The epidemiology of Gram negative bacteraemia at Tygerberg Hospital

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Thesis presented in partial fulfilment of the requirements for the degree Master of Science in Medical Microbiology in the Faculty of Medicine and Health Sciences at Stellenbosch University

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March 2018

Declaration

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Summary

Background

Escherichia coli and *Klebsiella pneumoniae* are common causes of Bloodstream Infections (BSI). β-lactam antibiotics, such as cephalosporins and carbapenems, are commonly used to treat these infections. Increasing resistance has been noted, usually due to plasmid mediated β-lactamases such as Extended-Spectrum β-lactamases (ESBLs) and carbapenemases. This study describes the antibiotic resistance profiles, outcomes and epidemiology of Gram negative BSIs in a tertiary hospital in Cape Town, South Africa.

Methods

Patients with *E. coli* (n=70) and *K. pneumoniae* (n=70) bacteraemia identified at Tygerberg Hospital between April 2015 and March 2016 were included. Identification and Antibiotic Susceptibility Testing (AST) were performed as part of routine testing. Patient data was obtained through record review. ESBL and carbapenemase genes were characterised by Polymerase Chain Reaction (PCR) and DNA sequencing. Isolates were typed using rep-PCR and Pulsed Field Gel Electrophoresis (PFGE). Chi-square and Mann-Whitney tests were used to estimate significance of correlation.

Results and discussion

45% of patients were male, and 30.7% were paediatric. 66.4% of BSI were hospital-acquired. *K. pneumoniae* accounted for 61.3% of hospital-acquired isolates; 72.3% of community-acquired isolates were *E. coli*. 55.7% of *K. pneumoniae* and 15.7% of *E. coli* were cephalosporin resistant (presumed ESBL); one *K. pneumoniae* isolate was carbapenem resistant. Increased antibiotic resistance and ESBL production was seen in hospital-acquired isolates.

ESBL genes were harboured in 35.7% of isolates; 50.7% contained β-lactamase genes and 13.6% no β-lactamase genes. Most TEM genes (98%) were β-lactamases; 47.4% of SHV genes were β-lactamases, 7% were ESBLs and 45.6% were SHV genes whose spectrum is uncertain. Isolates containing SHV genes with uncertain spectrum were phenotypically susceptible to cephalosporins, suggesting these enzymes do not have extended-spectrum activity. Multiple β-lactamase genes were present in 60% of *K. pneumoniae* isolates, and only 5.7% of *E. coli* isolates. CTX-M genes were the most common ESBL genes, with most (91.3%) of these belonging to group 1. CTX-M genes were found in combination more often than not (84.8%). No carbapenemase genes were detected.

Molecular and phenotypic resistance agreed in 95.3% of isolates. The 30-day mortality rate was 30%, with no association between mortality and hospital-acquired infection, or with ESBL production (phenotypic or molecular). Molecular and phenotypic resistance was associated with hospital-acquired isolates (P=0.001, P<0.001).

Both strain typing techniques showed substantial diversity among isolates, with minimal clustering; which suggests multiple clones in the hospital, precluding any possibility of assessing associations.

Conclusion

Increased resistance was observed in hospital-acquired isolates, and the association between hospital-acquired isolates and ESBL presence was significant, which is not unexpected. Isolates were genetically diverse and showed minimal clustering, suggesting that resistance may be due to horizontal transmission. Continuous efforts towards surveillance of the epidemiology and resistance patterns of circulating strains are required to monitor and guide antimicrobial stewardship, infection prevention and control (IPC) practises and empiric therapy.

Opsomming

Agtergrond

Escherichia coli en *Klebsiella pneumoniae* is alombekend om bloedstroominfeksies (BSI) te veroorsaak. Hierdie infeksies word grotendeels behandel met β-laktam antibiotika, soos kefalosporiene en “carbapenems”, Verhoogde β-laktam antibiotika weerstandigheid word toegeskryf aan plasmied-gemedieërde β-laktamases soos die “Extended-Spectrum β-lactamases” (ESBLs) en “carbapenemases”. Hierdie studie beskryf die antibiotiese weerstandsprofiele, uitkomste en epidemiologie van Gram negatiewe BSI’s in ‘n tersiêre hospitaal in Kaapstad, Suid-Afrika.

Metodes

E. coli (n=70) en *K. pneumoniae* (n=70) bakterieë vanaf kliniese monsters van Tygerberg Hospitaal tussen April 2015 en Maart 2016 was ingesluit. Spesie-identifikasie en antibiotiese-vatbaarheidstoetse was uigevoer as deel van roetine toetse. Pasiënt data was deur middel van mediese rekords verkry. ESBL en carbapenemase gene is met polimerasekettingreaksie (PKR) en DNA-volgordebepaling gekarakteriseer. Isolate is getipeer met rep-PKR en “Pulsed Field Gel Electrophoresis” (PFGE). Chi-kwadraat en Mann-Whitney toetse was toegepas om die statistiese betekenisvolheid van korrelasie te skat.

Resultate en bespreking

45% van pasiënte was manlik en 31.4% was pediatries. 66.4% van BSI was hospitaal-geassosieerd. 61.3% van die hospitaal-geassosieerd isolate was *K. pneumoniae*; 72.3% van die gemeenskaps-geassosieerde isolate was *E. coli*. 55.7% van *K. pneumoniae* en 15.7% van *E. coli* was kefalosporien-weerstandig (veronderstelde ESBL); een *K. pneumoniae* isolaat was carbapenem-weerstandig. ‘n Toename in antibiotiese weerstandiheid en ESBL-produksie was waargeneem in die hospitaal-geassosieerd isolate.

ESBL gene was teenwoordig in 35.7% van die isolate; 50.7% bevat β-laktamase gene en 13.6% bevat geen β-laktamase gene nie. TEM gene (98%) was grotendeels β-laktamases; 47.4% van die SHV gene was β-laktamases, 7% was ESBL en 45.6% was SHV gene waarvan die spektrum onbekend was. Isolate met SHV gene met onbekende spektrums was fenotipies vatbaar vir kefalosporiene, wat voorstel dat hierdie ensieme nie uitgebreide spektrum aktiwiteit gehad het nie. Verskeie β-laktamase gene was teenwoordig in 60% van *K. pneumoniae* isolate en 5.7% *E. coli* isolate respektiewelik. CTX-M gene was die mees algemeen ESBL gene en die meerderheid (91.3%) behoort aan groep 1, wat die

wêreldbekende CTX-M (CTX-M-15) bevat. CTX-M gene was grotendeels waargeneem in kombinasie (84.8%). Geen carbapenemase gene was gevind nie.

Molekulêre en fenotipiese weerstandigheid het in 95.3% van die isolate ooreengestem. Die 30-dag mortaliteit was 30%, met geen assosiasie tussen hospitaal-geassosieerde infeksie, of met ESBL produksie (molekulêr of fenotipies) nie. Molekulêre en fenotipiese weerstandigheid was geassosieer met hospitaal-geassosieerde isolate (P=0.001; P<0.001).

Beide tiperingstegnieke het aansienlike diversiteit bevestig tussen die isolate met minimale groepering. Dit dui daarop dat verskeie klone voorkom in die hospitaal, en dat enige moontlikheid om die assosiasies tussen tipe, weerstands-fenotipe of -genotipe of uitkoms bepaal kan word.

Gevolgtrekking

'n Toename in weerstandigheid was waargeneem in alle hospitaal-geasosieerde isolate. Die assosiasie tussen hospitaal geasosieerde islolate en die teenwoordigheid van ESBL's was statisties relevant. Isolate was geneties divers en het minimale groepering in identiteit vertoon, dit stel voor dat die verspreing horisontaal plaas kon gevind het. Verdere navorsing in epidemiologie en weerstandigheids patrone van verspreidende stamme is noodsaaklik om antimikrobiese verhoudings, infeksie beheer en empiriese terapie te kan bevorder.

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Acknowledgements

I would like to thank my supervisors, Prof Andrew Whitelaw and Dr Mae Newton-Foot for their guidance, mentorship and advice. Thank you for providing me with opportunities to grow and develop as a researcher. Thank you for all the time you have put in to make this possible.

Thank you to my parents for encouraging and supporting me throughout my academic career. Thank you for all the sacrifices you have made to make this possible. Thank you to my sister, Jessica, for her moral support and being my safeguard. Thank you to Nathan for your constant encouragement and positivity. And thank you to all my family and friends for their support.

To the technologists and consultants at the NHLS Medical Microbiology laboratory, thank you for the collection of isolates used in this study, as well as your assistance and training. Thank you to Nina du Plessis for all your assistance, and to all my fellow students in the division for your assistance, support and comradery. And a special thank you to Kristien Nel Van Zyl for her assistance with the translation of my summary.

I would like to thank the BARNARDS research group for the opportunity to be part of the research project and particularly for hosting me in Cardiff. It was a wonderful learning experience and your assistance with strain typing of my isolates is greatly appreciated. A special thank you to Rebecca Milton, Dr Maria Carvalho and Dr Mark Toleman for all your assistance and guidance.

Thank you Dr Moodley and Crystal Viljoen at the NICD for providing the control strains used in this study and Dr Innocent Karangwa at the Biostatistics unit from the Centre for Evidence-based Health Care at Stellenbosch University for his assistance with the statistical analysis of data. Thank you to the Sisters in the Infection Prevention and Control unit at Tygerberg Hospital, and particularly Sister Mocke, for their assistance in collecting patient information. Thank you to Wessel Kleinhans and Jeremy Goodway for their assistance in collecting isolate information and Dr Angela Dramowski for her advice regarding analysis and collection of clinical data.

Finally, thank you to the University of Stellenbosch and the Harry Crossley Foundation for providing personal funding towards this degree and to the NHLS Research Trust and the Harry Crossley Foundation for providing project funding.

List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
AST	Antibiotic Susceptibility Testing
BLAST	Basic Local Alignment Search Tool
BRICS	Brazil, Russia, India, China, and South Africa
CDW	Corporate Data Warehouse
CLB	Cell Lysis Buffer
CLSI	Clinical Laboratory Standards Institute
CRE	Carbapenem Resistant Enterobacteriaceae
CSB	Cell Suspension Buffer
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended-Spectrum β-lactamase
EU	European Union
HIS	Hospital Information System
HREC	Health Research Ethics Committee
ICU	Intensive Care Unit
IDT	Integrated DNA Technologies
IPC	Infection Prevention and Control
IRT	Inhibitor Resistant TEMs
kb	kilobase
LIS	Laboratory Information System
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic

NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NTC	No Template Control
PBP	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RE	Restriction Enzyme
REP	Repetitive Element Palindromic
RFLP	Restriction Fragment Length Polymorphism
SID	Simpson's Index of Diversity
ST	Sequence Type
TAE	Tris-Acetic acid-EDTA
TBA	Tryptone Bile Agar
TBE	Tris-Boric acid-EDTA
TE	Tris EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary Tract Infection
VNTR	Variable Number Tandem Repeat
WGS	Whole Genome Sequencing
WHO	World Health Organisation

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Chapter 1: Literature review

1.1 Antimicrobial resistance: an overview

Bacterial infections affect numerous people worldwide, including South Africans. Antibiotics have decreased the burden of bacterial infections and have become heavily relied upon [1]. There has been a notable increase in antibiotic resistance in bacteria and unfortunately, the growing resistance far outpaces the development of new therapies [2]. Drug resistance presents a treatment problem, as many bacteria have acquired one or more resistance mechanism. These strains are problematic due to the fact that they harbour multiple resistance genes encoding resistance to more than one type of antibiotic. Infections with these bacteria are difficult to treat due to the reduced number of treatment options [3].

The antimicrobial resistance rates vary widely across the globe, depending on the bacterial species, antibiotic group and location [4]. Many studies world-wide have cited an increase in resistance to third generation cephalosporins and carbapenems [4–6]. The increasing prevalence of resistance and multi-resistant bacteria has led to the use of combination therapies, expensive second-line antibiotics and older antibiotics, such as colistin, that are less appealing due to high levels of toxicity [7]. This may then place selective pressure on the bacteria and select for colistin resistant isolates, further exacerbating the resistance problem. Plasmid-mediated colistin resistance has already been cited and this is cause for great concern as the options for treatment are running out and development of truly pan-resistant bacteria may soon become a reality [8]. Expensive second-line antibiotics are not commonly used in low- and middle-income countries due to their cost, resulting in increased morbidity and mortality in these countries [1].

Extended-Spectrum β-lactamase (ESBL) producing bacteria were originally considered to be only healthcare-associated as infections and outbreaks of this organism were only seen in these settings. However, bacteria carrying these resistance mechanisms are becoming more common in community settings and have been seen in patients without previous exposure to healthcare settings [9].

Antibiotics are often overused, creating increased selection pressure for resistant bacteria and driving the evolution of resistance [10]. Studies have shown a link between antibiotic consumption and the emergence of resistant strains [10]. A study by Van Boeckel *et al.* looked at antibiotic consumption from 2000 to 2010 by analysing pharmaceutical sales data, and found that consumption of antibiotics increased by 35% over the investigated time period [1]. The largest absolute increase in consumption was observed in cephalosporins, broad-spectrum penicillins and fluoroquinolones; additionally cephalosporins and broad-spectrum

penicillins constituted 55% of the total antibiotics consumed [1]. They also noted significant increases in the use of carbapenems (45%) and polymyxins (13%) such as colistin [1]. This study also noted that consumption of antibiotics increased considerably in developing countries, with the highest rates being seen in the BRICS (Brazil, Russia, India, China, and South Africa) countries and French West Africa [1]. Distressingly, 76% of overall increase in global antibiotic use was attributed to the BRICS countries [1].

Other factors which contribute to antimicrobial resistance include the over the counter sale of antibiotics in some countries, self-medication with these drugs, non-adherence to the treatment recommended by doctors, which is common in Africa, and incorrect or unnecessary prescribing of antibiotics [10,11].

Antibiotics are extensively used in agriculture, particularly in livestock [10]. These antibiotics are used to promote growth and prevent infection, producing larger yields and higher quality product [10]. It is estimated that as much as 80% of the antibiotics sold in the United States of America are used by the livestock industry [10]. These antibiotics are ingested by humans when they consume these animal products, thereby increasing selection pressure within humans for antibiotic resistant bacteria [10]. Antibiotics are also excreted by animals in stool and urine, which along with the spraying of antibiotics on crops results in higher concentrations of antibiotics in the environment [10].

Several factors have been associated with increased risk of infection with an antibiotic resistant pathogen, including previous antibiotic use, recent or current hospitalisation, immunosuppressive drugs or disease, hospital overcrowding and international travel [11,12].

Infections with resistant organisms are costlier as patients have longer hospital stays and need expensive antibiotics. These infections may also result in prolonged exposure to invasive medical devices and procedures, affecting patient morbidity and further prolonging time spent in hospital. The hospitals need to spend money decontaminating the area when patients are infected with resistant bacteria, and ensure extra care is taken to prevent the spread of these resistant organisms to other patients. The increased prevalence of resistance combined with the absence of new antibiotics has resulted in improved efforts towards infection control as well as antibiotic stewardship [13]. The efficacy of antibiotics needs to be maintained so that we can continue to prevent infection in immunosuppressed patients and when performing invasive surgery and to treat bacterial infections [10].

Antimicrobial resistance has been described in a wide range of organisms, with increasing resistance in Enterobacteriaceae being of particular concern. Infection with these bacteria is frequent and development of multiple resistance mechanisms has seen therapeutic options

dwindling. Prevention of infections with Enterobacteriaceae may be particularly difficult as these bacteria are found in many environments and form part of the human microflora [14].

1.2 Enterobacteriaceae

Enterobacteriaceae are one of the largest groups of clinically important Gram negative bacilli. These organisms can be found in many environments, such as soil and water and also form part of the intestinal flora of various animals, including humans [14]. Clinically important species in this family include *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Shigella* spp. as well as *Salmonella* spp. This heterogeneous group of bacteria range from commensals to obligate pathogens. Some members of the Enterobacteriaceae family, such as *E. coli*, form part of our normal flora and usually only cause disease in immunocompromised hosts. Others such as *Shigella spp.*, are always associated with disease, i.e. obligate pathogens [14].

Enterobacteriaceae are a common cause of community- and healthcare- acquired infections, including Bloodstream Infections (BSI), Urinary Tract Infections (UTI), respiratory and wound infections [14,15]. Among the Enterobacteriaceae, *E. coli* and *K. pneumoniae* are frequently associated with bloodstream infections and commonly harbour β-lactam resistance mechanisms [16,17].

E. coli consists of a large diverse group of bacteria which occur naturally in the human gastro-intestinal tract. Most strains of *E. coli* are not known to cause illness in heathy hosts, although some strains are pathogenic and can cause serious illnesses, as a result of various virulence factors. Pathogenic *E. coli* may cause a variety of illnesses such as diarrhoea, UTIs, and pneumonia. *E. coli* is a common cause of bacteraemia and a major causative agent of food-borne illnesses [4]. *E. coli* is usually transmitted through ingestion of contaminated food, person-to-person contact, animal contact or contact with environments that are contaminated with faecal matter [18].

K. pneumoniae is said to be the most clinically relevant species within the *Klebsiella* genus as it is responsible for the majority of infections caused by *Klebsiella* species [19]. *Klebsiella* is often found in the gut of humans and animals and is known to be a cause of opportunistic infections in compromised hosts [20]. *K. pneumoniae* is frequently isolated in hospitals and has a notable ability to survive on hands, which facilitates its spread [19,20]. This species of bacteria often carries resistance genes such as β-lactamases, ESBLs and carbapenemases [21,22]. This characteristic makes infections with these bacteria more concerning and difficult to treat.

1.3 Bloodstream infections

Bloodstream infection (BSI), or bacteraemia, is defined as the presence of viable bacteria in the blood [23,24]. It is a major source of morbidity and death with mortality rates ranging from 10 to 70% [17,23,25,26]. A systemic review estimates bacteraemia to be amongst the top seven causes of death in European and North American countries, with 2 million episodes and 250 000 fatalities annually [27].

Bacteraemia may arise as a primary infection, or secondary to an infection at a defined anatomical site (e.g. urinary tract, lower respiratory tract) [25]. Patients who are immunocompromised or critically ill have an increased risk of developing a BSI [23]. BSIs can be classified into community- or hospital-acquired based on where the infection was contracted [28]. Studies have found that the different types of BSIs can differ in epidemiology, severity and mortality rate [17,23]. Sepsis arises when there is a systemic response from the host to the BSI [29]. This is characterised by increased heart and respiratory rate, elevated white blood cell count as well as an abnormally high or low body temperature [23]. 1.8 million cases of sepsis are reported every year, although this most likely an underestimation due to lack of reporting [23].

Over time, the aetiology of BSIs has changed; from Gram negative bacteria to Gram positive in the 1990s, and recently Gram negative bacteria have re-emerged as the predominant source of BSIs [28,30]. Enterobacteriaceae in particular are presently one of the major causes of BSIs [28].

Healthcare-associated infections have a higher incidence rate (at least double) in low income settings compared to high income settings [31]. African studies reported a prevalence of 15,5 patients per 100 compared to 7,1 reported by European studies and 4,5 reported by studies conducted in the United States of America. It has been estimated that healthcare-associated bacteraemia is the cause of 25 000 deaths of African children each year [32].

E. coli and *K. pneumoniae* are two major agents implicated in the cause of BSIs [33]. A study done in 2012 at Groote Schuur Hospital in South Africa, found that *E. coli* and *K. pneumoniae* were the most common Gram negative bacteria causing bacteraemia [17]. A retrospective study done at Tygerberg Hospital in South Africa described BSIs in paediatric patients from 2008 to 2013 [32]. This study found that Gram negative bacteraemia dominated, with *K. pneumoniae* and *E. coli* being the most common Gram negative pathogens. The study also described an association with healthcare-associated bacteraemia and Gram negative pathogens as well as a higher antimicrobial resistance in bacteria causing healthcare-associated bacteraemia. BSIs with Gram negative bacteria were associated with higher mortality, as observed in other studies carried out in Africa [34,35]. Concerningly, this

study observed a high rate of antimicrobial resistance among community-associated bacteria, particularly in *K. pneumoniae* and *E. coli.*

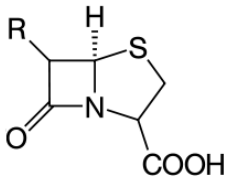
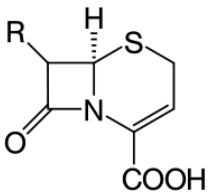
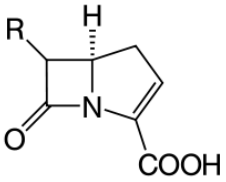
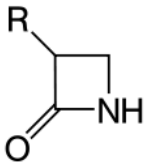
A study conducted on Kenyan children found that healthcare-associated BSIs had a mortality rate of 53%, compared to 24% in community-acquired BSIs [35]. They also observed a higher mortality rate when bacteraemia was caused by Gram negative bacteria, 61%, versus 38% for Gram positive infections.

It is necessary to identify the bacteria responsible for the BSI in order to effect appropriate treatment. These bacteria are usually identified by blood culture, as this is the current gold standard [23]. Blood from the patient is inoculated into the appropriate culture medium and incubated. This technique takes between 24 and 72 hours to obtain cultures and additional time to identify the bacterium and obtain the antibiogram, depending on the testing platform used. For this reason, empiric treatment is often relied on until more information is available, however some countries rely on empiric treatment alone if diagnostic facilities are not available [36]. The emergence and spread of various antibiotic resistance mechanisms has made the treatment of BSIs increasingly difficult and detrimental to patients [37]. This is because patients are often given incorrect empirical treatment leading to increased morbidity and mortality, especially when causative bacteria harbour resistance mechanisms such as ESBLs and carbapenemases [38]. It is therefore important to have current information regarding the epidemiology of circulating pathogens as well as their resistance rates.

1.4 β-lactam antibiotics

Infections with Enterobacteriaceae are usually treated using β-lactam antibiotics. β-lactams are a large group of antibiotics, which are all characterised by a β-lactam ring. These antibiotics are one of the oldest and most widely used antibiotic families [18,39]. The different groups within this family are distinguished by the structure of additional rings attached to the β-lactam ring [20]. Antibiotics in this group include penicillins, monobactams, cephalosporins and carbapenems. Table 1.1 summarizes the different classes of β-lactams.

Table 1.1: The different classes of β-lactam antibiotics and some of their properties (Adapted from Versalovic (2011) [18] & Vasconcillos (2010) [40])

Class	Penicillins (penams)	Cephalosporins (cephems)	Carbapenems (penems)	Monobactams
Core structure				
	β-lactam ring fused to a thiazolidine ring.	β-lactam ring fused to a dihydrothiazine ring.	β-lactam ring fused to a hydoxyethyl side chain.	β-lactam ring with various side-chains.
Spectrum of activity	Most Gram positive and some Gram negative and anaerobic organisms.	Grouped by generations based on their activity. Third generation (broad spectrum) are less active than first against Gram positive cocci but are more active against Enterobacteriaceae and *P. aeruginosa*.	Widest spectrum. Excellent activity against Gram positive species. Most potent β-lactam against anaerobes.	Active against aerobic Gram negative bacteria, including Enterobacteriaceae.
Examples	Ampicillin Cloxacillin Amoxicillin	Cefotaxime Cefepime Ceftazidime	Imipenem Meropenem Ertapenem	Aztreonam Tigemonam Tabtoxin

β-lactams act on a vital component of the bacterial cell, the cell wall. Specifically, they bind to enzymes called Penicillin-Binding Proteins (PBPs) which form an integral part of cell wall construction. These enzymes facilitate the final cross-linking of cell wall structures *N*-acetylmuramic (NAM) acid and *N*-acetylglucosamine (NAG); these two molecules form the building blocks of peptidoglycan and they form a rigid mesh which gives the cell wall its strength [14]. Binding of β-lactams to these enzymes results inhibition of cross-linkage and leads to the loss of the cell wall's integrity, ultimately leading to cell lysis [39]. β-lactams may also have a bactericidal effect by triggering autolytic enzymes in the cell envelope due to accumulation of cell wall precursors [41].

Penicillins were some of the first antibiotics discovered, changing the treatment of infections. Penicillins consist of a thiazolidine ring attached to a β-lactam ring [41]. While they are naturally synthesized by many *Penicillium* spp., many semi-synthetic variations are also manufactured [41]. Penicillins are active against many Gram positive bacteria, and have some

limited activity against Gram negative bacilli. Examples of these antibiotics include ampicillin and amoxicillin.

Monobactams are monocyclic β-lactams that consist of a single β-lactam ring with various attached side chains [41]. They are narrow spectrum antibiotics which target aerobic Gram negative bacteria [42]. Monobactams are stable against most plasmid and chromosomally mediated β-lactamase enzymes which hydrolyse β-lactam antibiotics [42]. Currently, aztreonam is the only monobactam in clinical use [41].

Cephalosporins are a group of β-lactams characterised by a six membered dihydrothiazine ring attached to the β-lactam ring [41]. Antibiotics in this group can be further classified into different generations (first to fifth) based on their spectrum of activity and stability against β-lactamases [43,44]. First generation cephalosporins, such as cefazoline, display activity focused on Gram positive bacteria. Second generation cephalosporins maintain activity against Gram positive cocci, but they have increased activity against Gram negative bacilli. Cefoxitin is an example of a second generation cephalosporin. Third generation cephalosporins demonstrate a considerable increase in activity against Gram negative bacilli, however some members have decreased action against Gram positive cocci. Commonly used cephalosporins of this generation include cefotaxime and ceftazidime. Agents classified as fourth generation cephalosporins have the widest spectrum of activity including most Gram negative bacilli and Gram positive cocci. These cephalosporins are also less susceptible to hydrolysis by β-lactamases. A well-known example of a fourth generation cephalosporin is cefepime. Third and fourth generation cephalosporins are considered to be extended-spectrum cephalosporins. The fifth generation cephalosporins, such as ceftaroline, are active against methicillin resistant staphylococci and also have broad-spectrum activity against Gram negative bacteria [44].

Penicillins and some cephalosporins are often combined with β-lactamase inhibitors to increase their activity against Gram negative bacilli, particularly those producing β-lactamases. β-lactamase inhibitors alone are weakly bactericidal but form a good adjuvant when combined with β-lactam antibiotics. This is due to their ability to breakdown β-lactamases, which prevents the hydrolysis of the β-lactam antibiotic, thereby retaining the activity of the β-lactam antibiotic despite the presence of a β-lactamase [44,45]. Examples of β-lactamase inhibitors include clavulanic acid, sulbactam and tazobactam. Examples of commonly used β-lactam-β-lactamase inhibitors include piperacillin-tazobactam and amoxicillin-clavulanate. These are a good carbapenem-sparing treatment option [45].

