Molecular characterisation of β-lactamase producing *Klebsiella pneumoniae* isolates

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Molecular characterisation of β-lactamase producing *Klebsiella pneumoniae* isolates

by

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Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained herein is my own original work and has not previously, in its entirety or in part, been submitted by me to this or any other tertiary institution for a degree. I further declare that all sources cited are acknowledged by means of a list of references.

Signed _____ this _____ day of _____ 2015

"One important key to success is self-confidence. An important key to self-confidence is preparation"

Arthur Ashe

DEDICATION

This dissertation is dedicated to my parents for their unconditional love, support and encouragement. You will forever be my inspiration for all you have done, for all those you have helped and for all you have accomplished despite the odds. I never have to look further than my own home to know what true greatness is.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

%	Percentage
g	Gram
h	hours
L	Litre
M	Molar
m/v	Mass of solute over volume of solution
mg	Milligram
mg.ml^{-1}	Milligram per millilitre
min	Minute
mL	Millilitre
mM	Millimolar
nm	Nanometre
ºC	Degree Celsius
rpm	Revolutions per minute
V	Volts
β	Beta
µl	Microlitre

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
***all*S**	Activator of the allantoin regulon
AMI-R	Aminoglycoside-resistant
BEL	Belgium extended-spectrum β-lactamase
BES	Brazilian extended-spectrum β-lactamase
BHI	Brain heart infusion
BSA	Bovine serum albumin
BSI	Blood stream infection
BTB	Bromothymol blue
CA	Community-associated
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system

CPS	Capsule polysaccharide
CRE	Carbapenem-resistant *Enterobacteriaceae*
CTX-M	Cefotaximase-Munich
CVP	Central venous pressure
DDST	Double disk synergy test
DNA	Deoxyribonucleic acid
DTB	Drugs and Therapeutics Bulletin
EDTA	Ethylene diamine tetra-acetate
EMB	Eosin-methylene blue
***ent*B**	Enterobactin
ESBL	Extended-spectrum β-lactamase
ESI MS	Electrospray Ionization Mass Spectrometry
FepA	Ferrienterobactin outer membrane receptor
FepA	Ferrienterobactin outer membrane receptor
FIDSSA	Federation of Infectious Diseases Societies of Southern Africa
***fim*H**	Fimbrial gene encoding type 1 fimbrial adhesion
***fyu*A**	Ferric yersiniabactin uptake
GERMS-SA	Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa
GES	Guyana extended-spectrum β-lactamase
hvKP	Hypervirulent *K. pneumoniae*
ICU	Intensive care unit
IMP	Imipenem
***int*A**	Putative integrase
***iro*N**	Ferric-catecholates receptor
***irp*1-*irp*2-*ybt*S-*fyu*A**	Yersiniabactin
IRT	Inhibitor resistant TEM
ISs	Insertion sequence elements
***kfu*BC**	Iron-uptake system
KPC	*Klebsiella pneumoniae* carbapenemase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lcn2	Lipocalin 2
LPS	Lipopolysaccharide
***mag*A**	Mucoviscosity-associated gene A
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multilocus variable-number tandem-repeat analysis

M-PCR	Multiplex-polymerase chain reaction
MR/K-HA	Mannose-resistant *Klebsiella*-like hemagglutinin
***mrk*D**	Type 3 fimbriae adhesion
MS	Mass spectrometry
NaCl	Sodium chloride
NDM-1	New Delhi metallo-ß-lactamase
NGS	Next-generation sequencing
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
Omp	Outer membrane protein
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PDR	Pan-drug-resistant
PER	*Pseudomonas* extended resistance
PFGE	Pulsed-field gel electrophoresis
PMSF	Phenyl methyl sulfonyl fluoride
PTA	Plasmid transfer assays
QS	Quorum sensing signal
RAPD	Random amplified polymorphic deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
rep-PCR	Repeat-based polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RND	Resistance nodulation cell division
***rpm*A**	Regulator of mucoid phenotype
***rpo*B**	RNA polymerase B
SDD	Selective digestive tract decontamination
SDS-PAGE	Sodium dodecyl sulphate polyacryamide gel electrophoresis
SFO	*Serratia fonticola*
SHV	Sulfhydryl variable
ST	Sequence type
TBE	Tris-borate-EDTA
TE	Tris-ethylene diamine tetra-acetate
TEM	Temoneira
TLA	Tlahuicas
uge	Uridine diphosphate galacturonate 4-epimerase
UPGMA	Unweighted pair group method using arithmetic average
***ure*A**	Related to the urease operon
USA	United States of America
UTI	Urinary tract infection

UV	Ultraviolet
VEB	Vietnam extended-spectrum β-lactamase
VIM	Verona integron-encoded metallo-β-lactamase
***wab*G**	Transferase
WHO	World Health Organization
XDR	Extensively drug resistant
YHPI	*Yersinia* high-pathogenicity-island

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

1. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, SAS Olorunju, MM Kock** (2014) High prevalence of the OXA-48-Like gene among-ESBL producing *Klebsiella pneumoniae* clinical isolates with *in vitro* suceptibillity to carbapenems. Submitted for publication to: *Journal of Antimicrobial Chemotherapy*

2. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, SAS Olorunju, MM Kock** (2014) Characterisation of β-lactamase producing *Klebsiella pneumoniae* isolates: Novel ST1632 in South Africa. To be submitted for publication to: *PLoS ONE*

CONFERENCE PRESENTATIONS

1. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, SAS Olorunju, MM Kock** (2012) Molecular detection of β-lactam resistance genes in *Klebsiella pneumoniae* isolates. Presented at the Faculty Day of the Faculty of Health Sciences, University of Pretoria, 28 to 29 August (Oral and poster presentation)

2. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, SAS Olorunju, MM Kock** (2013) Characterisation of β-lactamase producing *Klebsiella pneumoniae* isolates. Presented at the Faculty Day of the Faculty of Health Sciences, University of Pretoria, 20 to 21 August (Oral presentation)

3. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, SAS Olorunju, MM Kock** (2013) Characterisation of β-lactamase encoding *Klebsiella pneumoniae* subsp. *pneumoniae*. Presented at the 18th Biennial Congress of the South African Society for Microbiology (SASM) 2013 from 24 to 27 November in Bela Bela, Limpopo, South Africa. (Oral presentation)

4. **MB de Jesus, MM Ehlers, RF dos Santos, AW Dreyer, MM Kock** (2014) Characterisation of extended-spectrum beta-lactamase-encoding *Klebsiella pneumoniae* isolates from Pretoria, South Africa. Presented at the 114th General meeting of the American Society for Microbiology (ASM) 2014 from 17 to 20 May in Boston, Massachusetts, United States of America (Poster Presentation)

MOLECULAR CHARACTERISATION OF β-LACTAMASE PRODUCING *KLEBSIELLA PNEUMONIAE* ISOLATES

by

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SUMMARY

Klebsiella pneumoniae is an important nosocomial pathogen, which has rapidly acquired multidrug-resistance phenotypes, particularly towards β-lactam antibiotics through the expression of β-lactamases. The known β-lactams are classified into four Ambler molecular classes, namely classes A to D. In the South African public healthcare setting, a high prevalence of extended-spectrum β-lactamase (ESBL) producing *K. pneumoniae* has been recorded and carbapenemase resistance has been reported recently. The global emergence of carbapenemases has reduced treatment options available, which are further exacerbated by the unequalled novel antibiotic development over the years. *Klebsiella pnemoniae* is an important Gram-negative bacillus implicated in urinary, blood and respiratory infections and an important contributor to in-hospital mortality. The study encompassed the molecular detection of Ambler molecular classes A, B and D β-lactamase encoding genes in clinical ESBL-producing *K. pneumoniae* isolates and genetic characterisation.

The 100 unrepeated ESBL-producing *K. pneumoniae* isolates analysed in the study were collected from the National Health Laboratory Service (NHLS), Tshwane Academic Division. During routine testing, identification and antibiotic susceptibility were determined utilising the VITEK®2 Automated system (bioMérieux, France) and the β-lactamase genes were identified using both simplex and conventional multiplex-polymerase chain reaction (M-PCR)

assays. The clinical isolates were subsequently genetically characterised by pulsed-field gel electrophoresis (PFGE) utilising the Rotaphor VI system (Biometra, Germany) and clonal representatives further discriminated by multilocus sequence typing (MLST).

All three ESBL encoding genes, namely Sulfhydryl variable (SHV) (100%), Cefotaximase-Munich (CTX-M) (94%) and Temoneria (TEM) (81%), were detected as well as Oxacillinase (OXA)-1-Like (95%) and OXA-48-Like (94%) β-lactamase genes. The simplex and M–PCR assays results for β-lactamase detection in plasmid DNA in a representative portion of the isolates were found to be comparable to that detected in genomic DNA. No metallo-β-lactamases or *K. pneumoniae* carbapenemases (KPCs) were detected in the isolates expressing elevated carbapenem minimum inhibitory concentrations (MICs), as phenotypically determined by the VITEK®2 Automated system (bioMérieux, France). All the isolates were typeable by PFGE using *Xba*I, which discerned several pulsotypes. In addition, variable sequence types (STs) were identified by MLST, including the novel ST1632.

In conclusion, the PCR assays successfully detected five β-lactamase genes and all were highly prevalent within ESBL-producing *K. pneumoniae* isolates. Characterisation by PFGE revealed multiple pulsotypes, a few highly related clusters and different STs for the selected clonal representatives by MLST. The molecular data on β-lactamases accompanying *in vitro* susceptibility profiles is under-reported in South Africa. The current study has elucidated the high frequency of multiple ESBL-encoding isolates, the presence of OXA-48-Like β-lactamases in carbapenem susceptible and resistant isolates, as well as the presence of STs reported internationally and a novel ST1632. Future research should encompass research into resistance mechanisms working in concert with β-lactamase production resulting in carbapenem resistance, particularly in the absence of carbapenemases, due to waning treatment options. The combined effort of this study with that of future research can thus allow for trends in antimicrobial resistance to be tracked and may influence treatment by encouraging pre-emptive steps to be taken.

Keywords: *Klebsiella pneumoniae*, β-lactamase, Multiplex-PCR, Pulsed-field gel electrophoresis, Multilocus sequence typing

CHAPTER 1

Introduction

Klebsiella was initially named Friedlander's bacillus after its isolation from a patient who died of pneumonia in 1882 but was subsequently renamed (Shon *et al*., 2013). The evolution of the Gram-negative bacillus in an era of antibiotic use has resulted in a changed epidemiology, wherein *K. pneumoniae* commonly occurs in healthcare facilities, such as hospitals, and is responsible for a range of serious infections involving the abdominal cavity, bacteraemia, intra-vascular devices, lungs, urinary tract and soft tissues surgical sites (Shon *et al*., 2013). Treatment of *K. pneumoniae* infections has been complicated by the rapid and easy acquisition of antimicrobial resistance (Shon *et al*., 2013). Resistance determinants in *Enterobacteriaceae* are encoded on the chromosome, plasmids, integrons and transposons (Kocsis and Szabó, 2013). *Klebsiella pneumoniae* is an *Enterobacteriaceae* member which often expresses resistance towards β-lactam antibiotics, particularly through β-lactamase expression of which the most important are cephalosporinases, such as extended-spectrum β-lactamases (ESBLs) and carbapenemases (Kocsis and Szabó, 2013). The ESBL prevalence in South Africa is high and some of the carbapenemases detected include *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase (NDM), Oxacillinase (OXA)-48, OXA-181 and Verona integron-encoded metallo-β-lactamase (VIM) (Bamford *et al*., 2011; Brink *et al.*, 2012a; Brink *et al.*, 2012b; Brink *et al.*, 2013).

Extended spectrum β-lactamases typically confer resistance towards penicillins, first-, second- and third-generation cepahlosporins as well as aztreonam but remain mostly inhibited by clavulanic acid, an inhibitor (Khater and Sherif, 2014). Extended-spectrum β-lactamase-producers can additionally express resistance towards other antibiotics, such as aminoglycosides and fluoroquinolones, and are commonly treated with carbapenems (Giske *et al*., 2008; Gasink *et al.*, 2009; Rawat and Nair, 2010; Bush, 2012). The use of antibiotics, such as aminoglycosides, carbapenems, cephalosporins, fluoroquinolones as well as β-lactam/β-lactamase inhibitors has been identified as one of the several risk factors associated with carbapenem-resistant *Enterobacteriaceae* (CRE) infection (Gupta *et al.*, 2011; Brink *et al.*, 2012b). Carbapenem-resistance in clinical isolates can be mediated by several mechanisms of which carbapenemase expression is the most common, although carbapenem-resistance can also occur in ESBL-producing or AmpC over-expressing bacteria when

permeability is reduced, particularly due to outer membrane protein loss (Adler *et al.*, 2013; Patel and Bonomo, 2013). Treatment of CREs are often reliant on last resort antimicrobials, such as colistin, fosfomycin and/or tigecycline, which can be rendered ineffective due to antimicrobial resistance evolving or emerging (Giamarellou, 2010; Brink *et al.*, 2012b; Brink *et al.*, 2013; Van Duin *et al.*, 2013). Brink *et al.* (2013) reported the emergence of colistin-resistant OXA-181-producing *K. pneumoniae* in South Africa. The rise in carbapenemase-producers both locally and internationally poses a treatment problem as fewer efficacious antibiotics are available and all are threatened in light of the emergence of extensively drug resistant (XDR) and pan-drug-resistant (PDR) Gram-negative bacteria (Brink *et al.*, 2012b).

Characterisation of clinically relevant *K. pneumoniae* isolates has elucidated strains implicated in both community-associated and healthcare-associated infections, of which the former has displayed a metastatic spread uncommon for enteric Gram-negative bacilli (Yu *et al.*, 2007; Gupta *et al.*, 2011; Shon *et al.*, 2013). Clinical manifestations of infection and even geographical restriction of particular infections can be attributed to a myriad of factors, inclusively virulence factors and host-associated factors (Yu *et al.*, 2007; Bamford *et al.*, 2011). The hypervirulent *K. pneumoniae* (hvKP) strains, variants of the "classical" *K. pneumoniae*, typically cause pyogenic liver abscesses, pneumonia, meningitis and endophthalmitis in otherwise healthy individuals (Shon *et al.*, 2013). "Classical" *K. pneumoniae* strains have typically exhibited a propensity for multidrug-resistance acquisition, whereas hvKP has remained largely susceptible with only a few reports of MDR-hvKP (Shon *et al.*, 2013).

Emerging epidemiological trends for MDR bacterial infections are being influenced by leisure tourism, medical tourism, military conflict, natural disasters and migration leading to intercountry transfer of MDR resistant Gram-positive and Gram-negative bacteria (Rogers *et al.*, 2011). In a review by Rogers *et al.* (2011), the patients at risk of acting as both vectors and victims of healthcare-associated infections with MDR strains are categorised as civilian aeromedical evacuees, military aeromedical evacuees and medical tourists (Rogers *et al.*, 2011). Medical tourism from developed countries are increasingly directed to South and Central America, South Africa and Asia for several reasons, such as lower costs of treatment and to avoid treatment delays experienced in the public sector in the countries of origin (Rogers *et al.*, 2011). International travel to endemic countries, with ESBL- and carbapenemase-harbouring strains, presents a risk of MDR bacteria acquisition even without

healthcare contact (Rogers *et al.*, 2011; Van der Bij and Pitout, 2012). A metagenomic study of international travel effects on the human gut resistome determined the prevalence of specific resistance genes in travellers before and after travel and reported a high acquisition rate for the gene $bla_{CTX\text{-}M}$ (an ESBL) as well as *qnr*B and *qnr*S (quinolone resistance genes) (Wintersdorff *et al.*, 2014). Other risks for *K. pneumoniae* infections, particularly ESBL strains, include several host dependent factors, such as underlying complications and local antibiotic policies, which may influence colonisation patterns in hospital patients (Podschun and Ullmann, 1998; Yu *et al.*, 2007; Tsai *et al.*, 2010; Bamford *et al.*, 2011).

Once established in the hospital setting, the proliferation and spread of MDR strains can occur within and between hospitals (Diancourt *et al.*, 2005). The molecular characterisation of β-lactamases and the molecular typing of *K. pneumoniae* multidrug-resistant isolates thus provide insight into current resistance profiles and possible routes of transmission. Whether by evolution of local clones through genetic determinant acquisition or introduction of successful international clones and their β-lactamases, the increase in multidrug-resistant *K. pneumoniae* isolates can be associated with poor treatment outcome (Woodford *et al.*, 2011; Chmelnitsky *et al.*, 2013; Shon *et al.*, 2013). Typing and β-lactamase detection thus remain a necessary, albeit expensive, tool for epidemiological studies, which may be influential in determining local empirical treatment.

Aim

The aim of the study was to perform genetic typing of *K. pneumoniae* in clinical specimens utilising both PFGE and MLST techniques. The aim of the study also included determining the presence and prevalence of genes conferring β-lactamase production in *K. pneumoniae* clinical isolates.

Objectives

1. To collect 100 β-lactam resistant *K. pneumoniae* clinical isolates identified using the VITEK®2 automated system (bioMérieux, France)
2. To extract genomic DNA utilising a commercial DNA extraction kit
3. To extract plasmid DNA utilising a commercial plasmid DNA extraction kit

4. To detect β-lactamase encoding genes in *K. pneumoniae* total genomic DNA utilising a multiplex-PCR assay
5. To detect β-lactamase encoding genes in *K. pneumoniae* plasmid DNA utilising a multiplex-PCR assay
6. To characterise *K. pneumoniae* clinical isolates utilising the PFGE and MLST techniques
7. To analyse data

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CHAPTER 2

Literature review

2.1 Introduction

Klebsiella pneumoniae is an important Gram-negative bacilli of the *Enterobacteriaceae* family, which exists both in the environment and in the clinical setting (Grimont and Grimont, 2005; Brisse *et al.*, 2009; Nordmann *et al.*, 2011). The bacterium is a common nosocomial pathogen, which is implicated in both severe community and healthcare-associated infections, the latter of which may range from blood, respiratory to urinary tract infections (Yu *et al*., 2007; Brisse *et al.*, 2009; Rawat and Nair, 2010; Schroll *et al*., 2010; Nordmann *et al*., 2011). The increase in isolates circulating within the clinical setting harbouring extended-spectrum β-lactamase (ESBL) genes has led to severe consequences, such as treatment failure, increased mortality rates in patients with blood stream infections, increased hospital stays and hospital costs (Giske *et al*., 2008). The preferential treatment for the multidrug ESBL-producing *K. pneumoniae* implicated in severe infections are carbapenems but the rapid sporadic spread of carbapenem-resistant strains globally has led to reduced treatment options (Bush, 2012). Treatment is further limited by the unmet need for new antimicrobial agents' development matching the rate of antimicrobial resistance evolution in bacteria, as most newly developed or developing drugs belong to known antibiotic classes (Giske *et al*., 2008; Gasink *et al*., 2009; Giamarellou, 2010; Bush, 2012).

Several mechanisms contribute towards antimicrobial resistance and virulence in Gram-negative bacteria and may even work in concert to achieve multidrug resistance profiles (Clegg and Sebghati, 2001; Fertas-Aissani *et al*., 2013). Resistance mechanisms usually mediate resistance by inactivating the antimicrobial agent, modifying the antibiotic or its target and decreasing antimicrobial drug concentrations within the cell (Nordmann and Poirel, 2008; Fernández *et al*., 2011; Kumar *et al*., 2011). A form of enzymatic inactivation of antibiotics is the acquisition and encoding of β-lactamase genes within bacterial species, such as *K. pneumoniae*, which can be classified into Ambler classes A to D (Bush, 2010). A high percentage of ESBL-producing *K. pneumoniae* has been detected in South Africa with up to 75% of isolates testing positive for the ESBL phenotype (Bamford *et al*., 2011). Carbapenem

resistance in bacterial isolates can also exhibit multidrug resistance phenotypes and is a consequence of acquiring class A, B and D carbapenemases, which have all been detected in South Africa (Bamford *et al*., 2011). Carbapenem resistance or elevated minimum inhibitory concentrations (MICs) could also be the result of ESBL phenotypes combined with other resistance mechanisms, such as outer membrane porin loss in *K. pneumoniae* (Wang *et al*., 2009; Tsai *et al*., 2011).

Due to the severe consequences of multidrug-resistant *K. pneumoniae* infections, the importance of active surveillance and β-lactamase detection is emphasised to determine local antimicrobial susceptibility profiles (Storberg, 2014). Detection can include several manual, automated and molecular techniques, such as disk diffusion techniques, the VITEK®2 ESBL tests (bioMérieux, France) and polymerase chain reaction, respectively (Drieux *et al*., 2008; Rawat and Nair, 2010). Another important aspect of understanding transmission, outbreaks and management is the genetic typing of *K. pneumoniae* isolates (Johnson *et al*., 2007; Vimont *et al*., 2008; Berrazeg *et al*., 2013). Several typing techniques exist of which pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are the most frequently implemented to determine clones and the clonal relatedness, respectively (Diancourt *et al*., 2005; Johnson *et al*., 2007; Vimont *et al*., 2008; Woodford *et al*., 2011).

2.2 Epidemiology of multidrug-resistant *K. pneumoniae*

Klebsiella pneumoniae is an important nosocomial pathogen within the clinical setting and much like the other members of the *Enterobacteriaceae* the bacillus commonly expresses resistance towards β-lactam antibiotics, which is mainly accomplished through β-lactamase expression (Diancourt *et al*., 2005; Yu *et al*., 2006; Gasink *et al*., 2009; Bamford *et al*., 2011; Kocsis and Szabó, 2013). The most recognised β-lactamases are cephalosporinases (such as ESBLs) and carbapenemases (Kocsis and Szabó, 2013). *Klebsiella pneumoniae* is commonly isolated from the intensive care unit (ICU) and implicated in hospital outbreaks, which is exacerbated by the increase of antimicrobial resistance through ESBL production and global emergence of carbapenem-resistant bacterial strains (Yu *et al*., 2006; Gasink *et al*., 2009; Bamford *et al*., 2011; Tofteland *et al*., 2013). The carbapenem-resistant strains are resistant towards a wide range of antibiotics including the carbapenem antibiotic, which is preferentially used for the treatment of infections by ESBL-producing *K. pneumoniae* (Gasink *et al*., 2009). The lack of consistent studies or few studies reporting on the ESBL prevalence

and genes detected in some African countries, particularly within Eastern and Western Africa, makes it difficult to determine trends in antimicrobial resistance patterns (Storberg, 2014).

Infections caused by ESBL-producing bacteria can range from urinary tract infections to complicated sepsis (Rawat and Nair, 2010). *Klebsiella pneumoniae* has been identified as one of the six blood stream pathogens of significance in South African public hospitals (Bamford *et al*., 2011). Bamford *et al.* (2011) reported an increase in multidrug-resistant *K. pneumoniae* isolates in 2009 with 55% to 75% of isolates producing ESBLs in the public hospitals and 55% to 60% in the private hospitals. In 2011 a Sentinel surveillance report started through the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) reported 65% of the 1 601 *K. pneumoniae* referrals implicated in bacteraemia as ESBL-producers, 92% as tigecycline susceptible and 56% as ciprofloxacin susceptible [National Institute for Communicable Diseases (NICD), 2012]. The majority of ciprofloxacin resistant isolates (63%) were documented as ESBL-producers (NICD, 2012). In a 2013 surveillance report posted by the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA), 73% of *K. pneumoniae* isolates were found to be ESBL-producers and 2% were found to be ertapenem resistant, a carbapenem (FIDSSA, 2013). In the studies available from African countries, the most commonly identified ESBL is the CTX-M-15 encoding gene, which is often co-encoded with other ESBL genes (SHV and TEM) as well as Class D encoding genes (such as OXA-1 and OXA-48) (Storberg, 2014). In addition to the cephalosporinases detected, several carbapenemases have been detected in South African laboratories (Bamford *et al*., 2011; Brink *et al*., 2012a; Brink *et al.*, 2012b; Brink *et al*., 2013).

Carbapenem resistance in *Enterobacteriaceae* has been detected worldwide at alarming frequencies, including in Africa, Asia, Europe, North America and South America (Kitchel *et al*., 2009; Sisto *et al*., 2012; Tofteland *et al*., 2013; Storberg, 2014). The prevalence and geographical distribution of various *K. pneumoniae* strains differ but a particularly important strain involved in national and international epidemics is the sequence type (ST) 258 harbouring the *K. pneumoniae* carbapenemases (KPC) (Kitchel *et al*., 2009; Samuelsen *et al*., 2009; Grundmann *et al*., 2010; Coetzee and Brink, 2011). The most important carbapenemases belong to the Ambler Class A [*K. pneumoniae* carbapenemase (KPC)], Class B [metallo-β-lactamases (MBL), such as New Delhi metallo-β-lactamases (NDM-1)] and Class D [Oxacillinases, particularly OXA-48-type carbapenemases], which have all been

detected in South Africa (Hirsch and Tam, 2010; Coetzee and Brink, 2011; Sisto *et al*., 2012; Kocsis and Szabó, 2013; NICD, 2013).

2.3 Classification of *K. pneumoniae* isolates

The genus *Klebsiella* belongs to the *Enterobacteriaceae* family and can be subdivided into a range of species, including *Klebsiella granulomatis*, *K. mobilis*, *K. ornithinolytica*, *K. oxytoca*, *K. planticola*, *K. pneumoniae*, *K. singaporensis*, *K. terrigena*, *K. trevisanii* and *K. variicola* (Euzéby, 1997; Drancourt *et al.*, 2001; UniProt: Taxonomy; Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 2014). The bacterium *K. pneumoniae* can be further subdivided into *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* (Table 2.1) (Euzéby, 1997; UniProt: Taxonomy, 2014; Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 2012). *Klebsiella pneumoniae* is closely related to several other genera within the *Enterobacteriaceae* family, such as *Citrobacter*, *Escherichia*, *Enterobacter* and *Salmonella* (Brisse and Verhoef, 2001; Kumar *et al*., 2011).

Table 2.1: *Klebsiella pneumoniae* nomenclature (Euzéby, 1997)

Rank	Name
Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Enterobacteriales*
Family	*Enterobacteriaceae*
Genus	*Klebsiella*
Species	*pneumoniae*
Subspecies	*pneumoniae*

A study conducted by Drancourt *et al.* (2001) aimed at re-establishing and confirming the taxonomy of the genus *Klebsiella* determined the carbon assimilation patterns, 16S rDNA and β-subunit of RNA polymerase B (*rpo*B) sequences for eight *Klebsiella* species. Seven of the *Klebsiella* species, namely: *K. ornithinolytica*, *K. oxytoca*, *K. planticola*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. terrigena*, could be distinguished by the inability of the *K. pneumoniae* subspecies to

grow at 10°C or utilise L-sorbose as the sole carbon source (Drancourt *et al*., 2001). The 16S rDNA and *rpo*B sequence analyses furthermore indicated a 98.2% to 99.7% and 99.4% to 100% similarity, respectively, between the three *K. pneumoniae* subspecies and *K. granulomatis* (Drancourt *et al*., 2001). Sequence analysis of the *rpo*B gene is confirmatory for *K. pneumoniae* but is typically used for characterisation utilising MLST (Diancourt *et al*., 2005; Elhani *et al.*, 2010). *Klebsiella pneumoniae* is the most relevant and common species isolated from clinical specimens (Podschun and Ullmann, 1998).

2.4 General characteristics of *K. pneumoniae* bacteria

Klebsiella species are ubiquitous and can occur within two broadly defined habitats, namely the environment and mucosal surfaces of mammals, including humans (Podschun and Ullmann, 1998). In the environment it can be found to exist in surface water, sewage, soil and even on plants whilst on their human host the saprophyte can be located in the nasopharynx and the intestinal tract (Podschun and Ullmann, 1998). The human skin is not conducive for the growth of *Klebsiella* species and so is merely considered to be transiently present (Podschun and Ullmann, 1998).

Klebsiella pneumoniae presents typically as Gram-negative straight rods between 0.3 µm and 1.8 µm in size (Ørskov, 1984). The non-motile bacteria are lactose fermenting, facultative anaerobes that proliferate at 37˚C and produce characteristically mucoid colonies on carbohydrate rich media, attributed to the presence of a capsule (Ørskov, 1984; Drancourt *et al*., 2001). Biochemical reactions can be utilised for the identification and differentiation of *Klebsiella* species (Podschun and Ullmann, 1998).

2.4.1 Culture and metabolic characteristics

Klebsiella species are easily cultured on media suitable for *Enterobacteriaceae* bacteria, including: Nutrient agar, Tryptic casein soy agar, Bromocresol purple lactose agar, Drigalski agar, MacConkey agar, Eosin-methylene blue (EMB) agar and Bromothymol blue (BTB) agar (Grimont and Grimont, 2005). No additional growth factors are required by *K. pneumoniae*, which is capable of both fermentative and respiratory metabolism (Drancourt *et al*., 2001). The facultative anaerobe can have a variable mucoid appearance, which may vary

between different strains and be influenced by the composition of the medium used (Drancourt *et al*., 2001; Grimont and Grimont, 2005).

