potential reservoirs of infection, different transmission routes, and the role of community carriage in introducing resistant organisms into hospitals. which would be an important area of focus for future research.

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3.9 Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Chapter 3 has described the rising prevalence of carbapenem-resistant Enterobacterales and the molecular diversity of carbapenem-resistant *Enterobacterales* isolates in three KwaZulu-Natal hospitals. It shows widespread carriage of carbapenemase genes alongside carbapenemase-negative resistant isolates. These findings suggest that carbapenem resistance cannot be attributed solely to carbapenemase production. Chapter 4 extends these results by investigating the presence of other β -lactam resistance mechanisms, specifically extended-spectrum β -lactamase and ampC β -lactamase genes, to improve understanding of the molecular complexity underlying the resistance patterns observed in Chapter 3.

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3.12 Chapter 3: Supplementary Tables

Table S1: Primers used for the detection of carbapenemase genes

Target gene	Primer	Direction	Sequence (5'–3')	Amplicon size (bp)	Reference
<i>bla_{KPC}</i>	mc-kpc-f	Forward	CGGAACCATTCGCTAAACTCG	322	(11)
	mc-kpc-r	Reverse	AACAAATTGGCGGCGGCGT		
<i>bla_{IMP}</i>	mc-imp-f1	Forward	TCTCRATCTATCCCCACGTATGC	269	(11)
	mc-imp-r1	Reverse	GCGGACTTTGGCCAAGCTTCTA		
<i>bla_{NDM}</i>	mc-ndm-f	Forward	CGGTTTGGCGATCTGGTTTT	207	(11)
	mc-ndm-r	Reverse	GACCGGCAGGTTGATCTCC		
<i>bla_{VIM}</i>	mc-vim-f	Forward	GTTTGGTCGCATATCGCAAC	155	(11)
	mc-vim-r	Reverse	CCAATTGCTTYTCAATCTCCG		
<i>bla_{OXA-48-like}</i>	mc-oxa48-f	Forward	GCTCTGGAATGAGAATAAGCAGCA	125	(11)
	mc-oxa48-r	Reverse	TAACCACGCCCAAATCGAG		
<i>bla_{GES}</i>	mc-ges-f	Forward	CTGTGGCTAAAGTCCTCTATGGCG	94	(11)
	mc-ges-r	Reverse	GTCGCGTCTCCCGTTTGGTT		

β -lactamase (*bla*); *Klebsiella pneumoniae* carbapenemase (*KPC*); New Delhi metallo- β -lactamase (*NDM*); Verona integron-encoded metallo- β -lactamase (*VIM*); Imipenemase (*IMP*); Oxacillinase-48-like (*OXA-48-like*), Guiana-Extended-Spectrum (*GES*), forward (f), reverse (r).

Table S2: Multiplex PCR reaction mixture for carbapenemase genes (11)

Master mix for *bla_{KPC}*, *bla_{IMP}*, *bla_{NDM}*, *bla_{VIM}*, *bla_{OXA-48-like}*, *bla_{GES}*

Component	Volume per reaction (μ L)
DreamTaq Green PCR Master Mix (2 $\times$)	10.0
Forward primer (10 μ M) for each target gene	0.2×6
Reverse primer (10 μ M) for each target gene	0.2×6
Nuclease-free water	3.2
Template DNA	2.0
Total	20.0

β -lactamase (*bla*); *Klebsiella pneumoniae* carbapenemase (*KPC*); New Delhi metallo- β -lactamase (*NDM*); Verona integron-encoded metallo- β -lactamase (*VIM*); Imipenemase (*IMP*); Oxacillinase-48-like (*OXA-48-like*) and Guiana-Extended-Spectrum (*GES*)

Table S3: Bacterial distribution of CREs at IALCH (2016 – 2024)

Source	Number (n)	CRE Organisms						
		<i>Klebsiella pneumoniae</i> (n)	<i>Escherichia coli</i> (n)	<i>Enterobacter cloacae</i> (n)	<i>Proteus mirabilis</i> (n)	<i>Serratia marcescens</i> (n)	<i>Morganella morganii</i> (n)	Other <i>Enterobacterales</i> (n)
Blood Culture	1115	986	13	44	14	26	15	17
Catheter tip	217	170	1	4	23	7	2	10
Cerebrospinal Fluid (CSF)	72	55	0	8	2	3	2	2
Fine needle aspirates (FNA)	1	1	0	0	0	0	0	0
Rectal Swab & Stool	25	23	0	1	0	0	0	1
Pus	289	160	6	17	58	3	25	20
Sputum	774	643	9	25	36	27	21	13
Scrapings	1	0	0	0	1	0	0	0
Sterile sites	326	213	5	26	47	4	17	14
Tissue	75	28	0	8	19	2	7	11
Urine	857	655	17	39	68	15	39	24
Total	3752	2934	51	172	268	87	128	112

Table S4: Bacterial distribution of CREs at MGMH (2016 – 2024)

Source	Number (n)	CRE Organisms						
		<i>Klebsiella</i> <i>pneumoniae</i> (n)	<i>Escherichia</i> <i>coli</i> (n)	<i>Enterobacter</i> <i>cloacae</i> (n)	<i>Proteus</i> <i>mirabilis</i> (n)	<i>Serratia</i> <i>marcescens</i> (n)	<i>Morganella</i> <i>morganii</i> (n)	Other <i>Enterobacterales</i> (n)
Blood Culture	78	60	1	1	5	7	1	3
Catheter tip	2	1	0	0	1	0	0	0
Cerebrospinal Fluid (CSF)	1	0	0	0	1	0	0	0
Rectal Swab & Stool	44	1	4	3	13	0	21	2
Pus	384	33	8	13	265	2	30	33
Sputum	35	21	0	4	8	1	0	1
Sterile sites	3	0	0	1	1	0	0	1
Tissue	5	3	0	0	2	0	0	0
Urine	273	137	11	20	73	2	12	18
Total	825	256	24	42	369	12	64	58

Table S5: Bacterial distribution of CREs at DPKISMH (2016 – 2024)

Source	Number (n)	CRE Organisms						
		<i>Klebsiella pneumoniae</i> (n)	<i>Escherichia coli</i> (n)	<i>Enterobacter cloacae</i> (n)	<i>Proteus mirabilis</i> (n)	<i>Serratia marcescens</i> (n)	<i>Morganella morganii</i> (n)	Other <i>Enterobacterales</i> (n)
Blood Culture	9	5	1	1	1	0	0	1
Catheter tip	6	5	0	0	0	1	0	0
Rectal Swab & Stool	1	0	0	0	0	0	1	0
Pus	107	13	9	2	43	8	5	27
Sputum	23	10	1	1	6	0	1	4
Sterile sites	18	4	1	0	10	2	1	0
Tissue	28	2	2	1	8	1	3	11
Urine	51	16	1	11	10	3	5	5
Total	243	55	15	16	78	15	16	48

Table S6: CRE Co-harboursing gene combination across all Hospitals

Combination Type	IALCH (n, %)	MGMH (n, %)	DPKISMH (n, %)	Total (n, %)
<i>bla_{KPC}</i>	6 (1)	2 (4.2)	4 (4.7)	12 (1.7)
<i>bla_{IMP}</i>	3 (0.5)	0 (0)	0 (0)	3 (0.4)
<i>bla_{NDM}</i>	158 (26.2)	12 (25)	4 (4.7)	174 (24.2)
<i>bla_{VIM}</i>	18 (3.1)	0 (0)	0 (0)	18 (2.5)
<i>bla_{OXA-48-like}</i>	195 (33.2)	18 (37.5)	33 (38.8)	246 (34.2)
<i>bla_{GES}</i>	18 (3.1)	1 (2.1)	5 (5.9)	24 (3.3)
<i>bla_{NDM} & bla_{GES}</i>	3 (0.5)	4 (8.3)	5 (5.9)	12 (1.7)
<i>bla_{IMP}, bla_{KPC} & bla_{OXA-48-like}</i>	2 (0.3)	1 (2.1)	1 (1.2)	4 (0.6)
<i>bla_{IMP} & bla_{OXA-48-like}</i>	2 (0.3)	0 (0)	1 (1.2)	3 (0.4)
<i>bla_{NDM} & bla_{KPC}</i>	53 (9.0)	1 (2.1)	0 (0)	54 (7.5)
<i>bla_{KPC}, bla_{OXA-48-like} & bla_{VIM}</i>	1 (0.2)	0 (0)	0 (0)	1 (0.1)
<i>bla_{KPC} & bla_{OXA-48-like}</i>	1 (0.2)	0 (0)	2 (2.4)	3 (0.4)
<i>bla_{KPC}, bla_{NDM} & bla_{OXA-48-like}</i>	3 (0.5)	0 (0)	0 (0)	3 (0.4)
<i>bla_{NDM} & bla_{OXA-48-like}</i>	39 (6.6)	0 (0)	6 (7.1)	45 (6.3)
<i>bla_{KPC}, bla_{NDM} & bla_{GES}</i>	0 (0)	2 (4.2)	0 (0)	2 (0.3)
<i>bla_{GES} & bla_{IMP}</i>	0 (0)	1 (2.1)	0 (0)	1 (0.1)
<i>bla_{IMP} & bla_{KPC}</i>	0 (0)	1 (2.1)	3 (3.5)	4 (0.6)
No Carbapenemase Genes Detected	85 (14.5)	5 (10.4)	21 (24.7)	111 (15.4)
Total	587 (100)	48 (100)	85 (100)	720 (100)

β -lactamase (*bla*), *Klebsiella pneumoniae* carbapenemase (*KPC*), New Delhi metallo- β -lactamase (*NDM*), Verona integron-encoded metallo- β -lactamase (*VIM*), Imipenemase (*IMP*), Oxacillinase-48-like (*OXA-48-like*), Guiana-Extended-Spectrum (*GES*), Inkosi Albert Luthuli Central Hospital (IALCH), Mahatma Gandhi Memorial Hospital (MGMH), and Dr Pixley Ka Isaka Seme Memorial Hospital (DPKISMH).