Carbapenems are said to be one of the most important β-lactam antibiotics due to their broad spectrum of activity and high potency [46]. This is due to their efficient penetration

though the bacterial outer membrane as well as their high affinity for PBPs [44]. This group has a five membered ring attached to the β-lactam ring but it differs structurally from other β-lactams by its hydroxyethyl side chain [41]. This antibiotic is usually used to treat patients infected with multi-resistant organisms, as it is less affected by certain resistance mechanisms, including β-lactamases [47]. This is as a result of the antibiotic's unique structure [18]. Commonly used carbapenems include imipenem, meropenem and ertapenem [48].

1.5 β-lactam resistance

Bacteria often develop strategies to persist in the presence of antibiotics. This is a natural phenomenon in bacteria, but is accelerated by the increased use of antibiotics in humans and animals through natural selection of resistant bacteria [49]. These methods of resistance include antibiotic inactivation or modification (usually by enzymes), target alteration or preventing accumulation of the antibiotic inside the cell by decreasing permeability of the cell membrane/wall or increasing efflux [50]. The emergence of these resistance mechanisms in a bacterial population is associated with certain factors, the most important of which being mutation rate, horizontal transmission of resistance genes and the fitness costs that these gene/s could impose on the bacterium [51]. Increasing resistance in members of the Enterobacteriaceae family has resulted in multi-resistant and in some cases pan-resistant species i.e species resistant to all tested antibiotics. These once simple to treat organisms now pose a major problem as treatment options are considerably reduced due to increasing resistance [12].

β-lactam resistance in Enterobacteriaceae is most often due to the production of β-lactamase enzymes. These enzymes confer resistance to β-lactam antibiotics by hydrolysing the chemical bonds within the antibiotic's structure, specifically the bonds within the β-lactam ring [52] (Figure 1.1). This breaking of the β-lactam ring causes the loss of the activity or binding affinity of the β-lactam [18,52]. β-lactamases also belong to the same family of serine proteases as PBPs [14].

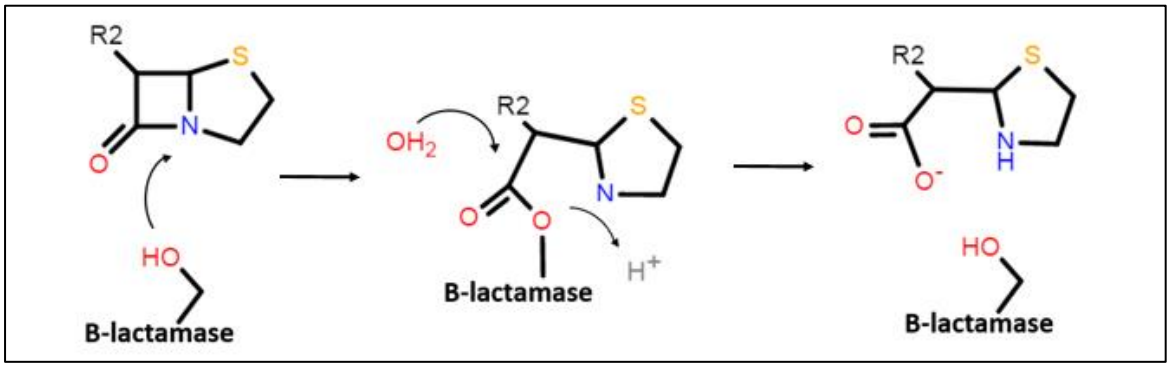


Figure 1.1: β-lactamase activity. β-lactamases hydrolyse β-lactam antibiotics which results in breaking of their β-lactam ring and consequently results in their loss of activity.

β-lactamases can be classified using two general schemes, the Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional classification system [22]. The Ambler classification scheme separates β-lactamases into four major classes, A to D, based on amino acid homology. Classes A, C and D are made up of serine β-lactamases, while class B is made up of metallo-β-lactamases [22]. The Bush-Jacoby-Medeiros classification scheme groups β-lactamases by their functional similarities. This scheme originally divided β-lactamases into 4 groups but has now been revised to 3 groups with multiple sub-groups [53]. While structural groupings like the Ambler scheme are simple and more objective, functional groupings assist in relating the enzyme's properties to their clinical role [53].

It has long been known that *K. pneumoniae* and *E. coli* produce β-lactamases, such as SHV-1 and TEM-1 respectively, which are able to hydrolyse amino-penicillins (such as amoxicillin and ampicillin), but not cephalosporins [9]. The cephalosporins were specifically designed to withstand hydrolysis by these enzymes and overcome the β-lactamase mediated resistance. [9].

1.5.1 Extended-spectrum β-lactamases

Extended-Spectrum β-lactamases (ESBLs) are β-lactamases which are able to hydrolyse an extended-spectrum of β-lactamase antibiotics including penicillins, 1st-, 2nd- and 3rd-generation cephalosporins and the monobactam, aztreonam. It is suggested that this resistance mechanism developed as a result of increased selection pressure due to the wide-spread use of extended-spectrum cephalosporins [54]. The first published report of this type of β-lactamase was in 1983, and since then many other ESBLs have been discovered [22]. ESBLs have been found in a range of Enterobacteriaceae, but they are most common in *K. pneumoniae* and *E. coli* [55]. Bacteria have developed two main approaches for the development of ESBLs, namely by the expansion of activity of β-lactamases and by acquiring genes encoding enzymes with ESBL activity, which can occur alone or in combination [56]. Different families of ESBLs have been described based on amino acid sequences, and the most commonly detected and clinically significant ESBLs belong to the TEM, SHV and CTX-M families [57].

TEM family

All TEM-ESBLs are derivatives of the β-lactamases TEM-1 and TEM-2. The TEM family is named after Temoneira, the first patient from whom a β-lactamase producing bacterium was isolated [22], in 1965. TEM-ESBLs have developed as a result of amino acid substitutions which have altered their substrate profile and isoelectric point. Some amino acid residues along the *blaTEM* gene have been said to more important for changes to the enzyme's

phenotype, particularly to create the ESBL phenotype [58]. These changes can be seen in the figure below (figure 1.2).

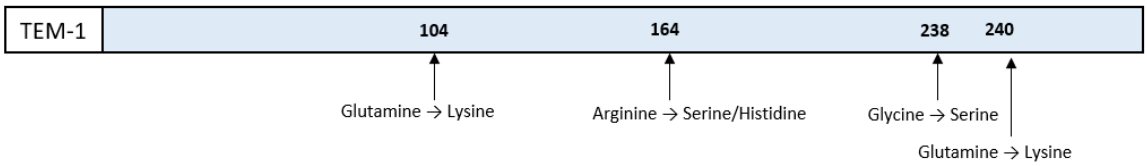


Figure 1.2: Amino acid substitutions in the TEM gene that are important to the ESBL phenotype. Adapted from Bradford (2001) [58].

SHV family

The SHV β-lactamases are named after the sulfhydryl variable. The progenitor of this family, SHV-1, is commonly found in *K. pneumoniae* [59]. The first ESBL-SHV, SHV-2, was discovered when an SHV enzyme was found to hydrolyse a third generation cephalosporin, cefotaxime [58]. Sequencing revealed a mutation resulting in a serine residue in place of a glycine at position 238. This mutation accounted for the extended-spectrum properties of this β-lactamase and is therefore found in the majority of *blaSHV* genes possessing an ESBL phenotype [58].

CTX-M family

The CTX-M family is a more recently discovered group of ESBLs, however they have disseminated worldwide and have quickly become the most prevalent ESBL [60]. CTX-M ESBLs were named due to their preferential hydrolysis of the antibiotic cefotaxime [59].

blaCTX-M genes are not closely related to *blaSHV* or *blaTEM* genes as they only show a 40% sequence similarity [55,59]. CTX-M ESBLs and β-lactamases of *Kluyvera* spp. are closely related and the adjacent gene sequences of both resistance genes also show similarity [59]. This suggests that CTX-M genes originated in *Kluyvera* spp. and mobilised onto a mobile element facilitating their dissemination to other bacterial species. CTX-M ESBLs also differ in their preferred substrates, as TEM and SHV ESBLs preferentially hydrolyse ceftazidime, while CTX-M ESBLs prefer cefotaxime [9].

CTX-M enzymes can be clustered into 5 sub-groups based on their amino acid sequences, namely CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CTX-M25 (Figure 1.3) [60]. These groups have been named after the first member in the group to be described [56]. The amino acid homology of different CTX-M ESBL groups differs by 10% or more [9]. CTX-M 15, belonging to group 1, has become the most commonly found CTX-M ESBL worldwide [9].

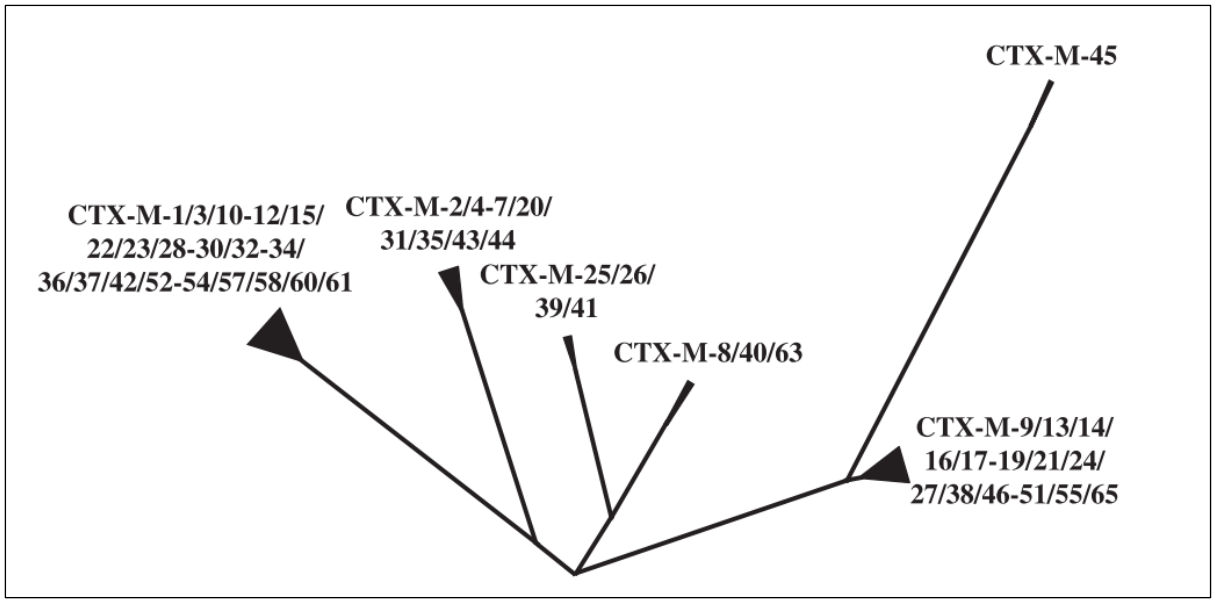


Figure 1.3: A tree diagram depicting the sub-groups of CTX-M enzymes [56]. The branches represent the five sub-groups of CTX-M ESBLs. From left to right they are CTX-M-1, CTX-M-2, CTX-M-25, CTX-M-8 and CTX-M-9.

1.5.2 Carbapenemases

Infections with ESBL producing organisms have a few treatment options, the most popular being β-lactam/β-lactamase inhibitor combinations and carbapenems [22]. Carbapenems are becoming increasingly favoured, leading to increasing carbapenem use and subsequent selection pressure for carbapenem resistance [61,62].

Bacteria may become resistant to carbapenems in a few ways, one of these being production of carbapenemases. This mechanism of resistance is potentially more concerning than the others, as it is more easily transferred to other bacteria via plasmids [63]. Other carbapenem resistance mechanisms include permeability defects and efflux pumps, sometimes combined with increased expression of β-lactamase enzymes that have limited carbapenemase activity in isolation [19]. Production of carbapenemases is considered to be the most clinically significant mechanism of carbapenem resistance [64].

Carbapenemase enzymes were first identified in the 1980s and since then have successfully disseminated globally [15]. Carbapenemase enzymes have a similar mode of action to other β-lactamases, and are considered to be the most powerful of the β-lactamases as they are able to hydrolyse most β-lactam antibiotics [63]. There are a number of different types of carbapenemase enzymes and they can be divided into different classification groups based on their amino acid homology [65]. The acquired carbapenemases can be grouped into the Ambler classes A, B and D [66]. The different carbapenemases found so far include KPC, NDM, VIM, GES, SME, OXA-48, IMP, NMC, GIM, SPM, SIM, IMI, CMX and CcrA.

Morrill *et al.* stated that KPC, NDM, IMP, VIM and OXA-48 are the mostly commonly found carbapenemases in Enterobacteriaceae and are also those with the greatest clinical importance [7]. The relatedness of these carbapenemase families has been demonstrated in the phylogenetic tree below (figure 1.4) and notable characteristics of these families have been laid out below in table 1.2.

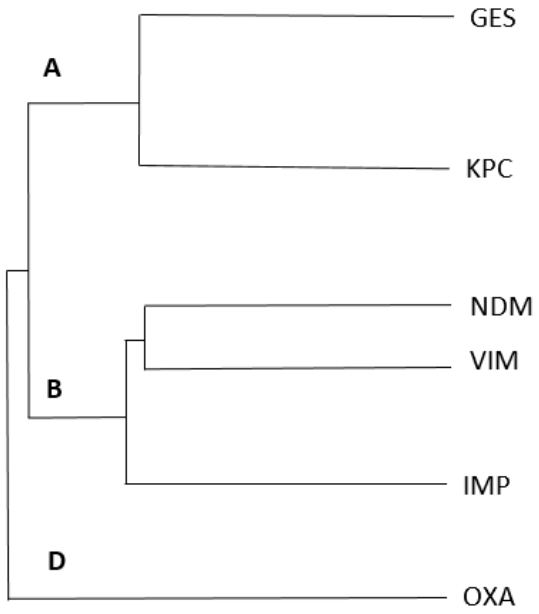


Figure 1.4: Phylogenetic tree demonstrating the relatedness of common carbapenemase genes. A, B and D refer to the Ambler classes. Adapted from Diene and Rolain (2014) [67].

Table 1.2: Characteristics of common carbapenemase families.

Carbapenemase family	First discovered	Class	Hydrolysis profile
KPC	Northern America 1996	Ambler class A Serine β-lactamases	Penicillins, carbapenems, all cephalosporins and aztreonam
IMP	Japan 1991	Ambler class B Metallo-β-lactamases	All β-lactams except aztreonam
VIM	Italy 1997	Ambler class B Metallo-β-lactamases	All β-lactams except aztreonam
NDM	India 2008	Ambler class B Metallo-β-lactamases	All β-lactams except aztreonam
OXA-48	Scotland 1993	Ambler class D Oxacillinases	Penicillins, carbapenems and first generation cephalosporins

The spectrum of hydrolysis of β-lactam antibiotics depends on the specific carbapenemase enzyme [48,66]. For example, aztreonam is hydrolysed by NMD, VIM and IMP, but not by KPC enzymes. KPC confers high-level resistance to carbapenems, whereas IMP and OXA-48 carbapenemases may need efflux pumps or low outer membrane permeability additionally to achieve high-level carbapenem resistance [66]. Carbapenem Resistant Enterobacteriaceae (CRE) are particularly concerning as they often carry additional resistance genes making them multi-resistant [68]. The prevalence of these enzymes has increased significantly over the past decade, warranting increased concern [69].

A review of carbapenemase resistance in South Africa reported that NDM and OXA-48 carbapenemases are most commonly observed [64].

KPC

The first KPC carbapenemase, KPC-1, was isolated from a *K. pneumoniae* isolate in Northern America [48]. The KPC enzymes belong to class A of the Ambler classification and are inhibited by clavulanic acid [66]. They have the ability to hydrolyse penicillins, carbapenems, cephalosporins and aztreonam [48]. KPC carbapenemases are powerful enzymes and do not need other resistance mechanisms to achieve high-level resistance [48]. There are 11 variants (KPC-2 to KPC-12) of the first KPC enzyme, KPC-1 [15].

IMP

IMP enzymes are grouped into class B of the Ambler classification, or the metallo-β-lactamases of the Bush-Jacoby-Medeiros classification. They were the first acquired metallo-β-lactamases, detected in Japan in 1991 [15]. Since then 33 variants have been identified [15]. They have a broad substrate profile which includes all β-lactams with the exception of aztreonam [48]. IMP carbapenemases are often found as part of gene cassettes situated inside integrons, along with other resistance genes [15].

VIM

VIM-1 was first reported in Verona, Italy in 1997. They are metallo or class B β-lactamases. They have a similar substrate profile to IMP enzymes, although they share little sequence similarity [48]. Like IMP enzymes, they are also found on gene cassettes inside integrons [15]. There are 33 known variants of this carbapenemase [15].

NDM

This type of metallo-β-lactamases was first discovered in 2008, isolated from a patient previously hospitalised in New Delhi, India [15]. This emerging group of carbapenemases has since been identified worldwide and 13 variants have been described [70]. These carbapenemases hydrolyse all β-lactams excluding aztreonam [70]. Many NDM producing

strains have been found to express additional resistance mechanisms such as ESBLs, AmpC cephalosporinases and other carbapenemases [15].

OXA-48

OXA-48 carbapenemases are classified as oxacillinases or Ambler class D β-lactamases. OXA-48 enzymes hydrolyse penicillins and carbapenems, but not extended-spectrum cephalosporins. Isolates harbouring OXA-48 carbapenemases usually do not show high levels of resistance to carbapenems [15]. This can make detection of these carbapenemases difficult in routine microbiology laboratories and may result in the underestimation of their prevalence [15].

1.5.3 Epidemiology of β-lactam resistance in Enterobacteriaceae

The distribution of resistant bacteria varies geographically, and differences may be due to patterns of antimicrobial use/misuse, healthcare practices, as well as environmental factors [4]. Understanding local epidemiology is important to guide empiric treatment, to understand potential transmission patterns, and to establish priorities for research and action. It can also assist in identifying risk factors and thereby strategies to prevent infection.

The European Centre for Disease Prevention and Control (ECDC) compiled a report from the European Antimicrobial Resistance Surveillance Network (EARS-Net) which included antimicrobial resistance data from 30 European Union countries from 2012 to 2015 [4]. This report noted that resistance to third generation cephalosporins increased significantly in *E. coli* and *K. pneumoniae* and a large portion of these isolates produced an ESBL. For *E. coli* in 2015, the resistance to third generation cephalosporin's ranged from 1.7% (Iceland) to 38.5% (Bulgaria), while the population weighted mean for all included countries was 13.1%. For *K. pneumoniae* in 2015, the resistance ranged from 0% (Iceland) to 75.5% (Bulgaria), with a mean of 30.3%. Overall, both organisms showed an increase in resistance to third generation cephalosporins between 2012 and 2015; from 11.9 % to 13.1 % in *E. coli* and from 25.8 % to 30.3 % in *K. pneumoniae* [4].

The rate of resistance to carbapenems observed in *E. coli* was quite low, with a population weighted mean of 0.1%. Many countries including Iceland, Belgium, Bulgaria, Finland, Lithuania and Slovakia saw no resistance to carbapenems in *E. coli.* Greece and Romania had the highest levels of carbapenem resistance in *E. coli* at 1.2% and 1.9% respectively. Carbapenem resistance in *K. pneumoniae* showed large variation between countries. Many countries reported resistance below 1% with a few countries, such as Iceland, Finland, Sweden and Lithuania reporting 0% resistance in 2015. The population weighted mean saw a significant increase from 6.2% in 2012 to 8.1% in 2015. Romania and Italy showed higher levels with 24.7% and 33.5% resistance respectively. Greece showed a concerningly high

level of resistance at 61.9%. It was also observed that generally countries with higher levels of resistance to a combination of third generation cephalosporins, aminoglycosides and fluoroquinolones also showed higher levels of carbapenem resistance.

A non-systematic literature review looking at ESBL producing Enterobacteriaceae in Africa reviewed 65 studies published from 2008 to 2012 and found an ESBL prevalence of 1.3 to 96% across Africa [71]. This review included studies with various specimens. Some of the included studies were carried out in hospital settings only, with others in hospital and community settings. A summary of the prevalence of the ESBLs found in different African countries can be seen in the table below (table 1.3).

Table 1.3: ESBL prevalence in African countries based on data from a review by Storberg (2014) [71].

	Country	ESBL prevalence (%)
Northern Africa	Algeria	16.4 – 31.4
	Egypt	11 – 42.9
	Guinea-Bissau	32.6
	Morocco	1.3 – 7.5
	Libya	16
	Tunisia	11.7 – 77.8
Eastern Africa	Ethiopia	62.8
	Kenya	37.4
	Rwanda	5.9 – 38.3
Central Africa	Cameroon	17.2 – 82.8
	Central African Republic	11.3
Western Africa	Ghana	49.4
	Mali	63.4 – 96
	Niger	40
	Nigeria	10.3 – 27.5
	Senegal	10
Southern Africa	South Africa	8.8 – 13.1

A meta-analysis of the available literature about the prevalence of ESBLs in Enterobacteriaceae in East Africa found rates of between 30.9-61.7%, with an average of 42% [11]. More specifically, the ESBL proportion estimate for the specific countries were Tanzania 38.8%, Kenya 45.8%, Ethiopia 30.9%, Uganda 61.7%, and Rwanda 38.3%.

A study in Ghana collected Enterobacteriaceae isolates from various sites in 2008 [72]. Overall, they found an ESBL prevalence of 49.3%. In *K. pneumoniae* and *E. coli* specifically, the prevalence was 61.5% and 43% respectively.

A study conducted in Morocco in 2013 examined the prevalence of ESBLs and carbapenemasas from rectal swabs [73]. The prevalence of ESBL producing Enterobacteriaceae was found to be 42.8%; for *E. coli* and *K. pneumoniae* the prevalence was 48.5% and 39.4% respectively. The prevalence of carbapenemases was 12.8%, all of which were OXA-48 carbapenemases.

A similar study examining the prevalence of ESBLs in Enterobacteriaceae from rectal swabs of children in Gabon, found an overall carriage rate of 45% [74]. Interestingly, this carriage rate increased with length of stay before the rectal swab was collected. When sampling at 48 hours after admission, the prevalence was 33.6%, this increased to 46.5% by 3-4 days, 66.6% by 5-6 days and 94.1% when swabbed 7 or more days after admission. This indicates possible nosocomial transmission of ESBL producing Enterobacteriaceae [74].

A report in 2011 that looked at BSIs at public sector hospitals across South Africa, found that 3-17% of *E. coli* isolates and 55-74% of *K. pneumoniae* isolates were ESBL producers. However the majority of these isolates (96-100%) were susceptible to carbapenems [6]. The specific data on ESBL production is not reported reliably, and therefore ESBL production is often inferred from cephalosporin resistance. This study also looked at rates of resistance in Tygerberg Hospital and found that 11% of *E. coli* isolates were ESBL producers (vs 16% average for all sites included in the study) and all *E. coli* were susceptible to carbapenems [6]. The prevalence of ESBLs was much higher in *K. pneumoniae* at 56%, and similarly, all isolates were susceptible to carbapenems.

The National Institute for Communicable Diseases (NICD) and Corporate Data Warehouse (CDW) at the National Health Laboratory Service (NHLS) compiled a report which examined antimicrobial resistance trends in South African hospitals [5]. The data was obtained from results generated by public health laboratories across South Africa for the year of 2015. The report stated that it did not see a significant increase in the resistance to the β-lactam group of antibiotics (including third generation cephalosporins and carbapenems) for *E. coli* from 2014 to 2015. Resistance to third generation cephalosporins was assumed indicative of ESBL production and was found to be 22% overall for *E. coli*. Levels of carbapenem resistance in *E. coli* remained low, ranging from 0% to 4%, with the majority of sites reporting a 0% resistance rate. Overall, antimicrobial resistance in *K. pneumoniae* was much higher, with resistance to third generation cephalosporins at 69%. The resistance rates to the carbapenem, ertapenem, remained unchanged from the previous year at 4%, while for meropenem and imipenem it increased from 3% to 6%. The majority of carbapenem resistant isolates (for all bacterial species) were submitted to the NICD to confirm the presence of carbapenemases and identify the type of carbapenemase. The majority (63%)

of the isolates were *K. pneumoniae* and the most common carbapenemase gene isolated was NDM (50%) followed by OXA-48 (27%).

A meta-analysis study examining the ESBL epidemiology in Enterobacteriaceae in East Africa, found CTX-M ESBL genes to be the most common (45.4-88.5%) followed by TEM (16-55%) and then SHV (3-64%) [11].

An investigation into the carriage of ESBLs in Enterobacteriaceae from rectal swabs of children in Gabon, found CTX-M to be the most commonly found ESBL [74]. The specific prevalence of genes found in *K. pneumoniae* was 89.6% CTX-M, 70.1% SHV and 85.1% TEM. For *E. coli,* the prevalence was 86.7% CTX-M, 6.7% SHV and 56.7% TEM. The most commonly found CTX-M was CTX-M-15.

A study carried out in South Africa in 2004 looked at ESBL genes present in *K. pneumoniae* isolates identified as ESBL producers by phenotypic testing [75]. This study gathered isolates from various sites across South Africa. SHV and TEM β-lactamases and ESBLs were the most common ESBLs, which were most commonly found together (66% of the isolates). Unfortunately, they did not distinguish between β-lactamase and ESBL TEM and SHV genes. Interestingly, no CTX-M enzymes were found in the isolates. This is different to other studies conducted which report CTX-M as the most common ESBL.

A study conducted in the Eastern Cape of South Africa from 2011- 2014 looked at ESBL production and screened for ESBL genes [76]. This study focussed on *Klebsiella* species, and found that 69% of *K. pneumoniae* isolates phenotypically presented as ESBL producers by disc diffusion. When examining the prevalence of ESBL genes in these isolates, they found most common profile was TEM, SHV and CTX-M (56%) followed by SHV alone (16%). No isolates contained carbapenemase genes, although only NDM and KPC genes were screened for. The study calculated the prevalence of TEM, SHV and CTX-M genes alone or in combination over the study duration, the total prevalence of TEM genes was 67%, SHV was 77% and CTX-M was 57%.

Comparison of results from different studies is challenging as reporting systems differ as well as study design. Variation may be seen as a result of the methods used to estimate resistance. Some studies report on phenotypic methods while other use molecular methods or a combination of both [11]. Site of infection and type of specimen may also play a role in the estimates of ESBL prevalence. It has also been said that stools tend to show a higher prevalence of ESBLs [71]. The setting of the study may also influence the estimates, and in hospital settings the particular ward may also influence the prevalence of ESBLs [11].

1.5.4 Molecular epidemiology of antibiotic resistance in *E. coli* and *K. pneumoniae*

Many studies have investigated the relationship between resistance genes and certain strains of *E. coli* and *K. pneumoniae*, known as Sequence Types (STs). STs have identical allelic profiles and may be considered bacterial clones. Specific clones carrying resistance genes have spread globally, these clones are considered to be highly successful or high-risk clones [51]. Certain STs of *E. coli* and *K. pneumoniae* commonly harbour specific resistance genes or combinations of resistance genes [77]. It has been suggested that resistance plasmids are essential in the rapid spread of high-risk clones and that these clones harbour resistance genes and therefore play a major role in the spread of these genes [51,77]. The resistance genes are spread vertically from generation to generation by the bacterium and can also be transferred horizontally to other bacteria [77].