Useful tests in determining enterobacterial taxonomy include carbon source utilisation tests, glucose oxidation test in the presence or absence of pyrroloquinoline quinone, gluconate- and 2-ketogluconate dehydrogenase tests and tetathionate reductase and β-xylosidase tests (Brisse *et al*., 2006). All *Klebsiella* strains are capable of utilising L-arabinose, D-arabitol, D-cellobiose, citrate D-fructose, D-galactose, D-glucose, 2-ketogluconate, maltose, D-mannitol, D-melibiose, D-raffinose, D-trehalose and D-xylose, whilst lactose and D-sorbitol can be used as a carbon source by all strains, except *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *ornithinolytica* (Grimont and Grimont, 2005). A unique characteristic to both *K. pneumoniae* subsp. *pneumoniae* and *K. mobilis* is the ability to oxidise glucose to gluconate using glucose dehydrogenase in the absence of pyrroloquinoline quinone (Grimont and Grimont, 2005). *Klebsiella pneumoniae* subsp. *pneumoniae* in addition possesses enzymes involved in the glycerol dissimilation pathway, namely glycerol dehydrogenase type I and 1,3-propanediol dehydrogenase, which permits fermentative growth on glycerol (Grimont and Grimont, 2005; Zhao *et al*., 2009; Marçal *et al*., 2009; Gao *et al*., 2014). The absence of the latter enzyme in other *Klebsiella* species renders them unable to do the same (Grimont and Grimont, 2005).

Klebsiella species are oxidase negative, catalase positive and often Voges-Proskauer test positive, with the exception of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* (Drancourt *et al*., 2001; Grimont and Grimont, 2005). Most strains can hydrolyse urea, reduce nitrates without the production of H_2S gas, as well as utilise glucose and citrate as carbon sources (Drancourt *et al*., 2001). In the case of fermentation of glucose a gas and an acid are produced (Drancourt *et al*., 2001). Glucose fermentation also results in the formation of acetoin and 2,3-butanediol (Grimont and Grimont, 2005).

2.4.2 Genomic characteristics

Klebsiella pneumoniae isolates' genome size ranges from 5.1 to 5.6 Mb with extensive genetic variation being reported among intraspecific strains due to genomic rearrangements (often as a result of chromosomal inversions, plasmids and mobile genetic elements) as well as strain-specific genes (Kumar *et al.*, 2011; Ramos *et al.*, 2014). The nine *K. pneumoniae*

subsp. *pneumoniae* whole genomes currently available on public databases [NCBI GenBank Entrez Genome database (http://www.ncbi.nlm.nih.gov/genome)] include: *K. pneumoniae* strain MGH78578, NTUH-K2044, 342, HS11286, KCTC2242, CG43, JM45, KP13 and 1084 (Gao *et al.*, 2014; Ramos *et al.*, 2014). In a study conducted by Kumar *et al.* (2011) where two *K. pneumoniae* strains were sequenced and compared with previously sequenced strains, 3 631 common proteins were identified and considered to be the core set of orthologous genes. A comparison with the information on known biological functions of 342 *K. pneumoniae* proteins revealed that 52.11% of the protein-encoding genes were dedicated to transport and binding proteins, energy metabolism, regulatory function and cell envelope, respectively (Kumar *et al.*, 2011). A five-gene cluster involved in anaerobic sugar metabolism that was also identified in two of the strains, namely strain 1162281 and JH1, was found to be similar to Gram-positive genera homologs (Kumar *et al*., 2011). In a separate study by Ramos *et al*. (2014), the Kp13 chromosome was compared to strains MGH78578, NTUH-K2044 and 342 and it was found to harbour a similar G+C content (57.5%, 57.5%, 57.7% and 57.3% respectively). The G+C content for Kp13 was on the other hand lower for the six plasmids, suggestive of DNA acquired through horizontal gene transfer (HGT) (Ramos *et al.*, 2014). At least 32 *K. pneumoniae* plasmids have been sequenced, which range in size from 3 kb to 270 kb (Bai *et al.*, 2013).

Microbial pathogens are capable of modifying inherent virulence or patterns of spread through evolutionary processes, which can often be mediated by HGT (Shon *et al*., 2013). The acquisition of pathogenicity islands and virulence plasmids are mechanisms by which *K. pneumoniae* may laterally transfer genes (Shon *et al*., 2013). Resistance genes could also be acquired by Gram-negative bacteria through recombination, integron-mediated mobilisation of gene cassettes or transposition (Girlich *et al*., 2012). An example of a lateral plasmid transfer mechanism is the acquisition of a large 180 to 220 kb virulence plasmid by hypervirulent *K. pneumoniae* (hvKP) strains that are not typical in "classical" *K. pneumoniae* strains and encode virulence factors, such as the RmpA (regulator of the mucoid phenotype) and iron acquisition factors (Shon *et al*., 2013).

Klebsiella pneumoniae has acquired multiple resistance genes over time (Girlich *et al*., 2012). The common statement that antibiotic use is solely to blame for increased antibiotic resistance over time is challenged by Projan (2007), who hypothesised that the ability of a bacterium to develop resistance could be a function of genome size because larger genomes have more

genetic information to draw from. In support of this school of thought, smaller genomes of some bacteria appear more specialised, such as *Treponema pallidum*, whereas those with larger genomes are more environmentally adaptable and versatile, such as *K. pneumoniae* and *Acinetobacter baumannii*, thus developing multidrug resistance more easily (Projan, 2007). Resistance genes acquired, particularly ESBL genes, are widely disseminated even between species, such as strains of *Escherichia coli*, *Enterobacter aerogenes*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Gniadowski, 2001).

2.5. Virulence factors and the role in pathogenesis of *K. pneumoniae*

The significant impact of *K pneumoniae* in the clinical setting as a healthcare-associated pathogen has prompted investigation into the factors implicated in its pathogenesis (Clegg and Sebghati, 2001). The factors aiding in basic pathogenesis of *K. pneumoniae* are the fimbrial and non-fimbrial adhesins, a capsule, siderophores (particularly enterobactin), urease, lipopolysaccharide (LPS), serum resistance as well as biofilm formation (Clegg and Sebghati, 2001; Clements *et al.*, 2008; Johnson *et al*., 2011; Fuursted *et al*., 2012; Hennequin *et al*., 2012; Fertas-Aissani *et al*., 2013). On the other hand, enhancing factors aiding invasion include other siderophores (aerobactin and yersiniabactin), catechols receptor, mucoid factor and hypermucoviscosity (Russo *et al*., 2011; Fertas-Aissani *et al*., 2013). The virulence of *K. pneumoniae* is further exacerbated by the additional, easy acquisition of β-lactamase encoding genes; however, successful infection is ultimately also reliant on a number of host dependent factors (Fertas-Aissani *et al*., 2013).

2.5.1 Adhesins

The prerequisite to an infection is often the mucosal pathogen's ability to adhere (Clegg and Sebghati, 2001; Struve *et al.*, 2008). *Klebsiella pneumoniae* expresses numerous fimbrial and non-fimbrial adhesins capable of recognising varied receptors, which in turn can facilitate the adherence to several target cells (Clegg and Sebghati, 2001). Fimbrial adhesins include mannose-sensitive type 1 fimbriae, type 3 fimbriae and plasmid-encoded fimbriae designated as KPF-28, while a non-fimbrial adhesin includes the CF29K factor (Podschun and Ullmann, 1998; Clegg and Sebghati, 2001; Grimont and Grimont, 2005; Schembri *et al.*, 2005; Schroll *et al*., 2010; Johnson *et al*., 2011). The above mentioned type 1 and type 3 fimbriae are

frequently detected in *K. pneumoniae* isolates (Stahlhut *et al*., 2009; Schroll *et al*., 2010; Ramos *et al.*, 2014).

The adhesive FimH subunit of the type 1 fimbriae plays an important role in *K. pneumoniae* and *E. coli* mediated urinary tract infections (UTIs), whereas type 3 fimbriae mediate adhesion to multiple host structures and are important in biofilm formation (Stahlhut *et al*., 2009; Schroll *et al*., 2010; Fertas-Aissani *et al*., 2013; Ramos *et al.*, 2014). The type 3 fimbriae are thus important in biofilm-associated infections, which typically affect catheterised patients (Schroll *et al*., 2010; Hennequin *et al*., 2012; Fertas-Aissani *et al*., 2013; Ramos *et al.*, 2014). The biofilm confers advantageous characteristics in the form of antibiotic tolerance by the matrix-enclosed bacterial populations as well as resistance to opsonisation and phagocytosis (Schroll *et al*., 2010; Fertas-Aissani *et al*., 2013). Additionally, the CF29K adherence factor is a non-fimbriate adhesin that does not occur in all strains of *K. pneumoniae* but has been associated with KPF-28 fimbriae (Clegg and Sebghati, 2001). The expression of the various fimbriae can be both beneficial in that it may facilitate attachment or disadvantage the bacterium due to the heightened host immune response that may be triggered, thus outlying the opportunistic nature of *K. pneumoniae* (Clegg and Sebghati, 2001).

2.5.2 Capsule

Klebsiella pneumoniae's ability to establish infection in a host is aided by the presence of capsular polysaccharides, which gives colonies the characteristic mucoid appearance and a mucopolysaccharide outside the capsule (Grimont and Grimont, 2005). Capsules can play an important role outside the human host by offering some protection against dessication in the environment or in the host by resisting complement-mediated lysis or phagocytosis (Cortés *et al*., 2002; Moranta *et al*., 2010; Clements *et al*., 2008). Resistance to phagocytosis was found to be higher in K1 and K2 capsular serotypes (Yu *et al*., 2008; Shon *et al*., 2013). Interference with complement activation and antiphagocytic properties may play a role in *K. pneumoniae*'s ability to cause primary, cavitating pneumonia (Regueiro *et al*., 2006; Ahmad *et al*., 2010; Moranta *et al*., 2010). The release of excessive capsular material may also have a neutralising effect against antibodies (Clegg and Sebghati, 2001).

At least 78 antigenically varied capsular types have been identified in *K. pneumoniae* (Pan *et al*., 2008; Turton *et al*., 2008; Brisse *et al.*, 2009; Pan *et al.*, 2013; Shon *et al.*, 2013).

Particular types may play a more significant role in virulence, such as the K2 capsule, which has frequently been isolated from clinical isolates implicated in urinary tract infections, pneumonia and bacteraemia (Clegg and Sebghati, 2001; Grimont and Grimont, 2005; Turton *et al*., 2008; Hennequin *et al*., 2012). *Klebsiella pneumoniae* strains implicated in respiratory tract infections can possess K1 to K6 antigens; however, other nosocomial strains may also possess antigens K2, K21, K55, K10 or K24 (Grimont and Grimont, 2005, Turton *et al*., 2008). The more recent emergence of liver abscess syndrome in countries, such as South Africa and Taiwan, is associated with virulent K1 and K2 hypermucoviscous strains with phenotypes attributed to the mucoviscosity-associated gene A (*mag*A) and the plasmid-encoded regulator of the mucoid phenotype (*rmp*A) gene (Yeh *et al*., 2007; Yu *et al*., 2007; Russo *et al*., 2011; Fertas-Aissani *et al*., 2013). The *mag*A gene is particular to serotype K1 (Yeh *et al*., 2007). The hypermucoviscosity phenotype, a virulence factor identifiable using the string test, is regulated by the *rmp*A gene, which has demonstrated a 1000-fold decrease in virulence during mouse lethality tests when knocked out (Yu *et al*., 2008; Cheng *et al*., 2010; Russo *et al*., 2011; Diago-Navarro *et al*., 2014).

2.5.3 Lipopolysaccharide

Surface saccharides that have been associated with *K. pneumoniae* virulence in a human host include an LPS and capsule (Clements *et al*., 2008). The LPS is a component situated in the outer membrane of bacteria and part of it forms the O-antigen of which there are only 12 differing antigens (Podschun and Ullmann, 1998; Grimont and Grimont, 2005). The O1 serotype is commonly detected in isolates that were capsule typeable (Clements *et al*., 2008). The proteins encoded by genes *wab*G (a transferase) and *uge* (uridine diphosphate galacturonate 4-epimerase) also contribute towards colonisation and virulence of *K. pneumoniae* through their association with LPS synthesis (Hennequin *et al*., 2012).

The complement cascade reaction is responsible for non-immune serum's bactericidal activity (Clegg and Sebghati, 2001). The LPS may be responsible for diverse serum-sensitivity between strains and the structure may affect complement components' ability to bind (Clegg and Sebghati, 2001; Fuursted *et al*., 2012). The ability to cause bacteraemia could therefore be facilitated in particular serum-resistant strains of *K. pneumoniae* (Clegg and Sebghati, 2001). The LPS may also aid in host cell association by creating a neutral surface charge

(Clements *et al*., 2008). The LPS may offer an advantage by overcoming repulsive forces and in that way aid specific adherence to eukaryotic cells (Clements *et al*., 2008).

2.5.4 Siderophores and Urease

The growth of *K. pneumoniae in vivo* necessitates essential elemental iron for which it competes with the host by producing high-affinity extracellular ferric chelators (iron-binding molecules) (Clegg and Sebghati, 2001; Russo *et al.*, 2011). A hypervirulent strain of *K. pneumoniae* was found to possess greater quantities of biologically active siderophores (Russo *et al*., 2011; Shon *et al*., 2013). The genes encoding siderophores include *ent*B (enterobactin), *iut*A (aerobactin), *irp*1-*irp*2-*ybt*S-*fyu*A (yersiniabactin) and *iro*N (ferric-catecholates receptor) (Russo *et al*., 2011). The *kfu* gene encodes a ferric iron uptake system in *Klebsiella*, which has demonstrated that the extracellular enzyme urease also has an important function in the growth of isolates implicated in urinary tract infections (Clegg and Sebghati, 2001).

Siderophores produced by *K. pneumoniae* include most commonly enterochelin and to a lesser extent aerobactin (Clegg and Sebghati, 2001; Grimont and Grimont, 2005). The latter is a hydroxamate siderophore, which has been implicated in virulence and has been associated with as much as a 100-fold increase in virulence in mouse lethality tests (Yu *et al*., 2008; Russo *et al.*, 2011). Another phenolate siderophore, called yersiniabactin, is encoded on the *Yersinia* high-pathogenicity-island (YHPI) and is activated in iron-depleted conditions (Hancock *et al*., 2008; Russo *et al.*, 2011). Yersiniabactin permits evasion of mucosal-secreted protein lipocalin 2 (Lcn2), which is capable of inactivating enterobactin (Bachman *et al*., 2011). Additionally, yersiniabactin promotes biofilm production by activating the outer membrane protein Ferric yersiniabactin uptake (FyuA) (Hancock *et al*., 2008). Yersiniabactin and aerobactin have both been indirectly implicated in reduced innate immune cells' killing ability by interfering with reactive oxygen species' production (Paauw *et al.*, 2009).

2.6 Clinical manifestations of *K. pneumoniae* infections

Klebsiella pneumoniae is both known as a commensal bacterium found in the environment and as an important healthcare-associated pathogen involved in a myriad of infections, ranging from blood, respiratory, urinary and intra-abdominal infections, in predominantly

incapacitated patients (Brisse *et al*., 2006; Struve *et al*., 2008; Brisse *et al.*, 2009; Schroll *et al*., 2010; Nordmann *et al*., 2011). Clinical presentations of disease caused by *K. pneumoniae* are affected by the quantity and type of virulence factors expressed, whereas the resulting infections can be divided into community-associated and healthcare-associated infections (Yu *et al*., 2007; Schroll *et al*., 2010).

Klebsiella pneumoniae mostly affects patients in the ICU and is an important contributor to in-hospital mortality (Gasink *et al*., 2009). In the clinical setting, *K. pneumoniae* is second only to *E. coli* in causing catheter-associated urinary tract infections and is one of six important blood stream pathogens in South Africa (Schroll *et al*., 2010; Bamford *et al*., 2011). On the other hand, *K. pneumoniae* is also responsible for diseases, such as community-associated pneumonia, pyogenic liver abscess, rhinoscleroma, atrophic rhinitis and less frequently: meningitis, necrotising fasciitis and prostatic abscess (Lu *et al.*, 2002; Wong *et al.*, 2004; Kohler *et al.*, 2007; Brisse *et al.*, 2009; Schroll *et al*., 2010). Rhinoscleroma and atrophic rhinitis are specifically described to be caused by *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae*, respectively (Brisse *et al.*, 2009).

Community-associated infections, such as pneumonia and liver abscess, meningitis or endophthalmitis have been identified in Taiwan and South Africa (Yu *et al*., 2007). *Klebsiella pneumoniae* implicated in community-associated meningitis led to mortality rates ranging from 30% to 83% in adult cases with added severe neurologic sequelae in survivors in South Africa (Russo *et al*., 2011). It was also noted that bacteraemic community-associated pneumonia due to *K. pneumoniae* had a poorer prognosis than *Streptococcus pneumoniae* mediated bactereamia (Lin *et al.*, 2010). Additionally, the death rate from community-associated *K. pneumoniae* pneumonia was determined to be as high as 54% and 56% in Taiwan and South Africa, respectively (Ko *et al.*, 2002). Two virulence factors of significance in the type of infections caused include the presence of a capsule, in particular K1 and K2 serotypes, as well as aerobactin production (Yu *et al*., 2007; Schroll *et al*., 2010). Serotypes K1 and K2 were detected in 14.3% and 38.8% of *K. pneumoniae* isolates implicated in bacteraemic community-associated pneumonia in a tertiary care hospital in Taiwan, from 2001 to 2008 (Lin *et al.*, 2010). The K1 and K2 serotypes appear more commonly in community-associated strains than in their healthcare-associated counterparts, as reported in Taiwan, South Africa and Singapore (Lin *et al.*, 2010). A study done by Yu *et al.* (2007) revealed that the mucoid phenotype, that is encapsulated *K. pneumoniae*, was

detected in 94% and 100% of isolates responsible for community-associated pneumonia and an invasive syndrome in Taiwan and South Africa, respectively.

Unlike their Gram-positive counterparts, invasive infections and metastatic spread are rare for extraintestinal Gram-negative pathogens, such as *K. pneumoniae* (Struve *et al.*, 2008; Russo *et al*., 2011). Hypervirulent strains of *K. pneumoniae* have on the other hand been identified and associated with community-associated liver abscesses as well as spread to bone, eyes, joints, kidneys, lungs, muscle/fascia, pleura, prostate, spleen, soft-tissue, skin and the central nervous system (CNS) (Struve *et al.*, 2008; Russo *et al*., 2011). *Klebsiella pneumoniae* is largely thought of as an opportunistic pathogen but the emergence of hypervirulent strains over the last decade have demonstrated the capacity to infect otherwise healthy individuals (Struve *et al.*, 2008; Russo *et al*., 2011).

The virulence factors expressed could contribute to the range of clinical manifestations of infections but the geographical restriction of certain manifestations could alternately be dependent on host factors typical to that region (Lautenbach *et al*., 2001; Yu *et al*., 2007; Bamford *et al*., 2011). Host factors could include the frequency of diabetes mellitus, genetic predilections, underlying prevalent diseases, alcoholism, socioeconomic determinants and the availability of quality healthcare (Lautenbach *et al*., 2001; Ko *et al.*, 2002; Yu *et al*., 2007; Bamford *et al*., 2011).

2.7 Treatment of *K. pneumoniae* infections

Appropriate therapeutic options are often determined based on the antibacterial spectrum, convenience of use and tolerability of antimicrobials, such as third- and fourth-generation cephalosporins (Endimiani *et al*., 2004). The factors influencing appropriate antimicrobial treatment are also dependent on local bacterial susceptibility patterns and patient risk profiles, which may ultimately determine the risk of infection with opportunistic and potentially antibiotic resistant pathogens (Micek *et al*., 2010). Multidrug-resistant bacterial strains, such as *K. pneumoniae*, *Pseudomonas aeruginosa* and *A. baumannii*, present a therapeutic conundrum due to its ability to undermine treatment, whilst also reducing appropriate antibiotic options available and causing a delay in appropriate treatment due to inefficient empirical treatment (Vaara, 2010; Woodford *et al.*, 2011).

2.7.1 Treatment of multidrug-resistant *K. pneumoniae* infections

The global emergence of multidrug-resistant Gram-negative bacilli is an unprecedented problem, which is exacerbated by the focus on improving existing classes of drugs instead of developing new classes of drugs with different targets over the last 50 years (Giamarellou, 2010; Bush, 2012). The rise in the rate of multidrug-resistant bacteria and the increasingly limited treatment options is exemplified by ever-prevalent ESBL-producing *K. pneumoniae* for which carbapenems were the mainstay treatment but are increasingly rendered ineffective by the sporadic emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) (Gasink *et al*., 2009, Bush, 2012; Van Duin *et al.*, 2013).

Typical characteristics of ESBL-producing members of the *Enterobacteriaceae* family include resistance to amino- and carboxy-penicillins, second generation cephalosporins and several third and fourth generation cephalosporins as well as monobactams (such as aztreonam) though some may remain susceptible to cephamycins (Gniadowski, 2001; Drieux *et al*., 2008; Lee *et al.*, 2012; Van der Bij and Pitout *et al*., 2012; Breurec *et al*., 2013). Extended spectrum β-lactamase producers additionally exhibit synergy between the former-mentioned antibiotics and clavulanate, a β-lactamase inhibitor and may exhibit additional resistance towards other antibiotics, such as fluoroquinolones, aminoglycosides, trimethoprim and sulfamethoxazoles (Drieux *et al*., 2008; Giske *et al*., 2008; Rawat and Nair, 2010; Kumar *et al.*, 2011). Treatment failure could be attributed to a drug's inability to reach therapeutic concentrations at the site of infection, particularly when the minimum inhibitory concentrations of the bacterium is close to the susceptibility breakpoint of drugs, such as ciprofloxacin sometimes used against Temoneria (TEM)-52 ESBLs (Endimiani *et al*., 2004). Emperical treatment should match information on pathogens distributed in the clinical setting and their respective susceptibility patterns so as to better ensure correct initial antimicrobial therapy [Drugs and Therapeutics Bulletin (DTB), 2008]. Delayed appropriate treatment can increase the likelihood of death (DTB, 2008).

In a retrospective study conducted by Micek *et al.* (2010) a better outcome was believed to be associated with correct initial combination antimicrobial therapy when empirically treating Gram-negative bacteria mediated sepsis as compared to monotherapy. In the aforementioned study, a combination of a antipseudomonal fluoroquinolone, such as ciprofloxacin, or an aminoglycoside with a carbapenem (imipenem and meropenem), piperacillin-tazobactam or

cefepime as initial treatment for severe Gram-negative bacterial infections offered a broader spectrum of activity (Micek *et al*., 2010). Additional retrospective studies further favour combination therapy in CRE infections for which treatment options have been reduced mainly to colistin, tigecycline, some aminoglycosides and fosfomycin (Giamarellou, 2010; Van Duin *et al*., 2013). Although fosfomycin appears active *in vitro*, there is little clinical experience with the drug as well as knowledge of adequate combinations for treatment without encouraging antimicrobial resistance (Falagas *et al*., 2010, Giamarellou, 2010; Raz, 2012). Tigecycline on the other hand, has demonstrated effectiveness against multidrug-resistant (MDR) *Enterobacteriaceae* and despite requiring dosage adjustments, due to low blood levels, has good clinical experience (Souli *et al.*, 2006; Giske *et al*., 2008; Giamarellou, 2010). An unfortunate drawback to tigecycline could include the selection of Gram-negative bacteria with efflux pump mutations (Giske *et al*., 2008; Bush 2012). Colistin has been recommended for use only in cases of known colistin-sensitive MDR strains or nosocomial and ICU late sepsis shock where MDR strains are suspected (Giamarellou, 2010). The use of colistin for a prolonged period (>13 days) of time has been suggested as responsible for the emergence of colistin-resistant or pandrug-resistant bacterial strains in some instances (Antoniadou *et al.*, 2007; Mentzelopoulos *et al.*, 2007; Brink *et al.*, 2013). The emergence of MBL and KPC strains of *K. pneumoniae* has rendered them resistant to all but one antibiotic, namely colistin (Souli *et al*., 2008; Souli *et al.*, 2010).

2.8 Antibiotic resistance mechanisms in *K. pneumoniae* isolates

Innate antimicrobial susceptibility could be impacted by adaptive responses, resulting in alterations to gene expression and cell physiology, which is induced in response to the pathogen's natural environmental stresses or within a host (Fernández *et al*., 2011; Poole, 2012a; Poole, 2012b; Bernier *et al*., 2013). Three modes of antibiotic resistance in bacteria, such as *K. pneumoniae*, include drug modification or enzymatic inactivation, antibiotic target modification or decreased concentrations of antimicrobial drugs within cells; possible by reduced permeability and increased efflux activity (Poole, 2004; Nordmann and Poirel, 2008; Page *et al.*, 2010; Fernández *et al*., 2011; Kumar *et al.*, 2011). These modes of action are encoded either intrinsically or acquired through mutation and resistance gene acquisition (Poole, 2004; Fernández *et al*., 2011). The adaptive responses are not only triggered by antibiotics but can occur as a response to environmental stresses and include: cessation of growth, stress-induced acquisition of resistance determinants, changes to target sites, altered

membrane barrier functions, induction of resistance-conferring mutations and promotion of biofilm formation (Fernández *et al*., 2011; Kumar *et al.*, 2011; Poole, 2012a). Ironically, some protective responses activated as a result of the stress caused by antimicrobial drugs, can lead to resistance towards these very same antimicrobial drugs (Poole, 2012a; Bernier *et al.*, 2013). Genetic elements conferring potential resistance genes are easily transferred horizontally both intra- and interspecies due to the close genetic semblance between bacteria of the *Enterobacteriaceae* family (Fernández *et al*., 2011; Kumar *et. al*., 2011).

2.8.1 Porin loss

Studies have investigated the effect of porin loss in the role of resistance (Carvalhaes *et al.*, 2010). Alterations in outer membrane proteins of both *K. pneumoniae* and *E. coli*, either due to mutations or deletion of porins, may limit influx of antimicrobial agents or alternately increase efflux (Page *et al.*, 2010). Besides the major OmpK35 and OmpK36 porins, the alternative OmpK37, PhoE and LamB porins may be expressed by *K. pneumoniae* (García-Sureda *et al*., 2011). The latter three porins' role in antimicrobial resistance has not been thoroughly investigated but is suspected to be important in the absence of OmpK35 and OmpK36 (García-Sureda *et al*., 2011).

The loss of either of the two porin outer membrane proteins OmpK35 and OmpK36 has been associated with increased MICs towards cefotaxime and cefoxitin, whereas the loss of both porin proteins could lead to elevated MICs for imipenem, meropenem and expanded-spectrum cephalosporins as well as reduced fluoroquinolone susceptibility (Wang *et al*., 2009; García-Sureda *et al*., 2011; Tsai *et al*., 2011). Alternately, the loss of porins or mutations in porin encoding genes within isolates expressing broad-spectrum β-lactamases or ESBLs can result in cefoxitin and cephalosporin resistance due to reduced permeability towards antimicrobial agents and can occasionally confer additional cross resistance to quinolones, aminoglycosides and co-trimoxazole (Gniadowski, 2001; Ehlers *et al*., 2009; Wang *et al*., 2009; Tsai *et al*., 2011). The loss of one of the outer membrane proteins, particularly OmpK36, has been associated with reduced carbapenem susceptibility or resistance in ESBL and AmpC β-lactamase producing *K. pneumoniae* isolates, as demonstrated in several studies (Wang *et al*., 2009; Carvalhaes *et al.*, 2010; Tsai *et al*., 2011). The loss of both porins combined with the aforementioned broad-spectrum β-lactamases or ESBLs could result in meropenem and

imipenem moderately resistant phenotypes as well as ertapenem resistance (Wang *et al*., 2009; Tsai *et al*., 2011).

While OmpK36 deficiency is implicated in increased antibiotic resistance, there is a consequential decrease in virulence and increased neutrophil phagocytosis susceptibility observed (Chen *et al*., 2010; García-Sureda *et al*., 2011; Tsai *et al*., 2011). The over-expression of the LamB porin has also been associated with OmpK36 deficiency and the expression thereof has been highlighted as important for the loss of OmpK36 (García-Sureda *et al*., 2011). An additional modification to the outer membrane aiding in resistance, other than porin loss, is the upregulation of capsule polysaccharide (CPS) production in *K. pneumoniae* (Campos *et al.*, 2004; Kocsis and Szabó, 2013). Increased polymyxin resistance in *K. pneumoniae* can be a result of CPS limiting the interaction of polymyxins with bacterial target sites (Campos *et al.*, 2004; Kocsis and Szabó, 2013).

2.8.2 Enzyme-mediated resistance

Resistance towards β-lactam antibiotics are mainly mediated by β-lactamase enzyme production, which is capable of hydrolysing third generation cephalosporins and monobactams (Gniadowski, 2001; Pitout *et al*., 2004; Monstein *et al*., 2007; Elhani *et al*., 2010; Page *et al.*, 2010). These enzymes are classified as ESBLs in the Ambler class A and other β-lactamases include class B metallo-β-lactamases, class C cephalosporinases and class D oxacillinases, which are discussed further in section 2.9.2 (Page *et al.*, 2010). Other factors at play besides ESBL production include cases of ESBL hyperproduction due to promoter up-regulation after direct mutation, inserted transposable elements in close proximity to the promoter and the capacity of a strain to coproduce more than one ESBL (Gniadowski, 2001).