3.13 Chapter 3: Supplementary Figures

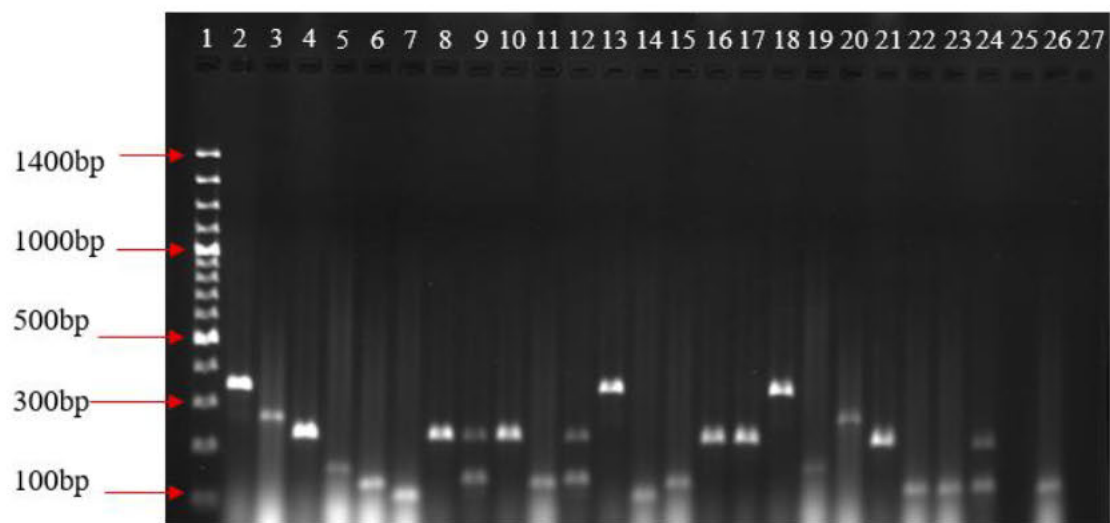


Figure S1: Gel electrophoresis showing PCR amplification of carbapenemase genes
Lane 1: 100bp DNA ladder or marker; Lane 2-7: positive control strains for *bla*_{KPC} (322 bp), *bla*_{IMP} (269 bp), *bla*_{NDM} (207 bp), *bla*_{VIM} (155 bp), *bla*_{OXA-48-like} (125 bp), *bla*_{GES} (94 bp); Lane 8-26: clinical isolates showing target amplicons; Lane 27: Negative control strain. Lane 8,10,16,17 and 21: *bla*_{NDM} positive isolates; Lanes 11, 15, 22,23,26: *bla*_{OXA-48-like} positive isolates; Lane 13 & 18: *bla*_{KPC} positive isolates; Lane 20: *bla*_{IMP} positive isolate; Lane 19: *bla*_{VIM} positive isolate; Lane 14: *bla*_{GES} positive isolate; Lane 9,12 and 24: *bla*_{NDM} and *bla*_{OXA-48-like} positive isolate; Lanes 25: no carbapenemase gene detected.

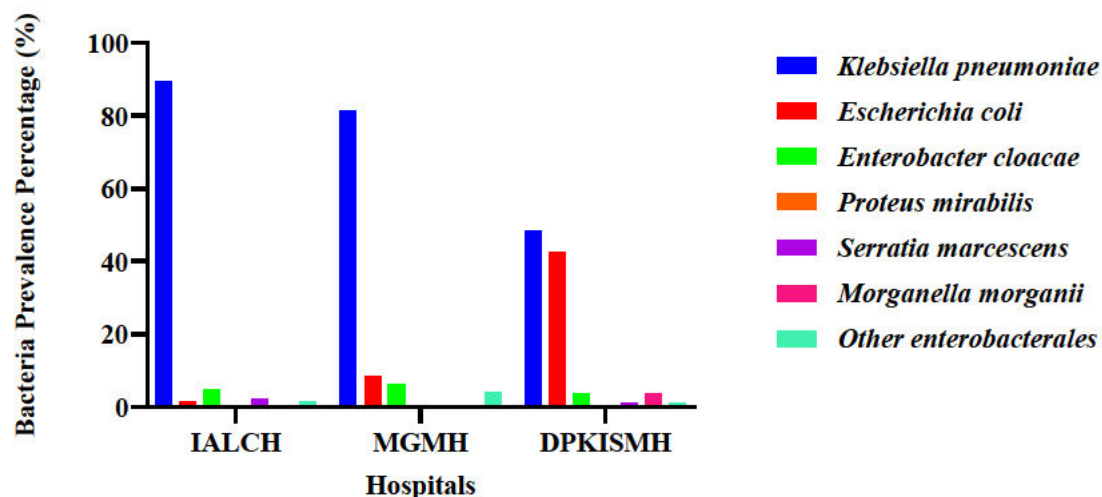


Figure S2: Prevalence of carbapenem-resistant bacterium from the 720 isolates per hospital (2016-2024)
(created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

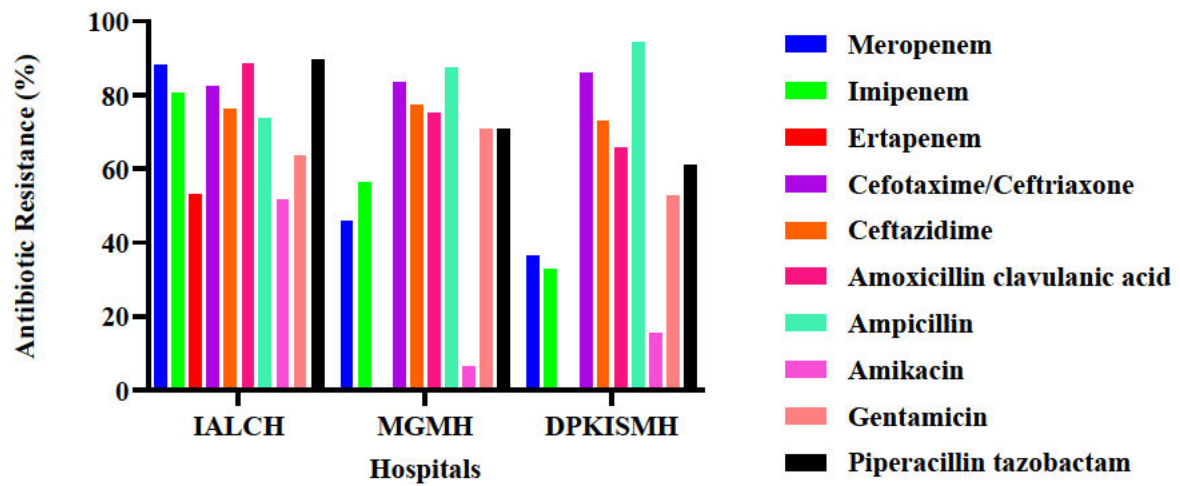


Figure S3: Antibiotic resistance among the 720 carbapenem-resistant isolates per hospital (2016-2024) (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

4. Chapter 4

Chapter 3 highlighted the increasing burden of carbapenem-resistant *Enterobacterales* in KwaZulu-Natal, including the molecular complexity of carbapenem-resistant *Enterobacterales* isolates present with carbapenemase genes. It also highlighted isolates that lack detectable carbapenemase genes, suggesting that additional resistance mechanisms contribute to the observed phenotypic carbapenem resistance. Chapter 4 looks at extended-spectrum β -lactamase and ampC β -lactamase gene presence among carbapenem-resistant *Enterobacterales* isolates, providing more insight into the accumulation and interaction of β -lactam resistance mechanisms in these organisms.

Presence of Extended-Spectrum β -lactamase & Ampicillin β -lactamase in a New Regional Hospital in KwaZulu-Natal, South Africa

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Abstract

Introduction

β -lactam resistance in *Enterobacterales* often arises from the coexistence and accumulation of multiple resistance mechanisms. These include extended-spectrum β -lactamases (ESBLs), plasmid-mediated AmpC β -lactamases, and carbapenemases. This combination of mechanisms makes it harder to interpret antimicrobial susceptibility testing, detect antimicrobial resistance in laboratories, and manage patients, especially in healthcare settings with high infection rates.

Methods

We analysed 720 non-duplicate carbapenem-resistant *Enterobacterales* isolates collected from three public hospitals in KwaZulu-Natal, South Africa, from 2016 to 2024. We used multiplex and singleplex polymerase chain reaction (PCR) assays to detect the genes *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and plasmid-mediated *bla*_{ampC}. We

confirmed representative amplicons through Sanger sequencing. We assessed patterns of gene co-carriage descriptively and compared them across hospital levels.

Results

We detected ESBL and/or ampC genes in 81.5% of the isolates. The most common gene was *bla_{TEM}*, followed by *bla_{CTX-M}*, *bla_{SHV}*, and plasmid-mediated *bla_{ampC}*. Most isolates, 96.4%, carried multiple β -lactamase genes, often alongside carbapenemase genes. Only 3.6% of isolates lacked any of the screened carbapenemases, ESBLs, or ampCs. We observed distinct patterns of gene co-occurrence across hospitals, indicating different levels of resistance complexity within the healthcare network.

Discussion

The high rate of multi-gene carriage shows the significant molecular complexity behind β -lactam resistance in *Enterobacterales*. The coexistence of ESBL, ampC, and carbapenemase genes leads to broad resistance to non-carbapenem β -lactams. This complexity creates challenges for routine laboratory detection and treatment decisions. Early signs of intricate resistance patterns in a newly established regional hospital suggest rapid spread within interconnected referral systems rather than isolated occurrences.

Conclusion

β -lactam resistance in *Enterobacterales* in KwaZulu-Natal results from the accumulation of multiple resistance genes. Detailed molecular diagnostics and ongoing genomic monitoring are crucial for understanding resistance profiles and supporting coordinated antimicrobial use and infection prevention efforts at all levels of care.

4.1 Introduction

Carbapenem-resistant *Enterobacterales* (CRE) have become common in healthcare facilities in KwaZulu-Natal, South Africa. Studies show increasing rates and widespread detection of *bla*_{NDM} and *bla*_{OXA-48-like} carbapenemases (1, 2). The presence of carbapenemase defines the carbapenem resistance phenotype but it does not describe the multidrug resistance seen in CRE isolates, such as resistance to non-carbapenem β -lactams. More evidence suggests that additional β -lactam resistance mechanisms, such as extended-spectrum β -lactamases (ESBLs) and plasmid-mediated Ampicillin β -lactamases (AmpCs), often exist alongside carbapenemases, which significantly shape resistance profiles and clinical results (3, 4).

The ESBLs, particularly those linked to β -lactamase (*bla*) Temoniera Beta-Lactamase (*TEM*), *bla* Sulphydryl Variable Beta-Lactamase (*SHV*), and *bla* Cefotaximase Beta-Lactamase (*CTX-M*) genes, have a global distribution. These enzymes provide resistance to third-generation cephalosporins and β -lactam/ β -lactamase inhibitor combinations. The spread of these genes occurs through mobile genetic elements that enable horizontal transfer between *Enterobacterales* species, leading to multiple resistance determinants (5). The global rise of ESBL-producing *Enterobacterales* strongly correlates with increased carbapenem use, which directly contributes to the rise and selection of CRE (1, 4).