Certain STs of *E. coli* , such as ST648, ST617, ST167, ST410, ST224, and ST117, have been linked to ESBL production [78]. Most notably *E. coli* ST131 has been linked to the worldwide dissemination of the CTX-M-15 gene [77,78]. This clone is known to carry additional resistance determinants and is therefore multi-drug resistant [51]. Studies have shown the increasing prevalence of this clone, not only in clinical settings but also in the community [78].

K. pneumoniae STs have also been linked to carriage of specific resistance genes, such as the association between ST258 and the carbapenemase KPC [77]. Studies have also found certain β-lactamase genes to be associated with one another as well as with other resistance genes, such as CTX-M-15, TEM-1, OXA-1 and the aminoglycoside modifying enzyme aac(6')-Ib-cr [79].

In some countries, resistant isolates mostly belong to these successful clones, while in others the resistant isolates are more diverse [77]. For example, in some countries the *E. coli* population is dominated by the clone ST131, while in other countries several clones are found [77]. It has been highlighted that even specific widespread clones may harbour different resistance genes based on their environment [77]. While there are trends in clonal lineages and the resistance genes they harbour, each country may have its own frequency of clones and resistance. This highlights the epidemiological importance of investigating the sequence type and genes conferring drug resistance in local populations.

Various strain typing techniques can be used to elucidate the population structure and clonal relatedness of circulating strains and may also assist in identifying the source of infections, which may be particularly helpful during an outbreak [80]. Strain typing of isolates in a particular setting is also helpful for surveillance as well as evaluating the efficacy of the local Infection Prevention and Control (IPC) strategies [81]. There are a number methods available, with molecular methods taking the forefront, due to their higher discrimination and ability to

type unculturable bacteria [81]. Frequently used molecular typing techniques make use of DNA-based methods that investigate chromosomal or plasmid DNA using probes, restriction enzymes, Polymerase Chain Amplification (PCR) and sequencing [81]. Some commonly used strain typing techniques include rep-PCR, Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST) and Variable Number Tandem Repeat (VNTR) analysis. The development of newer technologies such as microarrays and Whole Genome Sequencing (WGS) has greatly enhanced strain typing. Each strain typing method has different performance variables, such as discrimination, throughput, cost, speed and availability, which are considered before implementing a technique [81]. Often a combination of techniques is used to increase reliability of results.

1.6 Conclusion

Therapeutic decisions need to be made based on local epidemiology. Surveillance systems need to be established in order to determine the burden of infections with resistant bacteria, the specific risk factors for their acquisition and the outcome of infection with these bacteria [11]. This type of data from other settings may not be transferable to more resource limited African settings; therefore, surveillance studies need to be undertaken.

Action needs to be taken to ensure antibiotics are used as effectively and sparingly as possible. A balance must be found between curing infections now and conserving antibiotics so that they may also be effective for future use. It is important to make sure patients are receiving optimal doses of antibiotics and that antibiotic treatment is de-escalated to narrower spectrum agents or monotherapy after receiving species identification and antibiotic susceptibility results from the laboratory.

There is a great need to acquire as much information as possible about the epidemiology in our local area as well as the specific risk factors associated with these infections and place great importance on the value of infection prevention and control, so that we can prevent these infections as far as possible, treat them as effectively as possible and prevent their spread.

Aim and objectives

The aim of this study was to describe the epidemiology of *E. coli* and *K. pneumoniae* strains isolated from blood cultures at Tygerberg Hospital over the period of one year.

Objectives:

1. Detect and identify ESBL genes in the isolates using a PCR assay
2. Detection and identification of carbapenemase genes by PCR
3. Perform strain typing on collected isolates using rep-PCR and PFGE
4. Determine whether there is a link between strain type and resistance mechanisms
5. Differentiate community-acquired and healthcare-acquired infections and investigate the shared features among these groups.

Chapter 2: Prevalence and susceptibility of collected isolates

1. Introduction

Bloodstream infection (BSI), or bacteraemia, is defined as the presence of viable bacteria in the blood [23,24]. It is a notable cause of morbidity and mortality, and has fatality rates ranging from 10 to 70% [17,23,25,26]. BSIs can be categorised by where they were acquired, causative microorganism and focus of infection [82].

One method of classifying BSIs is based on where the infection was contracted, namely community- or hospital-acquired [28]. Community-acquired infections are those which were present or incubating at the time of admission to hospital, while hospital-acquired infections are those which occur after admission to the hospital, typically defined as acquisition 48 hours or more after admission [82]. These different types of BSIs can differ in epidemiology, severity and mortality, and importantly may influence the choice of empiric therapy [17,23].

BSIs may also be classified by the type of microorganism, and the microorganism/s causing infection may differ between settings [82]. Recently Gram negative bacteria, and the Enterobacteriaceae in particular, have re-emerged as a predominant cause of BSIs [28]. *Escherichia coli* and *Klebsiella pneumoniae* are two organisms implicated as major causes of BSIs [33]. Studies conducted in South Africa have found *E. coli* and *K. pneumoniae* to be the most common causes of Gram negative bacteraemia [17,32].

Bacteraemia may arise as a primary infection, or secondary to an infection at a defined anatomical site. The focus of infection may also be used to classify bacteraemia. Common foci of BSIs include urinary tract infections and lower respiratory tract infections [82]. Hospital-acquired BSIs are commonly associated with central venous catheters [82].

Identification of the causative organism is essential for effective treatment of BSIs, and is usually done by blood culture. Blood from the affected patient is cultured and the cultured organism is identified and tested for antibiotic susceptibility. These tests take 24 to 72 hours to obtain results; therefore empiric treatment is frequently relied on until more information is available. In some resource limited settings where there is little or no access to diagnostic facilities, empiric treatment is often relied on alone [36].

Correct and timely treatment has been found to reduce mortality from serious bacterial infections [83] . However, increasing rates of antibiotic resistance in bacteria has made the

empiric selection of appropriate agents increasingly challenging, and thus detrimental to patients [37]. This highlights the need for recent information about the epidemiology of circulating pathogens as well as their resistance rates. Studies have shown that improper empiric treatment results in increased morbidity and mortality, especially when these bacteria contain resistance mechanisms such as Extended Spectrum β-lactamases (ESBLs) and carbapenemases [38].

Surveillance of BSIs will help to estimate the burden of these infections, predict outcome and identify potential risk factors. The aim of this chapter was to investigate the antibiotic resistance and mortality rate of Gram negative BSIs in our setting and to differentiate between community-acquired and healthcare-acquired infections.

2. Materials and Methods

2.1 Sample selection

Isolates of *E. coli* and *K. pneumoniae* were obtained from positive blood cultures at the National Health Laboratory Service (NHLS) microbiology diagnostic laboratory in Tygerberg Hospital over 12 months, from April 2015 to March 2016. Only isolates from patients being treated at Tygerberg Hospital were included and isolates were convenience samples. These included blood cultures from adult and paediatric patients. The blood cultures were taken as part of regular patient investigation. All collected isolates were processed and identified using the laboratory's standard operating procedures. Isolate information was obtained from the Laboratory Information System (LIS) and stored in a secure database.

2.2 Sample processing

Blood culture bottles were incubated using the BacT/ALERT instrument (bioMérieux, France). Once a bottle was identified by the machine as positive, the bottle was removed from the machine and processed. Processing entails Gram staining, followed by culturing the organism to identify the species and generate the organism's antibiogram. Isolates were cultured using Tryptone Bile agar (TBA), MacConkey agar (MCC) and Chocolate agar (CHOC) and incubated overnight. This culture was then used to identify isolates using the VITEK®2 Advanced Expert System™ (bioMérieux, France) as per manufacturer's specifications. Antibiotic susceptibility testing was performed directly from the blood culture bottle by disc diffusion testing using the Kirby-Bauer method, using Mueller Hinton (MH) agar plates and antibiotic susceptibility discs (Mast Diagnostics, United Kingdom and Oxoid Limited, United States of America). Antibiotic susceptibility results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) guidelines (2016). If the isolates showed a highly resistant profile, were suspected ESBL producers or had inconsistent results,

antibiotic susceptibility profiles were confirmed using the VITEK®2 Advanced Expert System™ as per manufacturer's instructions. Resistance to the cephalosporin cefotaxime, was used as a marker for ESBL production.

The selected isolates were cultured on TBA to obtain pure colonies and then stored using MicroBank™ (Pro-Lab Diagnostic, United Kingdom) beads. This was done by taking a loop full of culture from the agar plates and inoculating the cryopreservative in the tube. The tube was then inverted 10 times and allowed to incubate on the bench for at least 2 minutes. Thereafter, the cryopreservative was removed using a pipette. The tube of beads was then stored in a labelled box at -80°C.

2.3 Patient information and statistical analysis

Specific patient information was obtained from the Hospital Information System (HIS), CLINICOM, and the LISs Disalab and TrakCare. This information included age, gender, ward, date of admission, duration of hospital stay and outcome.

BSIs were categorised as community-acquired or hospital-acquired based on where the infection was contracted. If the infection was present at admission or within 72 hours it was classified as community-acquired; if it was only observed after 72 hours in hospital it was classified as hospital-acquired. However, if the patient was previously hospitalised for two or more days within the past 90 days, the infection was classified as hospital-acquired [84]. A 72 hour cut off was opted for instead of a 48 hour cut off as only date of admission was available not the specific time, therefore this cut off would give a better indication of community- or hospital-acquired infections.

Statistical analysis was performed with the assistance of the Biostatistics unit from the Centre for Evidence-based Health Care at Stellenbosch University. IBM SPSS statistics version 25 was used for all statistical analysis. The chi-square test was used to estimate the significance of the correlation with statistical significance defined as <0.05.

Ethical approval for this study was obtained from the Human Research Ethics Committee (HREC) of Stellenbosch University (HREC# N14/06/069).

3. Results

3.1 Sample selection

One hundred and forty isolates (70 *E. coli* and 70 *K. pneumoniae*) were collected from April 2015 to March 2016. A total of 257 blood cultures cultured *E. coli* (n=142) and *K. pneumoniae* (n=115) over this period, therefore 54.4% of blood cultures were collected.

3.2 Patient demographics

Approximately one third of the patients (43/140; 30.7%) were paediatric, i.e. 15 years or younger [85], and two thirds were adult patients (97/140; 63.3%). The youngest patient was four days old while the oldest was 84 years old. Twenty-five (58.1%) of the paediatric patients were less than 12 months old. Forty-three (44.3%) adult patients were over 50 years, and 15 (15.5%) were 65 years or older. Sixty-three (45%) of patients were male, 76 (54%) were female and one patient was intersex. These patients were situated in various wards throughout Tygerberg Hospital (Table 2.1).

Table 2.1: Distribution of patients at Tygerberg Hospital.

Ward	Number of patients	
Adult emergency	29	20.7%
Adult surgery	26	18.6%
Paediatric medical	25	17.9%
Adult medical	17	12.1%
Obstetrics	12	8.6%
Adult ICU	10	7.1%
Paediatric ICU	7	5%
Paediatric surgery	6	4.3%
Paediatric emergency	6	4.3%
Out patient	2	1.4%

Ninety-three (66.4%) of the BSIs were hospital-acquired, while 47 (33.6%) were community-acquired (table 2.2). Of the hospital-acquired isolates, 57 (61.3%) were *K. pneumoniae* and 36 (38.7%) were *E. coli*. Of the community-acquired isolates, 13 (27.7%) were *K. pneumoniae* and 34 (72.3%) were *E. coli*. The distribution of *E. coli* and *K. pneumoniae* isolates in community- and hospital-acquired infections was statistically significant ($p<0.0001$).

Table 2.2: Distribution of hospital- and community-acquired isolates.

Type of BSI	Organism	Total
Hospital-acquired	57 *K. pneumoniae* (61.3%)	93 (66.4%)
	36 *E. coli* (38.7%)	
Community-acquired	13 *K. pneumoniae* (27.7%)	47 (33.6%)
	34 *E. coli* (72.3%)	

The duration of hospitalisation before and after the blood culture was taken was recorded. Patients spent an average of 11 days in hospital before the blood culture was taken, ranging from 0 to 133 days, with a median of 4.5 days. Patients spent on average 18 days in hospital after their blood culture was taken, with a median of 12 days. The number of days spent in hospital after the blood culture was taken ranged from 0 to 105 days.

The 30-day outcome for all patients is shown in figure 2.1. The crude 30-day mortality rate was 30% (42/140), although exact cause of death is not known. The crude mortality rate for the paediatric patients (32.6%; 14/43) was slightly higher than for adult patients (28.9%; 28/97) but this was not statistically significant. The mortality rate for *E. coli* and *K. pneumoniae* BSIs was similar, at 28.6% (20/70) and 31.4% (22/70) respectively.

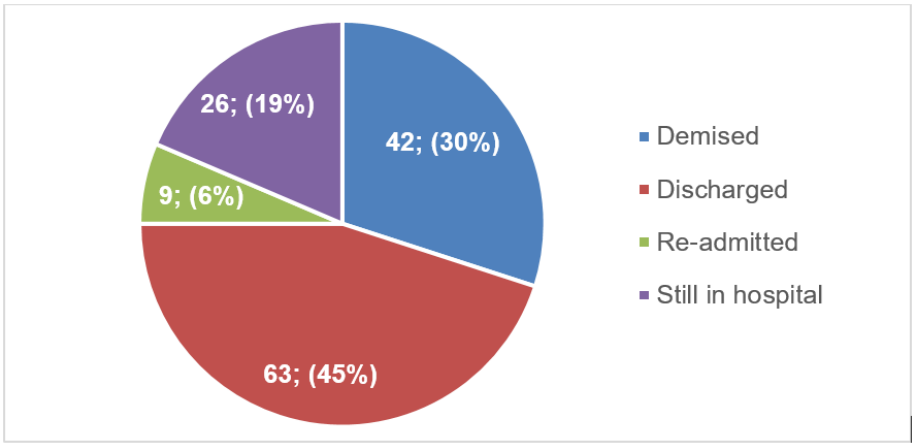


Figure 2.1: 30-day outcome of patients.

The crude mortality for hospital-acquired BSIs was 33.7% (31/92), while it was lower in community-acquired infections at 22.9% (11/48). This was also not statistically significant (p=0.183).

3.3 Phenotypic resistance

The antibiotic susceptibilities of the isolates can be seen in figure 2.2. *K. pneumoniae* isolates showed more resistance than *E. coli* to all antibiotics, except for co-trimoxazole, to which *E. coli* isolates were slightly more resistant. Little resistance to amikacin and ertapenem was seen in the isolates.

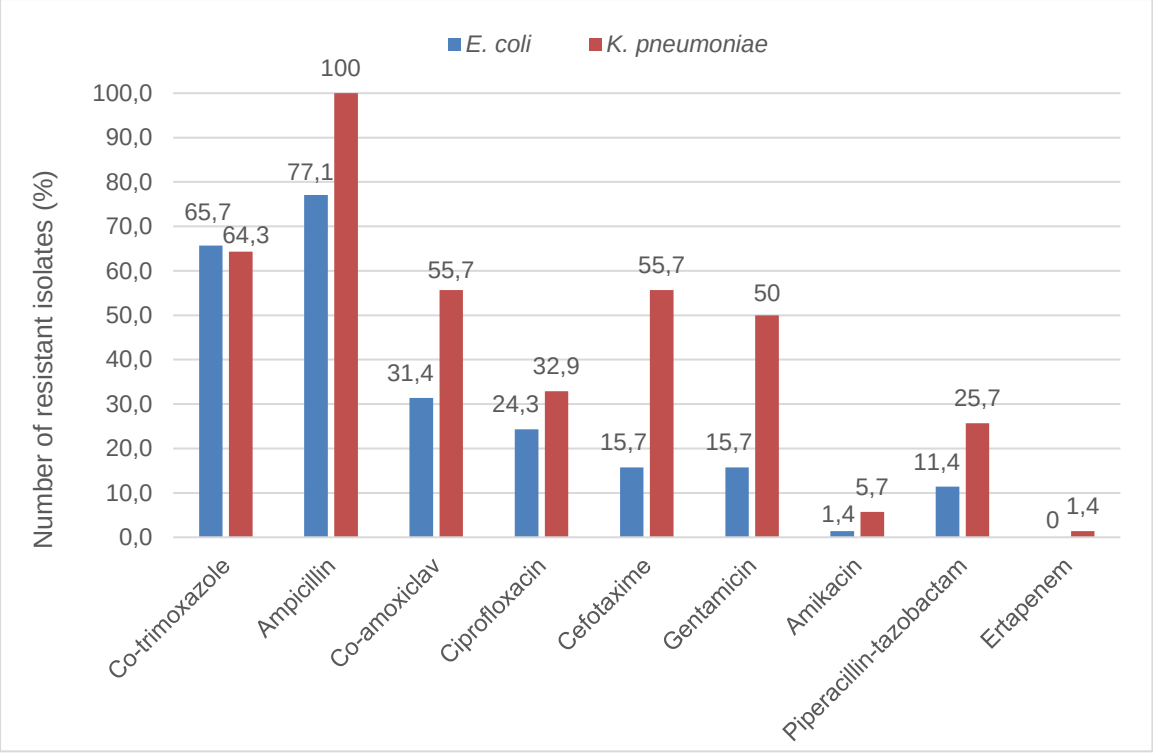


Figure 2.2: Antibiotic resistance results of all isolates.

Fifty isolates tested resistant to cefotaxime and were classified phenotypic ESBL producers. The resistance profile of ESBL producers compared to non-ESBL producers can be seen in figure 2.3. Increased resistance was seen in ESBL producers, particularly to co-trimoxazole, ampicillin, co-amoxclav, ciprofloxacin, gentamicin and piperacillin-tazobactam.

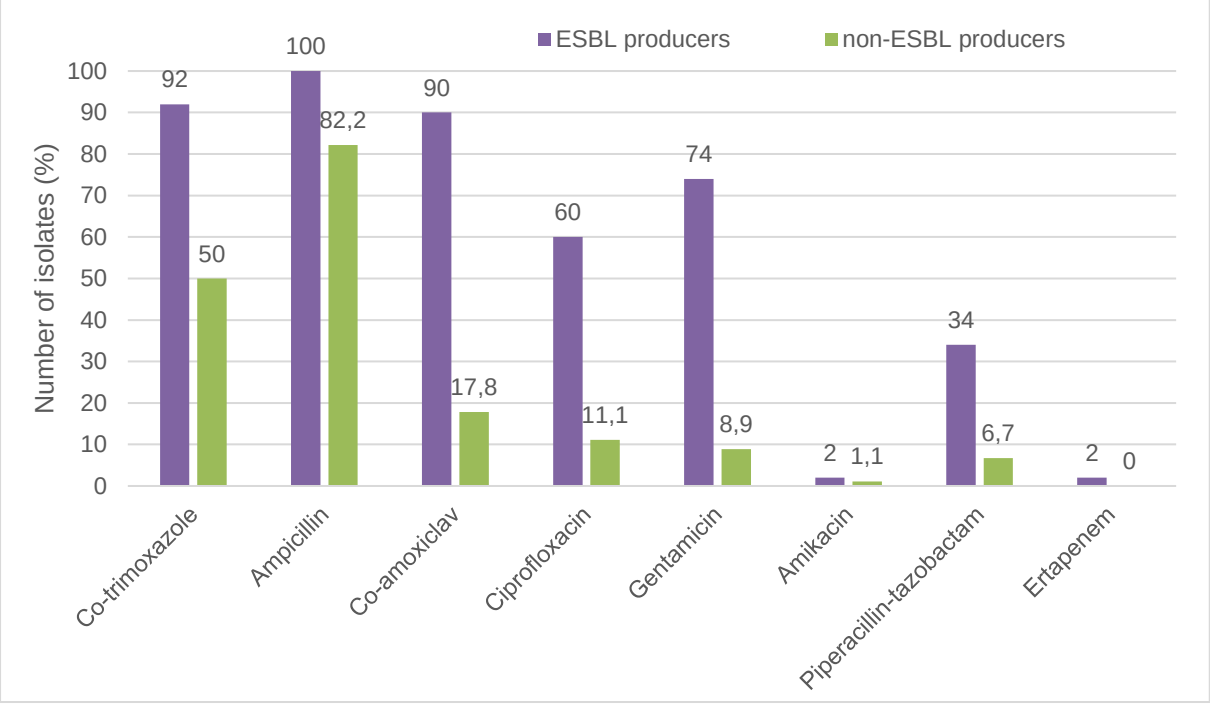


Figure 2.3: Comparison of antibiotic resistance of ESBL producers versus non-ESBL producers.

The resistance profiles of isolates classified as hospital- or community-acquired can be seen below (table 2.3). Phenotypic ESBL production in hospital-acquired isolates was higher at 46.2% (43/93), compared to community-acquired isolates at 14.9% (7/47). The association between hospital-acquired isolates and phenotypic ESBL production was statistically significant ($p<0.0001$).

Table 2.3: Antibiotic susceptibility results (sensitivity), shown as percentage of total number of isolates.

	E. coli (n=70)		*K. pneumoniae* (n=70)		Total (n=140)	
Antibiotic	**HA (n=36)**	**CA (n=34)**	**HA (n=57)**	**CA (n=13)**	**HA (n=93)**	**CA (n=47)**
Co-trimoxazole	25.0	44.1	26.3	76.9	**25.8**	**53.1**
Ampicillin	11.1	35.3	0.0	0.0	**4.3**	**25.5**
Co-amoxiclav	58.3	79.4	38.6	69.2	**46.2**	**76.6**
Ciprofloxacin	75.0	76.5	61.4	92.3	**66.7**	**80.9**
Cefotaxime	80.6	88.2	36.8	76.9	**53.8**	**85.1**
Gentamicin	88.9	79.4	43.9	76.9	**61.3**	**78.7**
Amikacin	97.2	100.0	93.0	100.0	**94.6**	**100.0**
Piperacillin-tazobactam	80.6	97.1	71.2	92.3	**74.2**	**95.7**
Ertapenem	100.0	100.0	98.3	100.0	**98.9**	**100.0**

4. Discussion

4.1 Patient demographics

Fifty-four percent of *E. coli* and *K. pneumoniae* isolates from blood cultures were collected. Although, not all *E. coli* and *K. pneumoniae* isolated over the one year time period were included due to isolates being collected by convenience, the collection of isolates is believed to be representative of the total population. A third of patients included in the study were paediatric, with 56.8% (25/44) of these patients being less than 12 months old. BSIs are known to affect patients at the two extremes of life [24], however not many patients of advanced age were seen in this study. Patients whose blood cultures were included in this study were located in various wards across Tygerberg hospital.

Two thirds of the isolates were classified as hospital-acquired. This may be influenced by the fact that Tygerberg is a tertiary hospital and patients are referred from other healthcare facilities, and usually have more complex pathology. Nonetheless, this provides an opportunity to reduce these infections through preventative measures, such as improved hand hygiene and removing intravenous lines as soon as possible [17].

Hospital-acquired isolates were predominantly *K. pneumoniae* (61.3%; 93/140), while community-acquired isolates were mostly *E. coli* (72.3%; 47/140). A study conducted at

another hospital in Cape Town, which investigated community- versus healthcare-acquired BSIs, also observed that more *K. pneumoniae* isolates were healthcare-acquired than community-acquired (15.8% vs 7.1%), and more *E. coli* isolates were community-acquired than healthcare-acquired (19.8% vs 9.9%) [17]. Likewise, a Tanzanian study on BSIs in children (0-7 years) observed more *K. pneumoniae* isolates to be healthcare-acquired than community-acquired (24.5% vs 12.3%), and more *E. coli* isolates to be community-acquired than healthcare-acquired (15.5% vs 9.4%) [34]. *K. pneumoniae* is a known nosocomial opportunistic pathogen that is often found in hospital settings and has notable ability to survive on hands, facilitating its spread [19].

Patients spent an average of 11 days in hospital before the blood culture was taken, and an average of 18 days after it was taken. It has been found that BSIs increase hospital stays by 10 days [86]. A lengthier hospital stay increases a patient's risk for subsequent infection, due to underlying morbidity and invasive procedures [87]. Unfortunately, this study did not include a control group to better define the additional length of stay for patients with BSI.

The 30-day mortality rate of 30% (42/140) is a crude estimate as the exact cause of death was not ascertained. However, 30-day crude mortality is a common outcome in similar studies and at least allows for some comparisons. The mortality in this study was higher than another study conducted in Tygerberg Hospital from 2008 to 2013, 20.4%, and studies conducted in the United States of America, 11.5-16%, Canada, 14-22.5%, Denmark, 12-16% and Spain 19-24% [27,32,82,88]. This may be because these studies included BSIs caused by Gram positive and negative bacteria and fungi. Studies have noted a lower mortality in Gram positive BSIs than Gram negative BSIs [32,34,35]. Aiken *et al.* found fungal infections to have a higher mortality than Gram positive BSIs, but still much lower than Gram negative BSIs, in contrast, Dramowski *et al.* found fungal bacteraemia to be associated with mortality. The mortality in this study was lower than reported in a Tanzanian study, 43.5%, and a Kenyan study, 61% [34,35]. Both studies focussed on paediatric patients and included patients with other severe co-morbidities such as HIV-infection and malnutrition. The Kenyan study only included hospital-acquired infections, which may explain why the mortality rate is higher. Blomberg *et al.* noted that expensive second-line antibiotics are not readily available in Tanzania.

The mortality rate was higher in hospital-acquired isolates (33.7%; 31/92) than community-acquired (22.9%; 11/48) isolates, although this was not statistically significant. Other studies have also found increased mortality in hospital-acquired infections as opposed to community-acquired [32,35,88,89]. A study conducted in Kenya reported an alarming mortality rate of 53% in hospital-associated BSIs versus 24% in community-acquired infections [35]. Hospital-acquired infections have an increased mortality rate as hospitalised patients often

have co-morbidities such as HIV-infection, cancer and tuberculosis, and may be immunocompromised due to the treatment they are receiving. Hospital-acquired bacteria are also often antibiotic resistant thereby complicating and delaying effective treatment.

4.2 Phenotypic resistance

High levels of resistance were seen to co-trimoxazole (65%; 91/140) and ampicillin (88.6%; 124/140). Most isolates remained sensitive to amikacin (96.4%; 135/140) and ertapenem (99.3%; 139/140), and piperacillin-tazobactam also retained a reasonable level of susceptibility (81.4%; 114/140).

Susceptibility data was only recorded as categorical and not Minimal Inhibitory Concentrations (MIC), as not all isolates were tested on the VITEK®2. This places limitations on the analysis of the data. Additionally, if an isolate is a suspected ESBL producer, all cephalosporins are recorded non-susceptible. A similar problem exists with co-amoxiclav, piperacillin-tazobactam, gentamicin and amikacin, which may be reported as non-susceptible by the VITEK®2 if an underlying resistance mechanism is detected.