2.8.3 Efflux pumps

Changes in membrane permeability and drug flux can be influenced by variable expression and regulation of the efflux pumps (Kumar *et al.*, 2011). Five families of bacterial efflux pumps have been classified based on several criteria, namely: the pump energy source, the exported product, the quantity of components and transmembrane-spanning regions (Poole, 2004; Poole, 2005). The five families include: the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the

small multidrug resistance (SMR) family and the multidrug and toxic compound extrusion (MATE) family (Poole, 2005). Within the *Enterobacteriaceae* Gram-negative bacteria, a significant bacterial efflux pump family is the RND (Poole, 2004; Poole, 2005; Kocsis and Szabó, 2013; Ramos *et al.*, 2014). The active expression of the chromosomal native AcrAB-TolC efflux pump of the RND family contributes to fluoroquinolone resistance in *E. coli, Enterobacter* spp. and *Klebsiella* spp. (Mazzariol *et al.*, 2002; Poole, 2005; Kocsis and Szabó, 2013). The AcrA protein is a membrane fusion protein, the AcrB an inner membrane pump protein and the TolC an outer membrane protein (Kocsis and Szabó, 2013). Additionally, resistance to aminoglycosides may also be as a consequence of efflux pump activity (Kumar *et al.*, 2011).

2.8.4 Biofilm formation

Bacterial cells can exist as single cells, the planktonic form, or within communities drawn together by a self-produced biopolymer matrix and attached to a surface (Mah and O'Toole, 2001; Hennequin *et al.*, 2012; Bernier *et al.*, 2013; Soto, 2013). The latter is referred to as a biofilm and confers survival advantages in the form of improved resistance to host immune defences, resistance to biocides, increased resistance to antimicrobial compounds and higher plasmid transfer rates within that environment, which could include antibiotic resistance genes (Mah and O'Toole, 2001; Schroll *et al.*, 2010; Fernández *et al.*, 2011; Hennequin *et al.*, 2012; Soto, 2013). The reduced antimicrobial drug effect against bacterial populations within a biofilm is largely unclear but could be as a result of several mechanisms acting in conjunction, such as: (i) poor compound diffusion, (ii) the slower growth and uptake of antibiotics by the bacteria in mature biofilm (>24 hours old), (iii) the production of antimicrobial inactivating enzymes, (iv) general stress responses, (v) the expression of efflux pumps and (vi) the presence of persister cells (Figure 2.2) (Mah and O'Toole, 2001; Ito *et al.*, 2009; Fernández *et al.*, 2011; Hennequin *et al.*, 2012; Bernier *et al.*, 2013; Soto, 2013).

Biofilm formation and swarming motility are both responses to environmental stimuli that induce coordinated microbial behaviour, which may in part be communicated by quorum sensing signals (Verstraeten *et al.*, 2008; De Araujo *et al.*, 2010; Fernández *et al.*, 2011). Biofilm formation in *K. pneumoniae* is influenced by cell density-dependent quorum sensing signalling *via* the non-specific bacterial type-2 QS regulatory molecules, AI-2 autoinducers

(De Araujo *et al*., 2010). The mannose-resistant *Klebsiella*-like (MR/K) hemagglutinins or "Mrk proteins" are encoded by the genes *mrk*ABCDF within an operon and form part of

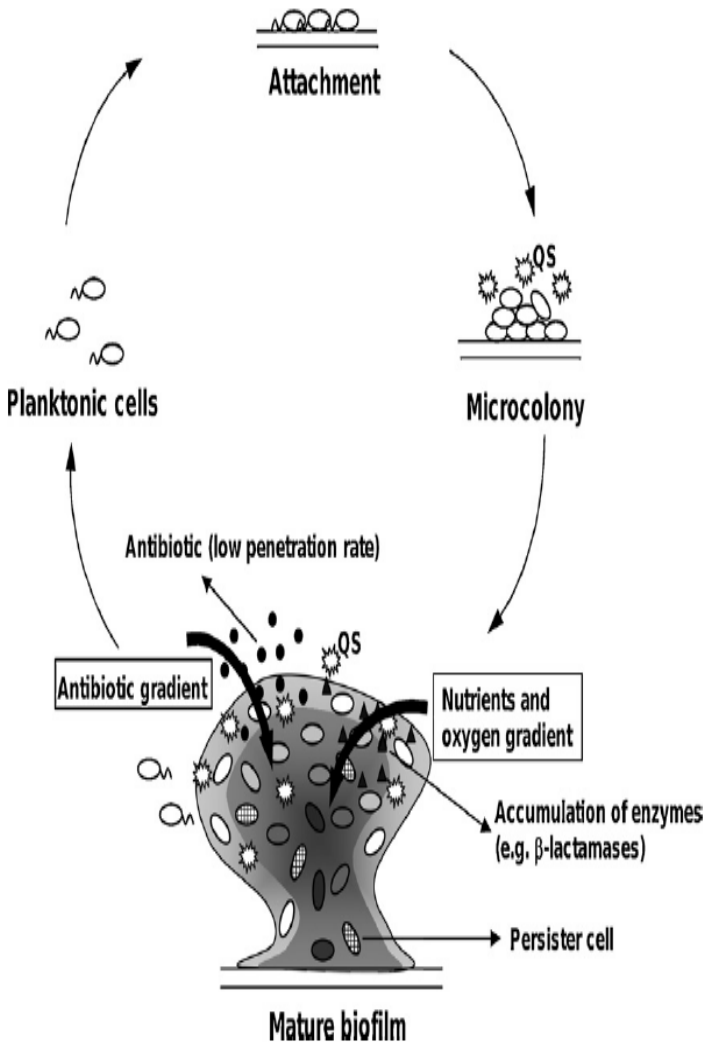

Figure 2.1: Diagrammatic representation of the formation of a biofilm and various mechanisms of resistance. The darkening grey gradient of shading represent the gradual decrease in oxygen and nutrients towards the base and the cells of lighter shades represent the more metabolically active cells towards the surface (QS: Quorum sensing signal) (Fernández *et al*., 2011)

type 3 fimbriae, which is important in mediating biofilm formation in *K. pneumoniae* (Wilksch *et al*., 2011). Antimicrobial drug resistance can increase up to 1000-fold for bacterial cells existing within the biofilm (Mah and O'Toole, 2001; De La Fuente-Núñez *et al*., 2013).

2.9 Extended spectrum β-lactamase production in *K. pneumoniae* isolates

Bacterial mechanisms of resistance can be described as either innate or acquired resistance, which in the latter case could be due to *de novo* mutations or the acquisition of genetic material by susceptible bacteria (Tenover, 2006; Davies and Davies, 2010; Stokes and Gillings, 2011). Genetic material encoding resistance mechanisms can be acquired through conjugation, transformation and transduction (Tenover, 2006; Davies and Davies, 2010; Stokes and Gillings, 2011). The acquisition of antimicrobial resistance by susceptible bacteria is problematic and exacerbated by antimicrobial use, which acts as a selective force encouraging its proliferation (Tenover, 2006; Davies and Davies, 2010; Stokes and Gillings, 2011).

In the clinical setting, *K. pneumoniae* and *E. coli* typically encode β-lactamase enzymes, which are capable of mediating resistance to β-lactam antibiotics (Livermore, 1995; Rawat and Nair, 2010). These enzymes inactivate the antimicrobial drug by hydrolysing the β-lactam ring thus preventing binding to its target, the penicillin binding proteins (PBPs) (Livermore, 1995). The ESBL-producers have activity against extended-spectrum cephalosporins, which includes the breakdown of cefotaxime, ceftazidime, ceftriaxone, cefuroxime and oxyimino-monobactam aztreonam (DTB, 2008).

2.9.1 The evolution of ESBL-producing *K. pneumoniae* isolates

The existence of β-lactamase enzymatic activity was first observed in 1940, which is prior to the implementation of penicillin for treatment (Bush, 2010). The existence of the β-lactamases was therefore naturally present within environmental isolates (Bush, 2010; Derbyshire *et al*., 2009; Ehlers *et al*., 2009). Broad-spectrum β-lactamases initially emerged in *E. coli* during the 1960s and 1970s but rapidly spread to other bacterial species, including within the *Enterobacteriaceae* family, which led to treatment using second and third generation cephalosporins (Livermore, 2008; Falagas and Karageorgopoulos, 2009; Kumar *et al.*, 2011). The first β-lactamase enzyme described in 1965 was the Temoneria (TEM)-1 enzyme and soon thereafter the Sulfhydryl variable (SHV)-1 β-lactamase, which can typically confer resistance to penicillins but not to cephalosporins (DTB, 2008). Temoneria- and SHV-type β-lactamase derivatives described as ESBLs were soon thereafter detected and found to have

activity against oxyimino-β-lactam antibiotics through minor active site modifications (Livermore *et al*., 2007; DTB, 2008; Rawat and Nair, 2010). Resistance to oxyimino-β-lactam antibiotics was recorded in 1982 shortly after the introduction of third generation cephalosporins in *K. pneumoniae* and *Serratia marcescens* (Knothe *et al*., 1983).

Hospital outbreaks of ESBL-producing bacteria, particularly *K. pneumoniae* and *E. coli*, are a threat that has existed for several years, since its first recorded outbreak in French hospitals in the 1980s (Sirot *et al.*, 1987; Philippon *et al.*, 1989; Lewis *et al*., 2007). Historically, the predominating β-lactamases encoded were of the TEM- or SHV-type, for example in the United States of America, but a shift has occurred with the Cefotaximase-Munich (CTX-M)-type being the most commonly detected ESBL (Lewis *et al*., 2007; Van der Bij and Pitout, 2012). Worldwide distribution of ESBL-producing *Enterobacteriaceae*, especially *K. pneumoniae* and *E. coli* encoding CTX-M, has been recorded with an increase in prevalence over the years (Hennequin *et al*., 2012). The predominating ESBL enzyme within clinical isolates mediating resistance can be geographically variable (Lewis *et al*., 2007). Non-ESBL resistant phenotypes are also still present in clinical isolates and are attributed to the production of broad-spectrum β-lactamases, such as TEM-1, TEM-2 and SHV-1 (Drieux *et al*., 2008). Alternately, high level resistance can be attributed to inhibitor-resistant β-lactamases, which are TEM derivatives or due to cephalosporinase production (Drieux *et al*., 2008).

Several other ESBL variants exist (Gniadowski, 2001). The only Ambler class D ESBLs are of the OXA-type enzymes of which OXA-1 has been frequently associated with other ESBL encoding genes and OXA-2 with PER-1 ESBLs (Gniadowski, 2001; Poirel *et al*., 2010; Poirel *et al*., 2011c). The result of the former OXA-1 association with other ESBLs, particularly with $bla_{\text{CTX-M}}$ genes, could be β-lactam-β-lactamase inhibitor combination resistance (Poirel *et al*., 2010).

2.9.2 Classification of β-lactamases

Enzyme-mediated resistance to β-lactam antibiotics was initially discovered in *E. coli* but has since spread to a large number of bacterial species in the form of over 890 unique β-lactamases (Bush, 2010). Both the chromosomal and plasmid encoded β-lactamases can be classified into either Bush-Jacoby-Medeiros functional groups based on hydrolysis and

inhibition characteristics or four Ambler molecular classes based on the proteins' amino acid sequences, as illustrated in Table 2.2 (Ambler, 1980; Bush, 2010). The former Bush-Jacoby-Medeiros classifies the β-lactamases into three groups and 16 subgroups (Ambler, 1980; Bush, 2010; Bush and Fisher, 2011; Kocsis and Szabó, 2013). Some resistance genes exist through natural selection of resistant clonal lineages or have been acquired through mobile genetic elements, such as plasmids, transposons and insertion sequence elements (ISs) (Samuelsen *et al*., 2009; Grundmann *et al*., 2010; Poirel *et al*., 2011a).

Table 2.2: Major groups of β-lactamases in Gram-negative bacteria (Bush, 2010)

Functional group	Molecular class	Common name	Resistance to β-lactams
1	C	Cephalosporinase	Penicillins, cephalosporins, carbapenems*, monobactams*
2b	A	Penicillinase	Penicillins, early cephalosporins, β-lactamase inhibitor combinations*
2be	A	Extended-spectrum β-lactamase	Penicillins, cephalosporins, monobactams, β-lactamase inhibitor combinations
2d	D	Cloxacillinase	Penicillins (including oxacillin and cloxacillin)
2df	D	Carbapenemase	Carbapenems and other β-lactams
2f	A	Carbapenemase	All current β-lactams
3	B	Metallo-β-lactamase	All β-lactams, except monobactams

*β-lactams that are resistant as a function of high β-lactamase production in combination with efflux and porin modifications

Ambler molecular classes A, C and D enzymes typically possess serine within the active site, while class B enzymes contain zinc (Livermore, 2008; Falagas and Karageorgopoulos, 2009; Hirsch and Tam, 2010; Kocsis and Szabó, 2013). Nine structural/evolutionary families have been described during the classification of ESBL variants (Gniadowski, 2001). The variants include Belgium extended-spectrum β-lactamase (BEL), Brazilian extended-spectrum β-lactamase (BES), CTX-M, Guyana extended-spectrum β-lactamase (GES), oxacillinase (OXA), *Pseudomonas* extended resistance (PER), *Serratia fonticola* (SFO), SHV, TEM, Tlahuicas (TLA) and Vietnam extended-spectrum β-lactamase (VEB) (Gniadowski, 2001, Naas *et al.*, 2008; Rawat and Nair, 2010; Kocsis and Szabó, 2013). Other β-lactamases of importance are carbapenemases detected in *Enterobacteriaceae*, which typically include the

OXA-48-type, KPC-type and MBL-type enzymes, Imipenem (IMP), Verona integron-encoded metallo-β-lactamases (VIM) and New Delhi metallo-β-lactamase (NDM) (Livermore, 2008; Nordmann and Poirel, 2013; Sisto *et al*., 2012).

Three definitions of ESBLs have been proposed, which include a classical definition, a broadened definition and an all-inclusive definition (Lee *et al.*, 2012). The classical definition originally defined an ESBL as derivatives of broad-spectrum TEM and SHV enzymes and later more functionally defined as β-lactamases of the Ambler class A or functional group 2be capable of hydrolysing extended spectrum cephalosporins and monobactams while still being inhibited by β-lactamase inhibitors and poorly hydrolysing cephamycins and carbapenems (Lee *et al.*, 2012). The classical definition did not on the other hand account for the β-lactamases with similar hydrolysis profiles and dissimilar evolutionary backgrounds, such as CTX-M, GES and VEB enzymes (Lee *et al.*, 2012). A broader definition by Livermore (2008), included TEM and SHV variants with weaker ESBL activity, the enzymes with similar hydrolysis but dissimilar sources, as well as β-lactamases possessing wider resistance to the parent types that do not fall within the 2be functional group (e.g. OXA variants and AmpC type mutants). The wider resistance observed is to oxyimino-cephalosporins (Lee *et al.*, 2012). Lee *et al.* (2012) have independently extended the broadened definition of ESBLs to include AmpC ESBLs from the Ambler class C; thus designating ESBLs as: aESBLs, cESBLs and dESBLs. The broadened definition is limited in that ESBLs with concurrent carbapenem and oxyimino-cephalosporin resistance are excluded.

Finally the all-inclusive definition classifies ESBLs into three classes: $ESBL_A$ (class A ESBLs), $ESBLS_M$ (miscellaneous ESBLs including as AmpC and OXA-type ESBLs) and $ESBL_{CARBA}$ (β-lactamases encompassing ESBLs with carbapenem hydrolysing activity) (Giske *et al*., 2009; Lee *et al.*, 2012). The GES-1 β-lactamase, for example, has hydrolysis profiles resembling that of other ESBLs but six GES β-lactamases have illustrated carbapenemase activity, inclusively: GES-2, -4, -5, -6, -11 and-14 (Patel and Bonomo, 2013). Bush *et al.* (2009) on the other hand felt the term $ESBL_{CARBA}$ as clinically confusing as ESBLs should be treatable with carbapenems and should thus remain more accurately classified as carbapenemases. Bush *et al*. (2009) further disputes the definitions set by Giske *et al*. (2009) by stating that AmpC-producers although treatable with carbapenems may develop resistance easily and should thus not be classified together with ESBLs. The all-

inclusive definition thus further excludes the clinical criteria in which ESBLs should have sensitivity to available β-lactamase inhibitors and current definitions of ESBLs, AmpC β-lactamases and carbapenemases should be kept independent (Bush *et al.*, 2009). The most common ESBL-encoding genes detected include SHV-, TEM- and CTX-M-type enzymes (Kocsis and Szabó, 2013).

2.9.2.1 Temoneria

The first β-lactamases identified in ESBL producers were the TEM and SHV enzyme families, which were originally found in the *Enterobacteriaceae* family but also in *Pseudomonas* species, *Neisseria gonorrhoeae* and *Haemophilus influenzae* (Brunton *et al.*, 1986; Gniadowski, 2001; Monstein *et al.*, 2007). The initially discovered broad-spectrum β-lactamases TEM-1/2 and SHV-1 are the source from which plasmid-encoded variants arose, known as extended-spectrum β-lactamases (Bradford, 2001; Endimiani *et al.*, 2004). Although initially named CTX-1 for its activity against cefotaxime, the first ESBL variant was identified in *K. pneumoniae* and named TEM-3 (Ghafourian *et al.*, 2014). More than 200 TEM-type β-lactamases have since been identified and most are classified as ESBLs (Kocsis and Szabó, 2013). Temoneria (TEM)-3, the first recorded ESBL capable of hydrolysing third-generation cephalosporins, differed from broad-spectrum TEM-2 by two amino acids, namely lysine instead of glutamic acid at residue 104 and serine instead of glycine at residue 238 (Sougakoff *et al.*, 1988; Lachmayr *et al.*, 2009; Kocsis and Szabó, 2013). Temoneria (TEM)-3 was additionally found to be inhibited by β-lactamase inhibitors (Lachmayr *et al.*, 2009; Kocsis and Szabó, 2013). Subsequent variants of the TEM β-lactamase exhibited inhibitor resistance, which was determined by amino acid changes at positions 39, 69, 165, 182, 244, 261, 275 and 276 (Lachmayr *et al.*, 2009; Kocsis and Szabó, 2013). The inhibitor resistant TEM (IRT) strains possess similar hydrolytic activity with the added benefit of resistance to β-lactamase inhibitors (Chaïbi *et al.*, 1999; Kocsis and Szabó, 2013).

2.9.2.2 Sulphydryl variable

The SHV-1 enzyme was derived from *Klebsiella* spp. chromosome and exhibited only narrow β-lactam hydrolysis until changes in the amino acid sequence resulted in variants with extended hydrolysis spectrums, including extended spectrum cephalosporins (Rasheed *et al.*, 1997; Bush and Fisher, 2011; Kocsis and Szabó, 2013). The SHV β-lactamases could have

possibly emerged due to the evolution of the K2 capsular variant of *K. pneumoniae* (Gniadowski, 2001). Most of the 171 SHV-variants identified are ESBLs and the SHV-38 β-lactamase has even exhibited carbapenemase activity (Poirel *et al.*, 2003; Kocsis and Szabó, 2013). The SHV-38 variant was identified in *K. pneumoniae*, is chromosomally encoded and exhibits hydrolysis of extended-spectrum cephalosporins and imipenem (Poirel *et al.*, 2003). Several inhibitor-resistant SHV-type enzymes have also been identified (Heritage *et al.*, 1999).

2.9.2.3 Cefotaximase-Munich

The Class A CTX-M β-lactamases were derived from chromosomally-encoded CTX-M in *Kluyvera* spp. and became the dominant plasmid encoded ESBL in *Enterobacteriaceae*, such as *E. coli* and *Klebsiella* spp (Rossolini *et al.*, 2007; Rawat and Nair, 2010; Breurec *et al.*, 2013). The enzyme is capable of higher cefotaxime hydrolysis than ceftazidime in the *Enterobacteriaceae* family and has a 40% amino acid sequence similarity to the SHV and TEM enzymes (Monstein *et al.*, 2007; Apisarnthanarak *et al.*, 2008; DTB, 2008).

All 140 known CTX-M enzymes reported are ESBLs and classified into five phylogenetic groups, based on sequence homology, the prevalence of which differs geographically (Paterson and Bonomo, 2005; Woodford *et al.*, 2006; Livermore *et al.*, 2007; Rawat and Nair, 2010; Kocsis and Szabó, 2013). The five groups are CTX-M-1, M-2, M-8, M-9 and –M-25 of which CTX-M-15 from the CTX-M-1 group is prevailing (Bonnet, 2004; Livermore *et al.*, 2007; Kocsis and Szabó, 2013; Ghafourian *et al.*, 2014). Flouroquinolone resistance has also been detected simultaneously within these CTX-M-type ESBL producers, as well as resistance towards aminoglycosides, classical tetracyclines, trimethoprim and sulphonamides (Pitout *et al.*, 2004; Livermore *et al.*, 2007; Brink *et al.*, 2012c).

2.10 Risk factors for ESBL-producing *K. pneumoniae* infections

With multidrug-resistant Gram-negative bacterial infections on the rise in the clinical setting, especially among immunosuppressed populations, such as cancer patients, a major concern is the consequence of ineffective treatment (Gudiol *et al.*, 2011). The clinical outcomes of inadequate empirical treatment with broad spectrum antibiotics with no activity against the isolated causative bacterium (*in vitro*) or a bacterium with additional antibiotic resistance can

lead to: (i) treatment failure, (ii) adverse patient outcomes, (iii) perpetuation of the increase in antimicrobial resistance and (iv) a financial burden to society (Micek *et al*., 2010; Gudiol *et al.*, 2011). The colonisation pattern in a patient after admission into hospital is largely influenced by the local antibiotic policy with increases in colonisation observed after 2 weeks, especially after treatment with broad spectrum antibiotics, which lead to higher attack rates by nosocomial *K. pneumoniae* (Podschum and Ullmann, 1998). The risk factors for colonisation with ESBL-producing *Enterobacteriaceae* have been extensively reported but varied according to populations investigated, case studies, controls and sample sizes (Ghafourian *et al.*, 2014). Generalised factors in at-risk patients commonly include severe illness, underlying medical conditions, recent surgery, haemodialysis, multiple or excessive antibiotic use, the use of medical devices, such as lines and tubes, prolonged hospitalisation, ICU admittance, admittance at long-term health facilities or nursing homes, and international travel to endemic areas (Ghafourian *et al.*, 2014).

The clinical manifestation of disease can be attributed to numerous host dependent factors, which may range geographically but it is also influenced by socioeconomic determinants and the quality of healthcare at hand (Lautenbach *et al*., 2001; Ko *et al.*, 2002; Yu *et al*., 2007; Bamford *et al*., 2011). Underlying complications or illness that may result in an increased risk of *K. pneumoniae* infection include malignancy, cirrhosis, biliary tract disorders, diabetes mellitus and alcoholism (Tsai *et al.*, 2010). Predisposing factors for blood stream infections (BSI) can include antineoplastic surgery, corticosteroids use, drainage, intravascular catheter use, intubation, prior surgery, prior antibiotic administration and urine catheter use (Endimiani *et al*., 2004; Kuster *et al*., 2010; Gudiol *et al.*, 2011). In a retrospective study conducted by Tsai *et al.* (2010), a higher rate of patients with healthcare-associated bacteraemia were infected with ESBL-producing *K. pneumoniae* as compared to patients with community-associated bacteraemia. The mortality rate was also found to be twice as high in patients with healthcare-associated bacteraemia (Tsai *et al.*, 2010). Urine catheters are also commonly associated with acquisition of multidrug-resistant *K. pneumoniae* due to the frequent isolation of these resistant strains in the bladder (Perez *et al*., 2010). Furthermore, multidrug-resistant infections requiring treatment with colistin, can be associated with risk factors, such as ventilator-associated pneumonia episodes or prior treatment with carbapenems for a prolonged period of time (Giamarellou, 2010).

Finally, an important risk factor that should be taken into consideration in modern society is the risk of acquiring ESBL-producing *K. pneumoniae* or *E. coli* when travelling to high-risk countries (Van der Bij and Pitout, 2012). The risk of infection with ESBL-producing *E. coli* and *K. pneumoniae* is particularly higher in the former scenario if antibiotics were consumed during travel, often for traveller's diarrhoea (Kuster *et al*,, 2010; Van der Bij and Pitout, 2012). During the travels, acquisition can occur in the absence of healthcare contact or along with leisure and medical tourism (Rogers *et al.*, 2011; Van der Bij and Pitout, 2012). The statement bears weight particularly when travel is directed to endemic areas, such as to Asian countries or Greece, Turkey and the United States of America (USA), which have ESBLs and carbapenemases (KPC, VIM, OXA-48 and NDM) (Van der Bij and Pitout, 2012).

2.11 Spread, prevention and control

The rise in antimicrobial-resistance among bacteria, such as those described as 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp.), has highlighted the need for new antibiotics due to the 'escape' from currently marketed antimicrobial drugs (Boucher *et al*., 2009). The impact of infections with ESBL-producing bacteria can include increased mortality rates, particularly in blood stream infections (BSI), as well as increase in length of hospitalisation and increased hospital costs (Giske *et al*., 2008). Principle reservoirs typically implicated in healthcare-associated outbreaks or spread includes the patients, the healthcare staff and the environment (such as sink drains) (Tofteland *et al*., 2013).

The faecal-oral route of transmission in cases of direct or indirect contact by healthcare workers is exacerbated in overcrowded conditions (Ghafourian *et al.*, 2014). The contact that staff have with patients during unassuming social interactions, such as taking a patient's blood pressure and the touching of inanimate objects in the patient's environment, could contribute to horizontal spread of pathogens, especially when elective hand hygiene practices are neglected (Allegranzi and Pittet, 2009; Giamarellou, 2010). The implementation of alcohol-based hand rubs and regular educational programmes are thus important steps in control measures undertaken (Allegranzi and Pittet, 2009). The role of post-acute care facilities in dissemination of multidrug-resistant strains is also stressed by Perez *et al*. (2010).

The gastrointestinal tract is often a reservoir and could serve as a source for infection (Schroll *et al*., 2010; Oostdijk *et al*., 2011). To reduce carriage and thus the rate of infection by at-risk hospitalised patients, there needs to be effective antimicrobial treatment, good hand hygiene, avoidance of unnecessary invasive procedures, such as central venous catheterisation, surveillance in the clinical setting, appropriate infection control policies and strict antimicrobial policies (Giamarellou, 2010, Rawat and Nair, 2010; Ghafourian *et al.*, 2014). Infection control measures undertaken can include: (i) increased barrier precautions, (ii) isolation of infected patients, (iii) appropriate antibiotic treatment duration and (iv) epidemiological standards for the handling of equipment as well as patient wounds (Podschun and Ullmann; 1998; Giamarellou, 2010, Rawat and Nair, 2010). A method thus investigated for its potential to reduce cross-contamination and infection rates in clinical settings, such as the ICU, is the effect of selective digestive tract decontamination (SDD) for the elimination of cephalosporin-resistant *Enterobacteriaceae* (De Smet *et al*., 2009; De Smet *et al*., 2011; Oostdijk *et al*., 2011; Oostdijk *et al*., 2012). The SDD approach has been associated with successful reduction of multidrug-resistant *Enterobacteriaceae* acquisition, lowering rates of bacteraemia in the ICU and even successful outbreak control of multidrug-resistant *Enterobacteriaceae* in the intestinal tract in smaller studies (De Smet *et al*., 2009; De Smet *et al*., 2011; Oostdijk *et al*., 2011; Oostdijk *et al*., 2012). No increased resistance was observed in cases of eradication failure in the former studies (Oostdijk *et al*., 2012). In contrast, a separate study, reporting on the emergence of OXA-48 and OXA-181 in South Africa, recorded the emergence of colistin-resistant OXA-181-producing *K. pneumoniae* after SDD with colistin (Brink *et al.*, 2013).

Several key shortcomings have been identified by the World Health Organization (WHO) in the combat against antimicrobial resistance (Leung *et al*., 2011). The issues are discussed under four topics which include: (i) lack of commitment and data; (ii) unconfirmed drug quality and irrational use; (iii) poor prevention and control of infections and (iv) languishing research into new antimicrobial agents (Leung *et al*., 2011). The resulting policy package recommended by the WHO thus initially suggests that governments adopt and finance comprehensive national plans with accountability and engaging civil society by creating public awareness (Leung *et al*., 2011). The second recommendation is based on improving surveillance and laboratory capacities, whilst the third advises local governments to guarantee an uninterrupted supply of essential, quality-assured medication (Leung *et al*., 2011). The regulation and promotion of the correct use of former-mentioned medication is also

emphasised along with good patient care (Leung *et al*., 2011). Finally, the last two recommendations involve improvement of infection prevention and control while encouraging research and development of new tools, including diagnostic tests and antimicrobials (Leung *et al*., 2011).