The AmpCs linked to the *bla* Ampicillin β -lactamase (*ampC*) gene complicate resistance by causing resistance to cephamycins and extended-spectrum cephalosporins while not being inhibited by clavulanate. These enzymes are increasingly found in *Enterobacterales* isolates and may be overlooked due to limitations in routine detection methods (6). AmpCs often coexist with ESBLs and carbapenemases, adding to broader β -lactam resistance by reducing the effectiveness of β -lactam/ β -lactamase inhibitor combinations (7).

Recent molecular studies have shown that CRE isolates often carry ESBL and/or ampC genes in addition to carbapenemases (7, 8), resulting in complex resistance patterns linked to resistance against aminoglycosides, fluoroquinolones, and β -lactam/ β -lactamase inhibitor combinations (8). The coexistence of these resistance genes is often facilitated by plasmids with multiple β -lactamase genes, enhancing persistence and spread in hospitals with high CRE burdens. The genetic complexity may obscure phenotypic carbapenem resistance, making it more challenging to interpret diagnostics and manage infection prevention and control (IPC) (7).

Molecular surveillance in South Africa has mainly focused on finding carbapenemase genes and monitoring CRE rates (9, 10). There has been less focus on the ESBL and AmpC resistance in CRE isolates (2, 5). National and hospital studies consistently show high rates of ESBL among *Enterobacterales* (2, 5, 11). Gene sequencing shows that carbapenem-resistance is not always caused by carbapenemase genes alone (12, 13). CRE isolates without detectable carbapenemases may carry ESBL or ampC genes with other resistance mechanisms, such as porin loss or efflux pump upregulation, leading to higher carbapenem minimum inhibitory concentrations (MICs) (7, 8). Identifying ESBL and ampC genes within CRE isolates provides insights into resistance

pathways, which may explain the discrepancies between phenotypic susceptibility tests and molecular findings.

We therefore aimed to explore resistance within carbapenem-resistant *Enterobacterales* isolates to identify ESBL and ampC genes that contribute to carbapenem-resistance that carbapenemase alone cannot fully explain.

4.2 Methods

4.2.1 Laboratory Methods

Bacterial Isolates, Study Setting, Bacterial Culture and Identification

We included a total of 720 non-duplicate isolates collected between 2016 and 2024. These isolates came from Inkosi Albert Luthuli Central Hospital (IALCH, quaternary, n = 587), Mahatma Gandhi Memorial Hospital (MGMH, regional, n = 48), and Dr Pixley Ka Isaka Seme Memorial Hospital (DPKISMH, regional, n = 85). All isolates were revived from cryobeads and cultured on 5% sheep blood agar to ensure purity and viability before molecular analysis. We incubated plates aerobically at 37 °C for 48 hours. Species identification and antimicrobial susceptibility profiles were generated using the Vitek®2 automated system (bioMérieux, Marcy-l'Étoile, France) to provide context for molecular findings. We interpreted antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) M100 guidelines (34th edition, 2024).

DNA Extraction

Deoxyribonucleic Acid (DNA) was extracted from cultures using the QuickExtract rapid protocol following the manufacturer's instructions (Lucigen®, Wisconsin, USA). A 100µl bacterial growth was suspended in 100 µL of extraction buffer and subjected to sequential heating steps (65°C for 5 minutes and then 98°C for 5 minutes) to break open the bacterial cells. Cellular debris was removed by centrifugation at 12,000rpm for 10 minutes, and the supernatant containing genomic DNA was stored at –20 °C until further use. DNA concentration and purity were assessed using a NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. ND-8000-GL) to ensure they were suitable for the downstream polymerase chain reaction (PCR) assays.

Extended-Spectrum β-Lactamase (ESBL) and ampC β-Lactamase (ampC) Polymerase Chain Reaction (PCR)

A Multiplex PCR assay was done to detect genes that code for ESBL, while a Singleplex PCR assay was done to detect plasmid-mediated ampC genes. The targeted ESBL genes included (*bla_{TEM}*, *bla_{SHV}* and *bla_{CTX-M}*). The targeted ampC gene was *bla_{ampC}* (14, 15). Primer sets and amplification conditions from previously validated protocols were used and optimised (Tables S1 and S2). The PCR assays were conducted using the Bio-Rad T100 Thermal Cycler (LASEC, Cape Town, South Africa, catalogue no. 1861096). Each Master mix was made up of 10.0µl of DreamTaq HS Green Master Mix (2×) (Thermo Fisher Scientific, Massachusetts, USA,

catalogue no. K9022), 0.2µl of Forward primer (10 µM) for each target gene, 0.2µl of Reverse primer (10 µM) for each target gene, 3.2µl of Nuclease-free water and 2µl Template DNA (20ng/µl)(16). PCR cycling conditions included an initial activation at 94°C for 3 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 60 seconds. A final extension step was done at 72°C for 3 minutes, after which specimens were held at 4°C (16).

Gel Electrophoresis

All PCR amplicons were analysed on 1.5% agarose gels (TopVision Agarose Tablets, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. R2802) prepared in 1× Tris-Borate Ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (TBE (10X), RNase-free, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. AM9865). Gels were run at 80V for 2 hours. A 100-base pair (bp) DNA ladder (GeneRuler Ready-to-Use DNA Ladders, Thermo Fisher Scientific, Massachusetts, USA, catalogue no. SM0323) served as the molecular size marker. DNA bands were visualised using DNA Gel Loading Dye (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. R0611) mixed with GelRed (GelRed® Nucleic Acid Stain 10000X Water, Sigma Aldrich, Missouri, USA, catalogue no. SCT123) under UV light using a BIO RAD GelDoc Go imaging system (LASEC, Cape Town, South Africa, catalogue no. 12009077). Amplicon sizes were interpreted based on expected fragment lengths listed in Supplementary Table S1 (14, 15). We described patterns of single, dual, and multiple resistance gene combinations to assess the molecular complexity of resistance in isolates.

Sanger Sequencing

Since no validated controls were available for the ESBL & ampC gene targets, representative positive amplicons for *bla_{SHV}*, *bla_{CTX-M}*, *bla_{TEM}*, and *bla_{ampC}* (Figures S1) were sent for Sanger sequencing to Inqaba Biotechnical Industries (Pretoria, South Africa) to confirm gene identity. They purified the amplicons and did sequencing using the Sanger method on an ABI 3500xl Genetic Analyser (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. 4405633), with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Massachusetts, USA, catalogue no. 4337455). A chromatogram for the forward and reverse primers was generated (ab1). Sequencing files were edited using BioEdit, which were then assessed for quality and trimmed (17, 18).

We analysed the resulting products against GenBank reference sequences for confirmation. Sequence analysis using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) revealed ≥98% nucleotide identity with known ESBL and ampC gene sequences available on GenBank (*bla_{SHV}* – LN558653.1, *bla_{CTX-M}* – FJ997865.1, *bla_{TEM}* – NG_050171.1 and *bla_{ampC}* – KF526117.1) (Figures S2 – S5). These sequences were used as controls for screening the remaining 720 specimens. All PCR procedures were conducted using a unidirectional workflow, with physically separate areas for pre- and post-amplification, and sterile aerosol-resistant tips were used throughout to minimise contamination.

4.2.2 Statistical Analysis

The co-occurrence of carbapenemase and ESBL & ampC genes was summarised using descriptive frequencies and percentages. The Chi-square (cell count ≥ 5) or Fisher's exact tests (cell count ≤ 5) were used to assess differences in the distribution of carbapenemase genes, ESBL and ampC genes, and the frequency of single, dual, and multiple gene combinations across the three hospitals. Statistical analyses were conducted using GraphPad Prism (version 10.6.1 (892) for Windows; GraphPad Software, Massachusetts, USA, www.graphpad.com), with p-values less than 0.05 considered statistically significant. Comparative analyses were conducted using the data between 2016 to 2024 from each hospital. Since DPKISMH is a newly opened hospital, only data from 2022 onward was available. However, the main goal of the study was to assess overall trends and distribution across the entire study period."

4.2.3 Ethical Approval

We obtained ethical approval for this study from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC/00007433/2024).

4.3 Results

4.3.1 Extended-Spectrum β -Lactamase (ESBL) and ampC β -Lactamase (ampC) Gene Distribution among *Enterobacteriales* Isolates per Hospital

Among the 720 isolates, 81.5% (587/720) tested positive for ESBL/ampC genes, *bla_{TEM}* was most prevalent at 74.9% (539/720), followed by *bla_{CTX-M}* at 47.2% (340/720), *bla_{ampC}* at 38.8% (279/720), and *bla_{SHV}* at 34.7% (250/720). Gene distribution patterns for ESBL and ampC varied in hospitals (Figure 1). The most prevalent gene was *bla_{TEM}*: IALCH 74.6% (438/587), MGMH 85.4% (41/48) and DPKISMH 70.6% (60/85).

A significant difference was observed between hospitals for the *bla_{CTX-M}* at IALCH v/s MGMH ($p = 0.0122$) and IALCH v/s DPKISMH ($p = 0.0013$), while MGMH v/s DPKISMH ($p = 0.9866$) was not significant. However, *bla_{SHV}* (IALCH v/s MGMH $p = 0.1529$, MGMH v/s DPKISMH $p = 0.4899$, IALCH v/s DPKISMH $p = 0.4575$), *bla_{TEM}* (IALCH v/s MGMH $p = 0.0947$, MGMH v/s DPKISMH $p = 0.0547$, IALCH v/s DPKISMH $p = 0.4281$), and *bla_{ampC}* (IALCH v/s MGMH $p = 0.1567$, MGMH v/s DPKISMH $p = 0.2632$, IALCH v/s DPKISMH $p = 0.9018$) did not show significant variation across hospitals. However, isolates without detectable ESBL/ampC genes showed a significant difference at IALCH v/s MGMH ($p = 0.0003$) and IALCH v/s DPKISMH ($p < 0.0001$), compared to MGMH v/s DPKISMH ($p = 0.6534$).

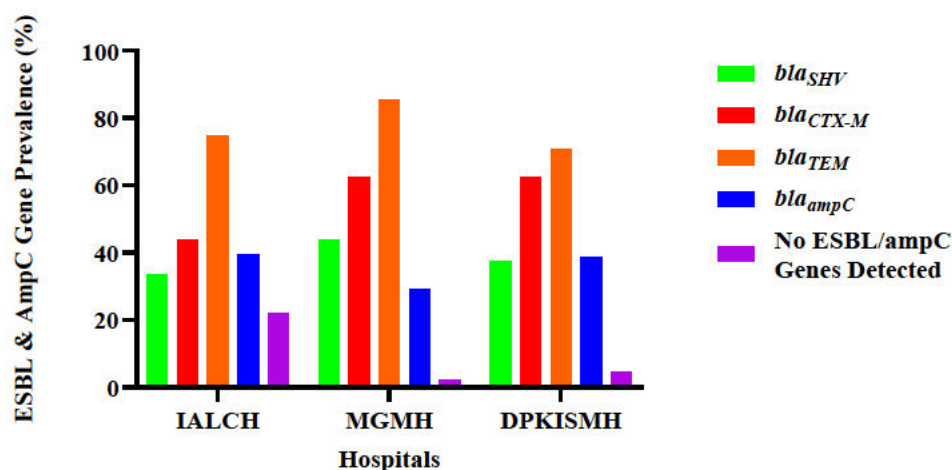


Figure 1: ESBL & ampC gene distribution per hospital (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

4.3.2 Co-harboured of ESBL & ampC genes

The most frequently detected ESBL genes were *bla_{TEM}* (23.9%, 172/720), *bla_{SHV}* (0.6%, 4/720), and *bla_{CTX-M}* (0.6%, 4/720), while the ampC gene *bla_{ampC}* (1.5%, 11/720) was detected at lower frequencies (Figure 2, Table S3).