Resistance to the third generation cephalosporin, cefotaxime, was used as a marker for phenotypic ESBL production. Fifty isolates (35.7%) were ESBL producers, 78% (39/50) of these were *K. pneumoniae* isolates and 22% (11/50) were *E. coli* isolates. This translates to 55.7% of *K. pneumoniae* isolates and 15.7% of *E. coli* isolates being ESBL producers. A study conducted in a hospital in Cape Town in 2012 found similar rates of ESBL production in blood culture isolates, 30%, although this was for all Enterobacteriaceae isolates [17]. A study done on BSIs at Tygerberg Hospital reported similar rates of ESBL production in *E. coli (*12-22%), but much higher rates in *K. pneumoniae* (76-78%) [32]. This may be as a result of only paediatric patients being included in the study.

A report on BSIs at public sector hospitals across South Africa in 2011, found that 3-17% of *E. coli* isolates and 55-74% of *K. pneumoniae* isolates were ESBL producers [6]. At Tygerberg Hospital, they reported 16% of *E. coli* isolates and 56% of *K. pneumoniae* isolates to be ESBL producers, which is akin to what our study found. A report looking at resistance in public hospitals across South Africa in 2015 reported a higher rate of resistance to third generation cephalosporins, at 22% for *E. coli* and 69% of *K. pneumoniae* [5]. The resistance rates they reported at Tygerberg Hospital were similar to what was found in our study, 13% for *E. coli* and 54% of *K. pneumoniae*. This report included all specimen types in the resistance rates. The European Centre for Disease Prevention and Control (ECDC) reported the average resistance to third generation cephalosporins for European Union (EU) countries; and found a similar resistance rate of 13% for *E. coli*, while for *K. pneumoniae* the resistance was much lower than found in this study at 30% [4].

The resistance profile of the subset of isolates classified as ESBL producers was determined and compared to non-ESBL producers (figure 2.3). An increase in resistance was seen to most antibiotics, i.e. co-trimoxazole, ampicillin, co-amoxiclav, ciprofloxacin, gentamicin and piperacillin-tazobactam. ESBL genes are often harboured on large plasmids that contain other resistance genes, resulting in resistance to other antibiotics such as aminoglycosides, tetracyclines, chloramphenicol and sulphonamides [90]. ESBL producing bacteria are also known to be more resistant to fluoroquinolones [90]. Studies conducted across Europe from 2004-2007 found ESBL production to be associated with resistance to other antibiotics such as to co-trimoxazole, tetracycline, gentamicin, and ciprofloxacin [60]. ESBL producers retained their sensitivity to amikacin and ertapenem. This is beneficial as these antibiotics are often used to treat ESBL producing organisms [17].

Resistance was compared between the hospital- and community-acquired isolates. Increased resistance was seen to most antibiotics in the hospital-acquired isolates, although most isolates remained sensitive to amikacin (94.6%; 88/93) and ertapenem (98.9%; 92/93). ESBL prevalence was much higher in hospital-acquired isolates (46.2%; 43/93), compared to community-acquired isolates (14.9%; 7/47). Increased resistance in hospital-acquired isolates is expected, as increased use of antibiotics in hospital settings places considerable selection pressure on circulating bacteria [17].

Although community-acquired isolates were more susceptible to antibiotics than hospital-acquired isolates, reduced susceptibility was still observed in these isolates. Resistance was observed to co-trimoxazole (46.8%; 22/47), ampicillin (74.5%; 35/47), co-amoxiclav (23.4%; 4/47), gentamicin (21.3%; 10/47), ciprofloxacin (15.2%; 9/47) and cefotaxime (14.9%; 7/47). Little resistance was seen to piperacillin-tazobactam (4.3%; 2/47) and no resistance was seen against amikacin or ertapenem. These resistance rates are lower than what was reported in a study in Cape Town looking at community- versus healthcare-acquired BSIs [17]. A study on paediatric BSIs in Tanzania recorded higher rates of resistance in community-associated *E. coli* and *K. pneumoniae* isolates, except for ciprofloxacin which had low levels of resistance (0-8%) [34]. Dramowski *et al.* noted a concerningly high level of antibiotic resistance in community-acquired *E. coli* and *K. pneumoniae* isolates from Tygerberg, although specific values were not given [32]. Antibiotic resistance in community-acquired bacteria may be due to previous antibiotic use or prior time spent in healthcare facilities. Previous antibiotic use is a known risk factor for resistance [91].

A review looking at the prevalence of ESBLs in BSIs in Africa found that African countries have a lower prevalence of ESBLs in community settings as compared to hospital settings [92]. African countries reported varying rates of resistance, with most reporting an increase in ESBL prevalence in hospital settings. Tunisia recorded community setting prevalence of 0.7-

7.3% and hospital setting prevalence of 11.7-77.8%, across five studies. Kenya and Ethiopia reported a prevalence of 11% in community settings and 42.9% in hospital settings. The review also recorded an ESBL prevalence of 0.3-4.7% for community-acquired isolates and 8.8-13.1% for hospital-acquired isolates in South Africa. This is much lower than what was observed in this study. This may be due to the fact that these studies were carried out from 2002 to 2009 and prevalence has since increased. A study which focused on BSIs at a hospital in Cape Town in 2011 reported a similar rate of ESBL producers in hospital-acquired Enterobacteriaceae isolates, 39.6%, but a much lower rate in community-acquired isolates, 5% [17].

Within the hospital-acquired isolates, reduced susceptibility to all antibiotics was observed for *K. pneumoniae* compared to *E. coli* (table 2.3). A considerable reduction in sensitivity was observed for cefotaxime (ESBL production) and gentamicin in *K. pneumoniae*, 36.8% and 43.9% respectively, in comparison to *E. coli*, 80.6% and 88.9%. Similar differences in ESBL production between hospital-acquired *E. coli* and *K. pneumoniae* BSIs were reported in a previous study carried out in Tygerberg Hospital [32]. They reported an ESBL production of 75.7% in *K. pneumoniae* and 21.7% in *E. coli*.

5. Conclusion

Two-thirds of isolates included in this study were hospital-acquired. Hospital-acquired BSIs were mostly caused by *K. pneumoniae*, while community-acquired infections were mostly caused by *E. coli*.

A crude mortality rate of 30% was reported for this study, which is higher than reported in other studies in Africa and Europe. A higher mortality was reported for hospital-acquired infections, although this was not significant. The reported mortality figures are crude; however it would be advantageous to gain further clinical information into cause of death to determine whether mortality is a direct cause of the BSI or as a result of other comorbidities. Additional clinical information may also assist to identify further risk factors for infection and mortality. A prospective study is underway to collect such data.

Isolates resistant to the third generation cephalosporin, cefotaxime, were presumed ESBL producers. Fifty isolates (35.7%) were ESBL producers, mostly made up of *K. pneumoniae* isolates. Increased antibiotic resistance was observed among these isolates, which may be as a result of multiple resistance genes being present on a single plasmid.

Increased resistance was observed among hospital-acquired isolates which was expected due to the increased selection pressure in hospital settings. Hospital-acquired *K. pneumoniae* isolates showed notably increased resistance, particularly to cefotaxime and

gentamicin. Fortunately, ertapenem and amikacin retained effectivity, and these antibiotics are often used as empiric therapy for hospital acquired infections.

The information generated is important to guide empirical treatment as there are epidemiological differences in hospital- and community-acquired isolates. Incorrect empiric therapy has been associated with increased mortality, particularly with ESBL producing bacterial infections [38,83].

It would be beneficial to collect additional information about previous antibiotic use to determine whether specific treatment is a risk factor for infection with ESBL producing organisms. Identification of risk factors may assist to prevent infections and focus preventative measures. Studies have found that using carbapenems for the treatment of infections with ESBL producing bacteria has resulted in lower mortality, although it is important to maintain the effectivity of carbapenems through antibiotic stewardship [32]. Thorough resistance information will assist in determining which antibiotics are viable for treatment in this setting, so that we may preserve agents such as carbapenems and colistin.

Chapter 3: Strain typing

1. Introduction

Strain typing is used to elucidate the relationships between microorganisms. Understanding this relatedness is important to identify the local epidemiology, but also to determine routes of transmission, sources of outbreaks, identify virulent strains and evaluate infection control measures [81].

There are several strain typing methods available, with molecular methods becoming increasingly prominent as a result of their higher discriminatory ability [81]. Some methods, such as Pulsed Field Gel Electrophoresis (PFGE) and Restriction Fragment Length Polymorphism (RFLP) analysis involve analysing chromosomal DNA with restriction enzymes. Others are amplification based like rep-PCR and Variable Number Tandem Repeat (VNTR) analysis or sequence based such as Multilocus Sequence Typing (MLST), microarrays and Whole Genome Sequencing (WGS) [81].

The choice of which strain typing method to use is dependent on performance variables and the epidemiological question to be answered [81]. Some performance variables to consider include repeatability, reproducibility, discriminatory power, throughput, ease of interpretation, time and cost. Two strain typing techniques are often combined to enhance or strengthen results. Typing technologies based on the whole genome of the microorganism yield better results in establishing the clonal relationships [93].

Rep-PCR entails the Polymerase Chain Reaction (PCR) amplification of repetitive extragenic palindromic (REP) elements which are highly conserved and dispersed throughout the bacterial genome [94]. It has been proposed that these elements are involved in mRNA stability, termination of transcription and chromosomal domain organisation [94]. Two degenerate primers are used to amplify genomic regions between the repeat elements [95]. Gel electrophoresis is used to separate the amplified DNA, generating a unique banding pattern which reflects the number of repetitive elements and length of DNA between them [96]. This banding pattern can be used as a genetic fingerprint for each bacterial isolate. This technique is favoured as it is much less time consuming and laborious than other typing techniques. However, rep-PCR is susceptible to minor changes in reagents and conditions, making its reproducibility and portability problematic [81].

PFGE was first described in 1984 and has since become a widely-used strain typing technique [97]. It is considered to be the gold standard for typing bacteria [81]. The restriction pattern of the whole genome is analysed using Restriction Enzymes (REs) with rare

recognition sites, resulting in a moderate number of large DNA fragments [97]. As DNA fragments larger than 20 kb move at the same speed through agarose gel under conventional electrophoresis with a constant electric current, PFGE utilises alternating electric currents at different angles, capable of separating large fragments of up to 10 Mb [93,97]. Continuously altering electrical currents re-orientates the DNA fragments and allows for separation of large fragments [93]. The banding patterns of isolates can then be compared; isolates that differ by one to three bands differ by a single genetic event and are said to be closely related. If isolates differ by four to six bands they are considered possibly related as they differ by two independent genetic events. Isolates that differ by more than six bands are deemed unrelated [97]. Unfortunately, this method is time-consuming and laborious and there are problems with reproducibility and portability across different laboratories [98].

The aim of this section of the study was to describe the epidemiology of the collected *Escherichia coli* and *Klebsiella pneumoniae* isolates by performing rep-PCR and PFGE. This information could then be used to describe the relatedness of the circulating strains in this setting.

2. Materials and methods

2.1 Bacterial Isolates

Strain typing was performed on all isolates collected as part of this study, including 70 *E. coli* and 70 *K. pneumoniae*; as described in Chapter 2.

2.2 DNA extraction

Bacterial isolates were retrieved from Microbank beads and cultured to obtain a pure culture. This was done by aseptically removing a single bead and streaking it onto a Tryptone Bile Agar (TBA) plate supplemented with horse blood (NHLS Media Laboratory, Greenpoint). The plate was incubated at 37°C overnight. A single colony from this plate was re-streaked onto a new TBA plate and incubated overnight at 37°C to obtain pure cultures. Colonies from this plate were used to perform a crude DNA extraction. An inoculum loop was used to remove culture from the plate which was then inoculated into 400 µl of nuclease free water in a 2 ml microcentrifuge tube (Eppendorf, Germany). This was vortexed to ensure a homogenous solution. The solution was heated at 95°C using a heating block (Accublock™, Labnet, United States of America) and then frozen at -20°C in a freezer for 30 minutes. The solution was thawed and then centrifuged at 14000 x g for 10 minutes to remove cell debris. The supernatant containing the DNA was removed and placed in a new 1.5 ml microcentrifuge tube. All DNA extractions were stored at -20°C.

2.3 Rep-PCR

Two degenerate primers were used to amplify repetitive consensus sequences (table 3.1); primers were synthesised by IDT (Integrated DNA Technologies, South Africa). The PCR was performed using extracted DNA and the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems, South Africa). All PCR reactions were performed using the ProFlex PCR system thermocycler (Applied Biosystems, United States of America). The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 40°C for 30 seconds and extension at 72°C for 8 minutes, followed by a final extension at 72°C for 16 minutes. The final primer concentrations were 0.8 pmol/µl for each primer. A reaction containing no template DNA (NTC) was included in each PCR run to ensure that there was no contamination. A control strain, ATCC25922 for *E. coli* and ATCC700603 for *K. pneumoniae*, was included in each gel to ensure reproducibility between PCR reactions and gels.

Table 3.1: Primers used for the rep-PCR strain typing [94].

Target	Primer	Sequence (5' – 3')	Product size (bp)	Source
REP	REP1R-I REP2-I	IIIICGICGICATCIGGC ICGICTTATCIGGCCTAC	Multiple (100 – 2000 bp)	Versalovic *et al.* (1991).

PCR products were separated on a 2% agarose gel run at 110V for 4 hours in a 1X TBE (89 mM Tris, 89 mM borate, 2.5 mM EDTA) buffer. TBE buffer was used instead of TAE as it is more resistant to heat produced by the high voltage and longer running time. KAPA universal DNA Ladder (Kapa Biosystems, South Africa) was used as a molecular size marker on either side of the gel. PCR products were stained using NovelJuice (GeneDireX, South Africa) and the image was visualised and captured using the UVItec Alliance 2.7 gel documentation system (UVItec, United Kingdom).

2.4 PFGE

PFGE was performed at the Division of Medical Microbiology at Cardiff University in the United Kingdom as part of a research exchange.

Preparation of bacterial isolates for shipment

Isolates were streaked onto blood agar plates and cultured overnight at 37°C. The culture was inoculated onto BIO-CULT AMIES + charcoal swabs (Liofilchem, Italy) which were sealed using parafilm and packaged in UN 13373 containers. The swabs were sent to the United Kingdom at room temperature. Once the swabs arrived they were stored at 4°C.

Making the agarose plugs

Bacterial isolates were streaked from swabs onto chromogenic agar to ensure purity. If the isolate was pure and the correct organism, i.e. *K. pneumoniae* or *E. coli,* a single colony from the plate was streaked onto Columbia agar supplemented with horse blood (E&O Laboratories limited, Scotland). Chromogenic agar could not be used as the pigmented culture may affect the PFGE result.

The agarose used (1.5%) to cast the plugs was prepared with 0.006 g of SeaKem Gold agarose (Lonza, Switzerland) and 400 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) per isolate tested. The mixture was microwaved until the agarose had dissolved (about 30 seconds) and then the flask was placed into an oven at 50°C for at least 15 minutes to equilibrate.

One millilitre of Cell Suspension Buffer (CSB) (100 mM Tris, 100 mM EDTA, pH 8.0) was dispensed into labelled 1.5 ml microcentrifuge tubes. Culture from the agar plates was inoculated into the CSB using a cotton swab to create a cell suspension with an optical density between 0.8-1.0 (600 nm). The optical density of the cell suspension was read by pipetting 800 µl of cell suspension into a cuvette and reading the optical density with a spectrophotometer (Jenway 7315 spectrophotometer). CSB was used to blank the spectrophotometer before use.

The plug moulds were prepared by sealing the bottom of the plug mould using autoclave tape and labelling the moulds appropriately. Four hundred microliters of cell suspension was transferred into a 1.5 ml microcentrifuge tube. Twenty microliters of Proteinase K (20 mg/ml) (Sigma-Aldrich, United States of America) was added to each tube and mixed gently by pipetting up and down. The flask containing agarose was removed from the incubator and placed on top of a heating block set at 50°C to prevent the agarose from solidifying. Four hundred microliters of agarose was added to the cell suspension/Proteinase K mixture and this was mixed by pipetting up and down. This mixture was immediately dispensed into the wells of the plug mould, taking care to avoid bubbles in the well. This process was then repeated with the remaining cell suspensions. The plugs were left to solidify at room temperature for 15 minutes.

Lysis and washing of the plugs

A master mix of Cell Lysis Buffer (CLB) (50 mM Tris, 50 mM EDTA, pH 8.0 and with 1% Sarcosyl) and Proteinase K was made up for the lysis of the plugs. Five millilitres of CLB and 25 µl Proteinase K was prepared per set of plugs. Five millilitres of the master mix was added to labelled 30 ml sterile universal containers.

Excess agarose was removed from the plug moulds using a scalpel sterilised with 70% ethanol and the tape was removed from the plug moulds. Plugs were pushed out of the mould into the labelled tubes, ensuring that the plugs were immersed in the CLB/Proteinase K mixture. Tubes were placed in a rack on a shaker and incubated at 50°C for 2 hours.

After incubation, the CSB was poured off, taking care that no plugs were lost. The tubes were filled with 15 – 20 ml of autoclaved water, preheated to 50°C. The tubes were then placed in a rack on a shaker and incubated at 50°C for 15 minutes. This wash step was repeated once.

The water in the tubes was poured off as before. Ten to fifteen millilitres of TE buffer preheated to 50°C was added to each tube. The tubes were returned to the shaker in the incubator for 15 minutes. The plugs were washed with heated TE buffer 3 more times. After the final wash, the TE buffer was poured off and 5 ml of fresh TE buffer was added to each tube and stored at 4°C until restriction digestion of the plugs.

Restriction digestion of DNA in the plugs

A master mix of diluted restriction buffer was made up of 20 µl of 10X FastDigest™ buffer (Thermo Scientific, United States of America) and 180 µl molecular grade water per isolate; and 200 µl of this master mix was added to labelled 1.5 ml microcentrifuge tubes.

An agarose plug was carefully removed from the TE buffer and placed on a sterile slide. The plug was cut into 4 equal slices using a scalpel that had been sterilised in 70% ethanol. The plug slices were transferred into the microcentrifuge tube (Eppendorf) containing the diluted restriction buffer, ensuring that all plug slices were at the bottom of the tube and covered by the diluted restriction buffer mixture. The plug slices were incubated at room temperature for 15 minutes. The buffer mixture was then removed from the microcentrifuge tubes (Eppendorf) using a pipette. A restriction enzyme master mix was made up using 175 µl molecular grade water, 20 µl of 10X FastDigest buffer and 5 µl XbaI enzyme (10 U/ µl) (Thermo Scientific) per set of plugs. Two hundred microliters of this master mix was added to each tube and the tubes were incubated in a heating block at 37°C for 2 hours.

After incubation, the digestion master mix was removed from the tubes using a pipette and 200 µl of 0.5 M EDTA, pH 8.0 was added to each tube to preserve the prepared plugs.

Casting and loading the gel

A 1% agarose gel was made by weighing 1.2 g of agarose (Sigma-Aldrich, United States of America) and placing it into a bottle with 120 ml of 0.5X TBE (prepared from 10X TBE stock). The mixture was microwaved until the agarose had dissolved. The bottle was then placed in

an oven set at 50°C to equilibrate for at least 15 minutes. The casting stand was prepared and fitted with a 15 well gel comb. Twenty microliters of ethidium bromide (0.5 µg/ml) was added to the gel and mixed before the gel was poured into the casting stand. The gel was left to set for 30 minutes at room temperature.

After the gel had solidified, the comb was removed from the gel. The CHEF DNA size standard Lambda Ladder (Bio-Rad, United States of America) was used as a ladder in each gel. A plug was removed from the tube and cut to the appropriate size. The plug slices with Lambda Ladder were loaded into the 1st and 15th well of the gel using a scalpel and disposable loop. Plug slices containing bacterial DNA were removed from the microcentrifuge tubes containing EDTA and loaded into the remaining wells in a similar manner. A specific bacterial isolate was used as a control in each gel to compare variation between gels; for *K. pneumoniae* it was GN10 and for *E. coli* it was GN8.

After the gel had been loaded with the agarose plugs, it was removed with the gel tray from the casting stand. Excess agarose was removed from the sides and bottom of the gel tray.

Electrophoresis

Two litres of 0.5X TBE was added to the electrophoresis chamber and 50 ml of ethidium bromide was added to the buffer. The CHEF DR-III system, pump and cooling unit (10°C) (Bio-Rad, United States of America) were turned on. The buffer was allowed to circulate through the system for at least 30 minutes before running the gel to allow it to cool. The buffer was used until the current reached 300V and was then replaced. After each replacement, the system was flushed with 0.5X TBE buffer and water. The gel frame was secured in the electrophoresis chamber. The prepared gel on the gel tray was placed inside the gel frame and the cover of the chamber was closed.

The CHEF DR-III system was set to the conditions below.

- Initial switch time: 5 seconds
- Final switch time: 45 seconds
- Voltage: 6 V
- Included angle: 120°
- Run time: 23 hours

After the electrophoresis was complete, the gel and gel tray were removed from the electrophoresis chamber and an image of the gel was captured using the Gel Doc-It imaging system (UVP, United States) and VisionWorksLS software (UVP).

2.5 Analysis of rep-PCR and PFGE gels

The gel images were uploaded into BioNumerics 7.5 (Applied Maths, United States of America). This program enables the analysis of gel images to create phylogenetic trees and determines the relatedness of strains. Images are uploaded into the program; the ladder is marked and the specific band sizes are entered. The banding patterns of the isolates are recognised and marked individually. This information is then used to generate a phylogenetic tree. The similarity coefficient used was Dice and the clustering method used was Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

For PFGE, clustering was assessed at 70% and 95% similarity thresholds as this is represents a difference of four to six bands (possibly related) and one to three bands (closely related) respectively [99]. For rep-PCR, many studies use 95% threshold to define isolates as closely related [100–102]. In this study, a second threshold of 70% was used to define isolates that are possibly related to correlate with the PFGE cut-off.

2.6 Comparison of typing methods

Simpson's index of diversity was used to compare the diversity between the two strain typing methods. This was calculated as 1 – D, where D represents Simpson's Index. Simpson's index was calculated using the following formula; $\frac{\sum n(n-1)}{N(N-1)}$ where n is the total number of isolates in a cluster and N is the total number of clusters. This index was chosen as it takes into account the number of clusters present as well as the number of isolates in each cluster, thereby evaluating the richness and evenness of the population. This index represents the probability that two randomly selected isolates will fall into different clusters [103]. The closer the value is to one, the higher the diversity**.**

3. Results

3.1 Rep-PCR

All 140 *E. coli* and *K. pneumoniae* isolates were typed by rep-PCR. An example of the gel images obtained can be seen in figure 3.1.

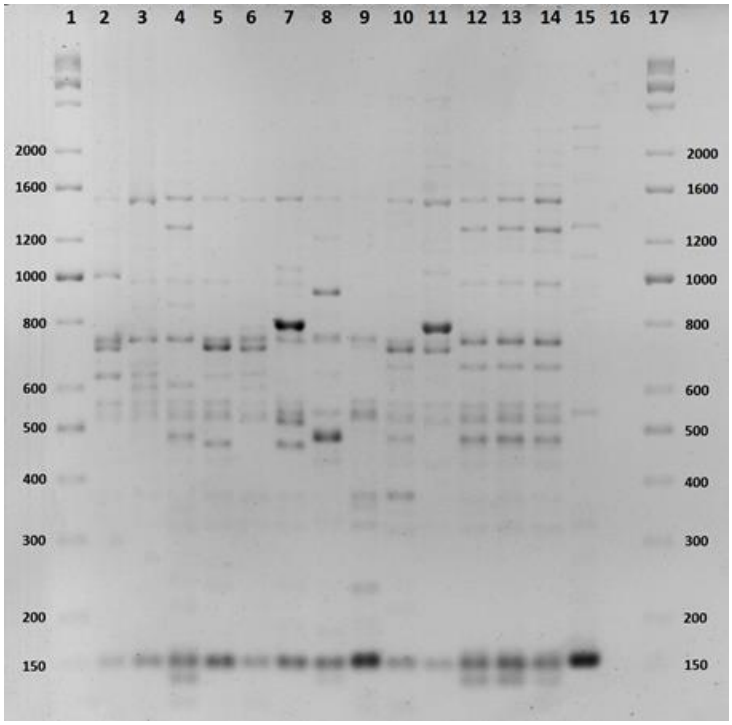


Figure 3.1: Rep-PCR gel image. Lanes 1 and 17 contain the KAPA universal DNA ladder. Lanes 2 -14 are clinical isolates. Lane 15 contains a control isolate, ATCC700603 and lane 16 is a NTC.

The phylogenetic trees generated for *E. coli* and *K. pneumoniae* can be seen in figure 3.2 and 3.3 respectively. Little clustering was seen in both the *E. coli* and *K. pneumoniae* isolates, with isolates not clustering according to the wards in which the patients were located, or between hospital- or community-acquired infections. For *E. coli*, a 70% threshold identified 16 clusters and 23 singletons. The largest cluster contained seven isolates, although most clusters (9/16) contained only two isolates. A cut-off of 95% identified one cluster of two isolates with identical banding patterns (GN58 and GN59) and 68 singletons. For *K. pneumoniae*, a threshold of 70% identified 16 clusters and 20 singletons. The largest cluster was made up of nine isolates, but most of the clusters (11/16) were made up of two isolates. A 95% threshold identified two clusters of two isolates and the remaining 66 isolates were singletons. Two pairs of identical isolates, GN90 and GN91, and GN27 and GN32, were identified.