2.12 Laboratory diagnosis of ESBL-producing *K. pneumoniae* isolates

In light of increasing antibiotic resistance among bacteria, surveillance of drug resistance patterns within clinical settings and clinically relevant pathogens is significant particularly when deciding on appropriate treatment for complicated infections (Bamford *et al*., 2011). The detection of ESBL-producing bacteria require tests that can accurately discern between ESBL producers and bacteria possessing alternative resistance mechanisms, such as inhibitor-resistant-β-lactamases, cephalosporinase overproduction and SHV-1 hyperproduction (Drieux *et al*., 2008).

2.12.1 Biochemical and phenotypic detection techniques

Characteristics associated with ESBL-producing *Enterobacteriaceae* include the synergy observable between the antibiotics amino- and carboxy-penicillins, second-generation cephalosporins and up to several third- and fourth–generation cephalosporins when combined with β-lactamase inhibitors, such as clavulanate (Drieux *et al*., 2008; Rawat and Nair, 2010). *Klebsiella pneumoniae* can encode all three ESBL-encoding genes whilst simultaneously encoding carbapenemases (Cabral *et al*., 2012). The characteristics associated with KPC and MBL carbapenemase production differ to ESBLs in that the KPC enzyme is capable of hydrolysing all β-lactams, whereas the MBL enzymes are capable of hydrolysing all β-lactams but not aztreonam (Nordmann and Poirel, 2013). The former KPC β-lactamase is partially inhibited by inhibitors, such as boronic acid, clavulanic acid and tazobactam, whereas the latter MBL enzymes are inhibited by Ethylene diamine tetra-acetate (EDTA) (Nordmann *et al*., 2012b; Nordmann and Poirel, 2013). Detection of these multidrug-resistant *K. pneumoniae* can be manually screened for utilising several techniques, which include culturing on chormogenic agar (such as ChromID® ESBL agar medium) (bioMérieux, France), Etest MBL (AB BioDisk Company, USA), MicroScan panels (MicroScan, USA), modified Hodge test, disk diffusion techniques on Mueller-Hinton agar and enriched medium

(such as tryptic-soy broth containing 2 mg.L^{-1} cefpodoxime) (Bauer *et al*., 1966; Kitchel *et al*., 2009; Elhani *et al*., 2010; Rawat and Nair, 2010; Bamford *et al*., 2011; Nordmann *et al*., 2011; Poirel *et al*., 2011b; Tofteland *et al*., 2013). Phenotypic techniques are often reliant on observable results, such as with the double disk synergy test (DDST), ESBL Etests (bioMérieux, France) and the combination disk method (Drieux *et al*., 2008).

Initially, the DDST following methodology specified by the Clinical and Laboratory Standards Institute (CLSI) guidelines was intended for the differentiation between ESBL-producing *Enterobacteriaceae* strains and strains overproducing cephalosporinase, but the combination of cefotaxime or ceftazidime with clavulanic acid can also be predictive of a CTX-M-producer, particularly in *E. coli* (Lewis *et al*., 2007; Drieux *et al*., 2008). The test makes use of a 30 µg disk of e.g. cefotaxime and a disk of amoxicillin-clavulanate (10 µg clavulanate) approximately 30 mm apart, or at 20 mm for greater sensitivity (Drieux *et al*., 2008; Rawat and Nair, 2010). The resistance breakpoints towards all third- and fourth-generation cephalosporins are not always apparent, regardless of whether disk diffusions in agar or automated systems are used (Drieux *et al*., 2008). False-negative results can occur when testing isolates encoding SHV-2, SHV-3 or TEM-12 (Rawat and Nair, 2010). Alternatively, the ESBL Etests are capable of quantifying synergy with one end of the strip containing gradients of cefotaxime, or ceftazidime, or cefepime and the other end a combination of the same former-mentioned antibiotic with 4 mg.L^{-1} clavulanate (Drieux *et al*., 2008). A limitation may include failure to detect ESBLs when ranges fall outside MIC ranges on the strip or misinterpretation of the inhibition ellipse (Carter *et al*., 2000; Leverstein-van Hall *et al*., 2002; Drieux *et al*., 2008). Another phenotypic testing method that can be utilised is based on broth microdilution assays, which includes the commercially available MicroScan panels (Dade Behring MicroScan, Sacramento, USA) that make use of dehydrated panels for microdilution antibiotic susceptibility (Rawat and Nair, 2010).

Cloxacillin has been added to agar media for the inactivation of cephalosporinases, an AmpC β-lactamase, whereas both clavulanate and EDTA have been added when MBLs are produced concurrently with ESBLs for the latter's identification and confirmation (Drieux *et al*., 2008). The detection of extended spectrum Ambler class D OXAs is on the other hand complicated due to weak inhibition and no inhibition observed towards clavulanate and EDTA, respectively (Naas and Nordmann, 1999; Drieux *et al*., 2008). A unique characteristic

attributed to most class D β-lactamases, including OXA-48-type enzymes, is the inhibition of activity by sodium chloride (NaCl) *in vitro* at a concentration of 100 mM (Poirel *et al.*, 2010).

Carbapenemases can on the otherhand also be screened for in at-risk patients using selective media, such as CHROMagar KPC medium (CHROMagar Ltd, France), Brilliance^{TM} CRE medium (Thermoscientific, UK) and SUPERCARBA medium (Nordmann and Poirel, 2013). Typically, methods of detecting carbapenemases make use of inhibition tests utilising boronic acid, clavulanic acid, EDTA and tazobactam (Carvalhaes *et al*., 2010; Nordmann and Poirel, 2013). Carbapenemase resistance in *Enterobacteriaceae* can be confirmed phenotypically using the modified Hodge test (MHT) according to CLSI guidelines, although several limitations have been recorded (Carvalhaes *et al*., 2010). Limitations include variable sensitivity and specificity recorded in the detection of carbapenemases other than KPC (>90% respectively) and the occurrence of false positive MHTs in the absence of carbapenemase production due to reduced susceptibility or resistance to carbapenems [Centers for disease control and prevention (CDC), 2008; Carvalhaes *et al*., 2010; CLSI, 2014]. The latter limitation could be as a result of isolates expressing alternative mechanisms of carbapenem resistance, such as ESBL production coupled with loss of porin proteins (Carvalhaes *et al*., 2010; CLSI, 2014). The MHT test demonstrated good sensitivity in the detection of OXA-48-producers (Poirel *et al.*, 2012). Inhibition-based carbapenemase detection is limited due to variable specificity and sensitivity (Nordmann and Poirel, 2013).

2.12.2 Automated detection of ESBLs

Automated systems used for the detection of ESBLs are the VITEK®2 ESBL test (bioMérieux, France) and the Phoenix ESBL test (Becton Dickinson, USA), both of which monitor the bacterial growth response to expanded-spectrum cephalosporins (Drieux *et al*., 2008; Rawat and Nair, 2010). The VITEK®2 ESBL test (bioMérieux, France) consists of cards with wells, whereas the automated Phoenix ESBL test (Becton Dickinson Biosciences, USA) consists of five wells containing a cephalosporin with or without clavulanic acid (Drieux *et al*., 2008). Another method that could be used for the detection of β-lactamase and carbapenemase activity is the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), which analyse carbapenem molecule hydrolysis, although its efficiency in detecting OXA-48 producers remains uncertain (Burckhardt and Zimmermann, 2011; Hrabák *et al*., 2011; Poirel *et al.*, 2012; Nordmann and Poirel, 2013).

2.12.3 Newer detection methods

Molecular investigations of outbreaks can be complicated when spurred by the spread of highly mobile plasmids (Tofteland *et al*., 2013). Antimicrobial resistance genes are often carried on varied plasmids, which have been implicated in multidrug-resistant Gram-negative bacteria outbreaks, as illustrated in a study by Tofteland *et al*. (2013), wherein the bla_{KPC} encoding plasmid was transmitted among varied strains and even species. Non-phenotypic tests, including molecular techniques, that are available for antibiotic gene detection and typing include: polymerase chain reaction (PCR) assays, Real-time PCR assays, Next-generation sequencing (NGS) methodologies, Microarrays, MALDI-TOF MS and PCR/Electrospray Ionization Mass Spectrometry (PCR/ESI MS) (Dallenne *et al.*, 2010; Voets *et al.*, 2011; Poirel *et al.*, 2012; Lupo *et al.*, 2013; Nordmann and Poirel, 2013; Veenemans *et al*., 2014). Molecular techniques, particularly PCR are the standard for detecting genes encoding ESBL, OXA-48-Like, VIM, KPC and NDM enzymes (Avlami *et al.*, 2010; Poirel *et al.*, 2011c, Poirel *et al.*, 2012). The detection of carbapenemases also includes the novel biochemical Carba NP test and a UV spectrophotometer-based technique (Bernabeu *et al*., 2012; Nordmann *et al*., 2012a; Nordmann and Poirel, 2013). The UV spectrophotometer-based method relies on the analysis of imipenem hydrolysis by extracted proteins from the isolate tested and demonstrates less variability in sensitivity (100%) and specificity (98.5%) as compared to inhibition-based methods (Bernabeu *et al*., 2012; Nordmann *et al*., 2012a; Nordmann and Poirel, 2013). Imipenem has also been used to detect carbapenemases using novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays (Kulkarni *et al*., 2014).

2.13 Typing of *K. pneumoniae* isolates

Genetic typing of *K. pneumoniae* isolates is important for outbreak investigations, investigating sources or reservoirs, understanding transmission, managing hospital infections and for epidemiological referencing (Johnson *et al*., 2007; Vimont *et al*., 2008; Berrazeg *et al*., 2013). Several typing methods exist for the characterisation of *K. pneumoniae* isolates, which can be subdivided into phenotypic and molecular methods but the appropriate method used is dependent on the question that needs answering (Diancourt *et al*., 2005; Woodford *et al*., 2011). Originally, typing methods for *K. pneumoniae* included phenotypic typing methods, such as Biotyping, Serotyping, Phage typing and Bacteriocin typing (Podschun and

Ullmann, 1998; Brisse and Verhoef, 2001; Brisse *et al*., 2004; Aanensen and Spratt, 2005; Diancourt *et al*., 2005; Johnson *et al*., 2007). The most popular serotyping method implemented in the past that gave the most reproducible results was capsule typing (Podschun and Ullmann, 1998). The technique was not; however, without its shortcomings as considerable serological cross-reactions could occur between the 77 capsule types (Podschun and Ullmann, 1998). Methods developed since then include molecular typing methods, such as amplified fragment length polymorphisms (AFLP), MALDI-TOF MS, MLST, multilocus variable-number tandem-repeat analysis (MLVA), NGS, PFGE, plasmid profiling, sodium dodecyl sulphate polyacryamide gel electrophoresis (SDS-PAGE), restriction fragment length polymorphism (RFLP), ribotyping and random PCR methods (Brisse and Verhoef, 2001; Brisse *et al*., 2004; Aanensen and Spratt, 2005; Diancourt *et al*., 2005; Brisse *et al*., 2006; Johnson *et al*., 2007; Woodford *et al*., 2011; Berrazeg *et al*., 2013; Arena *et al*., 2014). Random PCR methods include random amplified polymorphic DNA (RAPD) and repeat-based PCR (rep-PCR) (Brisse *et al*., 2006).

The PFGE molecular method is highly discriminatory and the gold standard typing method in the characterisation of *K. pneumoniae* isolates (Hansen *et al*., 2002; Johnson *et al*., 2007; Vimont *et al*., 2008). Pulsed-field gel electrophoresis discrimination is based on genomic DNA restriction utilising a rare-cutting restriction enzyme, such as *Xba*I for *K. pneumoniae* (Durmaz *et al*., 2009; Long *et al*., 2010; Woodford *et al*., 2011). The disadvantage of PFGE lies in that intra-laboratory reproducibility of results requires substantial managing and it is technically demanding (Vimont *et al*., 2008).

Multilocus sequence typing on the other hand is a useful technique utilised for determining the clonal relatedness between *K. pneumoniae* isolates and provides unambiguous, portable data (Aanensen and Spratt, 2005; Diancourt *et al*., 2005; Johnson *et al*., 2007). The MLST and MLVA methods are both described numerically and much like MLST, the MLVA data is portable (Woodford *et al*., 2011). In MLST the internal segments of seven housekeeping genes in *K. pneumoniae* are amplified and the variations in each sequence described as unique alleles, which will comprise the allelic profile of the isolate, otherwise known as a sequence type (ST) (Aanensen and Spratt, 2005; Woodford *et al*., 2011). The disadvantage lies in that the discrimination may not be defining enough for outbreak analysis but it is useful to compare to global epidemiology (Diancourt *et al*., 2005; Woodford *et al*., 2011). The MLVA, on the other hand, determines the number of repeat units at multiple loci and can be modified

to the desired resolution depending on the loci chosen, thus allowing for a higher resolution than PFGE (Woodford *et al*., 2011).

2.14 Commonly characterised *K. pneumoniae* strains

Sequence typing has allowed for the characterisation of *K. pneumoniae* strains and lead to the recognition of widespread multidrug-resistant clones (Woodford *et al*., 2011; Shon *et al*., 2013). Although a vast number of sequence types have been recorded globally, which can be accessed on public databases (such as www.pasteur.fr/mlst and http://pubmlst.org), a few important STs are frequently reported and discussed. Typing has elucidated widespread multidrug-resistant clones, such as *K. pneumoniae* ST 258, which is infamous for encoding KPC carbapenemases and the virulent *K. pneumoniae* clonal complex (CC) 23 (including ST23 and ST57) (Baraniak *et al*., 2009; Samuelsen *et al*., 2009; Woodford *et al*., 2011, Merlet *et al*., 2012; Chmelnitsky *et al*., 2013; Tofteland *et al*., 2013). Besides the "classical" *K. pneumoniae* strain STs, a few STs associated with hvKP strains include ST23 and ST57, which are associated with the K1 capsular serotype, as well as the ST86, ST375 and ST380, which are associated with the K2 capsular serotype (Merlet *et al*., 2012; Shon *et al*., 2013). It has been suggested that particular clones acquire resistance genes easily and may have evolutionarily changed similar genes acquired so as to maintain or improve bacterial fitness (Deschamps *et al*., 2009).

2.15 Summary

Enterobacteriaceae in the clinical setting have adapted to a harsh environment created by the use of antibiotics through several mechanisms, which include the expression of β-lactamases capable of hydrolysing penicillins as well as other β-lactam antimicrobials (Drancourt *et al*., 2001; Bush, 2010; Kocsis and Szabó, 2013). The β-lactamases commonly implicated in a range of serious infections by *K. pneumoniae* include cephalosporinases (particularly ESBLs) and carbapenemases (Kocsis and Szabó, 2013). Extended spectrum-producing *K. pneumoniae* forms part of the ESBL-producing *Enterobacteriaceae*, which is collectively listed as one of six dangerous pathogens by the Infectious Disease Society of America together with *A. baumannii*, *P. aeruginosa*, vancomycin resistant *Enterococcus faecium*, methicillin–resistant *Staphylococcus aureus* and *Aspergillus* species. Other mechanisms of

resistance and co-expression of several β-lactamases could work in concert to further extend the range of antimicrobial resistance by *K. pneumoniae*, often spurred on by excessive antimicrobial use in the clinical setting (Fernández *et al*., 2011; Kumar *et al.*, 2011; Poole, 2012a). The consequences of the broadening resistance among Gram-negative bacilli, particularly towards the commonly implemented carbapenem antimicrobials, are often increased mortality rates and hospital costs thus giving importance to tests with the capacity to discern between ESBLs, carbapenemases and other mechanisms of resistance being expressed (Drieux *et al*., 2008; Gasink *et al*., 2009; Micek *et al*., 2010). The typing of bacterial isolates is also a paramount step in determining infection sources and possible dissemination routes (Johnson *et al*., 2007; Vimont *et al*., 2008; Berrazeg *et al*., 2013).

The extensive and accurate knowledge of pathogens within local settings, as well as their susceptibility patterns can aid in determining or updating appropriate empirical treatment so as to prevent the higher odds of patient death associated with inadequate initial antimicrobial treatment (DTB, 2008). The regular monitoring of current resistance trends in regular bacterial contenders, such as ESBL-producing *K. pneumoniae*, in the South African clinical setting by molecular detection of β-lactamase encoding genes may compliment phenotypic susceptibility profiles *in vitro*. The molecular data along with typing of *K. pneumoniae* isolates can thus proffer invaluable information comparable on an international basis thus allowing trends in extending resistance to be inferred from international studies or to allow pre-emptive treatment regimes to be determined. The aim of the study was thus to determine the presence and prevalence of β-lactamase-encoding genes in ESBL-producing *Klebsiella pneumoniae* isolates as well as their genetic characterisation.

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CHAPTER 3

HIGH PREVALENCE OF THE OXA-48-LIKE GENE AMONG ESBL-PRODUCING *KLEBSIELLA PNEUMONIAE* CLINICAL ISOLATES WITH *IN VITRO* SUSCEPTIBILITY TO CARBAPENEMS

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3.1 SYNOPSIS

Klebsiella pneumoniae is a member of the *Enterobacteriaceae* family and an important nosocomial pathogen implicated in community and healthcare-associated infections responsible for high in-hospital mortality rates. The Gram-negative bacilli easily acquire multidrug-resistance profiles, such as those associated with extended-spectrum β-lactamase (ESBL)-producers and emerging carbapenem non-susceptible strains, which have effectively reduced treatment options available. High rates of ESBLs and several carbapenemases have been detected in South Africa. The detection of OXA-48-Like carbapenemases is phenotypically challenging, thus emphasising the development and use of molecular methods. The aim of the study was to identify β-lactamase genes of the molecular Ambler classes A, B and D in 100 ESBL-producing *K. pneumoniae* isolates utilising simplex and multiplex polymerase chain reaction (PCR) assays. Multiple β-lactamase genes were identified with up to five β-lactamase genes being encoded simultaneously, namely SHV, CTX-M, TEM, OXA-1-Like and OXA-48-Like enzymes. Sequencing of β-lactamase genes in four carbapenem susceptible and resistant isolates presumptively identified the OXA-48-Like genes as OXA-181 with no additional carbapenemases detected. The study reported the high prevalence and combined presence of multiple ESBL encoding genes, as well as the high frequency of OXA-1-Like and OXA-48-Like genes within ESBL-producers. The study highlights the detection of OXA-48 variants in both carbapenem susceptible and resistant isolates. Future research should encompass the detection of resistance mechanisms simultaneously expressed in ESBL encoding *K. pneumoniae* isolates with elevated carbapenem minimum inhibitory concentrations.

Key words: *Klebsiella pneumoniae*, β-lactamases, extended-spectrum β-lactamases, polymerase chain reaction, multidrug-resistance

3.2 INTRODUCTION

Klebsiella pneumoniae is an infamous Gram-negative bacillus of the *Enterobacteriaceae* family, which is implicated in mainly nosocomial infections responsible for increased in-hospital mortality rates, particularly among debilitated patients, such as intensive care unit (ICU) patients.[1,2] The infections may be distinguished as either community-associated or healthcare-associated and range from urinary, respiratory, blood stream and intra-abdominal infections.[1,3-7] The use of antibiotics to treat *Enterobacteriaceae* infections have gradually selected for resistant strains and may even have promoted the transfer of mobile genetic elements.[8] The rise in multidrug-resistant (MDR) strains implicated in infection may be partly due to the pressure caused by antimicrobial treatment eliciting protective responses, which may include acquisition of resistance determinants or resistance-conferring mutations, target site alterations and activation of biofilm formation.[9-14] The expression of β-lactamases, encoded on the chromosome and plasmids, confers resistance to β-lactam antibiotics commonly used in the clinical setting and can be classified into Ambler molecular classes A to D based on the proteins' amino acid sequences.[15]

The rise of MDR *K. pneumoniae* and *Escherichia coli* in the 1980s and 1990s expressing extended-spectrum β-lactamases (ESBLs) led to the administration of carbapenems in at-risk patients to avoid poor clinical outcomes.[16,17] The ESBLs may be encoded on plasmids carrying additional genes conferring MDR profiles, which include resistance towards aminoglycosides, tetracycline, quinolones, trimethoprim and sulphonamides.[18,19] The last decade has; however, seen a rise in the number of carbapenem-resistant *Enterobacteriaceae.*[17] Carbapenem resistance in clinical isolates can be mediated by several mechanisms, namely (i) expression of carbapenemases, (ii) penicillin binding protein alteration, (iii) augmented efflux activity and (iv) reduced permeability, particularly due to the loss of outer membrane proteins in isolates expressing an ESBL enzyme or over-expressing AmpC.[17,20] Carbapenemases can be simultaneously expressed with other β-lactamases and resistance genes, such as the NDM-1 which can be carried on a plasmid also encoding the *muc*AB genes implicated in ultraviolet (UV) resistance.[21]

The emergence and spread of carbapenem resistance present a public health threat in the form of severe infections with poor treatment outcomes, thus spurring research into carbapenem

resistance mechanisms implicated in *Enterobacteriaceae*, including within ESBL-producing *K. pneumoniae.*[20,22] Resistance in *Enterobacteriaceae* towards carbapenems is predominantly mediated by carbapenemase enzymes, which include the class A *Klebsiella pneumoniae* carbapenemase (KPC) and specific GES enzyme variants (e.g. GES-2, -4, -5 and -6); class B IMP, VIM and NDM-1 enzyme types; as well as class D OXA-48-Like enzymes.[23-27] The latter-mentioned OXA-48 and variant enzymes, such as OXA-181 and OXA-232, exhibit weaker but significant carbapenemase activity, which is elevated in *Enterobacteriaceae* with permeability defects.[7,28,29] The problematic detection of these OXA-48 β-lactamases variants is often due to only a slight decrease in carbapenem susceptibility that may be missed and the subsequent undetermined clinical implications of treatment of these carbapenemase-producers and the possibility of mutant selection resulting in elevated resistance, thus earning it the name "the phantom menace".[28]

The detection of carbapenemases in at-risk patients could require initial screening for which selective media exists, such as CHROMagar KPC agar (CHROMagar Ltd, France), Brilliance^{TM} CRE agar (Thermo scientific, UK) and SUPERCARBA medium for stool or rectal swabs.[30] The Modified Hodge test (MHT) could still be used as a confirmatory phenotypic test for suspected carbapenemase-expressing *Enterobacteriaceae* but notable limitations recorded include variable sensitivity and specificity when detecting carbapenemases other than KPC (>90% respectively), such as NDM carbapenemases (11%).[31,32] There are also instances of negative MHT results for some carbapenemase-producing *Enterobacteriaceae* and positive MHT results for isolates expressing alternative mechanisms of carbapenem resistance.[32] Novel methods for the detection of carbapenemases include the biochemical Carba NP test and a UV spectrophotometer-based technique, the latter of which has exhibited 100% sensitivity and 98.5% specificity unlike the variability observed for the inhibition-based methods.[30,33,34] Other recent non-phenotypic tests that have been developed for detection and characterisation of antibiotic resistance genes include: polymerase chain reaction (PCR) assays, Real-time PCR assays, Next-generation sequencing (NGS) methodologies, Microarrays, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy (MALDI-TOF MS) and PCR/Electrospray Ionization Mass Spectrometry (PCR/ESI MS).[30,35]

The rapid spread of resistance genes and determinants necessitates efficient and accurate detection methods, which may serve to guide antibiotic regimes implemented and to prevent

outbreaks.[30] The difficulty in detection of isolates encoding OXA-48 β-lactamases and relevant variants, which can exhibit variable resistance profiles due to additional resistance determinants, has emphasised the need for adequate screening and detection methods.[22,28] The OXA-48-Like producing isolates may additionally encode ESBLs.[28] Polymerase chain reaction assays are commonly utilised for the detection and confirmation of OXA-48-Like, VIM, KPC and NDM encoding genes.[28,36-38] The aim of the study was to: (i) detect β-lactam resistance genes within ESBL-producing *K. pneumoniae* clinical isolates and (ii) to determine the prevalence of the OXA-48-Like encoding genes.

3.3 MATERIALS AND METHODS

3.3.1 Study setting

A quantitative descriptive study was conducted on 100 ESBL-producing *K. pneumoniae* clinical isolates at the Department of Medical Microbiology, University of Pretoria/National Health Laboratory Service (NHLS). Ethical approval was granted for this study by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Protocol number S38/2012). A total of 162 *K. pneumoniae* isolates were collected from mid-May until August 2012 from which 100 consecutive, unrepeated isolates were selected for analysis and the remainder excluded due to repeats encountered from the same patients. These isolates were recovered from a variety of specimens sent for investigation at the NHLS, Tshwane Academic Division, a diagnostic laboratory servicing a tertiary academic hospital, three regional hospitals and 64 clinics and confirmed as ESBL-producers using the VITEK®2 system (bioMérieux, France). The VITEK®2 system (bioMérieux, France) along with VITEK®2 GN ID Cards (bioMérieux, France) was used for bacterial identification and the VITEK®2 AST-N133 Cards (bioMérieux, France) for susceptibility testing. The antibiotic susceptibility profiles were interpreted as susceptible, intermediate or resistant according to the 2010 CLSI guidelines.[39] The *K. pneumoniae* isolates were sub-cultured from the original culture plates onto blood agar plates and incubated (Hybridiser HB-1D, Techne Ltd, England) at 37°C (HF 212 UV, Shangai Lishen Scientific, China) for 18 to 24 h for subsequent storage and DNA extraction.

3.3.2 Genomic DNA and plasmid DNA extraction

The ZR Fungal/Bacterial DNA Miniprep kit [Zymo Research, Fermentas, United states of America (USA)] was used for total DNA extraction from each of the 100 *K. pneumoniae* clinical isolates. Plasmid DNA was extracted utilising the Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research, Fermentas, USA) from 15 *K. pneumoniae* clinical isolates, a small sample chosen to represent different combinations of β-lactamase genes encoded in individual isolates. Both extraction procedures were performed according to the manufacturer's instructions (Appendix B). The DNA was stored at 4^{o}C during short term storage or while in use and stored at -20^{o}C (Defy, South Africa) for long term storage for retesting when required.

3.3.3 Detection of β-lactamase genes in ESBL-producing *K. pneumoniae* isolates

Conventional multiplex- and simplex PCR assays were performed for the detection of β-lactamase genes utilising published primer sequences (Table 3.1).[37, 40-43] The PCR assays' reaction mixture was adjusted to a final volume of 25 µl, which was comprised of 12.5 µl QIAGEN® mastermix (QIAGEN® M-PCR Kit, Qiagen, Germany), 2.5 µl of primer mix of variable concentrations (Table 3.1) of the forward and reverse primer, 5 µl Q-solution, 3 µl template DNA and nuclease-free water (Qiagen, Germany) to make up the remainder of the volume. The PCR assays were performed on a G-storm thermocycler (Vacutec, UK) according to the optimal conditions specified by the QIAGEN Multiplex PCR manual (2008). The programme used was as follows: initial denaturation at 95^{o}C for 15 min, 35 cycles of denaturation at 95^{o}C for 30 sec, annealing at the specified temperature for each M-PCR assay (Table 3.1) for 1.5 min, extension at 72^{o}C for 1.5 min and a final extension at 72^{o}C for 10 min. The positive controls used included four ATCC cultures, namely *K. pneumoniae* ATCC 8303, *K. pneumoniae* ATCC BAA 1705, *K. pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 35218 as well as local clinical isolates positive for CTX-M, IMP, KPC, OXA-1-Like, OXA-48-Like, SHV, TEM, NDM-1 and VIM genes. A negative control was used to rule out template contamination by adding nuclease-free water instead of the 3 µl template DNA.

The amplified amplicons were analysed by comparison to a 100 bp molecular size marker ladder (Fermentas, Thermo Scientific, USA) after gel electrophoresis (Elite 300 power pack,

Wealtec, South Africa) at 90 V for 1.5 h on a 1% (m/v) MetaPhor™ agarose gel (Lonza, Rockland, USA) containing 5 µl of ethidium bromide (10 mg.ml^{-1}) (Promega, Madison, USA). The bands on the gel were subsequently visualised and digitally captured on an Ultra Violet light box (DigiDoc-It®, UVP product, USA).

3.3.4 Sequencing

Polymerase chain reaction (PCR) assays were used to amplify the β-lactamases in three *K. pneumoniae* isolates with elevated carbapenem MICs, as determined by the VITEK®2 system (bioMérieux, France) (Appendix C). The amplicons were subsequently sequenced in both directions for data analysis using the CLC Main workbench version 6.0 (CLCBio, Denmark) and by comparison to sequences in GenBank using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Due to the small size of the amplicons, gene identification was only presumptive for ESBL and carbapenemase genes targeted.

3.4 RESULTS

The specimens originated from male and female patients (age range: three days to 83 years old), of which 12% were infants (< 2 years) and 24% were elderly (≥ 60 years). The specimens from which *K. pneumoniae* was originally isolated were diverse, including urine (33%), pus swabs (12%), sputum (6%), tracheal aspirates (7%), blood cultures (22%), urine cliplocks (1%), lymph node (1%), tissue (6%), fluid collections (2%), CVP tips (8%) and urine catheter tips (2%).