Isolates with a single ESBL gene or a single ampC gene accounted for 26.5% (191/720) of isolates. Dual-gene combinations were detected in 17.1% (123/720) of isolates, with *bla_{CTX-M}* + *bla_{TEM}* 7.6% (55/720), and *bla_{TEM}* + *bla_{ampC}* 5.3% (38/720) being the most common. More than a third of isolates, 37.9% (273/720), carried three or more ESBL & ampC genes, frequently involving combinations of *bla_{TEM}*, *bla_{SHV}*, *bla_{CTX-M}*, and *bla_{ampC}*. 18.5% (133/720) of isolates tested negative for screened ESBL & ampC genes.

There were no significant differences between hospitals for isolates with single ESBL/ ampC genes (IALCH v/s MGMH $p = 0.7540$, MGMH v/s DPKISMH $p = 0.8489$, IALCH v/s DPKISMH $p = 0.4880$) or multiple gene combinations (≥ 3) (IALCH v/s MGMH $p = 0.2724$, MGMH v/s DPKISMH $p = 0.1820$, IALCH v/s DPKISMH $p = 0.5096$). However, dual-gene combinations showed significant differences at IALCH v/s MGMH ($p = 0.0087$) and IALCH v/s DPKISMH ($p < 0.0001$) but not at MGMH v/s DPKISMH ($p = 0.2162$). Isolates without detectable ESBL/ampC genes also had a significant difference at IALCH v/s MGMH ($p = 0.0011$) and IALCH v/s DPKISMH ($p = 0.0002$) but not at MGMH v/s DPKISMH ($p = 0.4451$).

Hospital-specific variations were observed. IALCH displayed the highest frequency of multi-gene carriage (predominantly *bla_{SHV}*, *bla_{TEM}*, *bla_{CTX-M}* in combination with other β -lactamase determinants such as *bla_{ampC}*). In contrast, MGMH and DPKISMH showed fewer complex resistance gene combinations but a higher relative proportion of dual-gene carriage. Notably, 18.5% of isolates were negative for all screened ESBL and ampC genes, suggesting the presence of alternative β -lactamase mechanisms such as non-ESBL-mediated resistance or non-ampC mediated resistance.

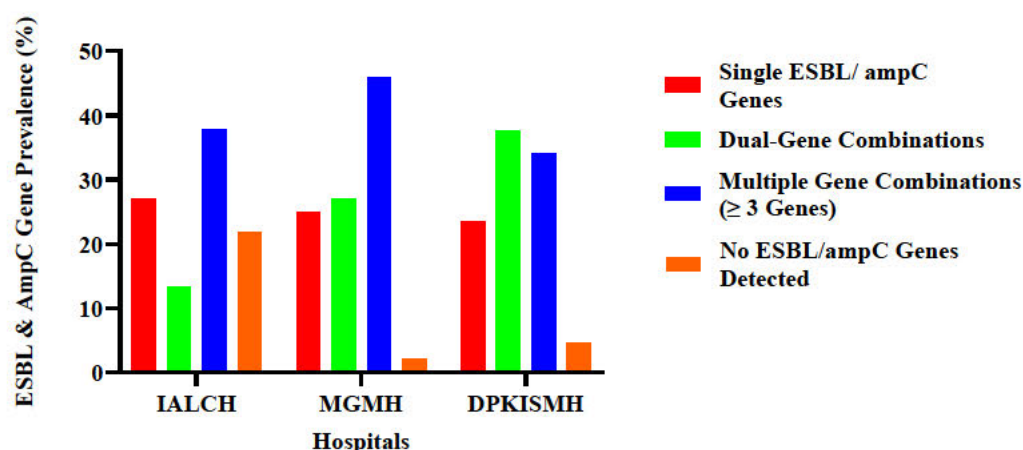


Figure 2: ESBL & ampC Co-harboursing gene combination distribution across all hospitals (created in GraphPad Software, Massachusetts, USA, www.graphpad.com)

4.3.3 Co-distribution of carbapenemase, ESBL & ampC genes

We integrated results from the ESBL and ampC PCR assays with carbapenemase gene data (*Klebsiella pneumoniae* carbapenemase (*KPC*), New Delhi metallo- β -lactamase (*NDM*), Verona integron-encoded metallo- β -lactamase (*VIM*), Imipenemase (*IMP*), Oxacillinase-48-like (*OXA-48-like*) and Guiana-Extended-Spectrum (*GES*)) from the same collection of isolates (See Chapter 3). We defined co-occurrence as the detection of one or more ESBL and/or ampC genes, with at least one carbapenemase gene, within a single isolate.

Most isolates, 96.4% (694/720) from our sample population, exhibited the presence of β -lactamase genes (carbapenemase, ESBL, and ampC) in various combinations, with 69.7% (502/720) co-harboursing carbapenemase and ESBL/ampC genes. In contrast, 14.9% (107/720) had only carbapenemase genes, and 11.8% (85/720) had only ESBL/ampC genes. Only 3.6% (26/720) showed none of the targeted β -lactamase genes (Table 1). At IALCH, 67.5% (396/587) of isolates had carbapenemase and ESBL/ampC genes, 18.1% (106/587) had only carbapenemase genes, and 10.7% (63/587) had only ESBL/ampC genes. MGMH had the highest rate of co-harboursing isolates at 89.6% (43/48), with 8.3% (4/48) having only ESBL/ampC genes and none having only carbapenemase genes. At DPKISMH, 74.1% (63/85) of isolates contained carbapenemase and ESBL/ampC genes, 21.2% (18/83) had only ESBL/ampC genes, and 1.2% (1/85) had only carbapenemase genes. The proportion of gene-negative isolates was similar to IALCH at 3.5% (3/85).

Our data shows that the combination of carbapenemase and ESBL/ampC genes is the main resistance pattern in all three hospitals. Distinct differences in the distribution of resistance genes were observed among the hospitals. In isolates possessing only carbapenemase genes, the proportion varied significantly at IALCH v/s MGMH ($p = 0.0002$) and IALCH v/s DPKISMH ($p < 0.001$) but not at MGMH v/s DPKISMH ($p > 0.9999$). For isolates with only ESBL & ampC genes, the proportions varied significantly at IALCH v/s DPKISMH ($p = 0.0057$) but not at IALCH v/s MGMH ($p = 0.8074$) and MGMH v/s DPKISMH ($p = 0.0871$). Isolates with

carbapenemase and ESBL/ ampC genes have proportions that differ significantly at IALCH v/s MGMH ($p = 0.0014$) and MGMH v/s DPKISMH ($p = 0.0332$) but not at IALCH v/s DPKISMH ($p = 0.2177$), with MGMH having the highest rate of multi-gene co-resistance. In contrast, the number of isolates without any target resistance genes did not vary significantly between sites (IALCH v/s MGMH $p > 0.9999$, MGMH v/s DPKISMH $p > 0.9999$, IALCH v/s DPKISMH $p > 0.9999$).

Table 1: Co-distribution of carbapenemase, ESBL & ampC genes among clinical isolates in the three hospitals

Categories	IALCH (n, %)	MGMH (n, %)	DPKISMH (n, %)	Total (n, %)
Carbapenemase Genes Only	106 (18.1)	0 (0)	1 (1.2)	107 (14.9)
ESBL/ampC Genes Only	63 (10.7)	4 (8.3)	18 (21.2)	85 (11.8)
Carbapenemase + ESBL & ampC Genes (≥ 1 gene)	396 (67.5)	43 (89.6)	63 (74.1)	502 (69.7)
No Carbapenemase, ESBL or ampC Genes Detected	22 (3.7)	1 (2.1)	3 (3.5)	26 (3.6)
Total	587 (100)	48 (100)	85 (100)	720 (100)

4.4 Discussion

Our study explores the molecular characteristics of ESBL and plasmid-mediated ampC genes found in carbapenem-resistant *Enterobacteriales* isolates from three hospitals in KZN, including the newly established DPKISMH. We used targeted multiplex and singleplex PCR assays to screen the carbapenem-resistant *Enterobacteriales* isolates for key ESBL and ampC genes. Our goal was to clarify the molecular complexity of β -lactam resistance in *Enterobacteriales* isolates and to describe the early emergence of these resistance mechanisms in a new regional hospital.

We found high levels of ESBL genes among the *Enterobacteriales* isolates, with *bla*_{TEM} being the most frequently detected family, followed by *bla*_{CTX-M} and *bla*_{SHV}. Several isolates carried more than one ESBL gene. Review and surveillance studies indicate that ESBL families (CTX-M, TEM, SHV) are the most common worldwide, with CTX-M often being reported as dominant in many regions, although local variations exist (19-21). Two Systematic reviews and region-specific meta-analyses show high ESBL prevalence across sub-Saharan Africa, but the frequency and dominance of gene families vary by country and setting (22, 23). Recent studies from South Africa indicate that ESBL genes are commonly found in *Enterobacteriales* and are often detected in parallel with carbapenemase genes; however, the ratios of TEM to CTX-M vary across studies and provinces (24, 25). Our observation that *bla*_{TEM} is the most common gene, rather than the globally dominant *bla*_{CTX-M}, likely reflects local plasmid reservoirs and the antibiotic selection pressures in these hospitals (19).

The deviation from the global CTX-M-dominant trend is not unusual; regional and hospital-level factors often determine which ESBL families are prevalent (19). The presence of multiple ESBL genes in single isolates aligns with the global finding that ESBLs are often carried on plasmids with other resistance genes, promoting co-carriage and multi-gene isolates (19). The detection of *bla*_{TEM} and *bla*_{SHV} genes in our study reflects the presence of β -lactamase gene families; however, these gene families include both narrow-spectrum and extended-spectrum variants (26, 27). Therefore, their presence was considered alongside established ESBL-associated genes such as *bla*_{CTX-M} when interpreting ESBL burden.