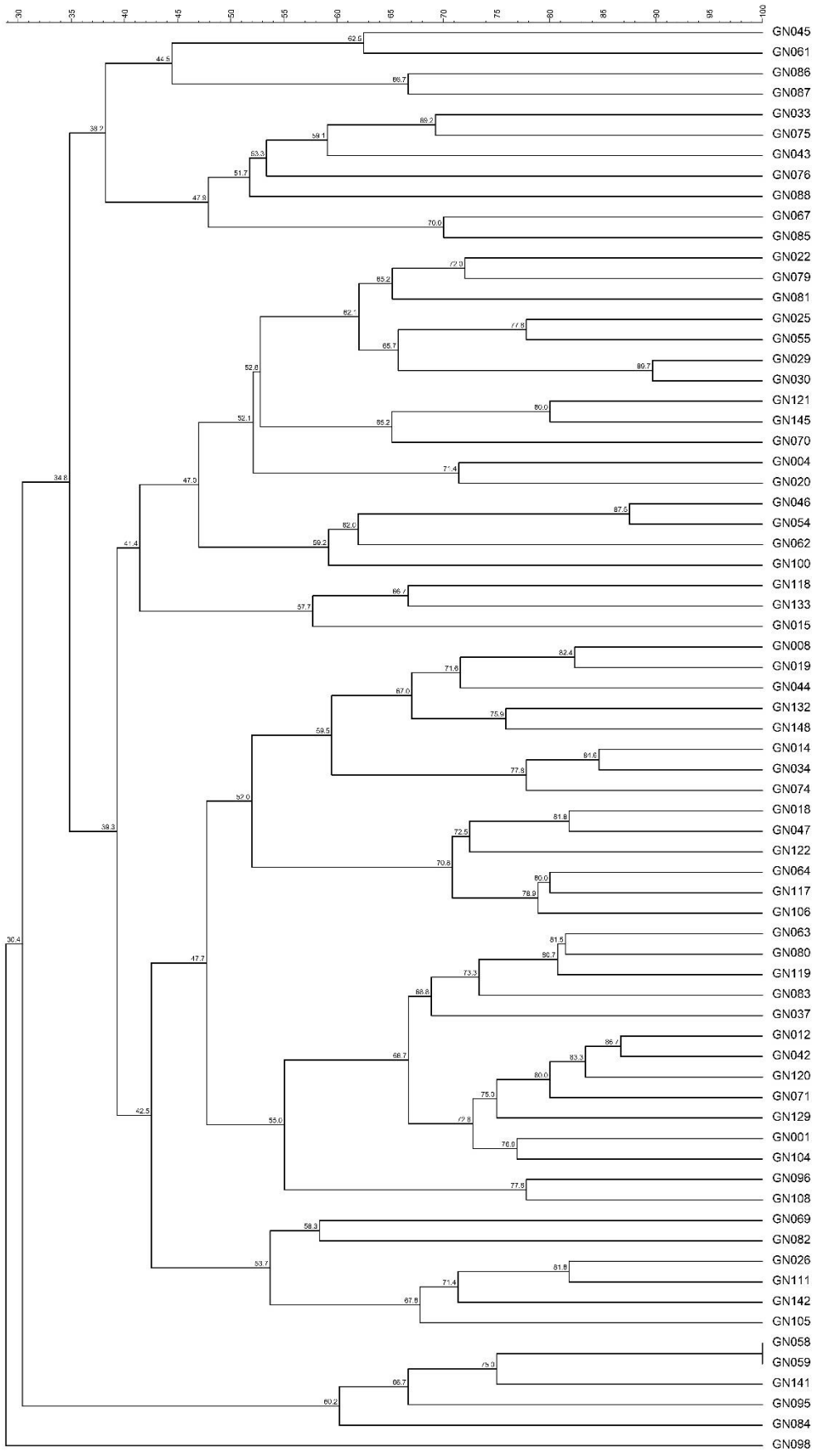


Figure 3.2: Phylogenetic tree generated from rep-PCR results for *E. coli* isolates.

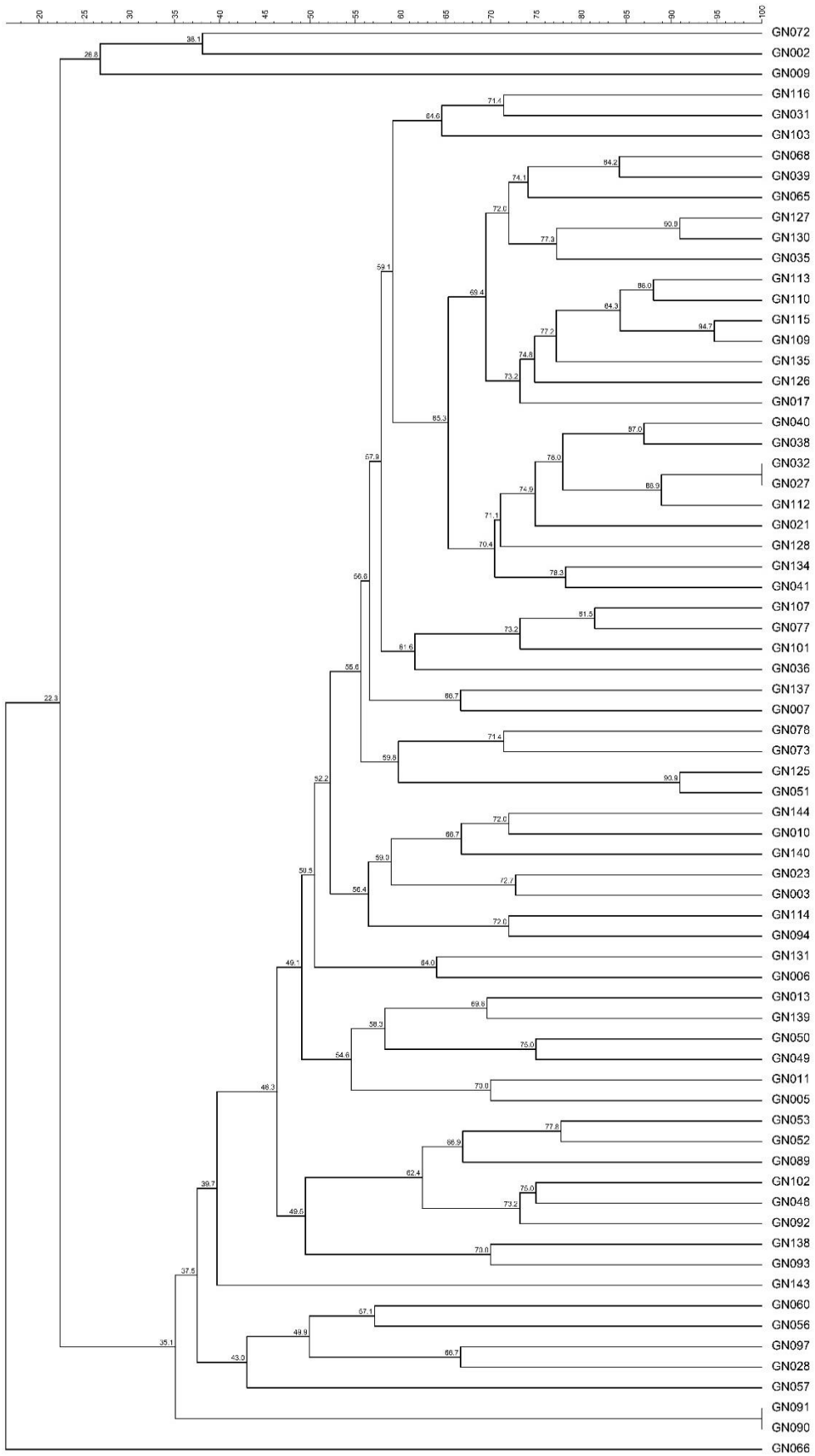


Figure 3.3: Phylogenetic tree generated from rep-PCR results for *K. pneumoniae* isolates.

3.2 PFGE

As isolates were being cultured from swabs shipped to Cardiff University, contamination was noticed among some isolates. Therefore, isolates were first swabbed onto selective chromogenic media to determine whether they were contaminated. Where possible, contaminated isolates were purified; unfortunately, 8 isolates, GN26, GN43, GN64, GN66, GN72, GN128, GN129 and GN139, could not be tested by PFGE as the swabs contained only contaminants. One *E. coli* isolate, GN67 failed PFGE twice despite re-extraction of DNA and was also excluded from analysis. Ultimately, 65 *E. coli* and 66 *K. pneumoniae* isolates were included in the PFGE analysis. An example of a PFGE gel image can be seen below in figure 3.4. All gels were run with the CHEF DNA size standard Lambda Ladder one either side to use as a marker when analysing gels. Unfortunately, the ladder was unclear in some gel images, therefore the bacterial isolate ran in every gel, i.e. GN8 (*E. coli*) or GN10 (*K. pneumoniae*), was used as the marker.

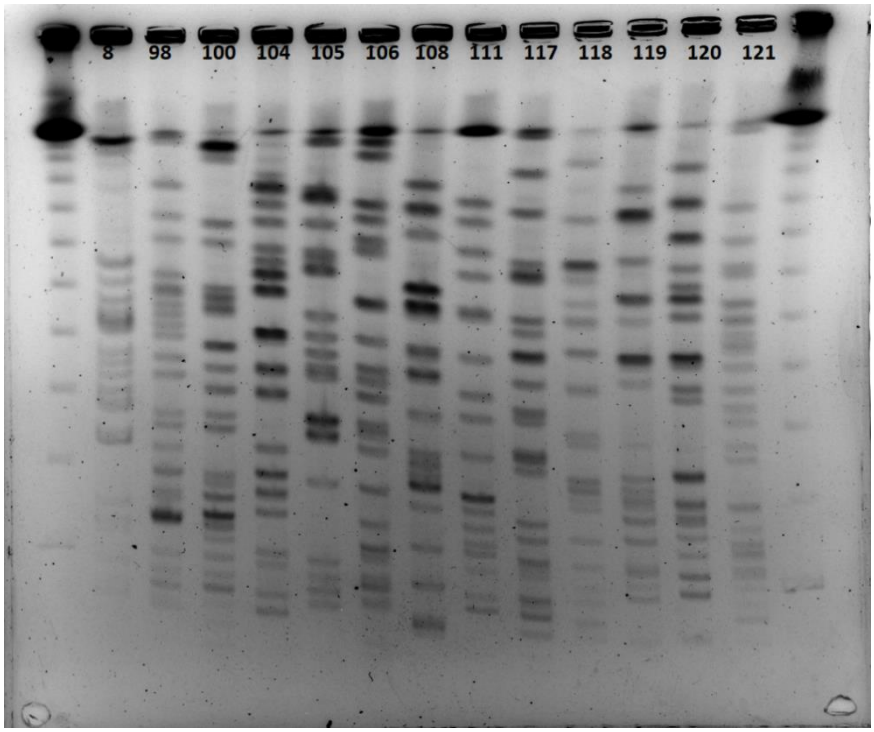


Figure 3.4: PFGE gel image. Lanes 1 and 15 contain the CHEF DNA size standard Lambda ladder. Lanes 2 -14 are clinical isolates. Lane 2 contains the control isolate for *E. coli*, GN8.

The phylogenetic trees for *E. coli* and *K. pneumoniae*, generated from the PFGE results, can be seen in figures 3.5 and 3.6 respectively. As with the rep-PCR results, minimal clustering was seen in *E. coli* or *K. pneumoniae* isolates. Isolates did not cluster according to the wards from which they were isolated, or between hospital- or community-acquired infections. When examining the phylogenetic tree of *E. coli* isolates using a cut-off value of 70%, there were 6 clusters of two isolates and 53 singletons. When using a cut-off of 95% there were two

clusters of two isolates and 61 singletons. Two isolates, GN58 and GN59 were identified as identical (and had also been identified as identical by rep-PCR). When examining *K. pneumoniae* using a cut-off of 70% there were 6 clusters and 50 singletons. The largest cluster was made up of 5 isolates, but most of the clusters (4/6) were made up of 2 isolates. When using a 95% cut-off there were two clusters of two isolates and 62 isolates singletons. Two isolates were identified as identical, namely GN50 and GN51 – which, interestingly, differs from the isolates identified as identical by rep-PCR.

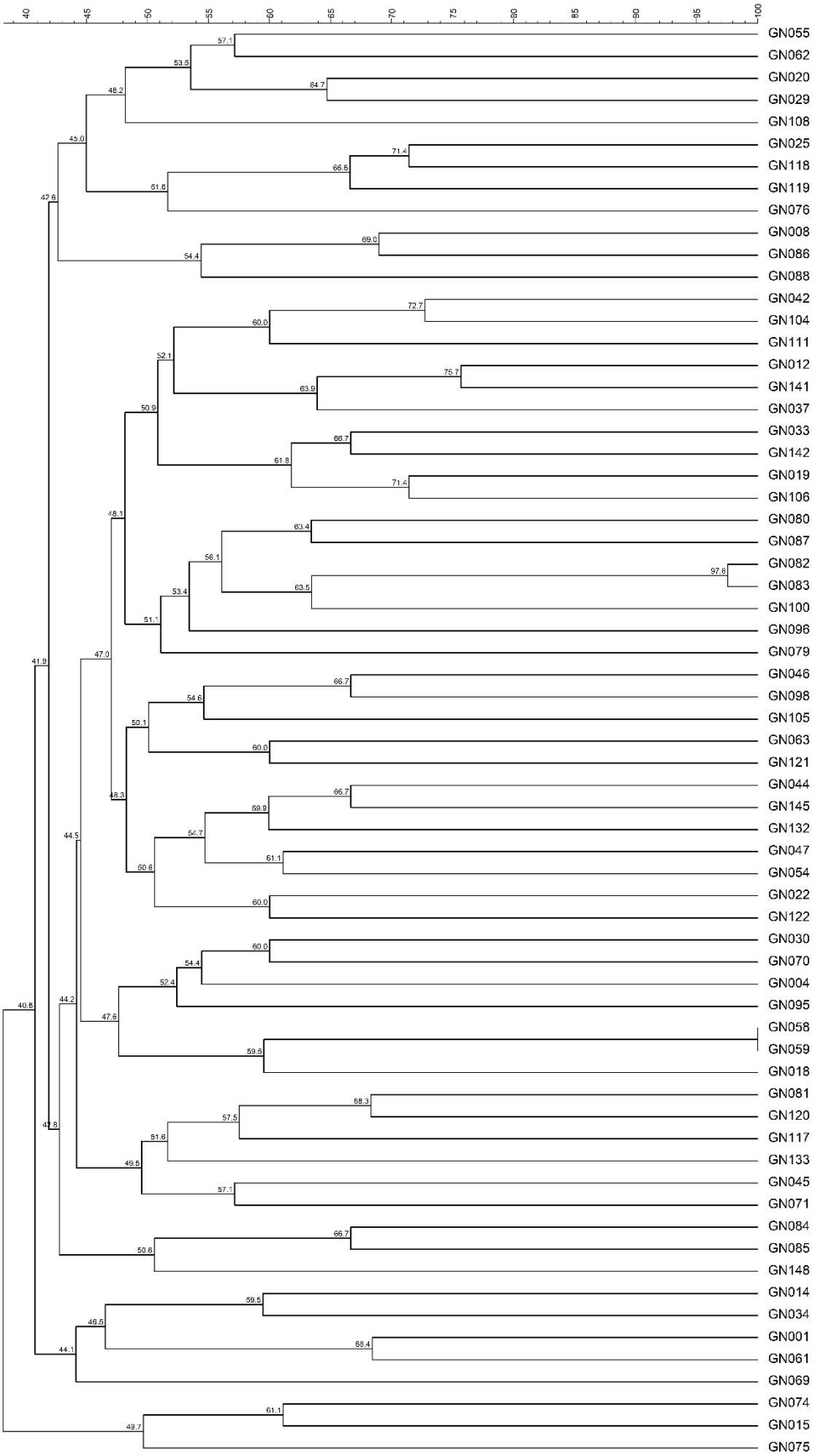


Figure 3.5: Phylogenetic tree generated from PFGE results for *E. coli* isolates.

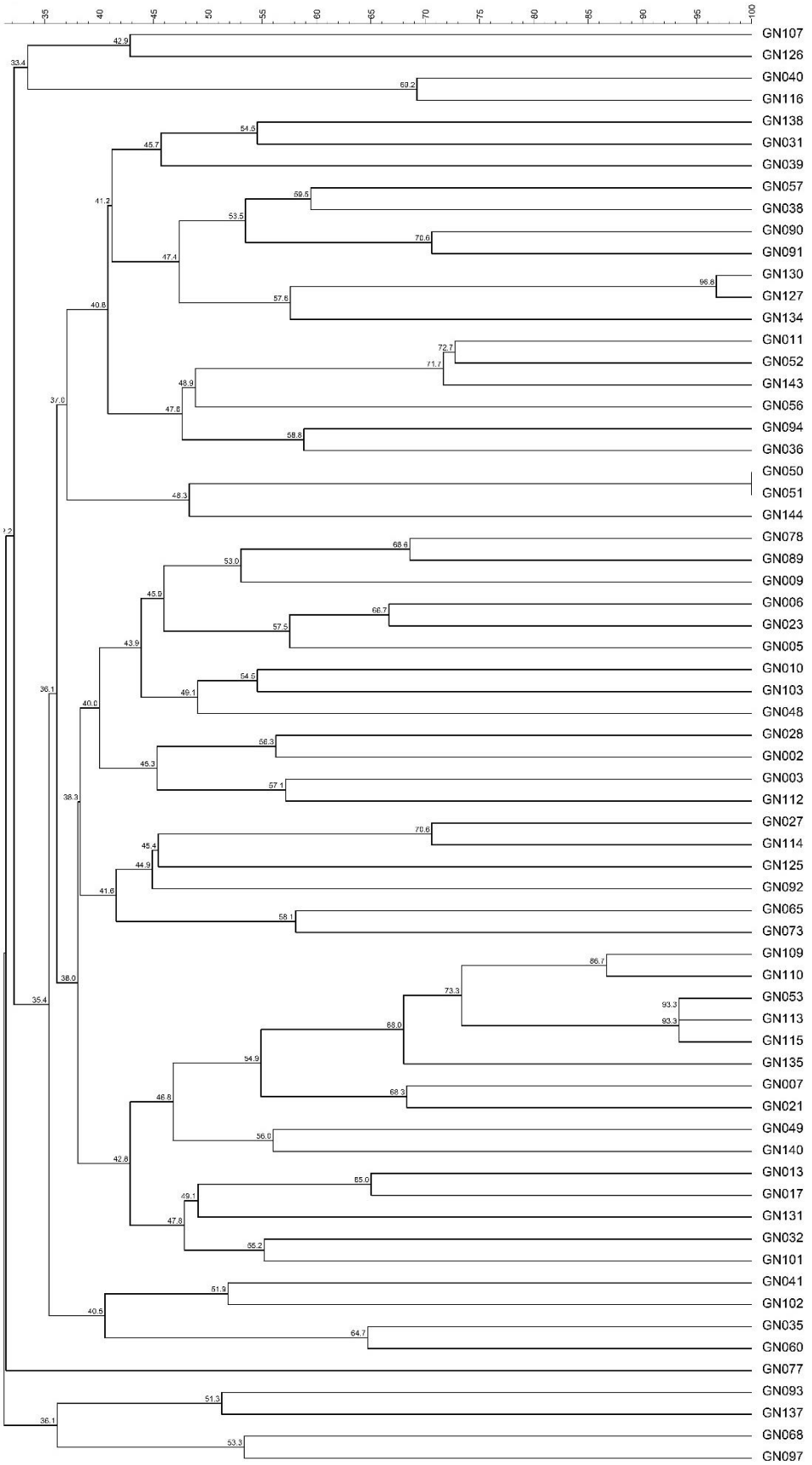


Figure 3.6: Phylogenetic tree generated from PFGE results for *K. pneumoniae* isolates.

3.3 Comparison of typing methods

The Simpson's Index of Diversity was calculated for *E. coli* and *K. pneumoniae* for each strain typing technique at two thresholds, 70% and 95% similarity. The Simpson's index of diversity was higher at a 95% similarity cut-off. This index was also higher for PFGE compared to rep-PCR (Table 3.2, Figure 3.7).

Table 3.2: Simpson's index of diversity for rep-PCR and PFGE.

Typing method and bacterial species	Total number of isolates	Cut-off	Number of clusters	Number of singletons	Simpson's index of diversity
Rep-PCR *E. coli*	70	70%	16	23	0.9150
		95%	1	68	0.9996
PFGE *E. coli*	65	70%	6	53	0.9965
		95%	2	61	0.9990
Rep-PCR *K. pneumoniae*	70	70%	16	20	0.8587
		95%	2	66	0.9991
PFGE *K. pneumoniae*	66	70%	6	50	0.9890
		95%	2	62	0.9990

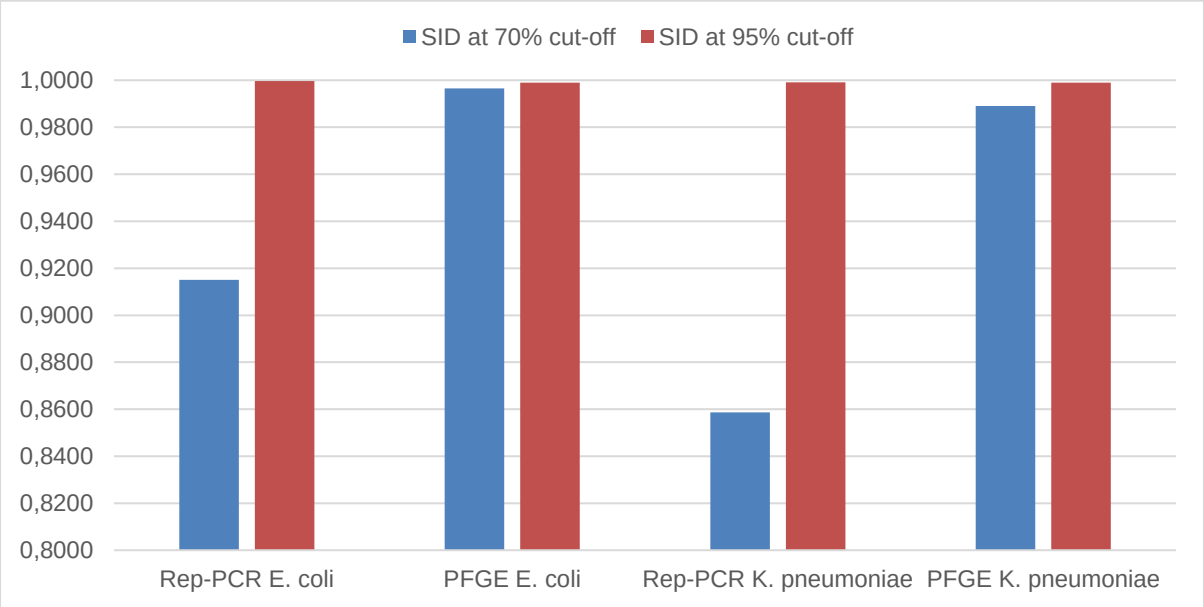


Figure 3.7: Simpson's Index of Diversity (SID) values of rep-PCR and PFGE for *E. coli* and *K. pneumoniae*.

4. Discussion

Strain typing of the *E. coli* and *K. pneumoniae* isolates collected from BSI at Tygerberg Hospital over the period of a year provides important epidemiological information about the bacterial strains causing bloodstream infections in our setting. PFGE remains the gold standard technique for strain typing. This method is laborious and requires four days to obtain a result, however it provides high levels of discrimination as it evaluates the entire bacterial genome. rep-PCR is a strain typing technique with a shorter turnaround time and simpler methodology. It is a viable alternative to PFGE for large sample groups and when information is required rapidly (e.g. a possible outbreak) [99]. Brolund *et al.* recommends using rep-PCR as a primary screening method to exclude unrelated isolates, then using a more discriminatory method to investigate related isolates [104].

Phylogenetic trees generated from both strain typing techniques show that the *E. coli* and *K. pneumoniae* isolates included in this study are genetically diverse. Two thresholds were used to analyse the relatedness of isolates; the cut-off of 70% represents isolates that are possibly related, while 95% represents isolates that are closely related [99]. As expected, increased discrimination and more diversity was seen at the 95% threshold for both typing techniques. For *E. coli* and *K. pneumoniae* with both strain typing techniques, the number of clusters decreased and the number of singletons increased when the threshold was raised from 70% to 95%; this was also demonstrated by the higher value of Simpson's Index of Diversity. However, these differences were more prominent in rep-PCR as compared to PFGE. This may be due to the fact that PFGE is a more discriminatory method than rep-PCR and divides the isolates into more genetic lineages [104]. Rep-PCR may be overestimating the genetic relatedness of these isolates. The increased discrimination of PFGE and increased clustering of isolates by rep-PCR has been observed in other studies [99,100,104].

Both rep-PCR and PFGE identified the *E. coli* isolates GN58 and GN59 as identical. For *K. pneumoniae* there were discrepancies in the isolates that were identified as identical by rep-PCR and PFGE. Rep-PCR denoted GN27 and GN32 as well as GN90 and GN91 as identical, while PFGE identified GN50 and GN51 as identical. According to the PFGE results, GN27 and GN32 are not closely related, although GN90 and GN91 are (70.6% relatedness); this likely reflects the better discriminatory ability of PFGE. Rep-PCR did not cluster GN50 and GN51 closely. Studies by Koroglu *et al.* and Hahm *et al.* also found discrepancies between the two methods in terms of identifying certain isolates as identical and certain isolates as singletons [100,105]. Hahm *et al.* evaluated a number of typing techniques, such as PFGE, rep-PCR, multiplex-PCR, ribotyping and Amplified Fragment

Length Polymorphism (AFLP), and found that the different methods did not group the isolates identically and isolates were found in different positions on the phylogenetic trees [105]. This indicates that the typing methods reveal different information about the phylogenetic relationships of the tested isolates. This is probably due to the fact that the various typing methods target different genetic regions.

In addition to the genetic diversity amongst isolates, isolates did not cluster according to the wards from which they were isolated, or between hospital- or community-acquired infections or by phenotypic ESBL production. Other studies have reported similar diversity amongst isolates. Studies in Taiwan and Thailand investigated the relatedness of ESBL producing *E. coli* and *K. pneumoniae* isolates from clinical specimens by PFGE and found the isolates to be genetically divergent [106,107]. Both studies examined only ESBL producing isolates from all clinical specimens, which were identified by disc diffusion. Lin *et al.* used a threshold of 75% to determine similarity, while Kiratisin *et al.* used a threshold of 80% to identify related isolates and a 60% threshold to identify distantly related isolates. Both studies concluded that ESBL producing *E. coli* and *K. pneumoniae* isolates are polyclonal in their setting and speculate that resistance genes are being transferred among isolates. A Spanish study looking at ESBL producing *E. coli* from bloodstream infections found the isolates to be unrelated by rep-PCR and PFGE [108]. The *E. coli* isolates with similar rep-PCR patterns were investigated further by PFGE, although the threshold values for similarity were not stated. This study concluded that patients were most probably colonised by the bacteria and developed infection whilst in hospital. Another Spanish study focused on ESBL producing *E. coli* and *K. pneumoniae* found isolates to be highly diverse by rep-PCR [109]. However, the study did find that *K. pneumoniae* isolates in some hospitals were clonally related, although they did not indicate in how many of the 40 healthcare centres this was observed. The authors noted that *K. pneumoniae* isolates are often involved in outbreaks.

On the other hand, various studies have demonstrated that bacteria isolated from infections are closely related. For example, an American study focused on ceftazidime resistant *E. coli* and *K. pneumoniae* isolates causing BSIs, found the isolates to be part of a poly-clonal outbreak, using PFGE [110]. The majority of the patients in this study were in nursing homes and the investigators speculate that cross-contamination occurred via a common source. A study conducted in South Africa looking at infections with ESBL producing *E. coli* found that almost half (45%) of the isolates belonged to the highly successful *E. coli* clonal complex ST131 [111]. Although most of the isolates were from urine specimens and the sample size was small (n=24).

MLST is another strain typing method which provides additional value as it identifies defined strain types and allows comparison to other epidemiological studies and MLST databases

[112]. Due to the large amount of sequencing required for this method, we had initially planned to perform MLST on selected representative isolates from the larger clusters (identified by PFGE or rep-PCR). However, the results of the typing showed no predominant clusters, and this approach was abandoned as it would have required performing MLST on the majority of isolates.