All the *K. pneumoniae* isolates were phenotypically classified as ESBL-producers by the VITEK®2 system (bioMérieux, France), with additional resistance to aminoglycosides (24%), exhibiting impermeability (to cephamycin) (4%), CTX-M-Like phenotypes (3%) and a single isolate (1%) as ertapenem resistant. The VITEK®2 system (bioMérieux, France) identified variable resistance profiles towards 20 different antibiotics for the clinical isolates. All 100 *K. pneumoniae* isolates were interpreted as resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime and cefuroxime axetil, while displaying variable profiles ranging from resistant, intermediate and susceptible for the remainder of the isolates as

demonstrated in Figure 3.1. Ninety-two isolates (92%) displayed resistance towards at least one antibiotic from more than two classes of antimicrobials.

During molecular detection, the M–PCR and simplex PCR assays detected all three ESBL associated genes, namely SHV (100%), CTX-M (93%) and TEM (81%), as well as additional β-lactamase encoding genes, particularly OXA-1-Like (95%) and OXA-48-Like (94%) genes (Figure 3.2; Figure 3.3). All five β-lactamases were found to be highly prevalent, with 70% of the isolates encoding all five genes, 23% encoding four genes and 7% encoding three genes (Figure 3.4). The most common combinations of β-lactamase genes detected simultaneously are illustrated in Figure 3.4. The 15 representative isolates' plasmid DNA extracted using the plasmid Zyppy^{TM} Plasmid Miniprep Kit (Zymo Research, Fermentas, USA) out of the total 100 isolates analysed had fewer β-lactamases when retested. The 15 isolates representing different combinations of β-lactamases differed as follows: (i) No OXA-48-Like genes were detected in any of the isolates, (ii) OXA-1-Like was no longer detected in two isolates (isolate 15 and 87), (iii) SHV was no longer detected in one isolate (isolate 20), and (iv) CTX-M was no longer detected in one isolate (isolate 71).

No KPC, IMI, IMP, NDM, VEB or VIM carbapenemases were detected. Sequencing of the β-lactamase genes within three isolates (isolates 56, 71 and 86) exhibiting elevated ertapenem MICs and ertapenem resistance presumptively identified the genes as OXA-181, OXA-1, CTX-M-15, TEM-1, SHV-12, SHV-1/28 and SHV-11. Isolates 56 and 71 had varying susceptibility profiles with resistance towards 12 (12/20) (isolate 56) and 16 (16/20) antibiotics out of a panel of 20 antibiotics while isolate 86 displayed resistance towards only six (6/20) antibiotics.

3.5 DISCUSSION

The rapid global spread of carbapenem-resistant *Enterobacteriaceae,* including *K. pneumoniae*, has greatly impacted treatment options; with only "last-resort" drugs, such as tigecycline, colistin, and/or fosfomycin still effective.[44] Resistance may; however, quickly evolve towards the former-mentioned drugs when implemented, as evidenced by the emergence of colistin-resistant OXA-181-producing *K. pneumoniae in vitro* in South Africa after selective decontamination of the gastrointestinal tract.[44-47] Extended-spectrum

β-lactamase profiles in multidrug-resistant *K. pneumoniae* isolates may differ from country to country and is largely underreported in Africa.[48] This study identified β-lactamase genes of the molecular Ambler classes A, B and D in clinical ESBL-producing *K. pneumoniae* isolates utilising M-PCR and simplex PCR assays and determined the prevalence of OXA-48-Like encoding genes. The current study is the first, to our knowledge, to report a high prevalence of OXA-48-Like genes detected within carbapenem-susceptible ESBL-producing *K. pneumoniae* isolates, according to the former 2010 CLSI guidelines.[39]

Few published reports are available on the prevalence of β-lactamases in *Enterobacteriaceae* in South Africa.[49] The publications and communications available report a high prevalence of ESBLs and the recent detection of GES carbapenemase variants, KPC, IMP, NDM, OXA-48, OXA-181 and VIM carbapenemases.[44,46,50-53] In South African hospitals, *K. pneumoniae* is an infamous bacterium with 55% to 74% of blood stream isolates in the public hospitals (in 2009) and 55% to 60% in the private sector testing positive for ESBL production.[49,54,55] More recently, the Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) posted a 2013 surveillance report recording 73% *K. pneumoniae* isolates as ESBLs and 2% ertapenem resistant.[56] The burden of carbapenemase-producing *Enterobacteriaceae* remains undetermined in South Africa and there is currently no platform for surveillance reports, thus a lack of local representative data.[57]

In the current study, five β-lactamases were identified molecularly and found to be highly prevalent in the ESBL-producing *K. pneumoniae* isolates, namely SHV, CTX-M, TEM, OXA-1-Like and OXA-48-Like genes. All the CTX-M positive isolates were confirmed to have CTX-M that could belong to groups CTX-M-1, CTX-M-2 or CTX-M-9 by the CTX-M Degenerate 1 primers (Table 3.1). The ESBL-producing *K. pneumoniae* strains originating from clinical settings in Gauteng appear to characteristically encode multiple β-lactamases. Comparably, Cabral and colleagues reported in 2012 that 21% of *K. pneumoniae* isolates harbouring β-lactamases and even carbapenemases encoded all three ESBL genes ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}).[58] In a study by Apisarnthanarak and colleagues in 2008, the isolates encoding multiple ESBL genes exhibited higher levels of resistance to diverse antibiotic classes and presented a greater risk of ineffective initial empirical antimicrobial therapy when compared to single-ESBL encoding counterparts.[59] The risk of mortality is augmented in the cases of delayed initial antimicrobial therapy.[16]

This study may be the first to report a high prevalence of OXA-48-Like (94%) β-lactamases within ESBL-producing *K. pneumoniae* falling within ertapenem-susceptible as well as non-susceptible ranges in South Africa. The study conducted by Brink and colleagues (2013) only identified 33 isolates of 240 ertapenem-non-susceptible isolates tested as harbouring OXA-48 (six isolates) and OXA-181 (27 isolates) genes.[44] Comparably, the reports within Africa mostly originate from North African countries where the OXA-48-Like carbapenemases are widespread.[28,44] The OXA-48 β-lactamase is known to hydrolyse penicillins and imipenem but not expanded-spectrum cephalosporins and the only variants possessing significant carbapenemase activity are OXA-181 and OXA-232.[22,26,29] These variants, OXA-181 and OXA-232, are known to exhibit modest rises in carbapenem MICs with the potential for significant increases in MICs in combination with other resistance factors.[29] The latter–mentioned variants may thus not be a major contributor to carbapenem resistance *in vitro* and the associated MICs may fall within susceptible ranges thus complicating its detection.[22,28] Alternatively, the presence of variant OXA-163, which posses a hydrolysis profile more akin to Ambler class A ESBLs, could be present in this study's isolates, particularly those with carbapenem susceptible profiles.[60]

The VITEK®2 automated system (bioMérieux, France) identified ESBL-producing *K. pneumoniae* isolates by determining susceptibility profiles and interpretations according to CLSI 2010 guidelines.[39] Isolate 71 was phenotypically identified as ertapenem-resistant by the VITEK®2 automated system (bioMérieux, France) and displayed intermediate resistance towards meropenem. Additionally, isolates 56, 64 and 86 displayed elevated MICs for ertapenem although still interpreted as carbapenem sensitive. Under the new 2014 CLSI guidelines[32] isolates 56 and 64 would be detected as ertapenem-resistant, whilst isolate 86 would be interpreted as ertapenem intermediate resistance. Ertapenem MICs have been found to commonly be more elevated than the other carbapenem counterparts thus making it a good indicator of carbapenemase activity.[30] Only OXA-48-Like β-lactamases, presumptively OXA-181, were identified in all three isolates (isolates 56, 71 and 86) using PCR assays and no additional carbapenemases. The β-lactamases detected in the former three isolates (isolates 56, 71 and 86) utilising M-PCRs are illustrated in Table 3.2. The loss of outer membrane proteins, such as OmpK36, can result in reduced susceptibility or resistance towards carbapenems in ESBL or AmpC β-lactamase producers.[61-63] The loss of both OmpK35 and OmpK36 in ESBL producers can result in moderate increases in meropenem and imipenem resistance but particularly ertapenem resistance.[62,63] The loss of porins in

ESBL-producers and broad-spectrum β-lactamases not only confers resistance to cefoxitin and cephalosporins but additional cross resistance towards quinolones, aminoglycosides and co-trimoxazole.[61-63]

The simplex PCR assays performed for downstream sequencing of the short amplicons was on other hand more sensitive in the detection of the β-lactamases in isolates 56, 71 and 86. An additional TEM and CTX-M encoding gene were identified in isolate 71 and an additional TEM encoding gene in isolate 86. All three isolates presumptively harboured TEM-1, OXA-1 and OXA-181, whilst isolates 56 and 71 also presumptively encoded CTX-M-15. Three SHV variants were identified, namely: SHV-1/28 (isolate 71), SHV-11 (isolate 86) and SHV-12 (isolate 56). In a study by Adler and colleagues (2013), decreased carbapenem susceptibility was found to be influenced by the expression of β-lactamases CTX-M-15, TEM-1 and OXA-1 in *E. coli* not exhibiting porin expression.[20] The *E. coli* experienced a 20% fitness cost in the total absence of porin expression and spontaneous gene amplification on the plasmid increased expression of β-lactamases.[20] The OXA-48-Like genes were only detectable by simplex PCR assays and sensitivity was greatly reduced in the M-PCR assay thus highlighting the need for a more sensitive M-PCR assay. The identity of the amplicon products was presumptively identified (due to the short gene segment targeted) only in these three isolates (isolate 56, 71 and 86) by sequencing and comparison to sequences in GenBank using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The same conditions and PCR assays were used to repeat β-lactamase gene detection in plasmid extracted DNA of 15 out of the 100 *K. pneumoniae* isolates representing varying profiles but the results were discrepant in that fewer ESBL-encoding genes and no OXA-48-Like genes were detected when compared to testing on total genomic DNA. The positive *Klebsiella pneumoniae* clinical isolate control encoding the OXA-48 gene was efficiently extracted and detected in the plasmid DNA extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Fermentas, USA). Comparably the OXA-48-Like genes were not detected in the plasmidial DNA extracted using the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Fermentas, USA) despite being present when testing the genomic DNA, whic was extracted using the ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research, Fermentas, USA). Although the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Fermentas, USA) was very easy and quick to use with good DNA purity, a limitation may arise due to the insert specification that DNA up to 25 kb could be extracted. The suitability of the Plasmid

Miniprep Kit (Zymo Research, Fermentas, USA) as a faster alternative extraction method would thus need to be determined for plasmids of a vast range in sizes, such as those reported in several studies to date (ranging from 3 kb to 270 kb).[58,64] For further evaluation the Plasmid Miniprep Kit (Zymo Research, Fermentas, USA) performance could be compared with other commercial plasmid extraction kits, such as the UltraClean Endotoxin-Free Mini Plasmid Prep Kit (Mo Bio Lab, USA) used by Cabral and collegues (2012).[58] Plasmids encoding ESBL genes and OXA-48-Like genes may differ in size, for example the OXA-48 gene has been associated with a self-transferable 62 kb plasmids with no additional resistance genes whereas OXA-181 may exist on varying plasmid scaffolds, such as a 7605 bp ColE2-type plasmid and a 83 577 bp IncT-type plasmid.[27,28] In the study by Cabral and colleagues (2012), plasmidial profiles of *K. pneumoniae* isolates harbouring both ESBLs and carbapenemases revealed that up to 10 plasmids with sizes ranging between <3.4 kb and >150 kb could be isolated from a single isolate.[58] The isolates in this study were not retested.

The current study elucidated a high rate of multiple β-lactamase carriage in the 100 ESBL-producing *K. pneumoniae* isolates sent for routine testing at the NHLS. A high percentage of the ESBL-encoding isolates in this study characteristically encoded multiple β-lactamases, have diverse phenotypic resistance profiles and feature the OXA-1-Like and OXA-48-Like genes prevalently for which the effect *in vivo* towards antibiotics is largely undetermined. The impact of using carbapenems as treatment for patients with ESBL isolates harbouring genes coding with low-level carbapenem resistance is unknown and may contribute to both a rise in the spread of carbapenem resistance and also associated mortality without effective treatment options. The study also demonstrated an apparent high association of OXA-48-Like and OXA-1-Like enzymes with the ESBL-producing *K. pneumoniae* strains. Polymerase chain reaction remains the gold standard for OXA-48 and OXA-181 detection.[22] Molecular methods, consisting predominantly of PCR methods, can be utilised for the accurate detection of carbapenemase encoding genes but sequencing is still required thereafter for variant identification or differentiation.[36,37]

In conclusion, the study highlights the current risk of local MDR *K. pneumoniae* developing resistance towards ertapenem and possibly other carbapenems, which are the mainstay drugs for the treatment of ESBL-producers, as well as the very high presence of OXA-48-Like genes in isolates interpreted as ertapenem-susceptible within the CLSI 2010 specified carbapenem MIC ranges.[39] The standardisation of M-PCR and simplex PCR assay protocols

are recommended to avoid deceptively low detection of β-lactamases, as the OXA-48-Like gene was only detectable in simplex PCR assays. Future studies should incorporate investigation into other resistance mechanisms to further understand the current and emerging resistance trends in *K. pneumoniae* isolates within local clinical settings, particularly those with elevated MICs towards carbapenems. The results also emphasise the need to determine effects and outcomes of current treatment regimes implemented in the light of current β-lactamase profiles and the regular, much-needed surveillance of phenotypic and accompanying genetic resistance profiles of *K. pneumoniae* strains.

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None to declare.

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LIST OF TABLES AND FIGURES

Table 3.1: Gene targets, primer concentrations and sequences targeting resistance genes in *K. pneumoniae* isolates

Gene target	Primer	Primer concentration (µm)	Primer sequence (5' to 3')	Amplicon size (bp)	Reference	PCR Annealling temp (°C)
Multiplex PCR I						
CTX-M Degenerate 1 primers	*bla*$_{CTX-M}$ (F) *bla*$_{CTX-M}$ (R)	0.4 0.4	SCSATGTGCAGYACCAGTAA CCGCRATATGRTTGGTGGTG	450	41	58
CTX-M Degenerate 2 primers	*bla*$_{CTX-M}$ (F) *bla*$_{CTX-M}$ (R)	0.2 0.2	CGC TTT GCC ATG TGC AGC ACC GCT CAG TAC GAT CGA GCC	307	41	
Multiplex PCR II						
CTX-M (Universal primers)	CTX-M-U1 CTX-M-U2	0.25 0.25	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	40	60.5
TEM	TEM-164.SE TEM-165.AS	0.3 0.3	TGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	40	
SHV	MultiTSO-S_for MultiTSO-S_rev	0.2 0.2	AGCCGCTTGAGCAAATTAAAC ATCCCGCAGATAAATCACCAC	713	42	
Multiplex PCR III						
VIM	MultiVIM_for MultiVIM_revc	0.5 0.5	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	42	58
KPC	MultiKPC_fo MultiKPC_rev	0.2 0.2	CATTCAAGGGCTTTCTTGCTGC ACGACGGCATAGTCATTTGC	538	42	
IMP	MultiIMP_for MultiIMP_rev	0.2 0.2	TTGACACTCCATTTACDG GATYGAGAATTAAGCCACYCT	139	42	
Multiplex PCR IV						
NDM-1	NDM-GBM-F NDM-GBM-R	0.7 0.7	CCC GGC CAC ACC AGT GAC A GTA GTG CTC AGT GTC GGC AT	129	43	60
OXA-1-Like (OXA-1, OXA-4 and OXA-30)	MultiTSO-O_for MultiTSO-O_rev	0.2 0.2	GGCACCAGATTCAACTTTCAAG GACCCCAAGTTTCCTGTAAGTG	564	42	
OXA-48-Like	OXA-F OXA-R	0.5 0.5	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	37	
Simplex PCR						
IMI	IMI-GS-F IMI-GS-R	0.2 0.2	GGTGTCTACGCTTTAGACACTGGCTC GCACGAATACGCGCTGCACCGG	536	43	60
VEB (VEB-1 to VEB-6)	MultiVEB_for MultiVEB_rev	0.3 0.3	CATTTCCCGATGCAAAGCGT CGAAGTTTCTTTGGACTCTG	648	42	60
OXA-48-Like	OXA-F OXA-R	0.5 0.5	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	37	58

*R = A or G; Y = T or C; S = G or C

**Y = T or C; D = A or G or T

Table 3.2: **VITEK®2 (bioMérieux, France) susceptibility profiles and molecular profiles of *K. pneumoniae* isolates exhibiting reduced carbapenem susceptibility and ertapenem resistance**

	***Klebsiella pneumoniae* susceptibility interpretations and molecular profiles**			
Antibiotics tested using the VITEK®2 system::	**Isolate 56**	**Isolate 64**	**Isolate 71**	**Isolate 86**
Ampicillin	R	R	R	R
Amoxicillin/Clavulanic Acid	R	R	R	R
Piperacillin/Tazobactam	R	R	R	R
Cefuroxime	R	R	R	R
Cefuroxime Axetil	R	R	R	R
Cefoxitin	R	R	R	S
Cefotaxime	R	R	R	I
Ceftazidime	R	R	R	I
Cefepime	I	I	R	I
Ertapenem	S** (2 mg/L)	S** (2 mg /L)	R (>=8 mg /L)	S** (1 mg /L)
Imipenem	S (<=1 mg /L)	S (<=1 mg /L)	S (<=1 mg /L)	S (<=1 mg /L)
Meropenem	S (<=0.25 mg /L)	S (<=0.25 mg /L)	I** (1 mg /L)	S (<=0.25 mg /L)
Amikacin	I	I	I	S
Gentamicin	S	S	R	S
Nalidixic Acid	R	R	R	S
Ciprofloxacin	R	R	R	S
Tigecycline	I	R	S	S
Nitrofurantoin	I	R	R	R
Colistin	R	S	S	S
Trimethoprim/Sulphamethoxazole	R	R	R	S
β-lactamases detected molecularly:	TEM, SHV, OXA-1-Like, OXA-48-Like	TEM, CTX-M, SHV, OXA-1-Like, OXA-48-Like	CTX-M, SHV, OXA-1-Like, OXA-48-Like	TEM, SHV, OXA-1-Like, OXA-48-Like

*S: Susceptible; I: Intermediate; R: Resistant

** Elevated carbapenem MIC

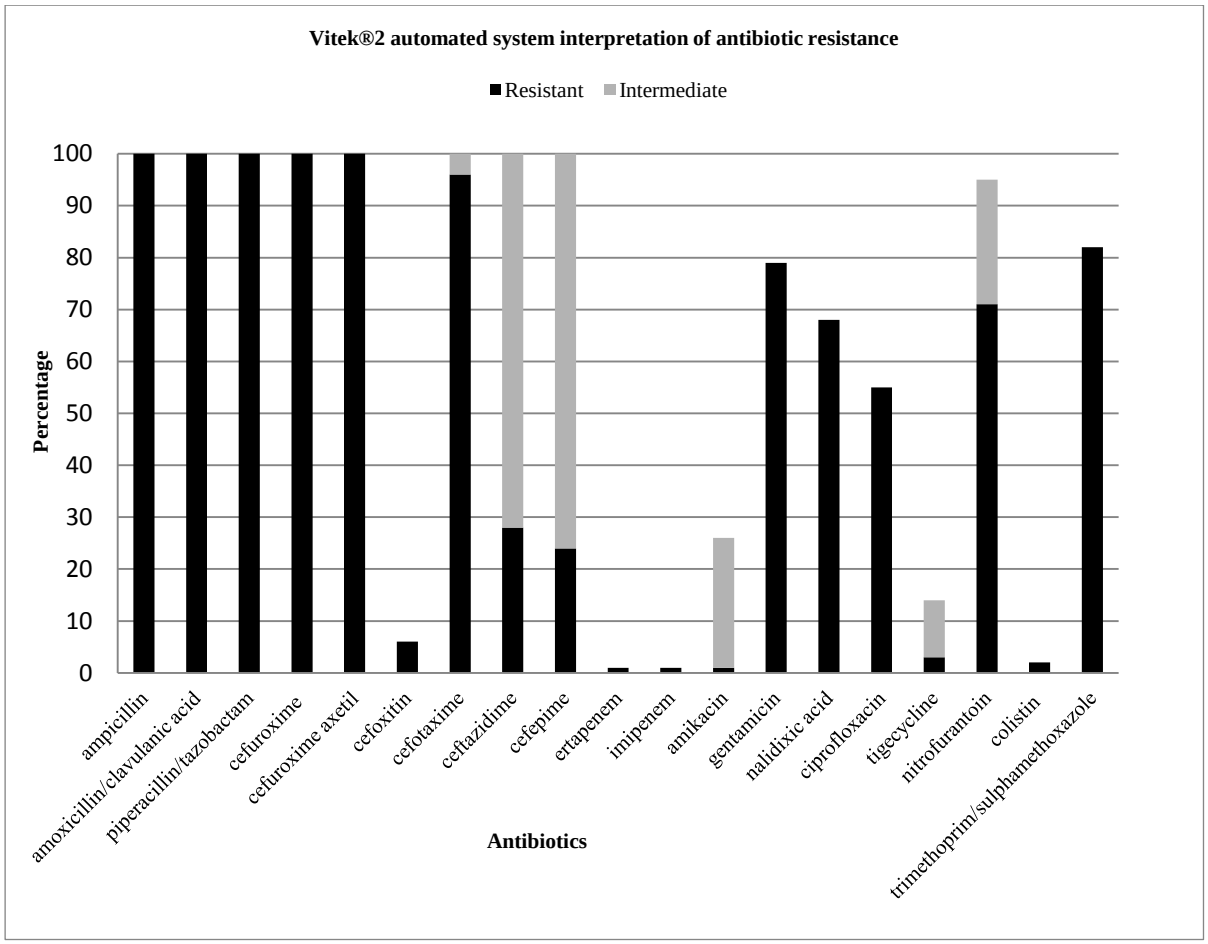

Figure 3.1: **VITEK®2 automated system interpretation of antibiotic resistance and intermediate resistance in *K. pneumoniae* isolates**

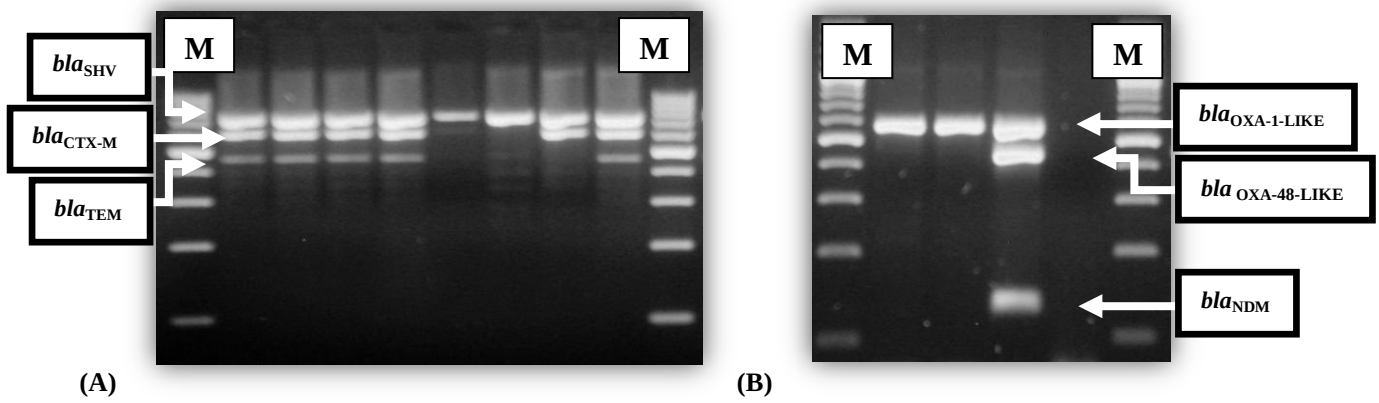

Figure 3.2: **Multiplex PCR assays for the detection of β-lactamase encoding genes in *K. pneumoniae*. (A) Gel electrophoresis image of multiplex PCR II assay illustrating five isolates positive for bla_{SHV} (713 bp), bla_{CTX-M} (593 bp) and bla_{TEM} (445 bp), one isolate positive for bla_{SHV} and bla_{CTX-M} and two positive for only bla_{SHV}. (B) Gel electrophoresis image of multiplex PCR IV assay illustrating two isolates positive for $bla_{OXA-1-Like}$ (564 bp), a positive control encoding $bla_{OXA-1-Like}$ (564 bp), $bla_{OXA-48-Like}$ (438 bp) and bla_{NDM} (129 bp) and a negative control (N)**

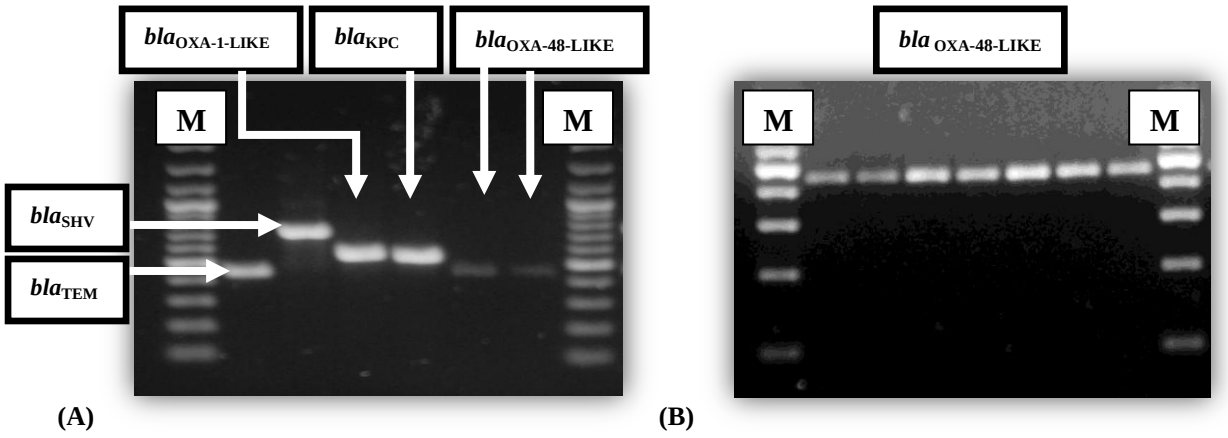

Figure 3.3: **Simplex PCR assays for the detection of β-lactamase encoding genes in *K. pneumoniae*. (A) Gel electrophoresis image of simplex PCR amplicons illustrating genes bla_{TEM} (445 bp), bla_{SHV} (713 bp), $bla_{OXA-1-Like}$ (564 bp), bla_{KPC} (538 bp), and $bla_{OXA-48-Like}$ (438 bp). (B) Gel electrophoresis image of simplex PCR amplicons illustrating gene $bla_{OXA-48-Like}$ (438 bp) in seven isolates**

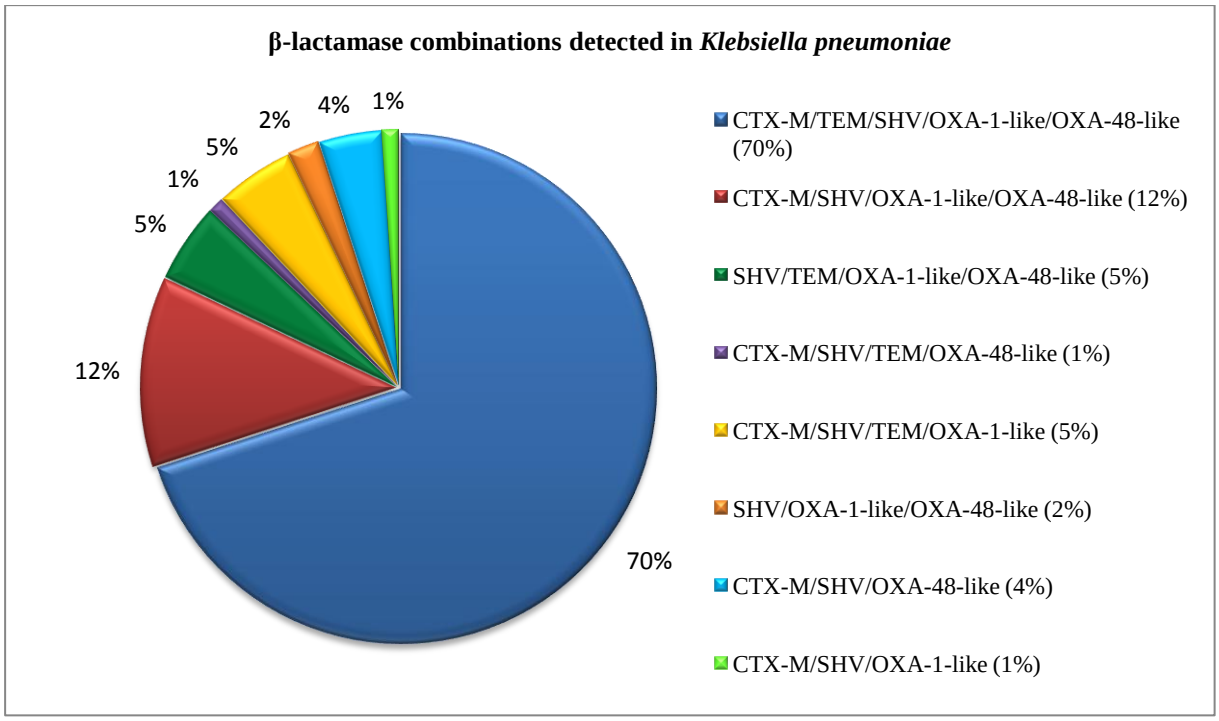

Figure 3.4: **Percentages of *K. pneumoniae* isolates encoding different combinations of multiple β-lactamases as detected by PCR**

CHAPTER 4

CHARACTERISATION OF β-LACTAMASE PRODUCING *KLEBSIELLA PNEUMONIAE* ISOLATES: NOVEL ST1632 IN SOUTH AFRICA

The editorial style of the PLOS ONE Journal was followed in this chapter

4.1 ABSTRACT

Genetic typing of *Klebsiella pneumoniae* is used for epidemiological referencing. In the clinical setting it can be useful in outbreak investigations, understanding transmission and managing hospital infections. Multi-drug resistant bacteria exist and proliferate either due to natural selection of clonal lineages or the transfer of mobile genetic elements, sometimes in response to antibiotic-use selective pressure. Pulsed-field gel electrophoresis (PFGE) is highly discriminatory and the gold standard typing method for the characterisation of *K. pneumoniae* isolates. The aim of the study was to genetically characterise *K. pneumoniae* isolates by PFGE and multilocus sequence typing (MLST). One hundred unrepeated ESBL-producing *K. pneumoniae* isolates were collected from the National Health Laboratory Service (NHLS). The PFGE was performed on a Rotaphor VI system (Biometra, Germany). Clonal representatives were further characterised by MLST. All the strains were typeable by PFGE using *Xba*I, which discerned multiple pulsotypes and MLST identified 10 different STs including a novel sequence type, ST1632. The diverse pulsotypes of *K. pneumoniae* isolates are not suggestive of clonal spread of particular strains. The MLST results further confirmed the variability among isolates tested and elucidated several STs, some of which have been identified internationally and often associated with carbapenem-resistance. Data on *K. pneumoniae* STs is still limited in the South African clinical setting, although the close monitoring of resistance profiles and characterisation of isolates is imperative for outbreak analysis, identification of prominent STs in clinical settings as compared to international counterparts and surveillance of expanding resistance.