We identified plasmid-mediated ampC genes in 38.8% of *Enterobacterales* isolates (*bla*_{ampC}-positive), suggesting that ampCs play a role in the overall β -lactam resistance, along with ESBLs and carbapenemases. Although some *Enterobacterales* species are known to have chromosomal ampC (27). The *bla*_{ampC} used in this study were based on previously published primers used in the detection of plasmid-mediated ampC genes. Therefore, the ampC genes identified in our study are interpreted as plasmid-mediated ampC rather than intrinsic chromosomal ampC that may be present in some *Enterobacterales* species (15, 28). Recent studies have noted a rising detection of plasmid-mediated AmpC enzymes in *Enterobacterales*, especially in *Enterobacter* and *Klebsiella* species, where AmpC can reduce the effectiveness of cephamycins and complicate treatment (29-31). African reports show AmpCs in clinical isolates at varying frequencies but are under-detected by standard phenotypic tests (32, 33). South Africa confirms the presence of AmpC but notes routine screening often underreports it, emphasising the need for targeted molecular screening to find the true prevalence and impact (34-36). Our detection of plasmid-mediated AmpCs in *Enterobacterales* isolates aligns with global and African observations that AmpC is a significant but under-recognised contributor to β -lactam resistance. It also highlights that targeted molecular screening can improve detection sensitivity (30, 31). AmpCs are not inhibited by clavulanate and may coexist with ESBLs and carbapenemases, explaining the resistance to β -lactam/ β -lactamase inhibitor combinations observed in *Enterobacterales* (37). These findings may support using AmpC screening in surveillance (37).

The majority of carbapenem-resistant *Enterobacterales* isolates (96.4%) contained carbapenemase genes plus one or more ESBL or ampC genes; dual gene and multi-gene co-harbours (≥ 3 β -lactamase genes) was common. Global studies often report the frequent co-occurrence of ESBLs/ampC with carbapenemases in CRE isolates, while molecular and phenotypic testing show that multi-gene carriage (CTX-M with NDM or OXA-48) is frequent and typically plasmid-mediated (8, 38, 39). African molecular reports similarly show the co-harbours of ESBL and carbapenemase genes, while emphasising the role of plasmid dynamics (22, 40). South African isolates often show co-carriage of carbapenemase genes (NDM, OXA-48-like) with ESBLs (24, 25). Our high rate of co-harbours reflects the well-documented global trend where plasmids carrying multiple β -lactamases spread across healthcare networks (8). The clinical implications are significant: co-harbours limits effective treatment options and complicates phenotypic detection, especially when one enzyme (e.g., OXA-48) produces low carbapenem MICs that can obscure resistance in routine tests. The

coharbouring of genes may also suggest clonal expansion or the sharing of mobile genetic elements (41, 42). Our data support the need for molecular screening in CRE surveillance (8).

Only 3.6% of isolates lacked all the screened β -lactamase genes, which shows that carbapenemase-negative isolates still contained ESBL and/or ampC genes. Global studies have documented non-carbapenemase CRE, where reduced susceptibility results from a combination of ESBL/ampC presence, porin loss, efflux pumps, or other membrane changes (7, 8). African studies have highlighted non-carbapenemase-producing-CRE (non-CP-CRE) cases and pointed out that porin mutations and the combination of ESBL/ampC with porin loss are mechanisms found in local isolates (43, 44). Carbapenemase-negative CRE in our data matches global trends, showing that multiple non-enzymatic or combined mechanisms can lead to carbapenem resistance, highlighting the limitations of only carbapenemase screening and the need for further molecular analyses (7, 8).

Combinations of resistance genes (ESBL/ampC with carbapenemase) were identified in all hospitals, including the rapid emergence of such profiles in the recently opened DPKISMH. Global outbreak and surveillance literature documents the fast spread of plasmid-borne resistance genes between facilities due to patient transfers and shared referral networks (45-47). The inter-facility spread and quick establishment of CRE clusters have been noted in various African contexts, driven by referral patterns and limited infection prevention resources (40, 48). Local studies indicate that referral networks and transfers between tertiary and regional facilities contribute to the spread of CRE; regional hospitals can quickly reflect the resistance ecology of referral centres (2, 49). The swift emergence of complex resistance profiles in a newly opened regional hospital aligns with documented pathways for inter-facility gene transfer and highlights the susceptibility of lower-tier hospitals to imported plasmid-borne resistance, confirming the importance of early infection prevention and molecular surveillance when new hospitals open or when referral connections are strong (2, 8, 49).

Our molecular findings align with current literature: CRE isolates frequently co-harbour ESBL/ampC genes and carbapenemases, plasmid-mediated gene sharing promotes rapid spread between hospitals, and non-carbapenemase mechanisms significantly contribute to carbapenem resistance (38, 50). The local pattern, particularly the prevalence of *bla_{TEM}*, demonstrates that regional molecular surveillance is critical because national or global dominant patterns (e.g., CTX-M dominance) do not always reflect local plasmid and selection dynamics (23, 51, 52). Our findings suggest the need for broader molecular diagnostics (such as multiplex panels), reinforced IPC and antimicrobial stewardship across referral networks, and routine molecular surveillance integrated with standard microbiology practices.

4.5 Strengths and Limitations

Our study provides a detailed investigation of ESBL and plasmid-mediated ampC genes found in *Enterobacteriales* isolates from three public hospitals in KZN. A significant strength is the inclusion of specimens from the newly opened DPKISMH, providing insight into the emergence of complex resistance mechanisms. The targeted PCR assays enabled the detection of key ESBL and ampC genes, allowing for the assessment of coexistence with carbapenemase genes. Additionally, confirmatory sequencing of selected amplicons reinforced the reliability of the PCR-based gene identification. However, our study does have limitations. Only available carbapenem-resistant *Enterobacteriales* isolates were used. Only selected ESBL and ampC genes were examined. No ATCC controls for ESBL and ampC genes were used. The primers used in this study were chosen from previously published assays, to specifically detect major β -lactamase, ESBL and plasmid-mediated ampC gene families such as bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{ampC}. These primers are highly specific towards their target genes, however they do not detect all known genotypes such as less common variants, which could have resulted in some variants not being detected. Our findings provide important insights into the molecular complexity of β -lactam resistance among CREs in KZN, despite the limitations.

4.6 Conclusion

Our study reveals that carbapenem-resistant *Enterobacteriales* detected in public hospitals in KwaZulu-Natal, including the new Dr Pixley Ka Isaka Seme Memorial Hospital, often carry ESBLs and plasmid-mediated ampC genes along with carbapenemases. The high presence of ESBL and ampC together reveals the complex nature of β -lactam resistance in CREs. It indicates that carbapenem resistance in our setting typically does not result from just one mechanism. The early detection of complicated resistance gene profiles in a new regional hospital suggests that carbapenem-resistant bacteria can be rapidly established, likely due to healthcare-associated transmission and the spread of genes via plasmids. Our results underscore the need for combined molecular monitoring, improved IPC methods, and better antibiotic treatment across all healthcare levels to reduce the spread of highly resistant *Enterobacteriales*.

4.7 Future Work

Other β -lactamase variants and non-enzymatic resistance methods, such as porin changes or increased efflux pump activity, should be further investigated. While our study characterised the presence of resistance genes and outlined antimicrobial susceptibility profiles, we did not perform a formal correlation analysis between the genotypic data and the phenotypic data. These analyses could show the clinical effects of specific resistance mechanisms, which is an important area for future research. The inclusion of contemporary susceptible and ESBL- or ampC-indicative strains should also be explored, along with testing alternative AST methods to ensure accurate results.

4.8 Funding

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4.9 Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

4.10 Acknowledgements

Dr Yesholata Mahabeer for the use of the Inkosi Albert Luthuli Central Hospital Isolates.

Chapter 4 has shown that extended-spectrum β -lactamase and ampC β -lactamase genes are common among carbapenem-resistant *Enterobacterales*. These genes often co-occur with carbapenemase genes and contribute to the development of complex resistance traits. These findings highlight the intricate nature of β -lactam resistance in KwaZulu-Natal hospitals. Chapter 5 synthesises the findings from all previous chapters to summarise the overall molecular characterisation of resistance, discuss the clinical and public health implications, and suggest recommendations for surveillance, antimicrobial management, and infection prevention and control.

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4.12 Chapter 4: Supplementary Tables

Table S1: Primers used for the detection of ESBL and ampC genes

Target gene	Primer	Direction	Sequence (5'–3')	Amplicon size (bp)	Reference
<i>bla_{SHV}</i>	mc-shv-f	Forward	GGTTATGCGTTATATTCGCC	867	(14)
	mc-shv-r	Reverse	TTAGCGTTGCCAGTGCTC		
<i>bla_{TEM}</i>	mc-tem-f	Forward	CGGATGGCATGACAGTAAGAG	274	(14)
	mc-tem-r	Reverse	TTGCCGGGAAGCTAGAGTAAG		
<i>bla_{CTX-M}</i>	mc-ctx-m-f	Forward	ATGTGCAGYACCAGTAARGT	593	(14)
	mc-ctx-m-r	Reverse	TGGGTRAARTARGTSACCAGA		
<i>bla_{ampC}</i>	mc-amp-c-f	Forward	ATTCGTATGCTGGATCTCGCCACC	396	(15)
	mc-amp-c-r	Reverse	CATGACCCAGTTCGCCATATCCTG		

β -lactamase (*bla*), Temoniera Beta-Lactamase (*TEM*), *bla* Sulphydryl Variable Beta-Lactamase (*SHV*), *bla* Cefotaximase Beta-Lactamase (*CTX-M*), Ampicillin β -lactamase (*ampC*), forward (f), reverse (r).

Table S2: Multiplex PCR master mix for ESBL gene detection and singleplex PCR master mix for ampC gene detection (16)

Master mix for *bla_{SHV}*, *bla_{TEM}*, *bla_{CTX-M}*,

Component	Volume per reaction (μ L)
DreamTaq Green PCR Master Mix (2 $\times$)	10.0
Forward primer (10 μ M) for each target gene	0.2×4
Reverse primer (10 μ M) for each target gene	0.2×4
Nuclease-free water	3.2
Template DNA	5.0
Total	20.0

β -lactamase (*bla*); Temoniera Beta-Lactamase (*TEM*); *bla* Sulphydryl Variable Beta-Lactamase (*SHV*); *bla* Cefotaximase Beta-Lactamase (*CTX-M*).