5. Conclusion

The collected isolates were typed by two strain typing techniques, providing some insight into the epidemiology of *E. coli* and *K. pneumoniae* strains causing BSIs in this setting. The results from both strain typing techniques demonstrate that the isolates in this study are genetically diverse, with little clustering. The diversity of circulating *E. coli* and *K. pneumoniae* strains suggests that patient-to-patient transmission of bacteria or contamination of the environment is not the main cause of BSIs. Due to the diverse bacterial strains causing infection, it makes it difficult to ascertain transmission routes and implement controls to reduce the number of BSIs. While the lack of a common circulating clone/s may suggest less ongoing transmission of the organisms (and thus suggests adequate infection prevention and control (IPC) measures), further studies would be required to better understand the transmission dynamics of these organisms, which may further inform IPC activities.

Chapter 4: Resistance genes

1. Introduction

Resistance to β-lactam antibiotics in Enterobacteriaceae is most often as a result of the production of plasmid mediated β-lactamase enzymes, such as Extended-Spectrum β-lactamases (ESBLs) and carbapenemases [52]. Understanding the molecular epidemiology of these resistance genes is important to define the impact of these resistance mechanisms and it may also assist in guiding correct empiric treatment [101].

The β-lactamases TEM-1, TEM-2 and SHV-1 are the progenitors for the TEM and SHV ESBLs respectively and are not able to hydrolyse the extended-spectrum β-lactams. While TEM- and SHV-related ESBLs may differ by only a few amino acids from their respective progenitors, these changes can result in a substantial change in enzymatic ability [22]. TEM and SHV β-lactamases can be divided into three groups based on their functional properties or phenotype [53,113]. The first group can hydrolyse penicillins and early cephalosporins and are strongly inhibited by β-lactamase inhibitors like clavulanic acid and tazobactam. Examples include TEM-1, TEM-2, TEM-12, SHV-1, SHV-11 and SHV-89. The second group has the same hydrolysis profile as the first but are resistant to clavulanic acid. TEM β-lactamases with this phenotype as also known as Inhibitor Resistant TEMs (IRT). TEM-30, TEM-31, SHV-10 and SHV-72 are examples of IRT TEMs. The third group is made up of ESBLs, which are able to hydrolyse penicillins, early and extended-spectrum cephalosporins but remain sensitive to inhibition by clavulanic acid. Examples include TEM-3, TEM-10, SHV-2 and SHV-115. All CTX-M ESBLs also fall into this group.

In earlier studies β-lactamase genes were analysed biochemically by isolating the protein and determining its isoelectric point by isoelectric focusing and then performing enzymatic studies to identify the hydrolysis and inhibition profile of the gene [65]. Currently molecular methods such as Polymerase Chain Reaction (PCR), sequencing, probes and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) are mostly used to identify β-lactamase genes.

Databases have been created to standardize the nomenclature for the growing number of β-lactamases (http://www.lahey.org/studies, http://www.laced.uni-stuttgart.de/) [53]. These databases contain information about TEM and SHV β-lactamases such as their phenotype, specific amino acid substitutions and GenBank accession numbers for the specific genes. These databases are valuable as they provides important information about the majority of the TEM and SHV β-lactamases. Unfortunately not all of these β-lactamases have been

characterised and some have been withdrawn or are invalid [113]. It is not clear whether measures are in place to address these deficits.

The aim of this component of the study was to determine the prevalence of ESBLs and carbapenamases in the collected set of isolates, and to further characterize the types of ESBLs and carbapenemases by a combination of PCR and sequence analysis.

2. Methods and materials

2.1 Bacterial isolates and controls

All *Escherichia coli* and *Klebsiella pneumoniae* isolates previously described in chapter two were screened for resistance genes. DNA was extracted from these isolates as described in chapter 3.

A previously characterised control strain containing all three ESBL genes was supplied by Dr C. Moodley from the NHLS National Institute for Communicable Diseases (NICD) satellite unit at Groote Schuur Hospital. Control strains containing all the screened carbapenemase genes were obtained from the NICD. Extracted DNA from these controls was combined to maximise space available for testing isolates.

2.2 PCR amplification of β-lactamase genes

All isolates were screened for the three β-lactamase gene families, *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M,}$ using a multiplex PCR. Specific primers were used for each family and an internal amplification control, *rpoB*, was included (Table 4.1). The internal amplification control indicates that the PCR reaction was successful and DNA quality is adequate. Primers were synthesised by IDT (Integrated DNA Technologies, South Africa). The KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems, South Africa) was used and 1 µl of bacterial DNA was used for each reaction. All PCR reactions were performed using the ProFlex PCR system thermocycler (Applied Biosystems, United States of America). The PCR amplification conditions were as follows: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 15 seconds, annealing at 67°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 3 minutes. Final primer concentrations were 0.2 pmol/µl for all primers except primers for *bla*$_{SHV}$ which were 0.24 pmol/µl. A no-template control (NTC) containing no bacterial DNA was included in each PCR to exclude contamination.

Gel electrophoresis of the PCR products was performed using the PowerPac Basic power supply system (Bio-Rad, South Africa). PCR products were separated using a 2-2.5% agarose gel run at 100V for 90 minutes in a Tris-acetic acid-EDTA (TAE) buffer, and using

KAPA universal DNA Ladder (Kapa Biosystems, South Africa) as a molecular size marker. Agarose gels were stained using NovelJuice (GeneDireX, South Africa) and the image was visualised and captured using the UVItec Alliance 2.7 gel documentation system (UVItec, United Kingdom).

Table 4.1: Primers used for the ESBL multiplex PCR [114,115].

Target gene	Primer	Sequence (5' – 3')	Product size (bp)	Source
***bla*$_{TEM}$**	TEM-164.SE TEM-165.AS	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	Monstein *et al.* (2007)
***bla*$_{SHV}$**	bla-SHV.SE bla-SHV.AS	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	747	Monstein *et al.* (2007)
***bla*$_{CTX-M}$**	CTX-M-U1 CTX-M-U2	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	Monstein *et al.* (2007)
rpoB	rpoB-F rpoB-R	AACCAGTTCCGCGTTGGCCTGG CCTGAACAACACGCTCGGA	1088	Hoffmann and Roggenkamp (2003)

2.3 TEM and SHV characterisation

All TEM and SHV amplification products (section 2.2) were sequenced to further characterise the genes. A single-plex PCR was performed using either the TEM or SHV primers listed in section 2.2 as appropriate, using KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems). The PCR amplification conditions were similar to the ESBL PCR: initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 3 minutes. In these single-plex assays the final primer concentrations were 0.4 pmol/µl for all primers. Gel electrophoresis was performed on all PCR products as described above in section 2.2, to ensure amplification was successful.

The PCR products were sent for sequencing at Inqaba Biotech (South Africa) using the forward primers. Sequencing results were analysed using the BioEdit Sequence Alignment Editor [116]. Sequences of amplification products for the TEM-related genes were compared to TEM-1, TEM-2 and ESBL TEM sequences downloaded from National Centre for Biotechnology Information (NCBI) [117]. SHV amplification product sequences were identified by translating the DNA sequences to amino acid sequences and analysing them through the BLAST database [118]. Amino acid sequences were used for SHV analysis as the DNA sequences contained numerous nucleotide substitutions and it was not known which were synonymous and which were nonsynonymous. If further discrimination was needed, sequences were compared to SHV sequences downloaded from NCBI [117].

2.4 Characterisation of CTX-M genes

All isolates that were positive for CTX-M genes in the β-lactamase PCR, were further tested to classify the CTX-M enzyme/s into the five CTX-M sub-groups. Specific primers for each of these groups were used, with Groups 8 and 25 using a shared reverse primer (Table 4.2). Primers were synthesised by IDT (Integrated DNA Technologies). The multiplex PCR was performed using the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems) and the ProFlex PCR system thermocycler (Applied Biosystems). The cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 3 minutes. Final primer concentrations were 0.2 pmol/µl for all primers. PCR products were run on an agarose gel and visualised as described in section 2.2. A NTC containing no bacterial DNA was included in each PCR performed to confirm that there was no contamination. Controls were not available; therefore selected amplification products were sent for sequencing to confirm correct amplification. These isolates could then be used as controls in successive PCR reactions. Isolates showing ambiguous results were retested using a singleplex PCR with each primer pair, using the cycling conditions listed for the multiplex PCR, and products were sent for sequencing at Inqaba Biotech (South Africa) to confirm results.

Table 4.2: Primers used for the CTX-M multiplex PCR [119].

bla_{CTX-M} subgroup	Primer	Sequence (5' – 3')	Product size (bp)
Group 1	CTX-M G1-F CTX-M G1-R	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	415
Group 2	CTX-M G2-F CTX-M G2-R	CGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	552
Group 8	CTX-M G8-F CTX-M G8/25-R	TCGCGTTAAGCGGATGATGC AACCCACGATGTGGGTAGC	666
Group 9	CTX-M G9-F CTX-M G9-R	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	205
Group 25	CTX-M G25-F CTX-M G8/25-R	GCACGATGACATTCGGG AACCCACGATGTGGGTAGC	327

2.5 Amplification of carbapenemase genes

A multiplex PCR was used to screen all isolates for selected carbapenemase genes. The carbapenemase genes *bla*$_{IMP}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$ and *bla*$_{VIM}$ along with an internal amplification control, *rpoB*, were amplified using specific primers (Table 4.3). Primers were

synthesised by IDT (Integrated DNA Technologies). The KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems) was used and the PCR conditions were optimised to a touchdown PCR to minimise non-specific binding of primers. All PCR reactions were performed using the ProFlex PCR system thermocycler (Applied Biosystems). The cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, 20 cycles of denaturation at 95°C for 15 seconds, annealing at 68°C for 30 seconds and extension at 72°C for 1 minute, followed by 10 cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute and lastly, a final extension at 72°C for 5 minutes. Final primer concentrations were 0.2 pmol/µl for all carbapenemase gene primers and 0.08 pmol/µl for internal control primers. A NTC was included in each PCR run, to ensure that there was no contamination present. Gel electrophoresis was performed on all PCR products as described in section 2.2. PCR products were run on an agarose gel and visualised as mentioned in section 2.2.

Table 4.3: Primers used for the carbapenemase PCR [102,115].

Target gene	Primer	Sequence (5' – 3')	Product size (bp)	Source
***bla*$_{IMP}$**	IMP-F IMP-R	CTACCGCAGCAGAGTCTTTGC GAACAACCAGTTTTGCCTTACC	591	Zowawi *et al.* (2014).
***bla*$_{KPC}$**	KPC-F KPC-R	ATCTGACAACAGGCATGACG GACGGCCAACACAATAGGTG	452	Zowawi *et al.* (2014).
***bla*$_{NDM}$**	NDM-F NDM-R	GCAGGTTGATCTCCTGCTTG ACGGTTTGGCGATCTGGT	203	Zowawi *et al.* (2014).
***bla*$_{OXA-48}$**	OXA-48-F OXA-48-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	Zowawi *et al.* (2014).
***bla*$_{VIM}$**	VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	Zowawi *et al.* (2014).
rpoB	rpoB-F rpoB-R	AACCAGTTCCGCGTTGGCCTGG CCTGAACAACACGCTCGGA	1088	Hoffmann and Roggenkamp (2003)

3. Results

3.1 Amplification of β-lactamase genes

A multiplex PCR adapted from Monstein *et al.* was used to screen all isolates for the β-lactamase genes *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ as well as an internal control, *rpoB* [114,115]. The PCR conditions were optimised to reduce non-specific binding as far as possible. A representative image of an electrophoresis gel can be seen in figure 4.1.

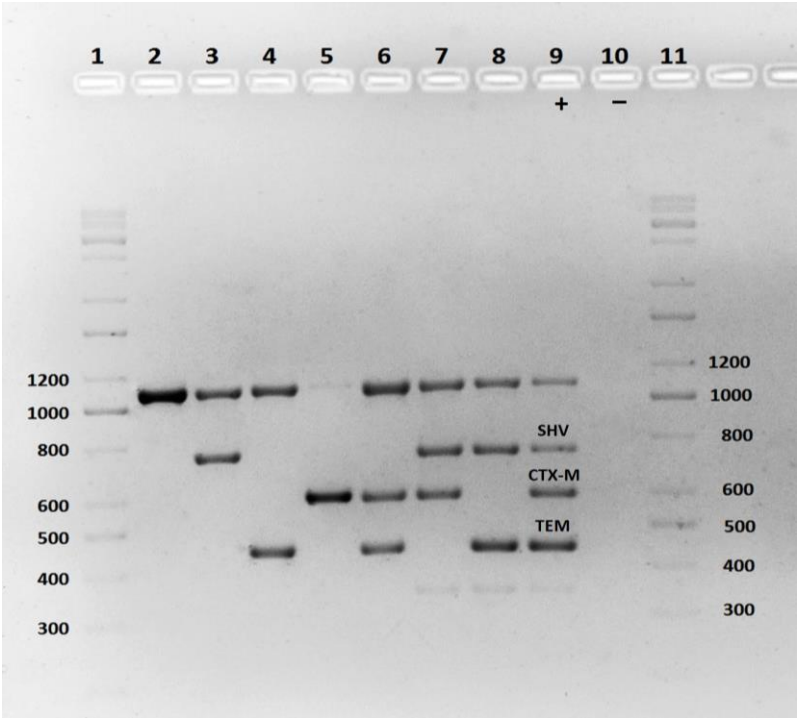


Figure 4.1: Multiplex PCR identification of β-lactamase genes. Lanes 1 and 11 contain the KAPA universal DNA ladder, lanes 2- 8 are clinical isolates; the positive control in lane 9 shows the desired amplification products for SHV, TEM and CTX-M genes and the internal control, *rpoB*, at 1100bp; lane 10 is the NTC.

The overall distribution of β-lactamase genes was as follows; in 53.6% (75/140) of isolates one β-lactamase family was detected, while 16.4% (23/140) of isolates contained two β-lactamase family genes and 16.4% (23/140) contained all three gene families. No β-lactamase genes were detected in 13.6% (19/140) of isolates (Figure 4.2).

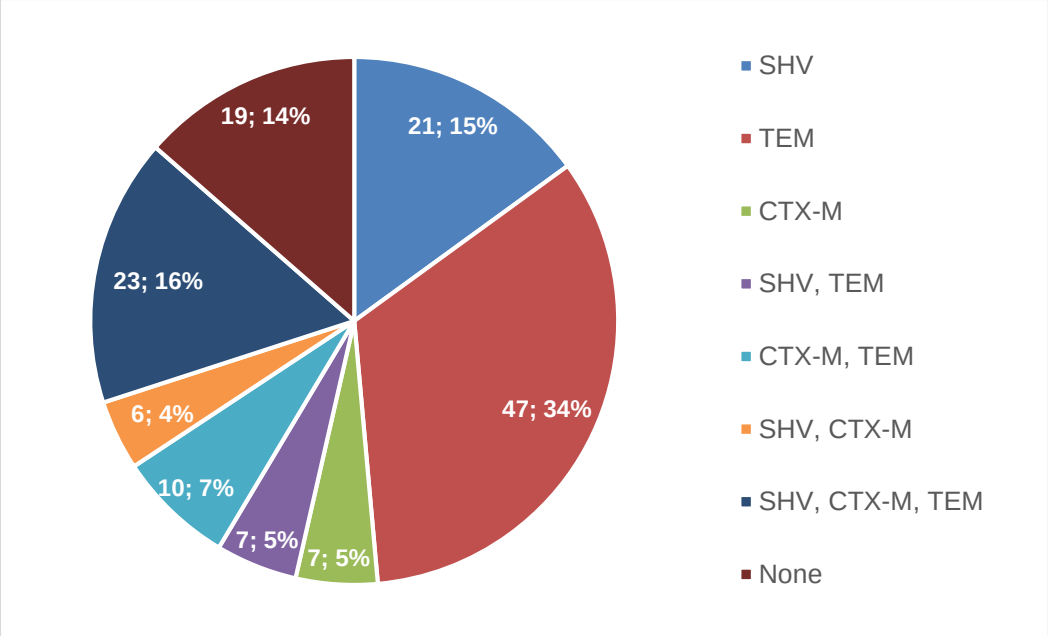


Figure 4.2: Graphical representation of the β-lactamase genes detected in isolates.

The distribution of β-lactamase genes differed between *E. coli* and *K. pneumoniae* isolates ($p<0.01$) (figure 4.3). *E. coli* isolates mainly contained TEM genes alone (45/70) or no β-lactamase genes (15/70). *K. pneumoniae* isolates showed more variation in β-lactamase genes with the most common profiles being a combination of all three β-lactamase genes (23/70) or SHV genes alone (21/70).

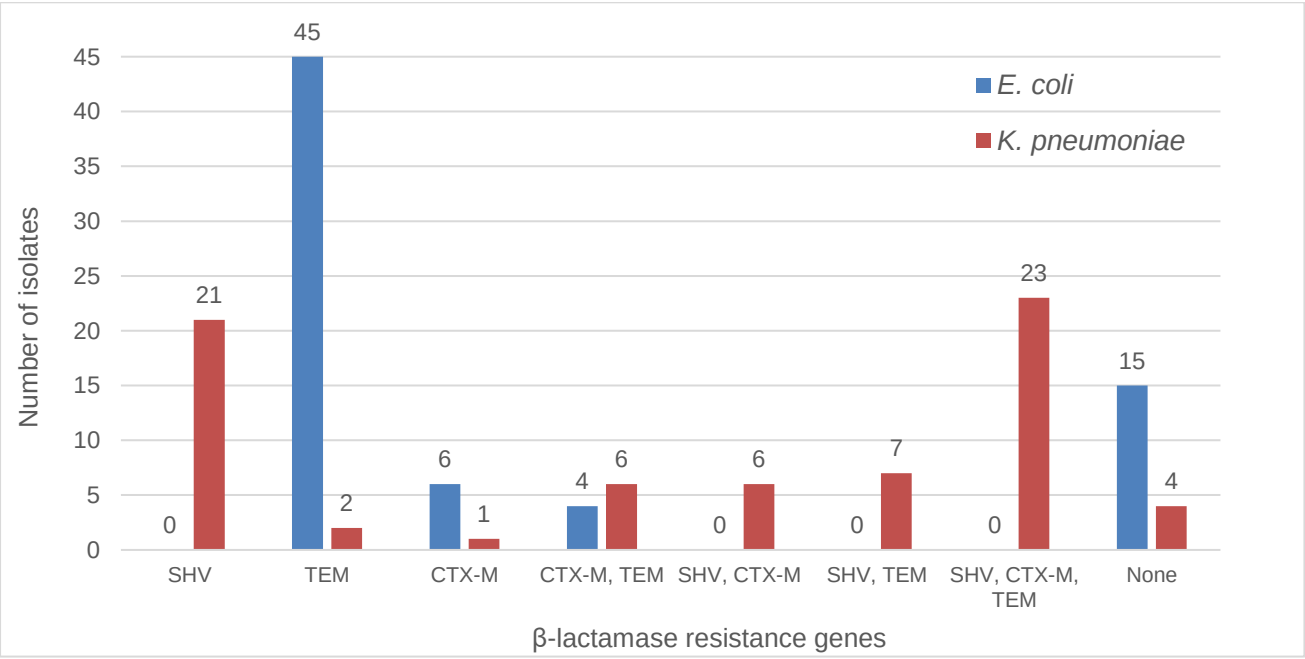


Figure 4.3: Distribution of β-lactamase genes in *E. coli* and *K. pneumoniae* isolates.

3.2 TEM and SHV characterisation

All TEM and SHV PCR products were sequenced to determine whether the genes were ESBLs or not. The results are summarised in table A.1 in appendix A. Eighty-seven isolates were positive for TEM genes, of which 86 amplification products were successfully sequenced and were included in the analysis. The sequence quality for isolate GN9 was too poor to analyse properly. The 86 sequences were compared to TEM-1, TEM-2 and ESBL TEM genes obtained from the GenBank database. Eighty-one (94.2%) of the TEM genes were found to be TEM-1, 3 (3.5%) had one nucleotide difference from TEM-1 and were found to be TEM-2. Only two (2.3%) TEM genes were ESBL TEM genes, however the specific TEM genes could not be identified as the sequenced products were too short.

Fifty-seven isolates were positive for SHV genes, all of which were successfully sequenced. These sequences were translated to amino acid sequences and identified using the BLAST database (table 4.4). The SHV amino acid sequences of a few isolates were identical to more than one SHV protein, such as SHV-14 and SHV-161 as well as SHV-1, SHV-28 and SHV-125. These isolates' SHV nucleotide sequences were then compared to those SHV gene sequences, downloaded from the NCBI database.

As displayed in table 4.4, the most common SHV genes were SHV-125 (42.1%) and SHV-11 (33.3%). According to the Lahey database, the SHV genes SHV-1, SHV-11, SHV-14, SHV-26, SHV-33, SHV-62 and SHV-76 are some of the genes that do not possess ESBL activity [53]. The SHV genes SHV-28, SHV-125, SHV-137 have not yet had their phenotype described. In table 4.4 the data was divided according to activity of the SHV genes. Therefore, 4/57 (7%) of the SHV genes from our study have previously confirmed ESBL activity, 27/57 (47.4%) have only narrow spectrum β-lactamase activity and for 26/57 (45.6%) of genes, it is not clear whether these gene products have extended-spectrum activity.

Table 4.4: Distribution of SHV genes.

ESBL activity	SHV gene	Nr of isolates	Isolates	Total nr of isolates
Non ESBL	SHV-1	1	GN116	27 (47.4%)
	SHV-11	19	GN5, GN11, GN13, GN27, GN32, GN39, GN40, GN41, GN50, GN51, GN56, GN78, GN92, GN101, GN112, GN134, GN135, GN137, GN143	
	SHV-14	3	GN10, GN93, GN140	
	SHV-26	1	GN36	
	SHV-33	1	GN128	
	SHV-62	1	GN68	
	SHV-76	1	GN125	
ESBL	SHV-2	1	GN23	4 (7%)
	SHV-5	1	GN94	
	SHV-126	2	GN21, GN38	
Unknown activity	SHV-28	1	GN103	26 (45.6%)
	SHV-125	24	GN6, GN7, GN17, GN28, GN31, GN35, GN48, GN49, GN53, GN57, GN60, GN65, GN73, GN77, GN97, GN109, GN110, GN113, GN115, GN126, GN127, GN130, GN139, GN144	
	SHV-137	1	GN3	

The β-lactamase PCR and sequencing results were analysed together and summarised below in table 4.5. The most commonly seen β-lactamase gene in *E. coli* was TEM β-lactamases; only 11 (15.7%) *E. coli* isolates contained ESBL genes. *K. pneumoniae* isolates had a variety of resistance gene profiles, with the most common β-lactamase genes being SHV ESBLs and SHV β-lactamases. Only four (5.7%) of *E. coli* isolates contained multiple β-lactamase genes, while 42 (60%) of *K. pneumoniae* contained multiple β-lactamase genes. A table showing the detailed distribution of β-lactamase and ESBL genes can be seen in appendix A (figure A.1).

Table 4.5: Distribution of β-lactamase genes with and without extended-spectrum activity among *E. coli* and *K. pneumoniae* isolates

		E. coli (n=70)	*K. pneumoniae* (n=70)	Total (n=140)
No β-lactamase genes		15	4	19
β-lactamase genes with no extended-spectrum activity [a]		44	27	71
β-lactamase/s genes with extended-spectrum activity [b]	TEM ESBL	1	1	2
	SHV ESBL	0	2	2
	CTX-M	10	34	44
	SHV ESBL & CTX-M	0	2	2
	Any ESBL	11 (15.7%)	39 (55.7%)	50 (35.7%)

[a]: This includes TEM-1, TEM-2, and all SHV genes either known or suspected not to have extended activity, as detailed in the text.

[b]: For isolates containing multiple genes where one has extended-spectrum activity and one or more, only the gene/s with extended-spectrum activity are shown.

3.3 Characterisation of CTX-M genes

All isolates that were positive for CTX-M genes in the β-lactamase PCR, were further tested to classify the CTX-M enzymes into the five different CTX-M sub-groups. Forty-six isolates demonstrated the presence of CTX-M genes in the multiplex β-lactamase PCR. Forty-two (91.3%) of these belonged to CTX-M group 1, three (6.5%) belonged to group 9 and one isolate, GN9, was untypeable. This isolate had abnormal bands present; subsequently each of the assays in the multiplex CTX-M PCR was performed as a singleplex assay to further investigate this result. The amplification products of the singleplex PCRs did not match what was seen in the multiplex CTX-M PCR. The singleplex PCR products were sent for sequencing; the amplified products were not β-lactamase genes as they were identified as a major facilitator superfamily (MFS) transporter, which is a membrane transport protein. The amplified products were interpreted as non-specific binding.

3.4 Amplification of carbapenemase genes

All isolates were screened for carbapenemase genes using a multiplex PCR including an internal control, *rpoB* [102,115]. The PCR was optimised as a touchdown PCR to reduce non-specific binding (figure 4.4). Carbapenemase genes were not detected in any of the isolates.

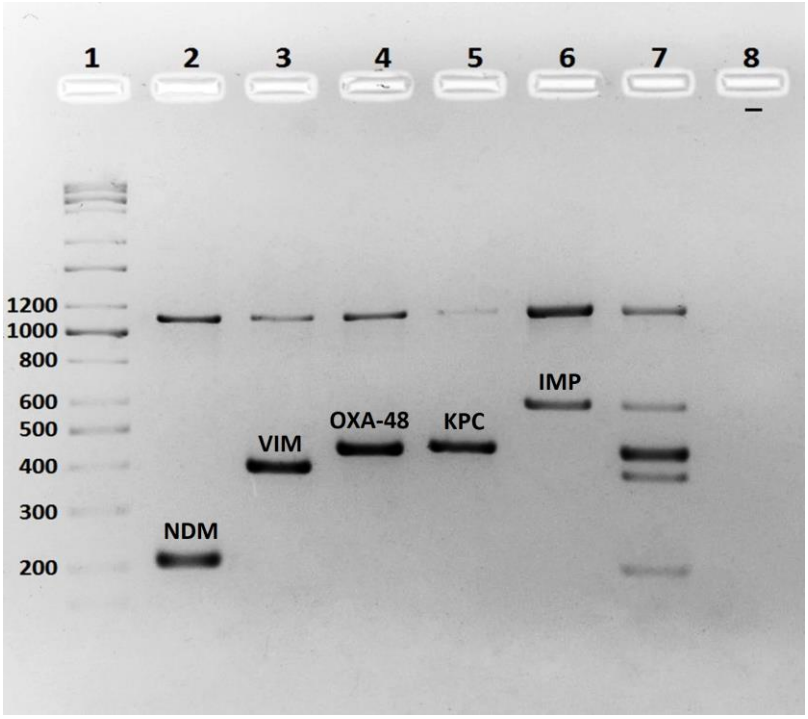


Figure 4.4: Multiplex PCR identification of carbapenemase genes. Lanes 1 and 11 contain the KAPA universal DNA ladder, lanes 2 to 6 contain the various control isolates. Lane 7 contains combination of all controls. Internal control, *rpoB*, at 1100bp.