Key words: *Klebsiella pneumoniae,* β-lactamases, pulsed-field gel electrophoresis, multilocus sequence typing

4.2 INTRODUCTION

Klebsiella pneumoniae is a Gram-negative saprophytic bacterium that exists within the environment and mammalian hosts, often as a coloniser of the gastrointestinal tract, skin and nasopharynx or implicated in nosocomial infections [1]. *Klebsiella pneumoniae* is implicated in both community-associated (CA) infections and healthcare-associated infections, which in the former case can present as liver abscesses with associated endophthalmitis and metastatic infections or in the latter case presents as pneumonia, blood stream, intra-abdominal or urinary tract infections [1,2]. *Klebsiella pneumoniae*'s rapid acquisition of plasmids encoding multidrug resistance genes, such as those encoding β-lactamases, led to the rise of multidrug-resistant (MDR) strains, which expressed extended-spectrum β-lactamases (ESBLs) and thereafter carbapenemases [1]. A rise in carbapenem resistance within Gram-negative bacterial pathogens, commonly within the *Enterobacteriaceae* family, is a global phenomenon that presents treatment problems due to the lack of efficacious antibiotics available against these and lack of novel antibiotics under development [3].

The rise of MDR *K. pneumoniae* has been aided by the extensive use of antimicrobial drugs, which could either select for multidrug-resistant strains or encourage spread of transferable genetic elements, such as plasmids and transposons [4]. The "success" of a bacterial strain has been described in terms of its capability of both vertical transmission of genetic elements encoding the resistance genes whilst spreading and increasing in prevalence, as well as horizontal transfer to other strains, species or even genera [5]. The proliferation and spread of resistant strains has been reported both within hospitals and to other hospitals [6]. The most disconcerting MDR *K. pneumoniae* strains recorded globally are carbapenem-resistant, which can be attributed to mutational changes resulting in: (i) loss of outer membrane porins in combination with over-expression of AmpC (Ambler class C) or ESBLs, (ii) increased drug efflux, (iii) penicillin binding protein alteration or (iv) more commonly due to carbapenemase expression (Ambler Class A, B and D β-lactamases) [3, 7-11]. *Enterobacteriaceae*, such as *Klebsiella pneumoniae* isolates, exhibiting carbapenemase activity have vast drug resistance profiles and often present serious health threats when infecting debilitated or immunocompromised patients [1,4]. Infections by carbapenemase-producers are severe and often require treatment using last resort drugs, such as colistin, fosfomycin and tygecycline,

although use of colistin is a risk factor for the emergence of colistin-resistant *K. pneumoniae*, which has already been observed in South Africa [12;13].

Over the years several phenotypic and molecular methods have been used for the characterisation and epidemiological typing of *K. pneumoniae*, including the isolates implicated in hospital outbreaks [6]. Typing techniques include but are not limited to: (i) amplified fragment length polymorphism (AFLP) analysis, (ii) multilocus sequence typing (MLST), (iii) multilocus variable-number tandem-repeat analysis (MLVA), (iv) pulsed field gel electrophoresis (PFGE), (v) restriction fragment length polymorphism (RFLP), (vi) matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and (vii) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [5,14].

Pulsed-field gel electrophoresis is the gold standard for *K. pneumoniae* typing although it is largely suitable for local outbreak investigation due to difficult interlaboratory reproducibility [6]. The MLST technique is on the other hand useful and comparable on international databases due to unambiguous, portable data produced but may lack in discriminatory power necessary for outbreak investigations [5,6]. Typing has elucidated a range of successful sequence types (STs) and clonal complexes (CCs) including bacterial strains with global dissemination, such as the carbapenemase-harbouring *K. pneumoniae* ST258, the community-acquired *K. pneumoniae* CC23 (including ST23 and ST57), the NDM-producing *Escherichia coli* ST101 and the ESBL-producing *E. coli* ST131 [4,5,15,16]. Typing of *K. pneumoniae* is not just a useful means for outbreak investigation but also for determining reservoirs and dissemination routes [14]. The aim of the study was to characterise ESBL-producing *K. pneumoniae* isolates, from Pretoria, utilising PFGE and MLST.

4.3 MATERIALS AND METHODS

4.3.1 Study setting

One hundred, unrepeated ESBL-producing *K. pneumoniae* isolates were obtained from the National Health Laboratory Service (NHLS) diagnostic laboratory, Tshwane Academic Division, after routine identification and antibiotic susceptibility testing using the VITEK®2 system (bioMérieux, France). The isolates originated from several specimens sent for

investigation at the NHLS, which services a tertiary academic hospital, three regional hospitals and 64 clinics. The isolates were collected over a three and a half month period (mid-May until August) in 2012 and characterised at the Department of Medical Microbiology, University of Pretoria/NHLS. These isolates had been previously tested using multiplex- and simplex PCR assays targeting β-lactamase genes utilising published primer sequences [17-21]. Ethical approval was granted for this study by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Protocol number S38/2012).

4.3.2 Pulsed-field gel electrophoresis (PFGE)

The PFGE DNA preparation protocol described by Gautom (1997) was modified and used for the genetic characterisation of the *K. pneumoniae* isolates using the Rotaphor VI (Biometra, Germany) system (Appendix B) [22]. The protocol was preformed as described briefly hereafter for both the *K. pneumoniae* isolates and the *Salmonella* serotype Braenderup (H9812) marker (ATCC BAA-664).

4.3.2.1 Plug preparation

The *K. pneumoniae* isolates were streaked from storage cultures onto MacConkey agar and a single colony was inoculated into 4 ml Brain heart infusion (BHI) broth (Merck, Germany), which was incubated [New Brunswick Scientific co. Inc, United States of America (USA)] at 37^{o}C for 16 h to 20 h. The resulting cell suspension in BHI broth (Merck, Germany) was adjusted to an approximate absorbance of 1.00 OD at 600 nm using a spectrophotometer (Perkin Elmer Lambda 25 UV/Vis spectrometer, USA) and 1 ml centrifuged (Spectrafuge 24D, Labnet International Inc., USA) at 4 200 x *g* for 2 min. The supernatant was discarded and the pellet re-suspended in 500 µl TE Buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8), which was subsequently heated to 37^{o}C in a digital dry bath (AccuBlock^{TM}, Labnet International Inc., USA). The suspension was mixed with an equal volume of a molten 500 µl 1.5% (m/v) SeaKem® LE Agarose (Lonza, Rockland, USA), which was prepared in 50 ml 1X TBE buffer [45 mM Tris borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, USA) and dispensed into a reusable PFGE plug mould (Biometra, Germany). The plugs were allowed to set for 15 min at room temperature (± 25^{o}C) after which each plug was pressed out into separate 15 ml sterile Cellstar® tubes (Greiner Bio-One, GmbH, Germany).

A volume of 6 ml PFGE lysis buffer (0.1 M EDTA, 10 mM Tris-HCl, pH 8, 1% sarcosyl) and 60 µl Proteinase K (20 $mg.ml^{-1}$) (Roche, Germany) was added to each 15 ml sterile Cellstar® tube (Greiner Bio-One, GmbH, Germany), which was incubated for 5 h at 36°C in a shaking incubator (New Brunswick Scientific co. Inc, USA). The solution was subsequently discarded and replaced with 6 ml of fresh PFGE lysis buffer (0.1 M EDTA, 10 mM Tris-HCl, pH 8, 1% sarcosyl) with 60 µl Proteinase K (20 $mg.ml^{-1}$) (Roche, Germany) prior to overnight incubation at 50°C (New Brunswick Scientific co. Inc, USA). The isolate plugs were transferred to a 2 ml microcentrifuge tube (Eppendorf AG, Germany) to which 2 ml PFGE storage buffer (10 mM EDTA, 10 mM Tris-HCl, pH 8) was added for storage between 2°C to 8°C.

Digestion with restriction endonucleases was performed on small standardised slices of agarose plugs, no more than 3 mm in width. The plug slices were subjected to two wash steps by adding 2 ml of PFGE storage buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Roche Applied Science, Germany) and were incubated at room temperature (± 25°C) for 40 min apiece. The solutions were decanted and volumes of 2 ml 10 mM Tris-HCl (pH 8) buffer (Sigma-Aldrich, USA) were used in three subsequent wash steps for 20 min apiece at room temperature (± 25°C). The plugs were thereafter submerged in a 70 µl solution comprising of 10 µl 10X FastDigest Buffer [including bovine serum albumin (BSA)] (Thermo Scientific, USA), 30 µl FastDigest *Xba*I (Thermo Scientific, USA) restriction endonuclease and 40 µl nuclease-free water (Qiagen, Germany). These plug slices were incubated at 37°C (digital dry bath, AccuBlock™, Labnet International Inc., USA) for 3 h before proceeding with PFGE.

4.3.2.2 Modified Rotaphor VI system pre-set programme

The Rotaphor Type VI system (Biometra, Germany) has several pre-set programmes. A 1% (m/v) SeaKem® LE Agarose (Lonza, Rockland, USA) gel was prepared in 0.25 X Tris-borate EDTA (TBE) (pH 8.5) buffer instead of SeaKem®Gold Agarose (Lonza, Rockland, USA) and the Rotaphor Type VI system (Biometra, Germany) pre-set programme adjusted accordingly. The *Salmonella* programme, from the eight pre-combined programme lists, was altered by lowering the voltage (220 V logarithmic to 160 V) and increasing the time of the run to 21 h by adding an additional 6 h to the original programme, which was comprised of three shorter 5 h programmes. Each short programme ran for the duration of 5 hrs, at 13˚C,

with an interval of 25 s linear to 2 s and at an 111˚C constant angle. The PFGE protocol for the Rotaphor Type VI system (Biometra, Germany) required a volume of 2.4 L of 0.25 X TBE (pH 8.5) buffer, which was freshly prepared every 2 weeks and stored at 4˚C. Upon programme completion, the gel was stained using ethidium bromide (10 mg.ml^{-1} stock solution) and destained. The gel image was viewed and digitally captured on an Ultra Violet transilluminator (DigiDoc-It®, UVP product, USA).

4.3.3 Multilocus sequence typing

The multilocus sequence typing protocol for *K. pneumoniae* targets seven housekeeping genes (*rpo*B, *gap*A, *mdh*, *pgi*, *pho*E, *inf*B and *ton*B) and is accessible at the Institut Pasteur MLST Databases (http://www.pasteur.fr/MLST) (Table 4.1) [6]. A single PCR assay reaction mixture was adjusted to a final volume of 25 µl comprised of 12.5 µl QIAGEN® mastermix (Qiagen, Germany), 0.5 mM final concentration of the forward and reverse primers in a 25 µl, 5 µl Q-solution (Qiagen, Germany), 2 µl template DNA and nuclease-free water (Qiagen, Germany) to make up the remainder of the volume. The template DNA used was extracted using the ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research, Fermentas, USA), as per the manufacturer's instructions. The PCR assays were performed on a G-storm thermocycler (Vacutec, UK) according to the optimal conditions specified by the QIAGEN Multiplex PCR manual (2008). The amplification programme used was as follows: initial denaturation at 95^{o}C for 15 min, 35 cycles of denaturation at 95^{o}C for 30 sec, annealing at temperatures ranging from 50^{o}C to 60^{o}C for different primer pairs (Table 4.1) for 1.5 min, extension at 72^{o}C for 1.5 min and a final extension at 72^{o}C for 10 min. The annealing temperatures for the *pgi* and *ton*B genes were modified to higher temperatures, 55^{o}C and 50^{o}C respectively (Table 4.1).

The resulting amplicons were analysed by comparison to a 100 bp molecular size marker ladder (Fermentas, Thermo Scientific, USA) after gel electrophoresis (Elite 300 power pack, Wealtec, South Africa) at 90 V for 1.5 h on a 1% (m/v) MetaPhor™ agarose gel (Lonza, Rockland, USA) containing 5 µl of ethidium bromide (10 mg.ml^{-1}) (Promega, USA) prior to sequencing. The sequencing of the amplified products in both directions was performed by Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

4.3.4 Data analysis

Fingerprint analysis of PFGE products was done using the GelCompar *II* (Applied Maths, Belgium) software programme. The Dice coefficient was used to determine the distance matrix from DNA profiles and the unweighted pair group method using arithmetic average (UPGMA) algorithm was used to construct a dendrogramme. Clusters were defined as isolates with a 70% similarity cut-off value and clonality (pulsotypes) at an 80% similarity cut-off value. Ten cluster representatives were selected and further processed by MLST. The sequenced MLST products were analysed using the CLC Main workbench version 6.0 (CLCBio, Denmark) and compared with the Pasteur MLST database (http://www.pasteur.fr/MLST) where unique ST numbers were assigned. A new allele was submitted to the MLST website to assign a novel ST.

4.4 RESULTS

The majority of the isolates tested were sent for analysis at the NHLS from two hospitals (Here named Hospital A and Hospital B) with 28 and 67 isolates each. A further two isolates originated from a third hospital (Hospital C), one from a fourth hospital (Hospital D) and two from clinics. All the isolates were successfully digested using the FastDigest *Xba*I (Thermo Scientific, USA) restriction endonuclease. Several diverse pulsotypes were identified, as illustrated in Figure 4.1. At a 70% similarity cut-off value, 76% of *K. pneumoniae* isolates could be grouped into 16 major clusters (Figure 4.1). At an 80% similarity cut-off, 19 clonal groups (pulsotypes) could be identified for 67% of the isolates (Figure 4.1). The 19 pulsotype groups ranged from two to eight isolates in a group.

Sequencing of 10 cluster representatives identified ST13 (isolate 98) (2-3-1-1-10-1-19), ST35 (isolate 1) (2-1-2-1-10-1-19), ST37 (isolate 63) (2-9-2-1-13-1-16), ST39 (isolate 16) (2-1-2-4-9-1-14), ST43 (isolate 86) (2-6-1-5-11-1-15), ST101 (isolate 71) (2-6-1-5-4-1-6), ST234 (isolate 56) (2-1-2-1-7-1-24), ST307 (isolate 54) (4-1-2-52-1-1-7), ST336 (isolate 10) (2-1-1-1-72-4-4) and the novel ST1632 (1-1-146-1-1-1-1) (isolate 57). The STs identified from Hospital A (28 isolates) were ST35, ST37 and ST101, while STs identified within Hospital B (67 isolates) were ST13, ST39, ST43, ST234, ST307 and ST1632. The ST336 originated from Hospital C. The CTX-M, OXA-1-Like, OXA-48-Like, SHV and TEM genes had previously been identified molecularly in the 10 representative isolates, as well as elevated

carbapenem MIC values in isolates 56, 64, 71 and 86 using the VITEK®2 system (bioMérieux, France).

4.5 DISCUSSION

The characterisation of all 100 isolates utilising PFGE discerned multiple pulsotypes, which demonstrated good typeability and reproducibility using the *Xba*I restriction endonuclease. The restricted genome bands were clearly visible and analysable using GelCompar *II* (Applied Maths, Belgium). The PFGE results identified several isolates that made the 80% to 100% similarity cut-off value demonstrating clonality and 10 isolates representing different clusters at a 70% cut-off value were all found to be different STs. One of the 10 isolates typed by MLST elucidated a novel ST with a *mdh* variant that has an A to C change at nucleotide 407, namely ST1632.

The protocol for *K. pneumoniae* typing using MLST was first established by Diancourt and colleagues (2005) in a study, which revealed 40 STs among 67 isolates [6]. Diancourt and colleagues (2005) concluded in the study that resistance was associated with multiple emergences in varying clones rather than the inter-hospital spread of a select few clones [6]. However, since MLST's introduction certain STs have been associated with international spread, such as the ST258 for which studies have suggested that spread has mainly been human-mediated rather than a result of acquisition of similar plasmids within a successful clone [5]. Comparably, diverse pulsotypes are illustrated in Figure 4.1 for this study, which corresponds to the diversity observed in MLST of the randomly selected cluster representatives. At a 70% cut-off value seven major clusters (≥5 isolates) were identified (clusters A, B, D, F, H, J and M) and nine minor clusters (<4 isolates) (clusters C, E, G, I, K, L, N, O, P) (Figure 4.1). At a 80% cut-off value three major pulsotypes were identified (within clusters A, D and M) (Figure 4.1). The three largest clusters identified at a 70% cut-off value contained 12, 10 and nine isolates (Clusters A, M and D respectively) (Figure 4.1). Cluster A isolates, to which isolate 16 (ST39) belongs, were isolated from hospitals A, B and a local clinic (Figure 4.1). Cluster B isolates, to which isolate 54 (ST307) belongs, originated from hospitals A, C and D. Cluster D to which isolate 98 (ST13) belongs, included isolates originating from only hospital A. Cluster F isolates, to which isolate 10 (ST336) belongs, originated from hospitals A, B and D. Cluster H isolates, to which isolate 1 (ST35) belongs, originated from only hospital B. Cluster J isolates originated from hospital A, B and a clinic.

Cluster M, to which isolate 71 (ST101) belongs, included 10 isolates originating from hospitals A and B. Several isolates, found across several clusters, are from the medical and pulmonology ward, internal medicine ward and the intensive care unit (ICU). Other varying wards found within the clusters dicussed include: paediatric, casualty, oncology, surgery, obstetrics, urology and immunology wards. Cluster A appears to originate predominantly from the paediatric wards (surgery ward and ICU), neonatal ICU or the medical and pulmonology ward. The minor cluster G appears to predominantly originate from casualty wards as well as the surgery and trauma wards, while cluster L isolates come from the paediatric ward, obstetrics ward and antenatal ICU. The 100 *K. pneumoniae* isolates were from diverse origins and could be grouped into 16 clusters or 19 plusotypes, which is not necessarily suggestive of clonal spread within and among the different hospitals but rather that some clusters, pulsotypes or STs may be established in these clinical settings.

Four of the STs in this study (ST13, 35, 37 and 39) were described in the first MLST development and evaluation study by Diancourt and colleagues (2005) for isolates originating from Curacao, Germany, Italy and Spain [6]. Most of the STs recorded in this study (ST13, 35, 37, 39, 43, 101, 234, 307 and 336) have also been identified in separate studies across the world. The ST35 was one of the multiple STs identified in Spain as a pathogen and coloniser in newborn and adult patients and found to harbour the CTX-M-15 ESBL enzyme [23]. The ST35 identified in the current study, was also previously found to harbour a CTX-M-Like enzyme during molecular screening (Figure 4.1). The *K. pneumoniae* ST37 strain identified in this study did not exhibit any elevated MICs towards carbapenems [as determined by the VITEK®2 system (bioMérieux, France)]. Comparably, the *Klebsiella pneumoniae* ST37 was identified in a hospital in Rome, Italy and six isolates were found to harbour an OmpK35 nonsense-mutation and a novel OmpK36 porin variant, which resulted in a characteristic ertapenem-resistant phenotype [24]. Seven of the 13 unrelated ESBL-producers tested in the same study presented with ertapenem-resistance, with six harbouring the OmpK36 gene variant and having additional elevated MICs for meropenem but not imipenem [24]. Notably, none of the ertapenem-susceptible isolates harboured the OmpK36 variant and 13 of the total 15 unrelated isolates tested also did not express OmpK35 due to a nonsense mutation [24]. On the other hand, the *K. pneumoniae* ST101 strain was also identified in this study, which exhibited comparably elevated MICs for ertapenem and meropenem with no reduced susceptibility towards imipenem, as was described by García-Fernández and colleagues (2010) [24].

The *K. pneumoniae* ST101 was identified in a countrywide cross-sectional study conducted in Italy [25]. The study identified *Klebsiella pneumoniae* as the most common carbapenem-resistant *Enterobacteriaceae* at 11.9% and that the majority of the CRE isolates (85%) referred were found to produce carbapenemases, which included KPC at 89.5%, VIM-1 at 9.2% and OXA-48 at 1.3% [25]. The majority of the KPC-harbouring *K. pneumoniae* isolates implicated in dissemination belonged to the CC258, including ST258 and ST512, and to a lesser extent the ST101 [25]. Reports of ST101 from Italy, Brazil and the United States have mentioned and highlighted the role of ST101 in the dissemination of KPC, OXA-48, OXA-181 and ESBLs [25-31]. Comparably, the ESBL-producing *K. pneumoniae* ST101 identified in this study was previously found to presumably harbour the OXA-181 β-lactamase (as identified by sequencing of the short amplicon fragment), an OXA-48 variant capable of modest carbapenem MIC rises, which may increase when working in concert with other resistance factors [32]. The ST101 additionally encoded the CTX-M and SHV ESBLs and OXA-1 β-lactamases (Figure 4.1). This profile was not unique as 70% of the 100 *K. pneumoniae* isolates had previously tested positive for five β-lactamases (CTX-M, TEM, SHV, OXA-1-Like and OXA-48-Like). The remaining isolates encoded varying combinations of four (24%) or three (7%) of these β-lactamases across the different pulsotypes.

In many African countries reports on molecular analysis of ESBL-producing *Enterobacteriaceae* through the use of methods, such as PCR, plasmid transfer assays (PTA) and PFGE are sparse or inconsistent [33]. In a study by Elhani and colleagues (2010) conducted in Tunisia, Northern Africa, the STs most found associated with the CTX-M-15 producing *K. pneumoniae* include: ST101, ST107 and ST147 [34]. The isolates carrying SHV-12 were on the other hand detected in ST25, ST35, ST48, ST107, ST133, ST309, ST320, ST321, ST323, ST324 and ST325 [34]. In another study conducted in the Lake Zone of Tanzania that characterised ESBL-producing *K. pneumoniae* also identified the ST101, along with the ST14, ST147 and ST348 [35]. This study thus elucidates yet another report with STs comparable to the former mentioned STs found internationally (such as ST101) and the novel ST1632 described in South Africa.

This study makes a valuable contribution to the otherwise sparse list of reports in Africa and focuses on the genetic typing of isolates so that parallel antibiotic resistance evolution or the

possible introduction of international STs can be assessed. The β-lactamase genes detected in all 10 representative isolates from South Africa are illustrated in Figure 4.1. The novel *K. pneumoniae* ST1632 isolate (isolate 57) belongs to minor cluster G from which all the isolates originated from Hospital A. It was isolated from blood culture from a 45 year old male in the surgery and trauma ward. This isolate displayed a phenotype interpreted by the vitek to be consistent with that of an ESBL and it was found to harbour genes encoding CTX-M-Like, SHV-Like and OXA-48-Like β-lactamases. Despite the presence of an OXA-48-Like encoding gene; no carbapenem non-susceptibility was observed. The isolates displaying ertapenem non-susceptibility or resistance in this study (isolates 56, 64, 71, 86) illustrated similar phenotypes to those reported by Giani and colleagues (2013) in the presence of the weakly carbapenem-hydrolysing OXA-181 that was presumably detected and no other detectable carbapenemases (Figure 4.1). The MICs for the carbapenem non-susceptible isolates in the Giani and colleagues (2013) study displayed resistance towards ertapenem and only intermediate or susceptible phenotypes towards the other carbapenems, particularly in isolates harbouring VIM, OXA-48 or no carbapenemases [25]. Isolates 56 and 64 belonged to the same cluster thus the ST was only determined for isolate 56 as well as isolate 71 and 86, which are all ertapenem-non-susceptible or -resistant isolates. *Klebsiella pneumoniae*'s ability to express resistance towards a vast range of antibiotics is problematic in that it has ultimately limited the efficacious drug therapy available to a few expensive antibiotics, which may not always be available in a developing country [35].

The spread of resistance can be mediated through extensive antibiotic use selecting for resistant *K. pneumoniae* strains and the spread of transferable genetic elements harbouring resistance genes [4]. The origin of resistant *K. pneumoniae* strains into the South African clinical environment remains largely unanswered, although reports exist on the risk factors for patient acquisition of ESBL-producers. Patients can be both the victim and vector of healthcare-associated infections with MDR strains, which is especially problematic due to current international travel to developing countries such as within South and Central America, South Africa and Asia as medical tourism [36]. Travel to endemic countries for carbapenemases and ESBL-producing *Enterobacteriaceae* is also a risk factor for acquisition of these, even in the absence of medical attention [36-39]. International travel effects on patient acquisition of resistance genes or resistant strains have been found to result in acquisition of ESBL genes (such as $bla_{\text{CTX-M}}$), quinolone genes (such as *qnr*B and *qnr*S) as well as carbapenemases (such as OXA-181 and NDM-1 β-lactamases) in *Enterobacteriaceae*

[38-40]. Carriage of these carbapenem-resistant *Enterobacteriaceae* has been reported lasting up to a month [39]. Alternatively, a review by Woodford *et al*. (2011), discussing high–risk multidrug-resistant Gram-negative clones in the dissemination of antibiotic resistance, makes mention of certain clones acquiring similar resistance genes in separate instances, which is further evolutionarily adjusted so as to not negatively impact on bacterial fitness [5, 41]. The results of the study and the techniques used, thus elucidate the presence of STs, such as ST101, which has been internationally identified as multidrug-resistant in multiple studies, thus outlying its potential for rapidly evolving resistance or possible international influence in local genetic epidemiology. The evolution of STs locally is also illustrated by the identification of the novel ST1632.

4.6 CONCLUSION

This is the first study describing the *K. pneumoniae* ST1632 in South Africa, for which limited molecular epidemiological data is available. The diverse pulsotypes and MLST STs within several clusters do not underline a dominant clone being spread within the clinical settings in Pretoria, although it is apparent that some clusters may be established. The study provides essential insight into the STs circulating within the clinical setting, which can be compared to international counterparts for which the evolution of resistance has been better tracked thus hinting at the potential of these STs to gain resistance under the right conditions, mainly through horizontal gene transfer, or to be implicated in hospital outbreaks.