Master mix for *bla_{ampC}*

Component	Volume per reaction (μ L)
DreamTaq Green PCR Master Mix (2 $\times$)	10.0
Forward primer (10 μ M) for the target gene	0.2
Reverse primer (10 μ M) for the target gene	0.2
Nuclease-free water	3.2
Template DNA	5.0
Total	20.0

β -lactamase (*bla*); Ampicillin β -lactamase (*ampC*)

Table S3: ESBL & ampC Co-harboured gene combination across all Hospitals

Combination type	IALCH (n, %)	MGMH (n, %)	DPKISMH (n, %)	Total (n, %)
<i>bla_{SHV}</i>	2 (0.3)	0 (0)	2 (2.4)	4 (0.6)
<i>bla_{CTX-M}</i>	1 (0.2)	1 (2.1)	2 (2.4)	4 (0.6)
<i>bla_{TEM}</i>	147 (25)	9 (18.8)	16 (18.8)	172 (23.9)
<i>bla_{ampC}</i>	9 (1.5)	2 (4.2)	0 (0)	11 (1.5)
<i>bla_{SHV} & bla_{TEM}</i>	3 (0.5)	2 (4.2)	3 (3.5)	8 (1.1)
<i>bla_{SHV}, bla_{TEM} & bla_{ampC}</i>	7 (1.2)	1 (2.1)	0 (0)	8 (1.1)
<i>bla_{SHV}, bla_{CTX-M} & bla_{TEM}</i>	33 (5.6)	15 (31.3)	13 (15.3)	61 (8.5)
<i>bla_{SHV} & bla_{CTX-M}</i>	3 (0.5)	0 (0)	1 (1.2)	4 (0.6)
<i>bla_{SHV}, bla_{CTX-M}, bla_{TEM} & bla_{ampC}</i>	143 (24.4)	2 (4.2)	7 (8.2)	152 (21.1)
<i>bla_{SHV} & bla_{ampC}</i>	4 (0.7)	0 (0)	2 (2.4)	6 (0.8)
<i>bla_{SHV}, bla_{CTX-M} & bla_{ampC}</i>	2 (0.3)	1 (2.1)	4 (4.7)	7 (1)
<i>bla_{CTX-M} & bla_{TEM}</i>	38 (6.5)	6 (12.5)	11 (12.9)	55 (7.6)
<i>bla_{CTX-M}, bla_{TEM} & bla_{ampC}</i>	37 (6.3)	3 (6.3)	5 (5.9)	45 (6.3)
<i>bla_{TEM} & bla_{ampC}</i>	30 (5.1)	3 (6.3)	5 (5.9)	38 (5.3)
<i>bla_{CTX-M} & bla_{ampC}</i>	0 (0)	2 (4.2)	10 (11.8)	12 (1.7)
No ESBL/ampC Genes Detected	128 (21.8)	1 (2.1)	4 (4.7)	133 (18.5)
Total	587 (100)	48 (100)	85 (100)	720 (100)

β -lactamase (*bla*), Temoniera Beta-Lactamase (*TEM*), *bla* Sulphydryl Variable Beta-Lactamase (*SHV*), *bla* Cefotaximase Beta-Lactamase (*CTX-M*), Ampicillin β -lactamase (*ampC*), Inkosi Albert Luthuli Central Hospital (IALCH), Mahatma Gandhi Memorial Hospital (MGMH), and Dr Pixley Ka Isaka Seme Memorial Hospital (DPKISMH).

4.13 Chapter 4: Supplementary Figures

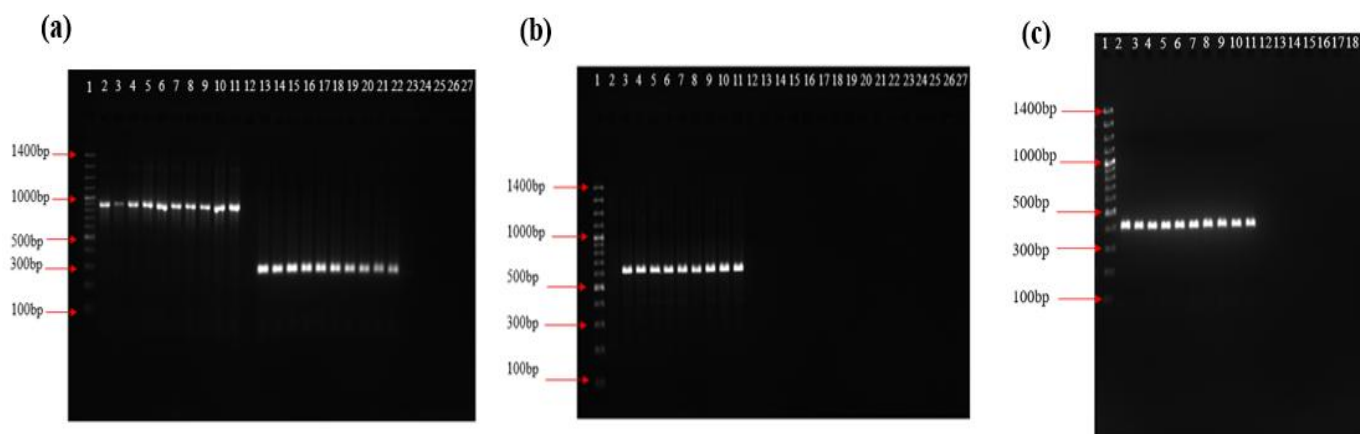


Figure S1: Gel electrophoresis showing PCR amplification of isolates showing the presence of (a) *bla_{SHV}*, *bla_{TEM}*, (b) *bla_{CTX-M}* and (c) *bla_{ampC}* that were sent for Sanger sequencing

- a) Lane 1: 100bp DNA ladder or marker; Lane 2 – 11: clinical isolates showing target amplicon *bla_{SHV}* appearing at 867 bp; Lane 12: Negative control strain for *bla_{SHV}*; Lane 13 – 22: clinical isolates showing target amplicon *bla_{TEM}* appearing at 274 bp; Lane 23: Negative control strain for *bla_{TEM}*
- b) Lane 1: 100bp DNA ladder or marker; Lane 2: Negative control strain for *bla_{CTX-M}*; Lane 3 – 11: clinical isolates showing target amplicon *bla_{CTX-M}* appearing at 593 bp.
- c) Lane 1: 100bp DNA ladder or marker; Lane 2 – 11: clinical isolates showing target amplicon *bla_{ampC}* appearing at 396 bp; Lane 12: Negative control strain for *bla_{ampC}*

Klebsiella pneumoniae partial SHV-1 gene for SHV beta lactamase, isolate AAIH-1

Sequence ID: [LN558653.1](#) Length: 870 Number of Matches: 1

Range 1: 87 to 865 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1428 bits(773)	0.0	778/780(99%)	1/780(0%)	Plus/Plus
Query 1	CCCTGCCGCTGGCGGTACACGCCAGCCGCGAGCCGCTTGAGCAAATTAACAAAGCGAAA	60		
Sbjct 87	CCCTGCCGCTGGCGGTACACGCCAGCCGCGAGCCGCTTGAGCAAATTAACAAAGCGAAA	146		
Query 61	GCCAGCTGTGCGGCCGCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACGCTGA	120		
Sbjct 147	GCCAGCTGTGCGGCCGCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACGCTGA	206		
Query 121	CCGCCTGGCGCGCCGATGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCG	180		
Sbjct 207	CCGCCTGGCGCGCCGATGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCG	266		
Query 181	GCGCAGTGCTGGCGCGGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATC	240		
Sbjct 267	GCGCAGTGCTGGCGCGGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATC	326		
Query 241	GCCAGCAGGATCTGGTGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGACGGCATGA	300		
Sbjct 327	GCCAGCAGGATCTGGTGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGACGGCATGA	386		
Query 301	CGGTCGGCGAACTCTGCGCCGCCGCCATTACCATGAGCGATAACAGCGCCGCAATCTGC	360		
Sbjct 387	CGGTCGGCGAACTCTGCGCCGCCGCCATTACCATGAGCGATAACAGCGCCGCAATCTGC	446		

Figure S2: Sequence alignment of *bla_{SHV}* gene showing $\geq 99\%$ nucleotide identity with reference sequence (GenBank accession: LN558653.1)

Escherichia coli strain D183CTXM beta lactamase-like (CTX-M-15) gene, partial sequence

Sequence ID: [FJ997865.1](#) Length: 550 Number of Matches: 1

Range 1: 1 to 513 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
941 bits(509)	0.0	512/513(99%)	1/513(0%)	Plus/Plus
Query 2	AAGTGAAGCG-ACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTT	60		
Sbjct 1	AAGTGAAGCGAACCGAATCTGTTAAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTT	60		
Query 61	AACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGC	120		
Sbjct 61	AACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGC	120		
Query 121	GCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGC	180		
Sbjct 121	GCGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGC	180		
Query 181	GGCCCGGCTAGCGTCACCGCGTTTCGCCGACAGCTGGGAGACGAAACGTTCCGTCTCGAC	240		
Sbjct 181	GGCCCGGCTAGCGTCACCGCGTTTCGCCGACAGCTGGGAGACGAAACGTTCCGTCTCGAC	240		
Query 241	CGTACCGAGCCGACGTTAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCT	300		
Sbjct 241	CGTACCGAGCCGACGTTAAACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCT	300		
Query 301	CGGGCAATGGCGCAAACTCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAA	360		
Sbjct 301	CGGGCAATGGCGCAAACTCTGCGGAATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAA	360		

Figure S3: Sequence alignment of *bla_{CTX-M}* gene showing $\geq 99\%$ nucleotide identity with reference sequence (GenBank accession: FJ997865.1)

Escherichia coli unnamed blaTEM gene for broad-spectrum class A beta-lactamase TEM-127, complete CDS

Sequence ID: [NG_050171.1](#) Length: 861 Number of Matches: 1

Range 1: 378 to 450 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
135 bits(73)	6e-28	73/73(100%)	0/73(0%)	Plus/Plus
Query 15	CATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCT	74		
Sbjct 378	CATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCT	437		
Query 75	AACCGCTTTTTTG	87		
Sbjct 438	AACCGCTTTTTTG	450		

Figure S4: Sequence alignment of *bla_{TEM}* gene showing $\geq 99\%$ nucleotide identity with reference sequence (GenBank accession: NG_050171.1)

Enterobacter hormaechei strain Ugd36 AmpC beta-lactamase ACT-20 (blaACT-20) gene, complete cds