4. Discussion

4.1 ESBL characterisation

A multiplex PCR was used to screen all isolates for the TEM, SHV and CTX-M β-lactamase genes; the addition of an internal control, *rpoB*, ensured that DNA quality was sufficient and amplification had taken place, and that the absence of any β-lactamase genes was true and not a result of a failed PCR. The addition of this internal control meant further optimisation of this PCR which involved increasing the annealing temperature to 67°C. The concentration of the SHV primers was also increased slightly to allow for better amplification. Some faint non-specific bands were observed at 350bp after the internal control was added.

The majority (86.4%; 121/140) of isolates contained at least one β-lactamase gene. From the β-lactamase PCR only, TEM genes are the most common, seen in 87 isolates, followed by SHV in 57 isolates and CTX-M in 46 isolates. A study conducted in Pretoria, South Africa found overall similar rates of β-lactamase genes; 87% of isolates had β-lactamase genes [120]. The most common profile observed in their study was TEM, SHV and CTX-M at 36% but the level of SHV genes was lower, 4% alone and 12% with TEM in comparison to 16 and 25%, respectively, in our study. The isolates studied by Ehlers *et al.* consisted mainly of *K. pneumoniae* and *E. coli* (83%) but also included other Enterobacteriaceae, such as *Citrobacter freundii, Morganella morganii, Enterobacter cloacae* and *Proteus penneri*, which may explain the dissimilarity in some results. Alternatively, this may reflect differences in the epidemiology between the two settings. The study used the same PCR assay as was used in the current study.

This multiplex PCR is advantageous as it allows for the collective detection of important β-lactamase genes. This provides important information about the molecular epidemiology of these genes. Unfortunately, it does not differentiate between non-ESBL and ESBL TEM and SHV genes, which is necessary to determine whether an isolate truly harbours ESBL genes. Therefore, all TEM and SHV genes were sequenced so that they could be differentiated into non-ESBL and ESBL TEMs and SHVs. It is necessary to investigate whether the genes are ESBLs as this will allow better comparison and interpretation of the data, and allow for correlation with results of phenotypic testing (which will be discussed in chapter 5). Previously, only isolates positive for CTX-M genes could be confidently identified as containing ESBL genes.

The majority of the TEM genes, 98%, were non-ESBL TEM genes, i.e. TEM-1 (94.2%; 72/86) and TEM-2 (3.5%; 3/86) genes. Therefore, in cases were these genes are found alone, these isolates are β-lactamase producers, not ESBL producers. This is consistent with the fact that TEM-1 is said to be the most common plasmid mediated β-

lactamase found in Gram negative bacteria [121]. In the two isolates with ESBL TEM genes, these genes were found alone, so these isolates were considered ESBL producers. Unfortunately, the amplified sequence was too short to further identify these ESBL genes.

Just under half of the SHV genes (47.4%; 27/57) were those that do not have ESBL activity, 7% (4/57) of SHV genes were ESBL SHV genes, while the spectrum of activity of 45.6% (26/57) of the SHV genes' is unknown. The most common non-ESBL SHV gene was SHV-11 (73.1%; 11/27). A review on SHV genes stated that SHV-11 is the most successful non-ESBL SHV variant [113]. Only one isolate contained the SHV-1 gene. It was expected that the majority of *K. pneumoniae* isolates would contain the non-ESBL SHV gene, SHV-1, as this β-lactamase is commonly harboured on the chromosomes of *K. pneumoniae* isolates [9]. It is possible that the isolates may harbour SHV-1 chromosomally in addition to another plasmid-encoded SHV gene, which may have been preferentially amplified due to a higher copy number. It is unlikely to be clonal expansion of an unusual strain, based on the strain typing results in chapter 3. The SHV genes SHV-28, SHV-125, SHV-137 have not yet had their phenotype described, and it is unknown whether these genes have ESBL activity or not.

The TEM and SHV sequences used to further identify the genes were only a portion of the gene, with the SHV sequence being 747bp and the TEM sequence being only 445bp, while the full TEM and SHV genes are over 850bp [58]. Having the full TEM and/or SHV sequences may have allowed better identification of these genes. The Lahey database was used to determine whether SHV genes have ESBL activity or not [53]. This database is useful in that it shows described TEM and SHV β-lactamase genes and the specific amino acid changes for each gene. However, numerous genes have not had their phenotype described so it is unknown whether these β-lactamase genes have ESBL activity. To add to the difficulties in using the database, some β-lactamase genes have been withdrawn or have more than one name. The profusion of research (including the current study) describing old and new β-lactamases makes curating and maintaining such databases more and more difficult. It is not clear whether this database will be updated and if further work is being done to investigate the genes that are not fully described.

Although analysis of the sequencing results was difficult, it did enable the assignment of TEM and SHV genes as ESBL or non-ESBL genes, thereby better interpreting the β-lactamase PCR results. When analysing the β-lactamase PCR and sequencing data together, the number of β-lactamase genes found in the isolates are 85 TEM β-lactamase genes and 27 SHV β-lactamase genes. The most commonly seen ESBL genes are CTX-M (46), followed by SHV (4) and then TEM (2). CTX-M genes were frequently observed in combination with other non-extended β-lactamase genes (36/46; 78.3%) as well as with

ESBL genes (3/46; 6.5%). Fifty isolates contained at least one ESBL gene, yet only 7 isolates had CTX-M genes in isolation. A study on ESBL producing *E. coli* and *K. pneumoniae* isolates observed co-production of CTX-M genes and other β-lactamase and ESBL genes in 81.3% and 97.6% of *E. coli* and *K. pneumoniae* isolates respectively [107].

Isolates contain multiple β-lactamase resistance genes as this may result in the isolate being able to hydrolyse an increased spectrum of antibiotics. β-lactamase TEMs are able to hydrolyse first generation cephalosporins, but have less activity against oxyimino cephalosporins [59]. CTX-M enzymes have greater activity against oxyimino cephalosporins, particularly cefotaxime [59]. Certain SHV and TEM genes, such as SHV-10 and TEM-103, have an inhibitor resistant phenotype, making them resistant to β-lactamase inhibitors such as clavulanic acid [58,122]. Therefore, a combination of genes may contribute to a bacterial strain having resistance to an increased range of β-lactam antibiotics. A Kenyan study on *E. coli* isolates spanning 18 years, observed β-lactamase genes in combination for 35.3% of isolates [122]. A study on ESBL producing Enterobacteriaceae in South Africa observed β-lactamase genes in combination in 58.5% of isolates [120]. Gürntke *et al.* observed the combination of TEM, SHV and CTX-M genes in 57.1% of *K. pneumoniae* isolates from bloodstream infections [123]. A study from Syria on the prevalence of β-lactamases in *E. coli* and *K. pneumoniae* isolates, observed a combination of β-lactamase genes in 64.8% of *E. coli* isolates and 100% of *K. pneumoniae* isolates [124].

Other studies focused on *E. coli* and *K. pneumoniae* have also reported that CTX-M genes are the most commonly found ESBL genes in their setting, although these studies cited much higher rates of CTX-M genes in their isolates (67-99.6%) [107,123]. A recent review on ESBLs stated that CTX-M ESBLs are now the most prevalent ESBLs globally [9]. CTX-M ESBLs in our study were more often found in *K. pneumoniae* (78.3%) than *E. coli* (21.7%). It was expected to find more CTX-M ESBLs in *E. coli* as they are said to be the most common hosts of CTX-M genes and the highly successful *E. coli* clone ST131 frequently harbours CTX-M-15 [9,77,108]. This may be explained by the fact that in this study *E. coli* isolates were mostly community-acquired while *K. pneumoniae* isolates were mostly hospital-acquired, and the community-acquired isolates did not harbour many ESBL genes (7/47).

A PCR to further classify CTX-M genes was performed on isolates containing these genes in the β-lactamase PCR. This was done to enable comparison to epidemiology in other studies. Optimisation of this PCR was more complex as controls were not available. Isolates showing amplification of the correct size were sequenced so that they could be used as controls in further PCR reactions.

Most CTX-M genes (42/46; 91.3%) were classified as group 1. Group 1 contains CTX-M-15, the most commonly found CTX-M gene worldwide and is therefore expected to be the most common group [9]. The obtained results correspond with what has been found in other studies in South Africa and other countries [101,111,120,125]. Ehlers *et al.* and Peirano *et al.* both carried out studies in South Africa and found CTX-M genes from group 1 made up 47% and 63.6% of CTX-M genes. A study from the United States reported group 1 CTX-M genes made up 67.7% of CTX-M genes, while a study from Burkina Faso reported 94% of CTX-M genes belonged to group 1. One isolate could not be further typed into its specific CTX-M group. Singleplex PCRs with each primer set, corresponding to the different CTX-M families, was performed on this isolate. The PCR products were sent for sequencing. Although amplification was seen emulating group 1, sequencing results could not confirm this.

Using the combined genotypic information to assign isolates as ESBL or non-ESBL producers, 50/140 (35.7%) of isolates contain ESBL genes, 71 (50.7%) isolates contain β-lactamase genes, 19 (13.6%) contain no β-lactamase genes. Of the 71 isolates containing β-lactamase genes, 12 of these contain SHV genes for which the spectrum of activity is unknown. The ESBL producing isolates are mostly made up of *K. pneumoniae* isolates (39/50; 78%) and the non-ESBL β-lactamase producers are mostly made up of *E. coli* isolates (59/78; 75.6%).

The prevalence of ESBL genes in our setting is much lower than what is observed in other studies from Africa and Asia (65-98%), however these studies only screened isolates that were phenotypically ESBL producers for ESBL genes [124,126–128]. A study investigating the carriage of β-lactamase genes in *E. coli* isolates collected in Kenya over 18 years, reported 23.9% of isolates carried ESBL genes and 38.9% of isolates carried β-lactamase genes [122]. This is lower than what was observed in our study, which may be due to the study including various clinical specimens and collection of isolates began in 1992 when carriage of β-lactamase genes may have been lower.

When looking at the distribution of β-lactamase and ESBL genes in *E. coli* and *K. pneumoniae*, two different profiles can be seen. For *E. coli* isolates, the most commonly seen genes are TEM β-lactamase genes alone (44/70; 62.9%), followed by the absence of any β-lactamase genes (15/70; 21.4%). A few isolates contained the CTX-M gene alone (6/70; 8.6%) and or in combination with TEM β-lactamase genes (4/70; 5.7%). None of the *E. coli* isolates contained SHV genes alone or in combination. Most *E. coli* isolates (59/70; 82.3%) have a non-ESBL genotype, while 11 (15.7%) do have ESBL genes.

In contrast, in *K. pneumoniae* isolates, the most commonly seen profiles are SHV β-lactamase genes (21/70; 30%), the combination of CTX-M and SHV- and TEM-β-

lactamase genes (21/70; 30%). The remainder of the profiles are seen in a couple of isolates. More than half of *K. pneumoniae* isolates (39/70; 55.7%) harbour ESBL genes, while only 31 (44.3%) isolates do not.

SHV ESBL genes were second most common ESBL genes found in this set of isolates, as in other studies [107,123]. These genes were commonly seen in *K. pneumoniae*, yet none were detected in *E. coli* isolates. A study conducted in Limpopo of South Africa found very similar results in their *E. coli* isolates [129]. TEM genes were observed in 95.1% of isolates, CTX-M genes were observed in 4.9% of isolates and no SHV genes were detected. Two studies from South Africa, covering multiple provinces found that SHV genes were most common in *K. pneumoniae* and observed the combination of SHV, CTX-M and TEM to be the most common profile seen in the isolates [57,76]. A study using data from SENTRY Antimicrobial Surveillance Program in the Asia-Pacific region, also found that TEM genes dominated in *E. coli* isolates, while SHV genes dominate in *K. pneumoniae* isolates [130].

4.3 Carbapenemase characterisation

All isolates were screened for carbapenemase genes. The genes screened for are the most clinically relevant and commonly found carbapenemase genes. Two multiplex PCRs were merged into one PCR which was further enhanced to include an internal control. Optimisation of this multiplex PCR was more complex and was ultimately optimised to a touchdown PCR, to reduce non-specific binding.

No carbapenemase genes were found in the isolates. Likewise, a study conducted in the Eastern Cape of South Africa and one conducted across various provinces in South Africa, looking at resistance in *K. pneumoniae*, saw no carbapenemase genes, although these studies only screened for NDM and KPC genes [57,76]. Carbapenemase resistance in South Africa is relatively low, although the absence of any of the carbapenemase genes was perhaps surprising as this resistance mechanism is increasing worldwide [4,5]. This data does however support another (as yet unpublished) study performed at Tygerberg Hospital where adult in-patients were screened for rectal carriage carbapenemases and one carbapenemase producing isolate was detected out of 439 samples (P. Nel, personal communication)

5. Conclusion

The presence of β-lactamase genes was observed in 86.4% of the isolates. Just more than a third of the isolates (35.7%) contained ESBL genes, 64.3% contained β-lactamase genes and 13.6% contained no β-lactamase genes. *E. coli* and *K. pneumoniae* showed different profiles with TEM β-lactamase genes being most common in *E. coli* isolates and SHV

β-lactamase genes and the combination of CTX-M and SHV- and TEM-β-lactamases in *K. pneumoniae*. Sequencing of the TEM and SHV isolates provided more information about the resistance genes. Analysis of the sequencing was difficult as only a portion of the gene was sequenced and the databases containing TEM and SHV sequences and information about each gene are not complete, especially for the more recently discovered genes. The incomplete SHV and TEM database meant that the phenotype of some SHV genes is not known. Comparison of the prevalence of ESBL genes found in this study compared to other studies can be difficult as it is not always known whether the detection method used by other studies discriminates between non-ESBL and ESBL genes. Most of the CTX-M genes were from group 1 which is consistent with the literature. No carbapenemase genes were observed in the isolates, which is positive for the efficacy of carbapenems in our setting, at least for now.

Chapter 5: Blending of clinical, resistance and epidemiological data

1. Introduction

Bloodstream infections (BSIs) are a notable cause of morbidity and mortality worldwide [131]. Increasing resistance in Gram negative bacteria has made treatment of infections with these bacteria increasingly difficult. Infection with resistant bacteria often results in poor clinical outcome which may be exacerbated by a delay in treatment with appropriate therapy [132]. These infections may also result in lengthier hospital stays and increased medical expenses [12,132].

The World Health Organisation (WHO) has said that the surveillance of antibiotic resistance is the cornerstone for evaluating the burden of resistance and providing the information needed to guide action plans regarding antibiotic resistance [133]. The WHO has also said that *Escherichia coli* and *Klebsiella pneumoniae* BSIs are priority specimens and pathogens for antibiotic resistance surveillance, as these infections are frequent and have shown an alarming increase in resistance, particularly to last resort antibiotics [133].

Resistance data from previous years can be compared to current data to determine whether there are any significant changes in resistance, particularly emerging resistance mechanisms such as Carbapenem Resistant Enterobacteriaceae (CRE). This information can be used to prevent the spread of concerning resistance mechanisms, although without additional clinical data this does not indicate the extent of the problem in the population [133].

Combining epidemiological, resistance and clinical data will provide a more complete picture of the antibiotic resistance problem. Understanding the molecular epidemiology of these resistance genes will assist in defining the impact of these resistance mechanisms and help to ascertain where infections with resistant bacteria are occurring [101]. It will also assist in guiding correct empiric treatment and highlight the importance of antibiotic stewardship and Infection Prevention and Control (IPC).

In this section clinical, resistance and epidemiological data from previous chapters was combined to better understand *E. coli* and *K. pneumoniae* BSIs.

2. Materials and methods

2.1 Statistical analysis

Statistical analysis was performed with the assistance of the Biostatistics unit from the Centre for Evidence-based Health Care at Stellenbosch University. IBM SPSS statistics version 25 was used for all statistical analysis. The chi-square test and Mann-Whitney test was used to estimate the significance of the correlation with statistical significance defined as $p<0.05$.

Molecular resistance was defined as isolates harbour at least one Extended-Spectrum β-lactamase (ESBL) gene; phenotypic resistance was defined as resistance to cefotaxime.

3. Results and discussion

3.1 Genotypic versus phenotypic resistance

Results indicating phenotypic and genotypic ESBL production (chapters 2 and 4) were compared. Isolates producing ESBL genes were determined via Polymerase Chain Reaction (PCR) and sequencing. Phenotypic ESBL producers were identified by Antibiotic Susceptibility Testing (AST) and were considered ESBL producers if they were resistant to the third generation cephalosporin, cefotaxime. As discussed in chapter 4, 25 of the 57 (43.9%) SHV genes have not yet had their phenotype described. There were 12 *K. pneumoniae* isolates in which these SHV genes were found alone or in combination with a TEM β-lactamase gene, it is unknown whether these SHV genes have ESBL activity or not, therefore these isolates were not included in further analysis.

The genotypic and phenotypic results of 122/128 (95.3%) isolates correlated, with 76 being non-ESBL producers and 46 being ESBL producers. Four (3.1%) of the of isolates were positive for ESBL genes but did not demonstrate phenotypic ESBL production. Two (1.4%) isolates did not contain ESBL genes but were phenotypically resistant to cefotaxime. The sensitivity of AST to detect ESBL genes was 92% and specificity was 97.4%. The sensitivity was lower than desired due to the four isolates which contained ESBL genes but were phenotypically negative for ESBL production. These four isolates include two isolates with TEM ESBL genes and two isolates with TEM β-lactamases and CTX-M ESBLs. It is possible that the TEM ESBLs may not possess extended-spectrum activity. This is a limitation of the analysis for TEM genes, as only a portion of the gene was sequenced it is not known exactly which TEM genes these isolates contain. It is unclear why two isolates with CTX-M genes would be phenotypically negative for ESBL activity as all CTX-M genes are said to have ESBL activity [53]. This has also been observed by a study conducted in India, and may be

due to the fact that isolates did not express the CTX-M gene or the isolate may be exposed to external effects that disrupt the gene [134]. Kiiru *et al.* noted that when OXA-1 and TEM-1 enzymes are co-produced with CTX-M enzymes, they mask the ESBL phenotype conferred by the CTX-M enzymes [122]. The two isolates in this study that were phenotypically ESBL negative but contained CTX-M genes, also contained TEM-1 genes, although presence of OXA β-lactamases was not investigated in this study, this could be investigated in future work. Two isolates harboured TEM ESBL genes but were cefotaxime resistant. This may be due to the production of other resistance mechanisms such as AmpC β-lactamases [135].

The *K. pneumoniae* isolates containing SHV genes with unclassified phenotype were further investigated. Twelve of the 25 isolates contained these genes in the absence of ESBL genes. These isolates were not phenotypically ESBL producers, therefore it can be speculated that these genes may only have β-lactamase activity. This hypothesis could be studied further using enzymatic assays or conjugation experiments with AST.

One isolate was resistant to the carbapenem ertapenem but did not demonstrate the presence of any of the carbapenemase genes that were screened for. This isolate may be resistant to ertapenem as a result of other less common carbapenemase genes or due to an alternative resistance mechanism, such as reduced porin expression along with the production of a β-lactamase with weak carbapenem activity [15,19]. Carbapenem resistance should be monitored as increasing levels of resistance has been noted in many countries [4].

3.2 Statistical analysis of patient data and resistance data

Patient data (chapter 2) and resistance results (chapter 4) were analysed to identify any associations. When looking at resistance and outcome, molecular resistance had a slightly higher mortality (33.9% vs 26.9%) as did phenotypic resistance (34.0% vs 27.8%). However, this difference was not significant and therefore, neither molecular nor phenotypic resistance was associated with mortality (p=0.373; p=0.441). While some studies have shown similar results, others have found that BSIs with ESBL producing bacteria to have significantly higher rates of mortality [16,123]. A review on Gram negative bloodstream infections has stated that it is not the presence of ESBL producing organisms, but rather the incorrect empirical treatment of these infections that results in increased mortality [83]. An Italian study on ESBL BSIs found that increased mortality was due to inadequate initial antimicrobial therapy and failure to identify the source of the infection [38]. Patients that received inadequate initial antimicrobial therapy had a mortality rate nearly three times higher than patients who received suitable treatment from the start. A study conducted in Tygerberg Hospital on paediatric BSIs found antimicrobial resistance was not associated with mortality, but attributed this to carbapenems being the empiric treatment option for hospital-acquired BSIs

[32]. Treatment of BSIs caused by ESBL producing organisms with carbapenems, is associated with a lower mortality [83]. This highlights the need to provide prompt adequate treatment to patients and the value of knowledge of the local distribution of pathogens and their antibiotic resistance rates.

Hospital-acquired isolates were associated with molecular resistance (p=0.001) as well as phenotypic resistance (p<0.001). A longer time spent in hospital before the blood culture was taken was also associated with molecular resistance (p<0.001) and phenotypic resistance (p<0.001). Bacterial strains circulating in hospitals are often resistant to antibiotics due to the high selection pressure for resistant bacteria resulting from high antibiotic use in hospitals. Increased length of hospital stay exposes patients to these resistant strains. Increased antibiotic resistance and ESBL production in hospital-acquired isolates was also observed in a study conducted at a hospital in Cape Town [17]. This highlights the importance of determining whether a BSI is hospital- or community-acquired when defining empirical treatment options.

Patients with infections due to phenotypically or genotypically ESBL-positive isolates had a slightly longer stay in hospital after the blood culture was taken, although this was not significant (p=0.664; p=0.172). Studies have noted increased hospital days due to infections with ESBL producing *E. coli* and *K. pneumoniae* [136,137]. However, Brenner *et al.* noted that only increased length of stay of patients in ICUs was statistically significant. Infection with resistant bacteria is expected to result in increased hospital stay due to decreased effectivity of antibiotics, delay in appropriate treatment and an increased need for surgery and other procedures [136]. All these factors also lead to increased cost of care.

The clinical data obtained was limited and therefore we could not analyse factors such as co-morbidities, antimicrobial treatment before and after microbiological results, and additional procedures performed on patients. This limited data also meant that we were unable to assess potential risk factors for infection with ESBL producing *E. coli* and *K. pneumoniae*.

3.3 Strain typing and resistance genes

As mentioned in chapter 3, the results from both strain typing techniques demonstrate that the isolates in this study are genetically diverse, with little clustering seen in both typing techniques. This precluded any attempt to associate specific resistance genes or phenotypes with specific strains. Phylogenetic trees supplemented with clinical and resistance data (chapter 2 and 4) can be found in appendix B (figure B.1-B.4).

The lack of clustering among isolates suggests that resistance is not being dispersed by proliferation of resistant strains, but rather due to the transfer of resistance genes and/or

plasmids [16,106]. This hypothesis could be further investigated in future work focussed on the resistance plasmids in these isolates. The lack of clustering may be a result of ongoing antibiotic selection pressure that has selected for multiple resistant strains which have now become endemic in our setting.

4. Conclusion

When comparing molecular and phenotypic resistance, 95.3% isolates correlated, 3.1% of isolates were positive for ESBL genes but did not demonstrate phenotypic ESBL production and 1.4% did not contain ESBL genes but were phenotypically resistant to cefotaxime.

Mortality was not associated with phenotypic and molecular resistance. Hospital-acquired isolates as well as increased stays in hospital before blood culture were associated with molecular and phenotypic resistance. Collected patient data was limited and additional clinical data may assist in evaluating antibiotic use in our setting as well as risk factors for infection with ESBL producing organisms. The genetically diverse isolate collection precluded any attempt to associate resistance genes or phenotypes with specific strains.

Understanding the prevalence of resistant strains in our setting is vital as this will help inform empiric therapy. Prompt adequate therapy of infections with resistant bacteria has been shown to reduce mortality. Continuous efforts are required to gather information on the molecular epidemiology of resistance genes to monitor antimicrobial stewardship efforts and ensure correct empiric treatment. Every effort should be taken to preserve the efficacy of cephalosporins and carbapenems in particular.

Conclusion

Escherichia coli and *Klebsiella pneumoniae* are common causes of both community- and hospital-acquired bloodstream infections (BSIs). Resistance to commonly used antibiotics, such as cephalosporins and carbapenems, threatens the treatment of these infections. Resistance to β-lactam antibiotics is often due to the production of β-lactamase enzymes, such as Extended-Spectrum β-lactamases (ESBLs) and carbapenemases.

The aim of this study was to describe the epidemiology of *E. coli* and *K. pneumoniae* strains isolated from blood cultures at Tygerberg Hospital over a period of one year. Commonly observed resistance mechanisms were investigated, and this data was combined with clinical and molecular typing data to elucidate the epidemiology and impact of these infections.

Patient and isolate information was collected to determine antibiotic resistance, mortality rates and differentiate between hospital- and community-acquired isolates and the shared features of these two groups. Two thirds of the collected isolates were hospital-acquired, which may be due to the fact that this study took place at a tertiary hospital. The hospital-acquired isolates were predominantly *K. pneumoniae*, while community-acquired isolates were mostly *E. coli*. The crude mortality was 30% and BSIs with hospital-acquired isolates were not associated with mortality.

Increased antibiotic resistance was observed in hospital-acquired isolates, particularly to co-trimoxazole, co-amoxiclav, cefotaxime, ciprofloxacin, gentamicin and piperacillin-tazobactam. Phenotypic ESBL production was defined as resistance to cefotaxime, which was observed in 35.7% of isolates. Phenotypic ESBL production was significantly more common in *K. pneumoniae* isolates (55.7%), compared to *E. coli* isolates (15.7%). One *K. pneumoniae* isolate was resistant to ertapenem. Increased antibiotic resistance was observed in isolates that were cefotaxime resistant (ESBL producer) as compared to cefotaxime sensitive isolates, fortunately these isolates remained relatively sensitive to amikacin (98%) and ertapenem (98%), which is re-assuring as these antibiotics are often used to treat infections with ESBL producing organisms.

Isolates were typed using rep-PCR and Pulsed Field Gel Electrophoresis (PFGE) to gain insight into the epidemiology. Phylogenetic trees generated from the typing results showed little clustering, indicating that the isolates were genetically diverse. This was also seen in the Simpson's index of diversity values which were close to 1, indicating that if two isolates were randomly selected they would most likely be unrelated. PFGE showed higher discrimination, however rep-PCR provides a typing method that is less laborious and time

consuming. The diversity of the isolates suggests that patient-to-patient transmission and contamination of the environment is not a major source of these organisms. Although this result is positive for infection prevention and control in our setting, it does make ascertaining transmission routes and implementing interventions to reduce BSIs with resistant organisms more difficult. Further studies may be required to investigate this.

Polymerase Chain Reaction (PCR) and sequencing was used to determine the prevalence of β-lactamases and carbapenamases in the collected isolates, and to further characterize the types present. The multiplex β-lactamase PCR identified β-lactamase genes in 86.4% of the isolates. TEM and SHV genes were further characterised by sequencing. The majority (98%) of TEM genes were narrow-spectrum β-lactamase TEMs, i.e. TEM-1 and TEM-2, while 2% were ESBL TEMs. Just under half of the SHV genes (47.4%) were narrow spectrum β-lactamase SHV genes, 7% were ESBL SHV genes, while the spectrum of activity of 45.6% of the SHV genes is unknown. These genes have not yet had their phenotype reliably described and it is not known whether they have an extended-spectrum of activity or not.