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FIGURE LEGENDS

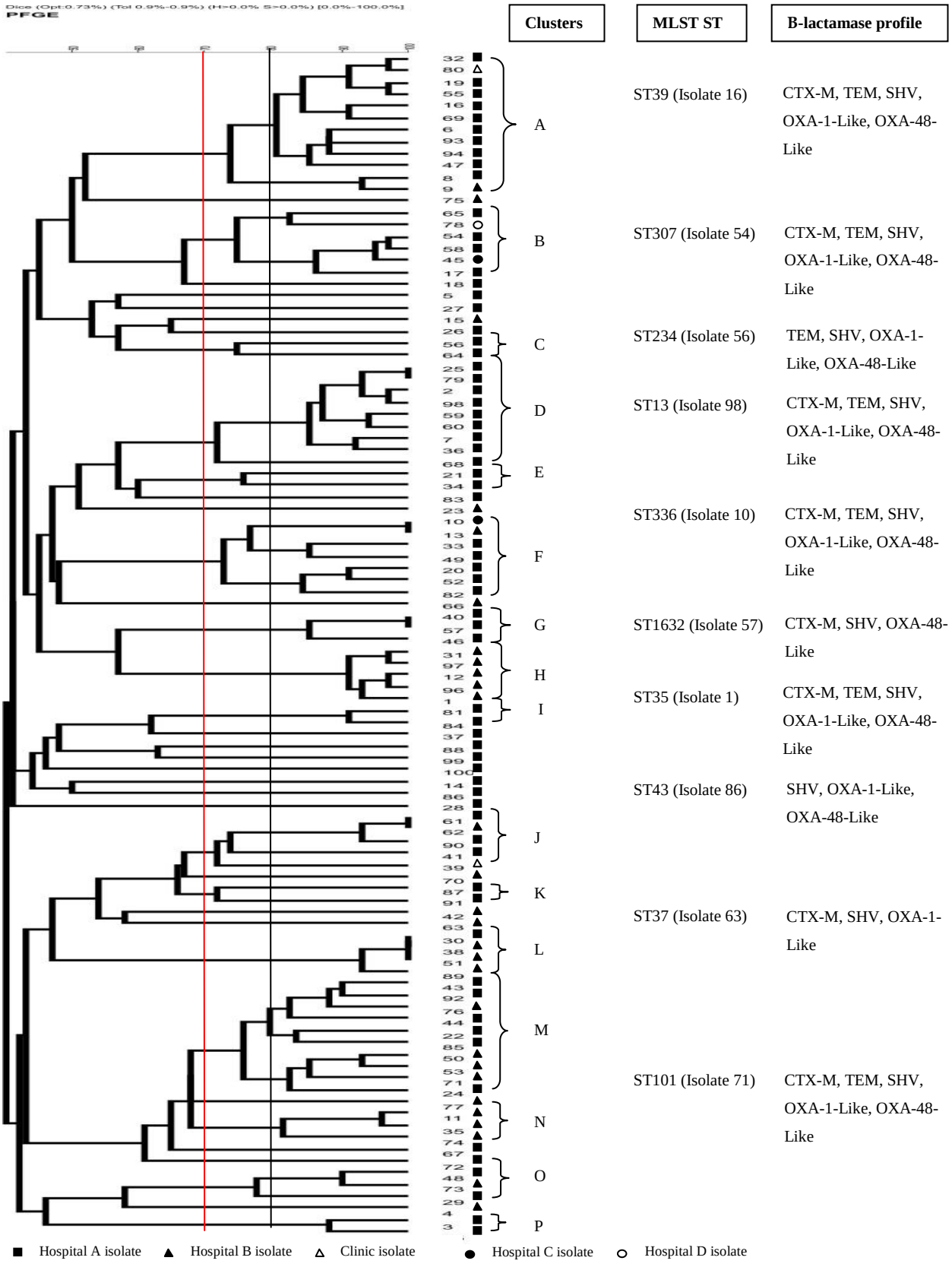

Figure 4.1: Dendrogramme of *K. pneumoniae* fingerprint analysis of PFGE products illustrating cluster groups at a 70% cut-off value, pulsotypes at an 80% cut-off value and STs of 10 isolates

TABLES

Table 4.1: ***Klebsiella pneumoniae*** **MLST PCR and sequencing primers of seven housekeeping genes (Diancourt *et al*., 2005)**

Locus	Primer name	Primer sequence	Amplicon size (bp)	Temperature (^{o}C)
***rpo*B**	rpoB Forward rpoB Reverse	GGC GAA ATG GCW GAG AAC CA GAG TCT TCG AAG TTG TAA CC	501	50
***gap*A**	gapA Forward gapA Reverse	TGA AAT ATG ACT CCA CTC ACG G CTT CAG AAG CGG CTT TGA TGG CTT	450	60
mdh	mdh Forward mdh Reverse	CCC AAC TCG CTT CAG GTT CAG CCG TTT TTC CCC AGC AGC AG	477	50
pgi*	Pgi1 Forward Pgi1 Reverse Pgi2 Forward (Seq) Pgi2 Reverse (Seq	GAG AAA AAC CTG CCT GTA CTG CTG GC CGC GCC ACG CTT TAT AGC GGT TAA T CTG CTG GCG CTG ATC GGC AT TTA TAG CGG TTA ATC AGG CCG T	432	55 **
***pho*E**	phoE Forward phoE Reverse	ACC TAC CGC AAC ACC GAC TTC TTC GG TGA TCA GAA CTG GTA GGT GAT	420	50
inf*B	infB Forward infB Reverse infB Forward (Seq)	CTC GCT GCT GGA CTA TAT TCG CGC TTT CAG CTC AAG AAC TTC ACT AAG GTT GCC TCC GGC GAA GC	318	50
***ton*B**	tonB Forward tonB Reverse	CTT TAT ACC TCG GTA CAT CAG GTT ATT CGC CGG CTG RGC RGA GAG	414	50**

* The PCR and sequencing primers differ and are distinguished by the abbreviation Seq for sequencing primers

** Elevated annealing temperatures used in comparison to original article

CHAPTER 5

CONCLUDING REMARKS

5.1 Conclusions

Klebsiella pneumoniae is a ubiquitous Gram-negative bacilli second only to *E. coli* in causing urinary tract infections, responsible for respiratory infections, such as pneumonia and one of six important blood stream pathogens in South Africa (Marchaim *et al*., 2008; Schroll *et al*., 2010; Bamford *et al*., 2011). Treatment of these infections are increasingly complicated by the rapid acquisition of antimicrobial resistance determinants among *Enterobacteriaceae*, which are often encoded on the chromosome and encoded or transferred by mobile genetic elements such as plasmids, integrons, transposons and insertion sequence elements (ISs) (Samuelsen *et al*., 2009; Grundmann *et al*., 2010; Poirel *et al*., 2011; Kocsis and Szabó, 2013; Shon *et al*., 2013). A successful pathogen is one able to transfer genetic elements harbouring resistance genes both vertically and horizontally to other strains or species, whilst also successfully increasing in prevalence (Woodford *et al.*, 2011). Beta-lactamase production is the leading mechanism of resistance in several Gram-negative bacteria, including *K. pneumoniae* (Vranakis *et al*., 2014). The expression of β-lactamases proffers resistance towards important β-lactam antibiotics and includes important contenders, such as extended-spectrum β-lactamase (ESBL) cephalosporinases and carbapenemases (Kocsis and Szabó, 2013). Few studies are available in Africa on multidrug-resistant, ESBL-producing *K. pneumoniae* isolates, despite the importance for understanding transmission and antimicrobial gene profiling (Breurec *et al*., 2013; Mshana *et al*., 2013).

This study revealed a high rate of multiple ESBL genes ($bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM}) being encoded simultaneously in *K. pneumoniae* isolates from South African clinical settings, as well as genes encoding OXA-1-Like and OXA-48-Like β-lactamases. The CTX-M-1 group, which includes CTX-M-15, is the prevailing ESBL recorded from faecal carriage studies from Guinea-Bissau, Madagascar, Niger, Cameroon, Tanzania and South Africa (Isendahl *et al*., 2012). In a study conducted in Tunisia, looking at ESBL-producing *K. pneumoniae* causing neonatal sepsis, the most prevalent ESBL encoded was CTX-M (76%) (Mshana *et al*., 2013).

Much like this study, a high rate of multiple β-lactamases was encoded with 70% encoding CTX-M-15 and TEM-1, 16% encoding CTX-M-15 and SHV-11 (Mshana *et al*., 2013).

The PCR assays used in this study allowed for initial molecular screening of *K. pneumoniae* isolates for important, globally-recognised β-lactamases (particularly ESBLs and carbapenemases) and their enzyme variants, which may have recently changed or emerged in the South African clinical setting. A limitation of the set of primers used in the study is the lack of differentiation between specific β-lactamase enzyme variants, thus requiring expensive downstream sequencing for specific β-lactamase gene identification. Additionally, the sensitivity of PCR assays used for the detection of β-lactamases in the current study, such as TEM, OXA-48-Like and OXA-1-Like genes, were found to be greatly influenced by minor technique changes implemented, such as spinning down and increasing the DNA volume to 3 µl as well as the choice of mastermix [preferably QIAGEN® M-PCR Kit (Qiagen, Germany)]. As a result, a previously unreported high frequency of OXA-48-Like genes were detected in ESBL-producing *K. pneumoniae* isolates (94%) of which three were sequenced in reduced ertapenem-susceptible or ertapenem-resistant isolates and presumptively identified as the OXA-181 variant. The OXA-48 variants identified in other studies, OXA-181 and OXA-232, have displayed weak but significant carbapenem hydrolysis particularly in the presence of permeability defects, whilst OXA-163 displays a hydrolysis profile more akin to that of an ESBL by being able to hydrolyse penicillins, ceftazidime and cefotaxime (Nordmann *et al.*, 2011; Poirel *et al*., 2011; Poirel *et al.*, 2012; Evans and Amyes, 2014). However, just as lack of expression can occur in isolates encoding carbapenemases, such as the *K. pneumoniae* carbapenemase (KPC) gene, likewise carbapenem resistance can be observed in clinical isolates negative for any carbapenemases, particularly when harbouring cefotaximases whilst presenting with porin loss (Peleg *et al.*, 2005; Cabral *et al*., 2012). The high presence of OXA-163 in these isolates could account for the carbapenem-susceptible phenotype observed in the OXA-48-Like encoding isolates although sequencing or specific primers are needed to confirm this. In a study by Breurec and colleagues, approximately 13% of the isolates from African and Southeast Asian towns from main health institutions were confirmed ESBL-producers phenotypically but did not possess CTX-M, SHV or TEM ESBL encoding genes, thus hinting at the possibility of a class D ESBL (Breurec *et al.*, 2013). In this study, primers targeting the ESBL and OXA-48-Like genes would need to amplify a larger segment of the gene for more accurate identification of the sequenced amplicon

product, especially when targeting gene variants, by comparison to the sequences in GenBank using the basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The β-lactamase PCR assays are ideal for the initial screening of ESBL-producing *K. pneumoniae* strains for epidemiological recording but would be costly for routine implementation in the clinical setting, particularly when including sequencing costs. On the other hand, the use of more specific PCR assays (targeting specific β-lactamase variants) are not advisable for initial molecular screening as new β-lactamase gene variants may be missed thus giving false epidemiological data. Optimally, the PCR assays targeting ESBL genes and carbapenemase genes are recommended for screening of ESBL-producing *K. pneumoniae* isolates in the clinical setting displaying elevated carbapenem MICs. Carbapenemases (such as KPC) can be produced concomitantly with ESBLs, for which molecular detection of the latter would be more sensitive than phenotypic methods (Cabral *et al*., 2012). Polymerase chain reaction remains the gold standard for the detection of class D OXA-48/OXA-181 carbapenemases (Shanthi *et al*., 2013).

The characterisation of the 10 *K. pneumoniae* isolates using MLST in this study elucidated important sequence types (STs), some of which are recognisable internationally, and the presence of a novel ST1632. Their associated molecular β-lactamase profiles were also determined. The presence of the STs identified in this study highlights several possible routes of introduction of multidrug-resistant *K. pneumoniae* strains in the clinical setting, which include antibiotic-driven selection and the possible introduction of international strains or genetic elements encoding resistance through patient acquisition during travel to endemic countries (Rogers *et al.*, 2011; Van der Bij and Pitout, 2012; Chmelnitsky *et al*., 2013; Ruppé *et al*., 2014; Wintersdorff *et al.*, 2014; Yaita *et al*., 2014). Additionally, there does not appear to be dominant *K. pneumoniae* clones among these ESBL-producers implicated in inter-hospital transmission but rather that some clones appear established in particular clinical settings or wards within a hospital. Unlike infamous carbapenemase-producing STs (for example ST258), ESBL-producing *K. pneumoniae* are likely multiclonally transmitted (Breurec *et al.*, 2013). In a study encompassing 135 *K. pneumoniae* isolates from African and Southeast Asian towns, the molecular profile comprised of 60 STs identified (including important strains ST11, ST14, ST15 and ST340) and a predominant CTX-M ESBL encoding gene present among ESBL-producers (Breurec *et al.*, 2013).

These few studies indicated that in sub-Saharan Africa, there appears to be a significant rise in antimicrobial resistance (Nyasulu *et al.*, 2012). Despite this, there appears to be a lack of antimicrobial resistance surveillance and published literature in South Africa despite the large HIV/AIDS affected population, which are susceptible to opportunistic infections leading to increased antimicrobial consumption (Nyasulu *et al.*, 2012). Widespread distribution and increasing reservoirs of β-lactamase and other resistance genes in *K. pneumoniae* isolates are important due to negative treatment outcomes predicted (Breurec *et al.*, 2013). The diversity and high number of resistance genes found in *K. pneumoniae* is indicative of an ever-growing resistance gene pool (Hoban *et al.*, 2014). Regular surveillance and characterisation can thus elucidate antimicrobial trends, both genetically and phenotypically and proffer invaluable information on (i) whether local infection control measures and treatment are adequate, (ii) whether a stricter global approach to infection control is needed to prevent transmission of international MDR strains, (iii) the potential evolution of microbial resistance and (iv) the impact of treatment on evolution of resistance, especially in ESBL-producers with low carbapenem-resistance *in vitro* due to OXA-48-Like β-lactamases.

5.2 Future research

The study of virulence genes, β-lactamase gene profiles and characterisation of multidrug-resistant *K. pneumoniae* has been largely underreported in the South African clinical setting (Nyasulu *et al.*, 2012). Future research should thus encompass deeper analysis of virulence factors implicated in the successful pathogenesis of *K. pneumoniae* working in concert with the existing β-lactamases, which attribute to its survival and proliferation within and outside its host. Virulence genes typically investigated include *uge* (encoding uridine diphosphate galacturonate 4-epimerase), *wab*G (involved in the biosynthesis of the outer core lipopolysaccharide), *ure*A (related to the urease operon), *mag*A (mucoviscosity-associated gene A), *mrk*D (type 3 fimbriae adhesion), *all*S (activator of the allantoin regulon), *kfu*BC (iron-uptake system), *rpm*A (regulator of mucoid phenotype) and *fim*H (fimbrial gene encoding type 1 fimbrial adhesion) due to their role in bacterial pathogenesis (Brisse *et al.*, 2009; Lascols *et al.*, 2013; Gao *et al.*, 2014). In addition, whole genome sequencing of important *K. pneumoniae* strains with multidrug resistance and the use of computational tools is an important next-step for elucidating gene characteristics, such as the virulence genes, through comparative genomics (Gao *et al.*, 2014; Lery *et al.*, 2014). Whole genome

sequencing of important nosocomial pathogens is a useful research tool that can be used for (i) new drug development, (ii) the development or improvement of diagnostic tests, (iii) surveillance of drug resistance, (iv) to direct infection control measures and (iv) to elucidate factors implicated in resistance emergence and persistence (Köser *et al.*, 2014).

Increasing resistance is often discussed in terms of selection and subsequent proliferation of multidrug-resistant strains or the horizontal transfer of genetic elements encoding resistance, such as plasmids (Chmelnitsky *et al*., 2013). A combination of proteomics and molecular techniques could thus be used for the characterisation of plasmids within outbreak *K. pneumoniae* isolates (Lau *et al.*, 2014). By targeting plasmid gene products, rapid real-time tracking of plasmids harbouring important β-lactamases (such as KPC) is possible using methods, such as the commercial matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) system and is beneficial for infection control efforts (Lau *et al.*, 2014). Alternatively, the MALDI-TOF MS system can also be used for the detection of carbapenemase activity *in vitro* of isolates harbouring carbapenemases, such as NDM-1, VIM-1, VIM-2 and KPC, as well as for the detection of other antibiotic resistance mechanisms (for example, the loss of outer membrane porins such as the OmpK36) (Burckhardt and Zimmermann, 2011; Hrabák *et al.*, 2013). Comparative studies of MDR bacterial proteomic information under specific *in vitro* conditions can be used for the identification of proteins associated with antibiotic resistance (Vranakis *et al*., 2014). Gel-based and gel-free proteomic methodologies that could be implemented include liquid chromatography and tandem MS (LC-MS/MS) or MALDI-TOF MS/TOF-MS (Vranakis *et al*., 2014). Proteomic techniques could additionally be used for the investigation of possible immunogenic *K. pneumoniae* antigens, such as FepA (Ferrienterobactin outer membrane receptor), OmpA (outer membrane protein A), OmpK36 (outer membrane porin) and the Colicin I receptor, for vaccine development (Kurupati *et al*., 2006; Lundberg *et al.*, 2013).

In light of *K. pneumoniae* isolates displaying elevated ertapenem MICs identified in this study the need to investigate the mechanisms of resistance in these ESBL-producers and ertapenem-non-susceptible isolates is imperative due to the negative treatment repercussions that could ensue. Improving the understanding of the progression of drug resistance and mechanisms involved could aid attempts to improve the efficacy of current antimicrobials, an alternative solution in light of the lack of new drugs under development in recent years (Gasink *et al*., 2009; Giamarellou, 2010; Bush, 2012; Vranakis *et al*., 2014).

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APPENDIX A

REAGENTS AND BUFFERS

1. Pulsed-field gel electrophoresis (PFGE) stock reagents

a) 0.5 M Ethylene diamine tetra-acetate (EDTA) (pH 8) (500 ml)

A mass of 93.5 g of EDTA (Merck, Germany) was dissolved in 400 ml of sterile distilled water. The volume was made up to 500 ml and the solution adjusted to pH 8. The stock solution was autoclaved at 121°C for 15 min.

b) 1 M Tris-HCl (pH 8) (500 ml)

A mass of 78.8 g Tris-HCl [Sigma-Aldrich, United States of America (USA)] was dissolved in 400 ml of sterile distilled water. The volume was made up to 500 ml and the solution adjusted to pH 8. The stock solution was autoclaved at 121°C for 15 min.

c) 10% N-Lauroylsarcosine sodium salt (Sigma-Aldrich, USA) (200 ml)

A mass of 20 g N-Lauroylsarcosine sodium salt (Sigma-Aldrich, USA) was dissolved in 100 ml of sterile distilled water. The volume was further made up to 200 ml using sterile distilled water.

d) Phenyl methyl sulfonyl fluoride (PMSF) 400 mM (10 ml)

A volume of 0.7 g PMSF (Roche, Germany) was added to 10 ml absolute Ethanol (Merck, Gauteng). The stock reagent was stored at -20˚C.

e) Proteinase K (20 mg.ml^{-1})

A volume of 250 mg of Proteinase K (Roche Applied Science, Germany) was dissolved in 12.5 ml of deionised water (sterile). The resulting 20 mg.ml^{-1} Proteinase K (Roche Applied Science, Germany) stock solution was transferred into 2 ml microcentrifuge tubes

(Eppendorf AG, Germany) in aliquots of 1 ml and 500 µl. The aliquots were stored at -20˚C and were only thawed once needed. After thawing, the enzyme was stored at 4˚C.

f) 5 X Tris-borate-EDTA (TBE) buffer (pH 8) (1 L)

Tris-borate-EDTA (TBE) buffer (pH 8) was prepared by dissolving 54 g of Tris-base (Sigma-Aldrich, USA) and 27.5 g of Boric acid (Merck, Germany) in 500 ml sterile distilled water. A volume of 20 ml of 0.5 M EDTA (pH 8) (Merck, Germany) was added and the solution made up to 1 L using sterile distilled water. The TBE buffer was autoclaved at 121°C for 15 m and stored at 4˚C for no longer than two weeks.

2. Pulsed-field gel electrophoresis reagents prepared from stocks

a) Tris-EDTA (TE) buffer (500 ml)

Tris-EDTA buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8) was prepared by adding 1 ml of 0.5 M EDTA (pH 8) (Merck, Darmstadt, Germany), 5 ml of 1 M Tris-HCl (pH 8) (Sigma-Aldrich, USA) and 494 ml of sterile distilled water. The TE buffer was stored at 4˚C.

b) Pulsed-field gel electrophoresis (PFGE) lysis buffer (1 L)

The PFGE lysis buffer (0.1 M EDTA, 10 mM Tris-HCl, pH 8, 1% sarcosyl) was prepared by adding 200 ml of 0.5 M EDTA (pH 8) (Merck, Germany), 10 ml of 1 M Tris-HCl (pH 8) (Sigma-Aldrich, USA) and 100 ml of 10% Sarcosyl (Sigma-Aldrich, USA). The volume was made up to 1 L by further adding 690 ml of sterile distilled water. The PFGE lysis buffer was stored at 4˚C.

c) Pulsed-field gel electrophoresis (PFGE) storage buffer (500 ml)

The PFGE storage buffer (10 mM EDTA, 10 mM Tris-HCl, pH 8) was prepared by adding 10 ml of 0.5 M EDTA (pH 8) (Merck, Germany), 5 ml of 1 M Tris-HCl (pH 8) (Sigma-Aldrich, USA) and 485 ml sterile distilled water. The PFGE storage buffer was kept at 4˚C.

d) 0.25 X TBE buffer (pH 8) (1 L)

The preparation of 0.25 X TBE buffer (pH 8), 50 ml of the 5 X stock was added to 950 ml of sterile distilled water. The solution was stored at 4˚C for no longer than two weeks.

3. 1 X TBE buffer (pH 8) (1 L) prepared for gel electrophoresis

The preparation of 1 X TBE buffer (pH 8), 200 ml of the 5 X stock was added to 800 ml of sterile distilled water. The solution was stored at 4˚C for no longer than two weeks.

APPENDIX B

DETAILED METHODOLOGY

1. Total DNA extraction utilising a ZR Fungal/Bacterial DNA Miniprep kit (Zymo Research, Fermentas, USA)

a) A volume of 1 ml overnight Brain heart infusion (BHI) (Biolab diagnostics, Merck) broth was added to a 2 ml microcentrifuge tube (Eppendorf AG, Germany) and subsequently centrifuged at 10 000 x *g* (Spectrafuge 24D, Labnet International, NJ) at ambient temperature (±25^{o}C) for 2 min to produce a pellet.

b) The supernatant was discarded and the pellet re-suspended in 200 µl PBS (pH 7.2) (GIBCO®, Invitrogen Ltd., USA) for total genomic DNA extraction according to the kit's instructions.

c) The 200 µl of bacterial suspension was added to the ZR BashingBead™Lysis Tube [Zymo Research, Fermentas, United States of America (USA)] as well as 750 µl Lysis solution before being secured in the Disruptor Genie™ and processed at maximum speed for 5 min.

d) The BashingBead™Lysis Tube (Zymo Research, Fermentas, USA) was centrifuged for 1 min at 10 000 x *g* (Spectrafuge 24D, Labnet International, NJ) at ambient temperature (±25^{o}C) and 400 µl of the supernatant transferred to a Zymo-Spin™ IV Spin Filter in a collection tube (Zymo Research, Fermentas, USA).

e) The Zymo-Spin™ IV Spin Filter (Zymo Research, Fermentas, USA) was subjected to centrifugation at 10 000 x *g* for 1 min after which 1.2 ml Fungal/Bacterial DNA Binding Buffer (Zymo Research, Fermentas, USA) containing beta-mercaptoethanol at a final dilution of 0.5% (v/v) was added to the filtrate in the former mentioned collection tube.

f) A volume of 800 µl of the solution was transferred to another collection tube containing a Zymo-Spin™ IIC Column (Zymo Research, Fermentas, USA), centrifuged for 1 min at 10 000 x *g* (Spectrafuge 24D, Labnet International, NJ) at ambient temperature (±25^{o}C) and subsequently repeated.

g) The flow-through was discarded and 200 µl of DNA Pre-Wash Buffer (Zymo Research, Fermentas, USA) was added before repeating centrifugation (Spectrafuge 24D, Labnet International, NJ).

h) A volume of 500 µl Fungal/Bacterial DNA Wash Buffer (Zymo Research, Fermentas, USA) was subsequently added to the Zymo-Spin™ IIC Column (Zymo Research, Fermentas, USA) and centrifuged at 10 000 x *g* (Spectrafuge 24D, Labnet International, NJ) at ambient temperature (±25°C) for 1 min.

i) The collection tube was discarded and the Zymo-Spin™ IIC Column (Zymo Research, Fermentas, USA) placed in a sterile 1.5 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) wherein DNA was eluted by addition of 100 µl of DNA Elution Buffer (Zymo Research, Fermentas, USA) and centrifugation (Spectrafuge 24D, Labnet International, NJ) at 10 000 x *g* at ambient temperature (±25°C) for 30 sec.

2. Plasmid DNA extraction utilising the Zyppy™ Plasmid Miniprep Kit (Zymo Research, Fermentas, USA)

a) Between 1.5 ml to 3 ml of overnight bacterial culture was used for extraction by adding 1.5 ml to an microcentrifuge tube (Eppendorf AG, Hamburg, Germany) which was centrifuged (Spectrafuge 24D, Labnet International, NJ) at 10 000 x *g* (Spectrafuge 24D, Labnet International, NJ) at room temperature (±25°C) for 30 sec.

b) The supernatant was discarded and an additional 1.5 ml overnight bacterial culture was added before repeating the former step.

c) Prior to starting extraction, the 7 X Lysis Buffer (Blue) (Zymo Research, Fermentas, USA) was incubated (New Brunswick Scientific co. Inc, USA) for 30 min at 37˚C.

d) The bacterial cells were resuspended in 600 µl TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8) and 100 µl of 7 X Lysis Buffer (Blue) (Zymo Research, Fermentas, USA) was added to the tube which was inverted four to six times.

e) Complete lysis was indicated by a change from an opaque to a clear blue solution after which 350 µl of Neutralization Buffer (Zymo Research, Fermentas, USA) was added and mixed in thoroughly within 2 min upon completion of the former step.

f) Complete neutralisation was indicated by a colour change from blue to yellow, after which the mixture was centrifuged (Spectrafuge 24D, Labnet International, NJ) at 11 000 x *g* (at ±25°C) for 2 to 4 min and 900 µl of the supernatant transferred to the Zymo-Spin IIN column (Zymo Research, Fermentas, USA) in a collection tube.

g) The Zymo-Spin IIN column (Zymo Research, Fermentas, USA) was subjected to centrifugation at 11 000 x *g* at ambient temperature (±25°C) for another 15 sec and the

flow through discarded before replacing the column back into the same collection tube.

h) A volume of 200 µl Endo-wash Buffer (Zymo Research, Fermentas, USA) was added to the column and centrifuged (Spectrafuge 24D, Labnet International, NJ) for 30 sec.

i) A volume of 400 µl Zyppy^{TM} wash Buffer (Zymo Research, Fermentas, USA) was added to the column and centrifuged (Spectrafuge 24D, Labnet International, NJ) at ambient temperature (±25^{o}C) for 1 min.

j) Finally, the column was transferred to a sterile 1.5 ml centrifuge tube to which 30 µl of Zyppy^{TM} Elution Buffer (Zymo Research, Fermentas, USA) was added and allowed to stand for 1 min before the final centrifugation step (Spectrafuge 24D, Labnet International, NJ) for 30 sec at 11 000 x g, all at ambient temperature (±25^{o}C).

k) The plasmid DNA was contained within the eluted solution, which was stored at -20^{o}C (Defy, South Africa).

3. Simplex and multiplex-PCR assays' amplicon analysis

a) A 1.8% (m/v) MetaPhor^{TM} agarose gel (Lonza, Rockland, USA) was prepared by dissolving 1.8 g of MetaPhor^{TM} agarose powder (Lonza, Rockland, USA) in chilled (4^{o}C) 100 ml 1 X TBE buffer [45 mM Tris-borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, USA) in a beaker.

b) The beaker was sealed and heated in a microwave oven for 2 min on the highest setting with intermittent swirling to avoid superheating and foaming over until the particles were dissolved completely.

c) The molten agarose was placed in a Hybridiser HB-1D oven (Techne Ltd, England) to cool and maintain the temperature at 50^{o}C.

d) A volume of 5 µl ethidium bromide (10 mg.ml^{-1}) (Promega, Madison, USA) was added to the molten agarose solution, which was subsequently poured into a casting tray with a comb and allowed to set at room temperature (± 25^{o}C).

e) The gel was placed at 4^{o}C for 20 min before the comb was removed.

f) The gel electrophoresis chamber (Elite 300 power pack, Wealtec, South Africa) was filled with 500 ml 1 X TBE buffer [45 mM Tris-borate, 1 mM EDTA, (pH 8)] (Sigma-Aldrich, USA).

g) The gel was placed in the gel electrophoresis chamber (Elite 300 power pack, Wealtec, South Africa) and the amplicons of each specimen, the positive and the negative PCR

controls were mixed with a 5 µl volume of Fermentas 6 X green loading dye solution (Fermentas, Thermo Fischer Scientific, USA), which was subsequently loaded into the wells in the agarose gel (Lonza, Rockland, USA).

h) The amplicons were subjected to gel electrophoresis (Elite 300 power pack, Wealtec, South Africa) at 90 V for 1.5 h in a 1 X TBE buffer [45 mM Tris-borate, (pH 8), 1 mM EDTA] (Sigma-Aldrich, USA).

i) Upon completion, the products were viewed on a UV transilluminator (UVP Doc It) and the sizes analysed by comparison to a 100 bp molecular size marker ladder (Fermentas, Thermo Fischer Scientific, USA).