Sequence ID: [KF526117.1](#) Length: 1146 Number of Matches: 1

Range 1: 405 to 764 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
636 bits(344)	8e-178	357/363(98%)	4/363(1%)	Plus/Plus
Query 1	CGGCCTTGCCGCTA-MGGTACCGGATGAGGTCACGGATAACGCCTCCCTGCTGCGCTTT	59		
Sbjct 405	CGGCC-T-GCCGCTACAGGTACCGGATGAGGTCACGGATAACGCCTCCCTGCTGCGCTTT	462		
Query 60	TATCAAAACTGGCAGCCGAGTGAAGCCTGGCACAACGCGTCTTTACGCCAACGCCAGC	119		
Sbjct 463	TATCAAAACTGGCAGCCGAGTGAAGCCTGGCACAACGCGTCTTTACGCCAACGCCAGC	522		
Query 120	ATCGGCTTTTTGGTGCCTGGCGGTCAAACCTTCCGGCATGCCCTATGAGCAGGCCATG	179		
Sbjct 523	ATCGGCTTTTTGGTGCCTGGCGGTCAAACCTTCCGGCATGCCCTATGAGCAGGCCATG	582		
Query 180	ACGACGCGGGTCTTAAGCCGCTCAAGCTGGACCATACCTGGATTAACGTTCCGAAAGCG	239		
Sbjct 583	ACGACGCGGGTCTTAAGCCGCTCAAGCTGGACCATACCTGGATTAACGTTCCGAAAGCG	642		
Query 240	GAAGAGGCGCATTACGCTGGGGCTATCGTGACGGTAAAGCGGTGCGGTTTCGCCGGGA	299		
Sbjct 643	GAAGAGGCGCATTACGCTGGGGCTATCGTGACGGTAAAGCGGTGCGGTTTCGCCGGGA	702		
Query 300	ATGCTGGATGCACAAGCCTATGGCGTGAAAACCAACGTGCAGGATATGGCGAACTGGGGT	359		
Sbjct 703	ATGCTGGATGCACAAGCCTATGGCGTGAAAACCAACGTGCAGGATATGGCGAACTGGG-T	761		
Query 360	CAT	362		
Sbjct 762	CAT	764		

Figure S5: Sequence alignment of *bla_{ampC}* gene showing $\geq 98\%$ nucleotide identity with reference sequence (GenBank accession: KF526117.1)

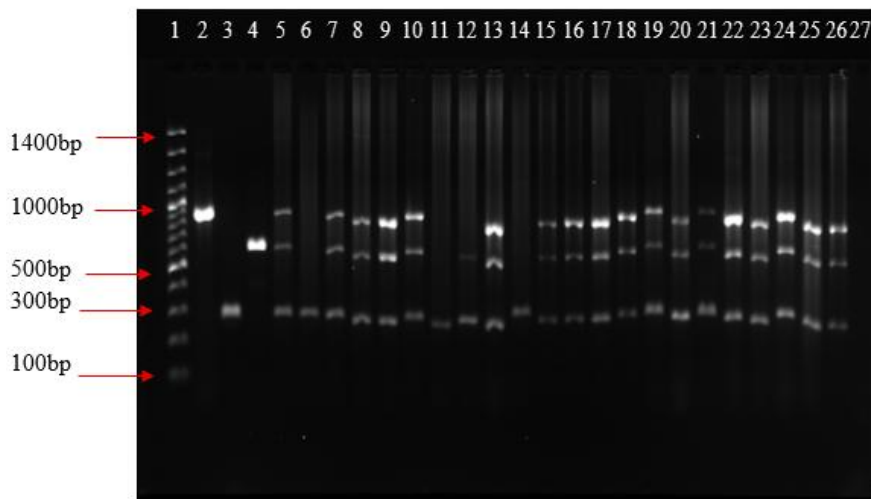


Figure S6: Gel electrophoresis showing PCR amplification of ESBL genes (*bla_{SHV}*, *bla_{CTX-M}*, *bla_{TEM}*)

Lane 1: 100bp DNA ladder or marker; Lane 2-4: positive control strains for *bla_{SHV}* (867 bp), *bla_{TEM}* (274 bp), *bla_{CTX-M}* (593 bp); Lane 5-26: clinical isolates showing target amplicons; Lane 27: Negative control strain. Lanes 5,7,8,9,10,13, and 15-26: *bla_{SHV}*, *bla_{TEM}* and *bla_{CTX-M}* positive isolates; Lanes 6,11,14: *bla_{TEM}*; and Lane 12: *bla_{TEM}* and *bla_{CTX-M}*.

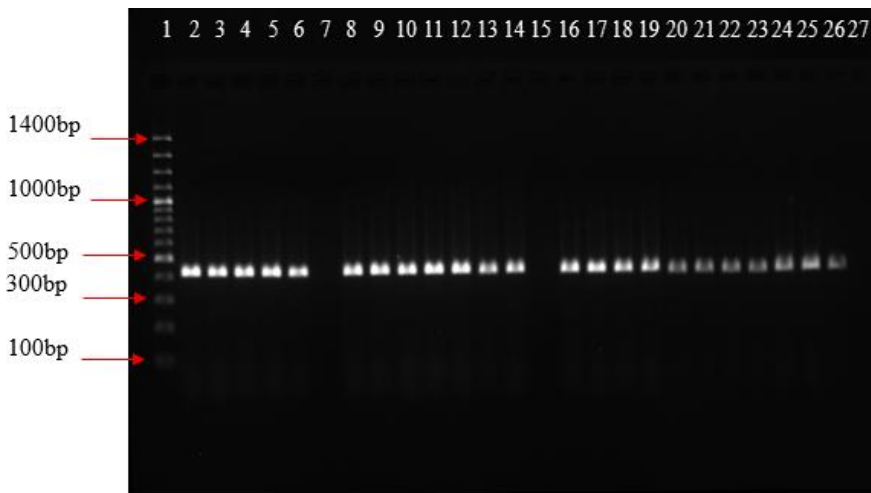


Figure S7: Gel electrophoresis showing PCR amplification of the ampC gene (*bla_{ampC}*)

Lane 1: 100bp DNA ladder or marker; Lane 2: positive control strains for *bla_{ampC}* (396 bp); Lane 27: Negative control strain.; Lane 2: positive control strains for *bla_{ampC}* (396 bp) Lanes 3-6;8-14; 16-26: clinical isolates showing target amplicon *bla_{ampC}*. Lane 7 & 15: clinical isolates showed no presence of the target amplicon *bla_{ampC}*.

5. Chapter 5: Synthesis

Chapter 5 synthesises the findings of the dissertation. Chapter 1 introduced the problem of carbapenem-resistant *Enterobacterales* (CRE) and outlined the aims and objectives. Chapter 2 reviewed the global, national, and provincial literature, highlighting key resistance mechanisms. Chapter 3 provided original data on the prevalence of CRE and molecular characterisation of carbapenem-resistant *Enterobacterales*, identifying the presence of carbapenemase genes in three KwaZulu-Natal hospitals. Chapter 4 expanded the work by identifying extended-spectrum β -lactamase and ampC β -lactamase genes, revealing significant co-carriage and molecular complexity. Chapter 5 synthesises these findings to draw conclusions and offer suggestions to improve molecular characterisation, infection prevention and control (IPC), and the use of appropriate antibiotics in KwaZulu-Natal.

5.1 Synthesis

Our study aimed to assess the prevalence and molecular characterisation of carbapenem-resistant gram-negative bacteria, focusing on carbapenem-resistant *Enterobacterales* (CRE), in a new regional hospital in KwaZulu-Natal (KZN), South Africa. The study objectives were to (1) determine the prevalence of CRE in a new regional hospital compared with other regional hospitals in KZN, (2) identify the main species of CRE in the new regional hospital in KZN, (3) characterise the antibiotic resistance profiles of isolates using phenotypic and genotypic techniques, and (4) describe the genetic sequencing of the detected target genes to confirm the genes of interest.

We found that CRE prevalence was 11.7% of all *Enterobacterales* across three KZN hospitals. The rates were 13.1% at the quaternary hospital (IALCH), 8.1% at the established regional hospital (MGMH), and 11.0% at the newly opened regional hospital (DPKISMH). Notably, all sites showed increasing trends in CRE prevalence over time, with a sudden rise soon after DPKISMH opened, indicating an early presence of resistant bacteria. Recent surveillance data indicate a steady rise in CRE prevalence, especially in bloodstream infections (1-3). The World Health Organisation (WHO), Global Antimicrobial Resistance and Use Surveillance System (GLASS) reported an increase from 2.9% in 2017 to 6.7% by 2023(3-5). Studies in Africa in the past five years show a growing detection of CRE beyond tertiary centres, with more involvement from regional and district hospitals (6-9). South African data from the Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa (GERMS-SA) and the National Institute of Communicable Diseases (NICD) highlight a rising prevalence of CRE nationwide, particularly in referral networks (10, 11). The prevalence in our study closely matches global and national observations, which supports that CRE are now spreading to regional hospitals, which play a key role in persistence and spread within interconnected referral systems in KZN (9, 12).

We identified a variety of CRE bacteria at the new regional hospital, including *K. pneumoniae*, *E. coli*, *E. cloacae*, *P. mirabilis*, *S. marcescens*, *M. morgani*, etc. *P. mirabilis* was the most common bacterium at the regional hospitals, while *K. pneumoniae* was the leading bacterium at the quaternary centre. Recent global studies consistently identify *K. pneumoniae* and *E. coli* as the most common CREs, especially in invasive infections (13, 14). African systematic reviews since 2020 also show *K. pneumoniae* as the dominant CRE but increasingly highlight the role of other *Enterobacterales* in community and regional contexts (15, 16). South African studies have recognised *P. mirabilis* as an emerging CRE in regional hospitals, often linked to urinary tract and wound infections (12). The CRE distribution seen in our study reflects emerging evidence from Africa and South Africa that CRE patterns differ by healthcare level (15, 17). The prominence of *P. mirabilis* in regional hospitals suggests that local factors related to ecology and antibiotic use influence bacterial selection (18), which reinforces the need for surveillance at the hospital level rather than solely depending on data from tertiary centres (19).

Phenotypic and genotypic techniques were used to characterise the antibiotic resistance profiles of isolates. Phenotypic tests showed significant multidrug resistance among *Enterobacterales* isolates, with higher resistance levels observed at the quaternary hospital compared to the regional hospitals. Resistance was detected not just to carbapenems but also to third-generation cephalosporins, β -lactam/ β -lactamase inhibitor combinations, and aminoglycosides. These results highlight the limited treatment options for CRE infections across all hospital levels (20). Genotypic analysis identified *bla*_{OXA-48-like} as the most common carbapenemase gene, followed by *bla*_{NDM} and *bla*_{KPC}. Low frequencies of *bla*_{VIM}, *bla*_{IMP}, and *bla*_{GES} were also found. Our results also showed a clear shift in the carbapenemase landscape in IALCH from *bla*_{NDM} to *bla*_{OXA-48-like} from 2016 to 2023. Importantly, 15.4% of CRE isolates did not show detectable carbapenemase genes, indicating alternative resistance mechanisms. Additional screening revealed widespread presence of ESBL and ampC genes, especially *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{ampC}, and *bla*_{SHV}. Overall, 96.4% of isolates had at least one β -lactamase gene, while only 3.6% lacked all the resistance determinants tested.