The majority of the CTX-M genes (91.3%) were classified as group 1 CTX-M's, which contains the most commonly found CTX-M gene worldwide, CTX-M-15. No carbapenemase genes were detected in any isolates. South African studies on the prevalence of carbapenemase genes have detected low frequencies of these genes as compared to studies in other countries.

Based on PCR and sequencing data, 35.7% of isolates contained ESBL genes, 50.7% contained narrow-spectrum β-lactamase genes and 13.6% of isolates were negative for β-lactamase genes. The most common ESBL gene was CTX-M, which was frequently detected in combination with other ESBL (6.5%) and β-lactamase genes (78.3%).

Phenotypic ESBL production (cefotaxime resistance) was compared to molecular detection of ESBL genes; the results correlated for 95.3% of isolates. Interestingly, two isolates containing CTX-M genes were phenotypically cefotaxime susceptible, this may be due to the gene not being expressed. This could be investigated further in future work. Twelve *K. pneumoniae* isolates harboured one or other of the SHV genes with unclassified phenotypes, in the absence of any other ESBL gene. These isolates were all susceptible to cefotaxime, suggesting that these genes may not be able to hydrolyse extended-spectrum beta lactams such as third generation cephalosporins. This hypothesis could be investigated further by enzymatic assays and/or conjugation studies combined.

Statistical analysis of clinical and resistance data was performed to determine whether there was a correlation between results. Mortality was higher in patients with isolates

phenotypically (34% vs 27.8%) or genotypically (33.9% vs 26.9%)) positive for ESBLs, although this was not statistically significant. Hospital-acquired isolates and longer stays in hospital before blood culture was taken were associated with the presence of ESBLs. This is not surprising as high antibiotic use in hospitals selects for resistant bacterial strains. Infection with resistant bacteria was not associated with increased length of hospital stay. Studies have suggested that mortality due to infection with resistant organisms is due to inappropriate empiric therapy, which highlights the importance of local epidemiological data and surveillance of antibiotic resistance.

Unfortunately, the patient information collected in this study was limited and cause of death, co-morbidities of patients and previous antibiotic use were not known. This information may assist in evaluating risk factors for bloodstream infection with ESBL producing bacteria. Obtaining information about treatment before and after laboratory results will assist to evaluate empiric treatment and whether antibiotic treatment is adjusted according to bacterial species and resistance information. The finding that the mortality rate for cefotaxime resistant and susceptible isolates was not statistically different may indicate that the local empiric regimen for hospital-acquired infections (which were associated with the resistant phenotype) is appropriate. However, this assumption needs to be tested more thoroughly, and a prospective study collecting both treatment and outcome data, as well as data from uninfected controls, is underway.

Strain typing revealed the *E. coli* and *K. pneumoniae* isolates at Tygerberg Hospital to be genetically diverse. The lack of clustering precluded any attempt to associate specific traits with particular strains. The diversity of strains harbouring β-lactamase genes suggests that resistance may be due to the transfer of resistance genes and/or plasmids. Further studies could investigate this hypothesis and enable a better understanding of the transmission dynamics of resistant organisms, which may assist in focusing Infection Prevention and Control (IPC) efforts.

Continuous efforts are required to investigate the epidemiology of circulating strains and their resistance patterns to monitor antimicrobial stewardship efforts, guide action plans and ensure correct empiric treatment. It is pertinent that the efficacy of cephalosporins and particularly carbapenems are preserved as far as possible.

Appendix

Appendix A

Table A.1: Distribution of β-lactamase and ESBL genes in *E. coli* and *K. pneumoniae* isolates.

	E. coli	*K. pneumoniae*	Total
TEM β-lactamase only	44	1	45
TEM ESBL only	1	1	2
SHV β-lactamase only	0	21	21
SHV ESBL only	0	0	0
CTX-M only	6	1	7
CTX-M, TEM β-lactamase	4	6	10
SHV β-lactamase, CTX-M	0	6	6
SHV ESBL, CTX-M	0	0	0
SHV β-lactamase, TEM β-lactamase	0	5	5
SHV ESBL, TEM β-lactamase	0	2	2
SHV β-lactamase, CTX-M, TEM β-lactamase	0	21	21
SHV ESBL, CTX-M, TEM β-lactamase	0	2	2
None	15	4	19
	70	**70**	**140**

Appendix B

Phylogenetic trees of *E. coli* and *K. pneumoniae* for rep-PCR and PFGE, supplemented with clinical and resistance information. Phenotypic resistance refers to resistance to cefotaxime.

Key: M = Male; F = Female; HA = Hospital-acquired; CA = Community-acquired; S = Susceptible; R = Resistant; TEM-E =TEM gene with extended-spectrum activity (ESBL); SHV-N = SHV without extended-spectrum activity; SHV-E = SHV with extended-spectrum activity; SHV-U = SHV with uncertain extended-spectrum activity.

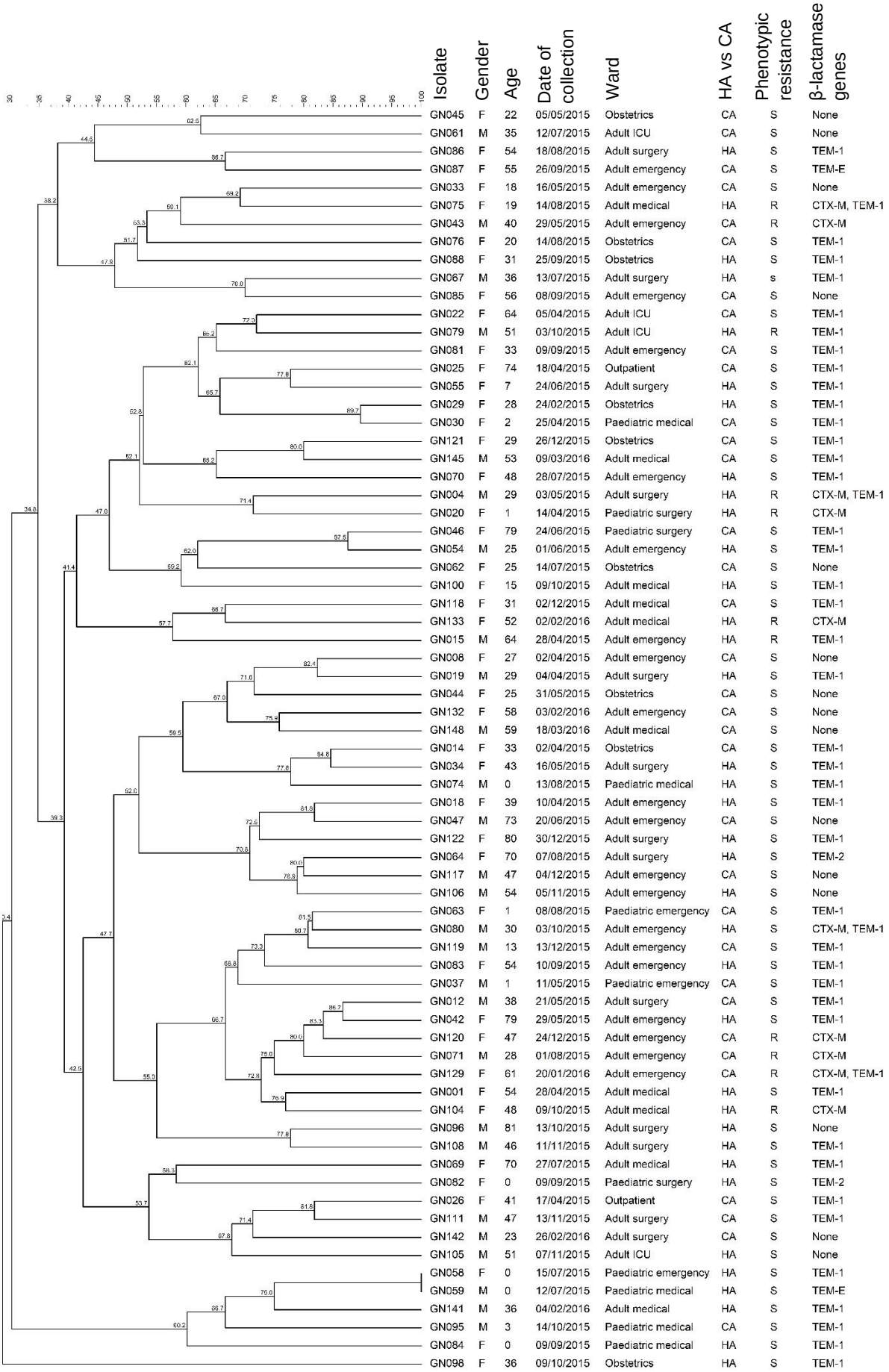
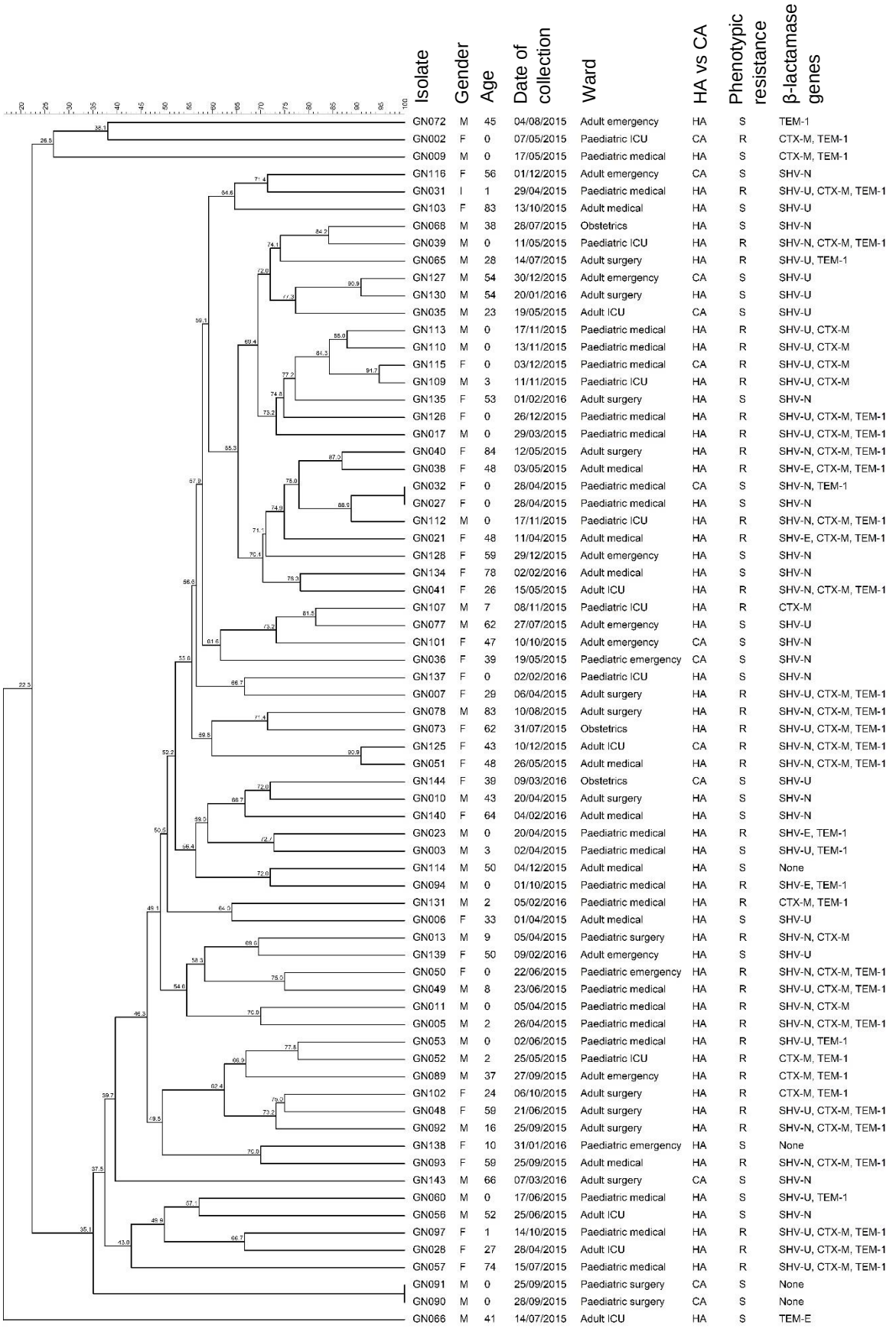


Figure B.1: Phylogenetic tree generated from rep-PCR results for *E. coli* isolates, supplemented with clinical and resistance data.



Isolate	Gender	Age	Date of collection	Ward	HA vs CA	Phenotypic resistance	β-lactamase genes
GN072	M	45	04/08/2015	Adult emergency	HA	S	TEM-1
GN002	F	0	07/05/2015	Paediatric ICU	CA	R	CTX-M, TEM-1
GN009	M	0	17/05/2015	Paediatric medical	HA	S	CTX-M, TEM-1
GN116	F	56	01/12/2015	Adult emergency	CA	S	SHV-N
GN031	I	1	29/04/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN103	F	83	13/10/2015	Adult medical	HA	S	SHV-U
GN068	M	38	28/07/2015	Obstetrics	HA	S	SHV-N
GN039	M	0	11/05/2015	Paediatric ICU	HA	R	SHV-N, CTX-M, TEM-1
GN065	M	28	14/07/2015	Adult surgery	HA	R	SHV-U, TEM-1
GN127	M	54	30/12/2015	Adult emergency	CA	S	SHV-U
GN130	M	54	20/01/2016	Adult surgery	HA	S	SHV-U
GN035	M	23	19/05/2015	Adult ICU	CA	S	SHV-U
GN113	M	0	17/11/2015	Paediatric medical	HA	R	SHV-U, CTX-M
GN110	M	0	13/11/2015	Paediatric medical	HA	R	SHV-U, CTX-M
GN115	F	0	03/12/2015	Paediatric medical	CA	R	SHV-U, CTX-M
GN109	M	3	11/11/2015	Paediatric ICU	HA	R	SHV-U, CTX-M
GN135	F	53	01/02/2016	Adult surgery	HA	S	SHV-N
GN126	F	0	26/12/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN017	M	0	29/03/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN040	F	84	12/05/2015	Adult surgery	HA	R	SHV-N, CTX-M, TEM-1
GN038	F	48	03/05/2015	Adult medical	HA	R	SHV-E, CTX-M, TEM-1
GN032	F	0	28/04/2015	Paediatric medical	CA	S	SHV-N, TEM-1
GN027	F	0	28/04/2015	Paediatric medical	HA	S	SHV-N
GN112	M	0	17/11/2015	Paediatric ICU	HA	R	SHV-N, CTX-M, TEM-1
GN021	F	48	11/04/2015	Adult medical	HA	R	SHV-E, CTX-M, TEM-1
GN128	F	59	29/12/2015	Adult emergency	HA	S	SHV-N
GN134	F	78	02/02/2016	Adult medical	HA	S	SHV-N
GN041	F	26	15/05/2015	Adult ICU	HA	R	SHV-N, CTX-M, TEM-1
GN107	M	7	08/11/2015	Paediatric ICU	HA	R	CTX-M
GN077	M	62	27/07/2015	Adult emergency	HA	S	SHV-U
GN101	F	47	10/10/2015	Adult emergency	CA	S	SHV-N
GN036	F	39	19/05/2015	Paediatric emergency	CA	S	SHV-N
GN137	F	0	02/02/2016	Paediatric ICU	HA	S	SHV-N
GN007	F	29	06/04/2015	Adult surgery	HA	R	SHV-U, CTX-M, TEM-1
GN078	M	83	10/08/2015	Adult surgery	HA	R	SHV-N, CTX-M, TEM-1
GN073	F	62	31/07/2015	Obstetrics	HA	R	SHV-U, CTX-M, TEM-1
GN125	F	43	10/12/2015	Adult ICU	CA	R	SHV-N, CTX-M, TEM-1
GN051	F	48	26/05/2015	Adult medical	HA	R	SHV-N, CTX-M, TEM-1
GN144	F	39	09/03/2016	Obstetrics	CA	S	SHV-U
GN010	M	43	20/04/2015	Adult surgery	HA	S	SHV-N
GN140	F	64	04/02/2016	Adult medical	HA	S	SHV-N
GN023	M	0	20/04/2015	Paediatric medical	HA	R	SHV-E, TEM-1
GN003	M	3	02/04/2015	Paediatric medical	HA	S	SHV-U, TEM-1
GN114	M	50	04/12/2015	Adult medical	HA	S	None
GN094	M	0	01/10/2015	Paediatric medical	HA	R	SHV-E, TEM-1
GN131	M	2	05/02/2016	Paediatric medical	HA	R	CTX-M, TEM-1
GN006	F	33	01/04/2015	Adult medical	HA	S	SHV-U
GN013	M	9	05/04/2015	Paediatric surgery	HA	R	SHV-N, CTX-M
GN139	F	50	09/02/2016	Adult emergency	HA	S	SHV-U
GN050	F	0	22/06/2015	Paediatric emergency	HA	R	SHV-N, CTX-M, TEM-1
GN049	M	8	23/06/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN011	M	0	05/04/2015	Paediatric medical	HA	R	SHV-N, CTX-M
GN005	M	2	26/04/2015	Paediatric medical	HA	R	SHV-N, CTX-M, TEM-1
GN053	M	0	02/06/2015	Paediatric medical	HA	R	SHV-U, TEM-1
GN052	M	2	25/05/2015	Paediatric ICU	HA	R	CTX-M, TEM-1
GN089	M	37	27/09/2015	Adult emergency	HA	R	CTX-M, TEM-1
GN102	F	24	06/10/2015	Adult surgery	HA	R	CTX-M, TEM-1
GN048	F	59	21/06/2015	Adult surgery	HA	R	SHV-U, CTX-M, TEM-1
GN092	M	16	25/09/2015	Adult surgery	HA	R	SHV-N, CTX-M, TEM-1
GN138	F	10	31/01/2016	Paediatric emergency	HA	S	None
GN093	F	59	25/09/2015	Adult medical	HA	R	SHV-N, CTX-M, TEM-1
GN143	M	66	07/03/2016	Adult surgery	CA	S	SHV-N
GN060	M	0	17/06/2015	Paediatric medical	HA	S	SHV-U, TEM-1
GN056	M	52	25/06/2015	Adult ICU	HA	S	SHV-N
GN097	F	1	14/10/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN028	F	27	28/04/2015	Adult ICU	HA	R	SHV-U, CTX-M, TEM-1
GN057	F	74	15/07/2015	Paediatric medical	HA	R	SHV-U, CTX-M, TEM-1
GN091	M	0	25/09/2015	Paediatric surgery	CA	S	None
GN090	M	0	28/09/2015	Paediatric surgery	CA	S	None
GN066	M	41	14/07/2015	Adult ICU	HA	S	TEM-E

Figure B.2: Phylogenetic tree generated from rep-PCR results for *K. pneumoniae* isolates, supplemented with clinical and resistance data.

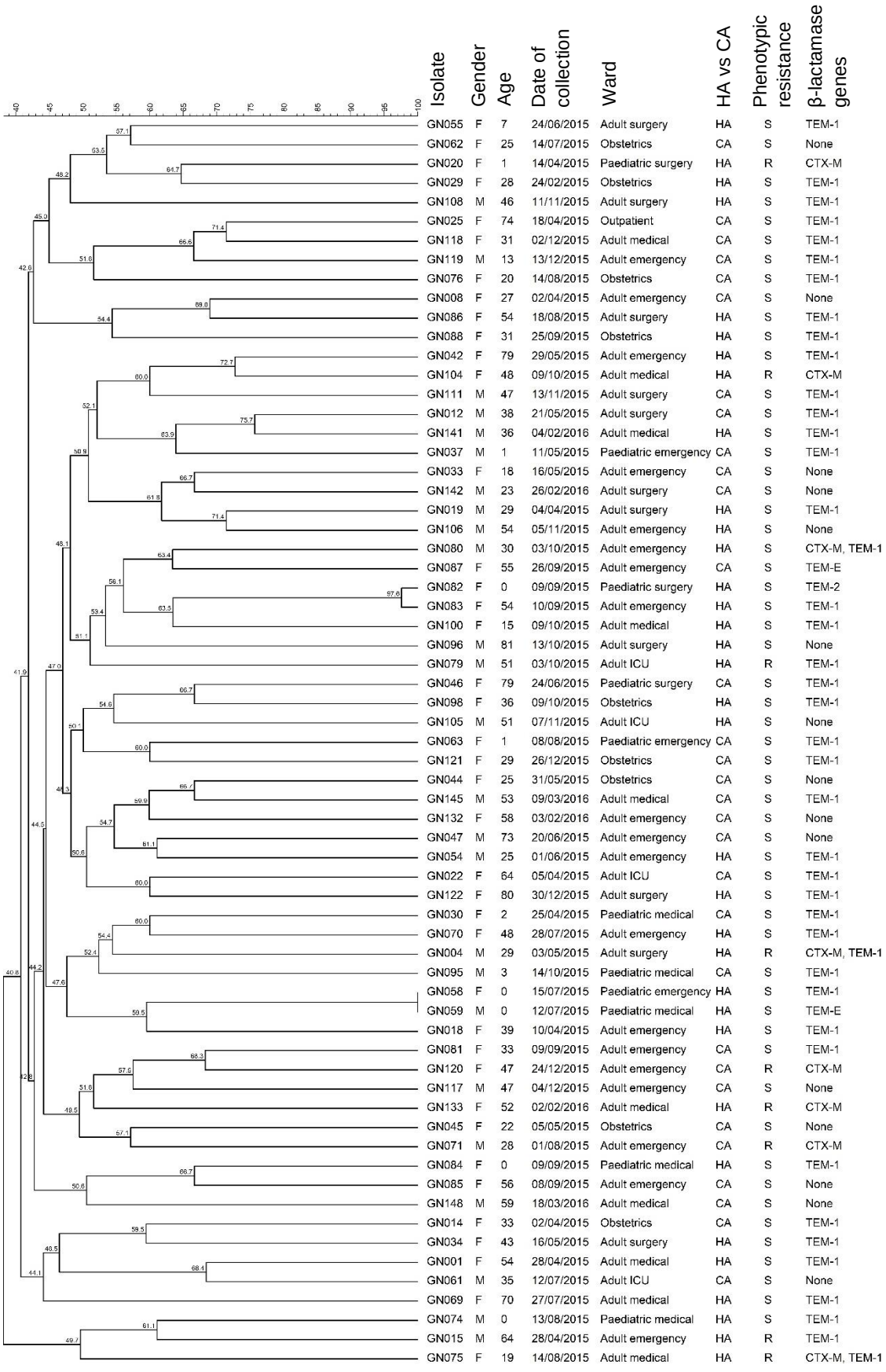


Figure B.3: Phylogenetic tree generated from PFGE results for *E. coli* isolates, supplemented with clinical and resistance data.

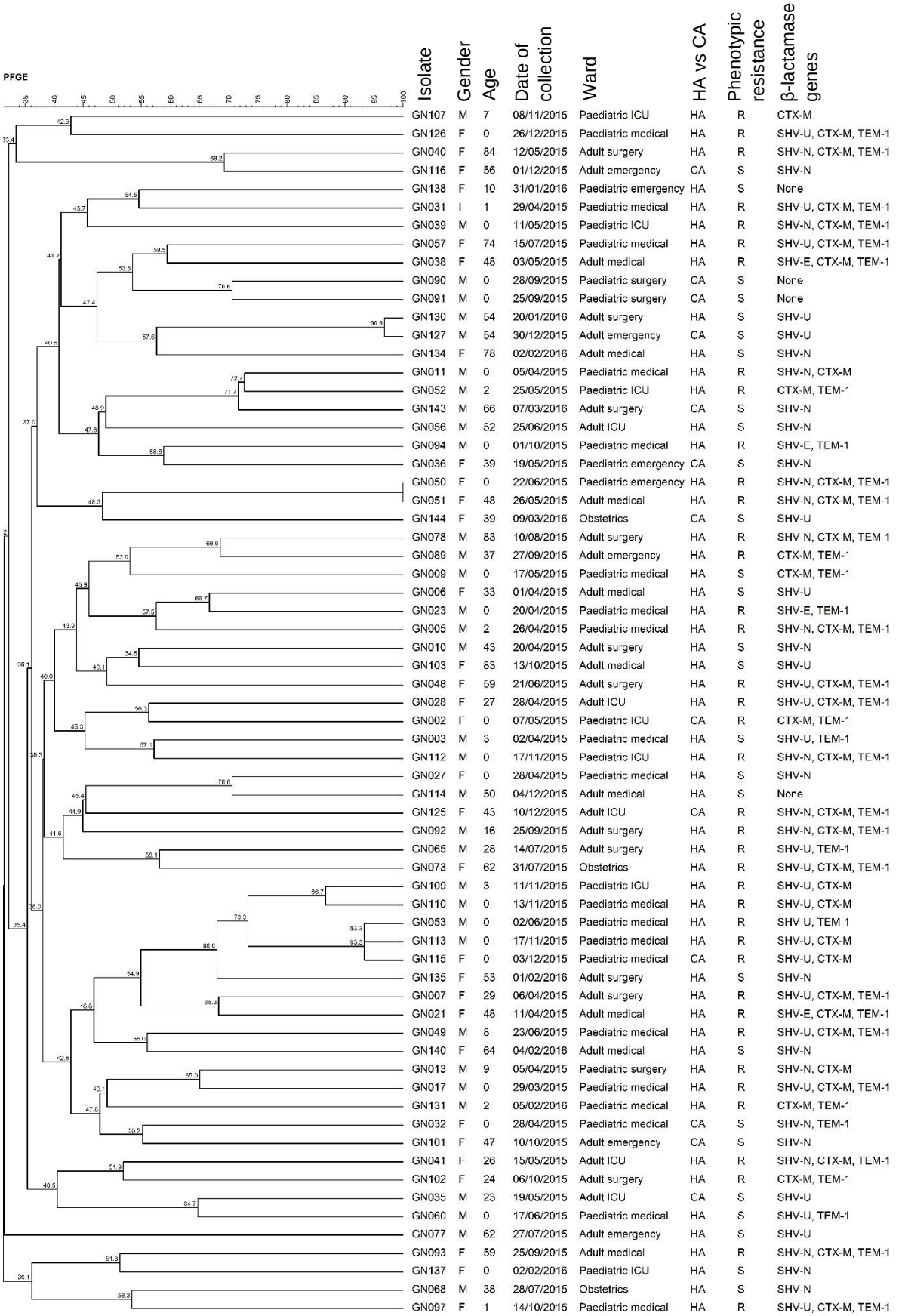


Figure B.4: Phylogenetic tree generated from PFGE results for *K. pneumoniae* isolates, supplemented with clinical and resistance data.

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