Recent global studies confirm the prevalence of *bla*_{OXA-48-like} and *bla*_{NDM} in many areas, especially in Africa, parts of Europe and Asia (15, 21, 22). South African surveillance findings also show an increase in OXA-48-like carbapenemases (11), which also aligns with international findings that describe the quick spread of OXA-48 variants in hospital networks (23). South African studies highlight the challenges in diagnosing OXA-48-like enzymes and carbapenemase-negative CRE (12, 24). African research increasingly reports complex co-carriage of carbapenemase, ESBL, and ampC genes (7, 15). The high rate of multi-gene carriage aligns with plasmid-mediated spread and horizontal gene transfer (25). Our study findings align with global and African data (7, 15, 21, 22) but uniquely show that complex resistance profiles can rapidly establish in a newly opened regional hospital, which highlights that resistance complexity emerges rapidly in cases of referral-linked transmission.

We described the genetic sequences of the detected target genes to confirm the genes of interest. Sanger sequencing of selected *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{ampC} amplicons confirmed a nucleotide identity of $\geq 98\%$

with reference sequences in GenBank. The sequencing confirmed the reliability of PCR and ensured confidence in subsequent molecular interpretations. Globally, confirming resistance genes through sequencing is essential for accurate surveillance (26, 27). A study highlights the importance of verifying sequences to prevent misclassification of β -lactamase genes (28). African and South African studies increasingly use sequencing to enhance molecular surveillance, although access remains limited in many areas (29, 30).

Our work shows that targeted molecular screening for carbapenemases, ESBLs, and ampC genes may be integrated into routine diagnostic practices in South African hospital laboratories. The addition of multiplex PCR assays to standard susceptibility testing in hospital laboratories may enable them to detect high-risk resistance profiles earlier, improving infection prevention and control and supporting antibiotic stewardship decisions. Regional hospital laboratories may benefit from periodic molecular surveillance to spot emerging resistance trends before large-scale outbreaks occur.

A key strength of our work is the inclusion of multiple hospital levels over an extended period, offering a broad view of CRE patterns. The combination of phenotypic testing and molecular characterisation enhanced the reliability of our findings. However, limitations include the retrospective nature of the laboratory data and the lack of American Type Culture Collection (ATCC) controls for the target ESBL and ampC genes. Additionally, clinical outcome data were not included, limiting the assessment of patient-level impacts.

5.2 Conclusion

Our work shows that CREs are well-established across hospital levels in KwaZulu-Natal, including a newly opened regional facility. Resistance is driven by complex, co-existing molecular mechanisms that can emerge rapidly, reflecting global and national trends. Our findings highlight the urgent need for integrated molecular surveillance, improved infection prevention and control strategies, and coordinated antimicrobial stewardship across South African healthcare systems.

5.3 Recommendations

Further work should be done to determine additional resistance mechanisms causing carbapenemase-negative CRE. Community circulation and subclinical colonisation of CREs requires further investigation. Clinical data such as patient outcomes, prior antibiotic exposure, and comorbidities need to be further clarified. The clonal spread of CREs between hospitals needs to be assessed. Other β -lactamase variants and non-enzymatic resistance methods, such as porin changes or increased efflux pump activity, should be further investigated.

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6. Appendices

6.1 Appendix 1: Ethical Approval Letter



09 September 2024

Mr Diyothan Pillay (215007837)
School of Laboratory Medicine & Medical Science
Medical School

Dear Mr Pillay,

Protocol reference number: BREC/00007433/2024
Project title: Molecular epidemiology of Carbapenem resistant Gram - negative bacteria in a regional hospital in KwaZulu - Natal, South Africa
Degree: Masters

EXPEDITED APPLICATION APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 09 September 2024. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 09 September 2024. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2024), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 08 October 2024.

Yours sincerely,



Prof S Singh
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor S Singh

UKZN Research Ethics Office Westville Campus, Govan Mbeki Building

Postal Address: Private Bag X54001, Durban 4000

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Website: <http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx>

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6.2 Appendix 2: Gatekeeper Letter



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Inkosi Albert Luthuli Central Hospital
800 Vusi Mzimela (Bellair) Road, Mayville, 4091
Tel: +27 (0)31 240 2787
Reference:



Practice No: 5200296

22 August 2024

To whom it may concern

Permission to Use Clinical Data from Routinely Analysed Samples for MSc Project

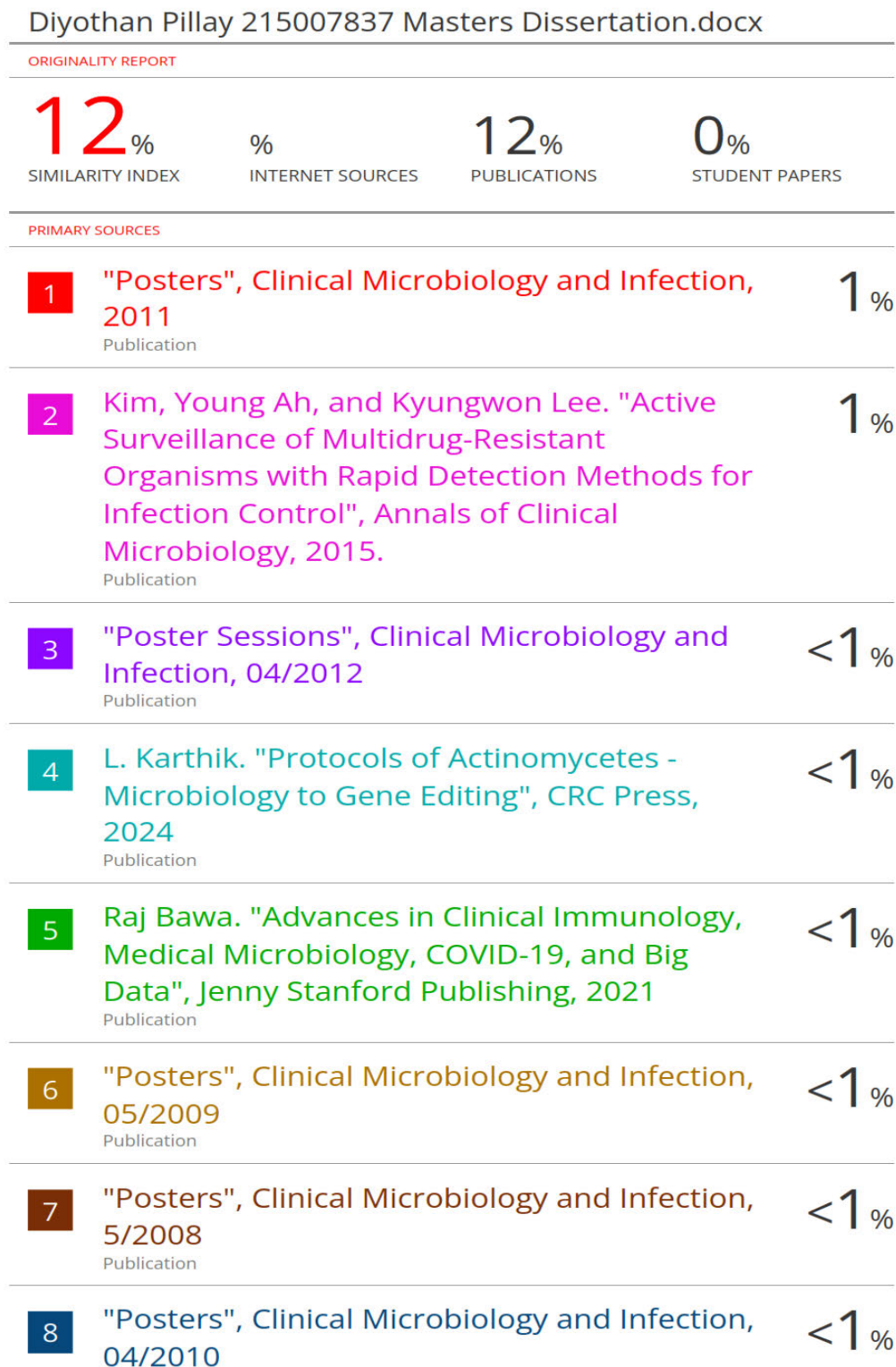
This letter serves to allow Mr Diyothan Pillay use of the relevant clinical data from routinely analysed samples at the specified NHLS Microbiology laboratories for his MSc project entitled: "Molecular epidemiology of Carbapenem resistant Gram - negative bacteria in a regional hospital in KwaZulu - Natal, South Africa". He will be given access to the data via me, as I am his supervisor.

Yours sincerely,



Dr Prathna Bhola (MBChB, FCPATH(SA)Micro, PhD)
Senior Pathologist (Medical Microbiology)
Mahatma Gandhi Memorial Hospital
KZN Academic Complex
National Health Laboratory Services
University of KwaZulu-Natal
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6.3 Appendix 3: Turn-it-in Report



6.4 Appendix 4: Carbapenem-Resistant Enterobacterales: A Narrative Review of Prevalence, Resistance Mechanisms, and Molecular Characterisation Manuscript Submission Letter

1/20/26, 7:18 PM

Gmail - Manuscript submitted to Health Science Reports



Diyothan Pillay [REDACTED]

Manuscript submitted to Health Science Reports

1 message

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Reply-To: Health Science Reports <healthsciencereports@wiley.com>
To: Diyothan Pillay [REDACTED]

Tue, Jan 20, 2026 at 7:07 PM

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6.5 Appendix 5: The Molecular Characterisation of Carbapenem-Resistant Enterobacterales in a New Regional Hospital in KwaZulu-Natal, South Africa Manuscript Submission Letter

1/20/26, 7:51 PM

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Diyothan Pillay [REDACTED]

Manuscript submitted to MicrobiologyOpen

1 message

MicrobiologyOpen <iahmad@wiley.com>
Reply-To: MicrobiologyOpen <oa-microbiology@wiley.com>
To: Diyothan Pillay <[REDACTED]>

Tue, Jan 20, 2026 at 7:50 PM

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6.6 Appendix 6: Presence of Extended-Spectrum β -lactamase & Ampicillin β -lactamase in a New Regional Hospital in KwaZulu-Natal, South Africa Manuscript Submission Letter

1/20/26, 8:22 PM

Gmail - Manuscript submitted to International Journal of Microbiology



Diyothan Pillay <[REDACTED]>

Manuscript submitted to International Journal of Microbiology

1 message

International Journal of Microbiology <ijm.office@wiley.com>
Reply-To: International Journal of Microbiology <vttharanend@wiley.com>
To: Diyothan Pillay <[REDACTED]>

Tue, Jan 20, 2026 at 8:21 PM

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Dear Mr. Diyothan Pillay,

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