4. Pulsed-field gel electrophoresis genotyping of *K. pneumoniae*

Pulsed-field gel electrophoresis required plug preparation, endonuclease digestion of the plugs and the running of the isolates using the Rotaphor VI (Biometra, Germany) system. The procedures were discussed hereafter.

4.1 Plug preparation and storage protocol for *K. pneumoniae*

a) The PFGE DNA plug preparation protocol was modified from that described by Gautom (1997) and used to characterise 100 clinical *K. pneumoniae* isolates.

b) Single colonies from MacConkey plates were picked and used to inoculate 4 ml BHI broth (Merck, Germany), which was incubated (New Brunswick Scientific co. Inc, USA) at 37^{o}C for 16 h to 20 h.

c) The absorbance of the cell suspension was adjusted to an approximate absorbance of 1.00 OD at 600 nm using a spectrophotometer (Perkin Elmer Lambda 25 UV/Vis Spectrophotometer, USA).

d) Volumes of 1 ml were transferred to 2 ml microcentrifuge tubes (Eppendorf AG, Germany) for centrifugation (Spectrafuge 24D, Labnet International, NJ) at 4 200 x *g* for 2 min at ambient temperature (±25^{o}C).

e) The supernatant was discarded and the pellet re-suspended in 500 µl TE Buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8), which was subsequently heated to 37^{o}C in a digital dry bath (AccuBlock^{TM}, Labnet International) for 10 min.

f) Melted 500 µl 1.5% (m/v) SeaKem® LE Agarose (Lonza, Rockland, USA) was prepared by dissolving 0.75 g powder in 50 ml TE buffer [45 mM Tris-borate, (pH 8),

1 mM EDTA] (Sigma-Aldrich, St. Louis, USA) and was thereafter cooled to approximately 55˚C in an incubator (Hybridiser HB-1D, Techne Ltd, England).

g) The 500 µl cell suspension was mixed with an equal volume of the molten 500 µl 1.5% (m/v) SeaKem® LE Agarose (Lonza, Rockland, USA) and poured into a reusable PFGE plug mould (Rotaphor VI; Biometra, Germany), which was allowed to set for 15 min at room temperature (± 25^{o}C).

h) Each plug was pressed out into separate 15 ml sterile Cellstar® tubes (Greiner Bio-One, GmbH, Germany) which were filled with 6 ml PFGE lysis buffer (0.1 M EDTA, 10 mM Tris-HCl, pH 8, 1% sarcosyl) and 60 µl Proteinase K (20 mg.ml^{-1}) (Roche, Germany) and incubated for 5 h at 36^{o}C in a shaking incubator (110 rpm, New Brunswick Scientific co. Inc, USA) (HF 212 UV, Shangai Lishen Scientific Equipment, China).

i) The solution was thereafter decanted and replaced with 6 ml fresh PFGE lysis buffer (0.1 M EDTA, 10 mM Tris-HCl, pH 8, 1% sarcosyl) with 60 µl Proteinase K (20 mg.ml^{-1}) (Roche, Germany) prior to overnight incubation at 50^{o}C.

j) The plugs were transferred to a 2 ml microcentrifuge tube (Eppendorf AG, Hamburg, Germany) with 2 ml PFGE storage buffer (10 mM EDTA, 10 mM Tris-HCl, pH 8) for storage up to 4 months at 2^{o}C to 8^{o}C.

4.2 *Xba*I restriction endonuclease digestion of PFGE plugs

a) Small standardised slices of each agarose plug, approximately a quarter of the plug, were cut using a scalpel and transferred into 2 ml microcentrifuge tubes (Eppendorf AG, Germany).

b) The plug slices were subjected to two wash steps by adding 2 ml of PFGE storage buffer containing 1 mM phenyl methyl sulfonyl fluoride (PMSF) (Roche Applied Science, Germany) and were kept at room temperature (± 25^{o}C) for 40 min apiece.

c) In the three subsequent wash steps, a volume of 2 ml 10 mM Tris-HCl (pH 8) buffer (Sigma-Aldrich, USA) was added to each microcentrifuge tube (Eppendorf AG, Germany), which was kept at room temperature (± 25^{o}C) for 20 min apiece.

d) After completion of the wash steps, the plug slices were transferred to new 2 ml microcentrifuge tubes (Eppendorf AG, Germany) and immersed in a 70 µl solution containing 10 µl 10 X FastDigest Buffer [including Bovine serum albumin (BSA)]

(Thermo Scientific, USA), 20 µl FastDigest *Xba*I (Thermo Scientific, USA) restriction endonuclease and 40 µl nuclease-free water (Qaigen, Germany).

e) The plug slices were incubated for three hours at 37°C (Digital dry bath, AccuBlock™, Labnet International) after which it was loaded into a cast agarose gel for PFGE.

4.3 Pulsed-field gel electrophoresis using the Rotaphor VI (Biometra, Germany) system and product analysis

a) A 1% (m/v) agarose gel was prepared by adding 3 g of SeaKem® LE Agarose (Lonza, Rockland, USA) to 300 ml 0.25 X TBE (pH 8.5) buffer, which was heated in a microwave until thoroughly dissolved and cooled in an incubator (Hybridiser HB-1D, Techne Ltd, England) to set at 50°C.

b) The gel was poured into a levelled gel support tray to which a gel casting frame was secured and allowed to set with an 18 well comb in place for 40 min.

c) The comb was subsequently removed and the gel casting frame detached.

d) Each plug slice previously subjected to enzymatic digestion, including the *Salmonella* serotype Braenderup (H9812) marker (ATCC BAA-664), was loaded into the wells and positioned to the bottom-front.

e) All the wells were sealed with melted 1% (m/v) SeaKem® LE Agarose (Lonza, Rockland, USA) prepared in a 0.25 M TBE (pH 8) buffer.

f) The uneven gel surface that formed due to adhesive forces, referred to as the agarose rampart, was subsequently cut away and the tray was placed in the Rotaphor Type VI system (Biometra, Germany) electrophoresis chamber containing 2.4 L of 0.25 M TBE (pH 8) buffer.

g) The thermostatic circulator required 2.8 L of distilled water and was used to maintain an optimal temperature range between 10°C to 13°C.

h) At the end of the PFGE run the gel was stained in 1 L distilled water containing 80 µl ethidium bromide (10 $mg.ml^{-1}$ stock solution) (Promega, Madison, USA) for 1 h and destained in distilled water for 30 min.

i) The gel image was viewed and captured on a UV transilluminator (UVP Doc It).

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APPENDIX C

CLINICAL DATA AND DETAILED RESULTS

Table C1: Patient associated information for the *Klebsiella pneumoniae* isolates

Isolate number	VITEK®2 Description	Specimen	Patient age	Patient sex	Additional clinical Information	Clinical Ward
1	ESBL	Endotracheal Aspirate	50 Y	M	N/A	ICU
2	ESBL	Endotracheal Aspirate	35 Y	M	Acute Renal Failure	Medical and Pulmonology
3	ESBL; AMI-R	Urine	81 Y	M	N/A	Internal medicine
4	ESBL; AMI-R	Urine	24 Y	F	Urinary Tract Infection	Antenatal clinic
5	ESBL; AMI-R	Pus Swab	42 D	M	N/A	Paediatric surgery
6	ESBL	CVP Tip	4 D	M	N/A	Paediatric surgery
7	ESBL	Tissue	41 Y	M	N/A	Orthopaedic
8	ESBL	Urine	19 D	M	N/A	Orthopaedic
9	ESBL	Blood Culture	33 D	M	N/A	Neonatal ICU
10	ESBL	Tracheal Aspirate	22 Y	F	N/A	Surgery
11	ESBL; AMI-R	Blood Culture	25 Y	F	N/A	Internal medicine
12	ESBL; AMI-R	Pus Swab	18 D	F	N/A	Neonatal ICU
13	ESBL	Sputum	36 Y	F	N/A	ICU
14	ESBL; AMI-R	Blood Culture	3 D	M	Sepsis	General paediatrics
15	ESBL	Blood Culture	29 Y	M	N/A	ICU
16	ESBL	Tissue	32 Y	M	Arm Sepsis	Orthopaedic
17	ESBL	Urine	47 Y	F	N/A	General surgery
18	ESBL	Pus Swab	54 Y	F	N/A	Urology and gynaecology
19	ESBL	Sputum	37 Y	F	N/A	Medical and Pulmonology
20	ESBL	Urine	77 Y	M	N/A	Medical and Pulmonology
21	ESBL; AMI-R	Urine	29 Y	F	Tuberculosis Meningitis	Neurology
22	ESBL; Imperm (Ceph)	Urine	62 Y	M	Liver Failure	Internal medicine
23	ESBL	Tracheal Aspirate	48 Y	M	N/A	Medical out patients
24	ESBL	Pus Swab	24 Y	M	N/A	Internal medicine
25	ESBL	CVP Tip	45 Y	F	N/A	High care multi-discipline

Table C1: Patient associated information for the *Klebsiella pneumoniae* isolates (Continued)

Isolate number	VITEK®2 Description	Specimen	Patient age	Patient sex	Additional clinical Information	Clinical Ward
26	ESBL; AMI-R	Tissue	28 Y	M	Septic leg	Internal medicine
27	ESBL	Pus Swab	34 Y	F	Gun shot wound	Neurosurgical
28	ESBL	Blood Culture	82 Y	M	Post Laparotomy	High care multi-discipline
29	ESBL; AMI-R	Urine	63 Y	F	Abdominal pain	Casualty
30	ESBL	Blood Culture	19 Y	F	Haematuria	General paediatrics
31	ESBL	Urine	26 Y	F	N/A	High care
32	ESBL; AMI-R	Blood Culture	14 D	F	Bacteremia	Neonatal ICU
33	ESBL; AMI-R	Blood Culture	3 Y 2 M	F	N/A	Oncology
34	ESBL	Blood Cultures	68 Y	F	N/A	High care multi-discipline
35	ESBL	Urine	46 Y	M	Urinary Tract Infection	Medical out patient
36	ESBL	Blood Culture	81 Y	F	Pyrexia	Surgery and Trauma
37	ESBL; AMI-R	Pleural fluid	31 Y	M	Empyema	Orthopaedic
38	ESBL	Urine	34 Y	F	N/A	Obstetrics
39	ESBL (CTX-M Like)	Pus swab	42 Y	M	Sepsis, Bed sores	N/A
40	ESBL	Sputum	59 Y	M	Pneumonia	Casualty
41	ESBL	Tissue	5 Y 5 M	M	Empyema	Neonatal ICU
42	ESBL	Pus Swab	34 Y	F	N/A	Immunology
43	ESBL	Urine	79 Y	M	N/A	Internal medicine
44	ESBL	CVP Tip	46 Y	F	N/A	Surgery
45	ESBL; AMI-R	Sputum	27 Y	F	Pulmonary T.B.	Surgery
46	ESBL	Sputum (luki)	75 Y	F	N/A	Casualty
47	ESBL	Blood Culture	3 M	M	Bowel perforation; sepsis	Paediatric medical ICU
48	ESBL	Catheter tip	26 Y	M	N/A	Internal medicine
49	ESBL; AMI-R	Urine	13 Y	M	N/A	Paediatric nephrology
50	ESBL	Urine	30 Y	M	N/A	Orthopaedics

Table C1: Patient associated information for the *Klebsiella pneumoniae* isolates (Continued)

Isolate number	VITEK®2 Description	Specimen	Patient age	Patient sex	Additional clinical Information	Clinical Ward
51	ESBL	Blood culture	28 Y	F	N/A	Obstetrics
52	ESBL; Imperm (Ceph)	Pus Swab	52 Y	M	N/A	Urology
53	ESBL	Urine	59 Y	M	Urinary Tract Infection	Urology out patient
54	ESBL	Urine	26 Y	F	N/A	Internal medicine
55	ESBL; AMI-R	Urine	7 D	M	Sepsis	Paediatric medical ICU
56	ESBL	Tracheal Aspirate	23 Y	F	N/A	Medical and Pulmonology
57	ESBL	Blood Culture	45 Y	M	N/A	Surgery and Trauma
58	ESBL	Urine cliplock	61 Y	F	N/A	Internal medicine
59	ESBL	Urine	32 Y	M	Congestive Cardiac failure	Internal medicine
60	ESBL	Urine	61 Y	M	N/A	Surgery and Trauma
61	ESBL	Blood Culture	10 D	F	Colon Atresia; Spiking temperature	Paediatric surgery
62	ESBL	Lymph Node	29 Y	F	Tuberculosis Cervical Lymphadenopathy	Internal medicine
63	ESBL (CTX-M Like)	Pus Swab	60 Y	M	Wound sepsis	Drip room/ ear, nose and throat
64	ESBL; AMI-R; Imperm (Ceph)	Tracheal Aspirate	31 Y	F	N/A	Casualty
65	ESBL; AMI-R	Tissue	80 Y	M	Septic hip	Orthopaedic
66	ESBL	L ABD Fluid Collection	44 Y	M	N/A	ICU
67	ESBL	Urine	28 Y	M	Diabetes	Orthopaedic
68	ESBL	Urine	45 Y	F	N/A	Internal medicine
69	ESBL	CVP Tip	28 Y	F	N/A	Medical and Pulmonology
70	ESBL	Urine	63 Y	F	N/A	Urology and gynaecology
71	ESBL; Carbap (Metallo or KPC)	Blood Culture	41 Y	F	N/A	ICU
72	ESBL	Blood Culture	36 Y	F	Sepsis	Labour
73	ESBL	Urine	42 Y	M	Pulmonary T.B.	Immunology
74	ESBL	Urine	73 Y	F	N/A	Medical out patients
75	ESBL	Urine	61 Y		N/A	Internal medicine

Table C1: Patient associated information for the *Klebsiella pneumoniae* isolates (Continued)

Isolate number	VITEK®2 Description	Specimen	Patient age	Patient sex	Additional clinical Information	Clinical Ward
76	ESBL	Urine	20 Y	M	Bilateral Kidney Stone	Casualty
77	ESBL; AMI-R	Blood culture	48 Y	M	N/A	ICU
78	ESBL; AMI-R	Urine	71 Y	F	N/A	Medical
79	ESBL	CVP Tip	69 Y	F	Renal Failure	Medical and Pulmonology
80	ESBL; AMI-R	Urine	83 Y	F	Paraplegia	Not Specified
81	ESBL	Sputum	34 Y	F	Renal Failure	Nephrology
82	ESBL	CVP Tip	33 Y	F	N/A	Internal medicine
83	ESBL; AMI-R	Urine	55 Y	F	Urinary Tract Infection	Internal medicine
84	ESBL; AMI-R	Urine	20 Y	F	Chronic Renal Impairment	Nephrology
85	ESBL	Blood Culture	49 Y	F	Sepsis	Surgery
86	ESBL	Blood Culture	26 Y	M	N/A	Surgery and Trauma
87	ESBL (CTX-M Like)	Urine Catheter tip	1 Y 9 M	M	Burns; Sepsis	Neonatal ICU
88	ESBL	Blood culture	19 Y	F	N/A	Surgery and Trauma
89	ESBL	Pus swab	24 Y	F	N/A	Antinatal ICU
90	ESBL; AMI-R; Imperm (Ceph)	Tracheal alavage	69 Y	F	Ventilator associated LRTI	Medical and Pulmonology
91	ESBL	Tissue	52 Y	M	N/A	Cardiothoraic surgery
92	ESBL	Blood culture	24 Y	M	N/A	Surgery and Trauma
93	ESBL	Urine	77 Y	F	N/A	Medical and Pulmonology
94	ESBL	Pus swab	9 D	M	N/A	Paediatric surgery
95	ESBL; AMI-R	Urine	67 Y	M	N/A	Main casualty
96	ESBL	Pus swab	57 Y	M	Septic burn on leg	Spinal unit
97	ESBL	CVP tip	62 Y	M	CVP tip removed	Spinal unit
98	ESBL	Blood Culture	69 Y	F	N/A	Medical and Pulmonology
99	ESBL	Urine	36 Y	F	N/A	Antinatal
100	ESBL	Fluid	38 Y	F	Sepsis	Medical and Pulmonology

Table C2: VITEK®2 automated system (bioMérieux, France) antibiotic susceptibility interpretations for *Klebsiella pneumoniae* isolates

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
1	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	S	R
2	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	I	R	S	R
3	R	R	R	R	R	S	R	I	I	S	S	S	R	R	R	R	S	R	S	R
4	R	R	R	R	R	R	R	I	I	S	S	S	I	S	R	S	R	S	S	R
5	R	R	R	R	R	S	R	I	I	S	S	S	I	S	R	S	S	S	S	S
6	R	R	R	R	R	S	R	I	R	S	S	S	S	R	S	S	S	R	S	R
7	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
8	R	R	R	R	R	S	R	R	R	S	S	S	S	R	S	S	S	R	S	R
9	R	R	R	R	R	S	R	I	I	S	S	S	S	S	S	S	S	R	S	S
10	R	R	R	R	R	S	R	R	R	S	S	S	S	S	R	R	S	R	S	R
11	R	R	R	R	R	S	R	R	R	S	S	S	I	R	R	R	S	R	S	R
12	R	R	R	R	R	S	R	I	R	S	S	S	I	R	R	R	S	R	S	R
13	R	R	R	R	R	S	R	I	I	S	S	S	S	S	R	R	S	R	S	R
14	R	R	R	R	R	S	R	I	I	S	S	S	I	S	R	S	S	R	S	R
15	R	R	R	R	R	S	I	R	I	S	S	S	S	R	S	S	S	R	S	R
16	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	R
17	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
18	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
19	R	R	R	R	R	S	R	I	I	S	S	S	I	R	S	S	S	R	S	R
20	R	R	R	R	R	S	R	R	R	S	S	S	S	R	R	S	S	S	S	R
21	R	R	R	R	R	S	R	I	R	S	S	S	I	R	R	R	S	R	S	R
22	R	R	R	R	R	R	R	R	R	S	S	S	S	R	R	R	S	R	S	R
23	R	R	R	R	R	S	I	I	I	S	S	S	S	S	R	S	S	R	S	S
24	R	R	R	R	R	S	R	R	R	S	S	S	S	R	R	R	S	R	S	R
25	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	I	R	S	R

AMP: Ampicillin
AMC: Amoxicillin/Clavulanic Acid
TZP: Piperacillin/Tazobactam
CXM: Cefuroxime
CXM-AX: Cefuroxime Axetil
FOX: Cefoxitin
CTX: Cefotaxime
CAZ: Ceftazidime
FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
GEN: Gentamycin
NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
R: Resistant
S: Susceptible
I: Intermediate
A: Information absent

Table C2: VITEK®2 automated system (bioMérieux, France) antibiotic susceptibility interpretations for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
26	R	R	R	R	R	S	R	R	I	S	S	S	I	R	R	S	S	S	S	R
27	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	I	S	R
28	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	S	R
29	R	R	R	R	R	S	R	I	R	S	S	S	I	R	R	R	S	R	S	R
30	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	S
31	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	S
32	R	R	R	R	R	S	R	I	I	S	S	S	I	S	S	S	S	R	S	S
33	R	R	R	R	R	S	R	R	R	S	S	S	I	R	S	S	S	R	S	R
34	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	I	R	S	R
35	R	R	R	R	R	S	R	R	I	S	S	S	S	R	R	R	S	R	S	R
36	R	R	R	R	R	S	R	R	I	S	S	S	S	R	R	R	I	R	S	R
37	R	R	R	R	R	S	R	I	I	S	S	S	I	R	S	S	S	R	S	R
38	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	S
39	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	S	S	I	S	R
40	R	R	R	R	R	S	R	I	I	S	S	S	S	S	R	R	I	R	S	R
41	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	R
42	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
43	R	R	R	R	R	S	R	I	R	S	S	S	S	R	R	R	S	R	S	R
44	R	R	R	R	R	S	R	R	R	S	S	S	S	R	R	R	S	R	S	R
45	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	R	S	I	S	R
46	R	R	R	R	R	S	R	I	I	S	S	S	S	S	R	R	I	R	S	R
47	R	R	R	R	R	S	R	R	R	S	S	S	S	S	S	S	S	R	S	S
48	R	R	R	R	R	S	R	R	I	S	S	S	S	R	R	R	S	I	S	R
49	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	S	S	I	S	R
50	R	R	R	R	R	S	R	I	I	S	S	S	S	S	R	R	S	R	S	S

AMP: Ampicillin
AMC: Amoxicillin/Clavulanic Acid
TZP: Piperacillin/Tazobactam
CXM: Cefuroxime
CXM-AX: Cefuroxime Axeti
FOX: Cefoxitin
CTX: Cefotaxime
CAZ: Ceftazidime
FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
GEN: Gentamycin
NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
R: Resistant
S: Susceptible
I: Intermediate
A: Information absent

Table C2: VITEK®2 automated system (bioMérieux, France) antibiotic susceptibility interpretations for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
51	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	S
52	R	R	R	R	R	R	R	R	I	S	S	S	S	R	R	R	R	R	S	R
53	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
54	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
55	R	R	R	R	R	S	R	R	R	S	S	S	I	S	S	S	S	I	S	S
56	R	R	R	R	R	R	R	R	I	S	S	S	I	S	R	R	I	I	R	R
57	R	R	R	R	R	S	R	I	I	S	S	S	S	S	S	R	I	R	S	R
58	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
59	R	R	R	R	R	S	R	I	R	S	S	S	S	S	R	S	S	I	S	R
60	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	I	S	R
61	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	I	S	R
62	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	R
63	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	S	S	R	S	S
64	R	R	R	R	R	R	R	R	I	S	S	S	I	S	R	R	R	R	S	R
65	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	R	S	R	S	R
66	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	I	S	R
67	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
68	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	R	R
69	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	R
70	R	R	R	R	R	S	R	R	I	S	S	S	S	R	S	S	S	I	S	R
71	R	R	R	R	R	R	R	R	R	R	S	I	I	R	R	R	S	R	S	R
72	R	R	R	R	R	S	R	I	R	S	S	S	S	R	R	R	S	R	S	S
73	R	R	R	R	R	S	R	R	I	S	S	S	S	S	R	R	S	R	S	R
74	R	R	R	R	R	S	R	R	I	S	S	S	S	S	R	R	S	R	S	R
75	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	S	S	S	S	R

AMP: Ampicillin
AMC: Amoxicillin/Clavulanic Acid
TZP: Piperacillin/Tazobactam
CXM: Cefuroxime
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FOX: Cefoxitin
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CAZ: Ceftazidime
FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
GEN: Gentamycin
NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
R: Resistant
S: Susceptible
I: Intermediate
A: Information absent

Table C2: VITEK®2 automated system (bioMérieux, France) antibiotic susceptibility interpretations for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
76	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
77	R	R	R	R	R	S	R	R	R	S	S	S	I	R	R	R	S	R	S	R
78	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	R	S	R	S	R
79	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	I	R	S	R
80	R	R	R	R	R	S	R	I	I	S	S	S	I	R	S	S	S	R	S	R
81	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	S	R
82	R	R	R	R	R	S	R	R	I	S	S	S	S	R	S	S	S	R	S	R
83	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	R	S	I	S	R
84	R	R	R	R	R	S	R	I	I	S	S	S	I	R	R	R	S	I	S	R
85	R	R	R	R	R	S	R	I	R	S	S	S	S	R	R	R	S	R	S	R
86	R	R	R	R	R	S	I	I	I	S	S	S	S	S	S	S	S	R	S	S
87	R	R	R	R	R	S	R	I	I	S	S	S	S	S	R	S	S	R	S	S
88	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	R	S	R
89	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	S	S	R	S	S
90	R	R	R	R	R	R	R	R	R	S	S	S	I	R	S	S	S	R	S	R
91	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	R	S	R
92	R	R	R	R	R	S	R	R	R	S	S	S	S	R	R	R	S	R	S	R
93	R	R	R	R	R	S	R	I	I	S	S	S	S	R	S	S	S	I	S	R
94	R	R	R	R	R	S	R	R	R	S	S	S	S	R	R	S	I	R	S	R
95	R	R	R	R	R	S	R	R	R	S	S	S	I	R	R	R	S	R	S	R
96	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	S	R
97	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	S	I	S	R
98	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	R	I	I	S	R
99	R	R	R	R	R	S	R	I	I	S	S	S	S	R	R	S	S	I	S	S
100	R	R	R	R	R	S	I	I	I	S	S	S	S	R	S	S	S	I	S	S

AMP: Ampicillin
AMC: Amoxicillin/Clavulanic Acid
TZP: Piperacillin/Tazobactam
CXM: Cefuroxime
CXM-AX: Cefuroxime Axeti
FOX: Cefoxitin
CTX: Cefotaxime
CAZ: Ceftazidime
FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
GEN: Gentamycin
NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
R: Resistant
S: Susceptible
I: Intermediate
A: Information absent

Table C3: VITEK®2 automated system (bioMérieux, France) antibiotic MICs for *Klebsiella pneumoniae* isolates

Isolate nr	Antimicrobial MIC profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
1	>=32	16	64	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	16	2	1	64	<=0.5	>=320
2	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	128	<=0.5	>=320
3	>=32	8	32	>=64	>=64	<=4	>=64	4	4	<=0.5	<=1	<=0.25	>=64	>=16	>=32	>=4	<=0.5	256	<=0.5	80
4	>=32	>=32	>=128	>=64	>=64	32	>=64	16	4	<=0.5	<=1	<=0.25	16	<=1	>=32	<=0.25	>=8	32	<=0.5	>=320
5	>=32	8	8	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	16	<=1	8	1	<=0.5	32	<=0.5	40
6	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	4	>=16	4	<=0.25	1	128	<=0.5	>=320
7	>=32	16	>=128	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	256	<=0.5	>=320
8	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	<=0.5	128	<=0.5	>=320
9	>=32	8	<=4	>=64	>=64	<=4	>=64	8	2	<=0.5	<=1	<=0.25	<=2	<=1	4	<=0.25	1	128	<=0.5	<=20
10	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	2	>=512	<=0.5	>=320
11	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	8	>=16	>=32	>=4	2	256	<=0.5	>=320
12	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	16	>=16	8	2	<=0.5	128	<=0.5	>=320
13	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	2	256	<=0.5	>=320
14	>=32	8	<=4	>=64	>=64	<=4	>=64	4	2	<=0.5	<-=1	<=0.25	16	<=1	16	<=0.25	<=0.5	128	<=0.5	>=320
15	>=32	16	8	4	4	<=4	<=1	>=64	2	<=0.5	<=1	<=0.25	<=2	>=16	4	<=0.25	1	128	<=0.5	>=320
16	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	<=0.5	128	<=0.5	>=320
17	>=38	16	32	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	128	<=0.5	>=320
18	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	256	<=0.5	>=320
19	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	<=2	<=0.25	1	128	<=0.5	>=320
20	>=32	16	<=4	>=64	>=64	<=4	>=64	>=64	>64	<=0.5	<=1	<=0.25	<=2	>=16	8	<=0.25	<=0.5	<=16	<=0.5	>=320
21	>=32	16	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	8	>=16	16	2	1	128	<=0.5	>=320
22	>=32	>=32	>=128	>=64	>=64	32	>=64	>=64	>=64	>=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	>=512	<=0.5	>=320
23	>=32	4	<=4	8	8	8	<=1	2	<=1	<=0.5	<=1	<=0.25	<=2	<=1	8	<=0.25	1	128	<=0.5	40
24	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	32	<=0.5	<=1	<=0.25	<=2	8	>=32	>=4	<=0.5	>=512	<=0.5	>=320
25	>=32	16	>=128	>=64	>=64	<=4	32	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	128	<=0.5	>=320

AMP: Ampicillin
AMC: Amoxicillin/Clavulanic Acid
TZP: Piperacillin/Tazobactam
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CXM-AX: Cefuroxime Axetil
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CTX: Cefotaxime
CAZ: Ceftazidime
FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
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NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
A: Information absent

Table C3: VITEK®2 automated system (bioMérieux, France) antibiotic MICs for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
26	>=32	8	<=4	>=64	>=64	<=4	8	>=64	<=1	<=0.5	<=1	<=0.25	16	>=16	8	1	<=0.5	<=16	<=0.5	>=320
27	>=32	16	8	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	4	<=0.25	<=0.25	64	<=0.5	>=320
28	>=32	16	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	>=16	16	2	<=0.5	64	<=0.5	>=320
29	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	8	>=16	>=32	>=4	<=0.5	128	<=0.5	>=320
30	>=32	16	32	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	4	<=0.25	<=0.5	128	<=0.5	<=20
31	>=32	16	>=128	>=64	>=64	<=4	>=64	4	>=64	<=0.5	<=1	<=0.25	<=2	>=16	8	2	<=0.5	256	<=0.5	>=320
32	>=32	16	64	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	32	<=1	4	<=0.25	<=0.5	128	<=0.5	<=20
33	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	32	>=16	4	<=0.25	1	128	<=0.5	>=320
34	>=32	>=32	64	>=64	>=64	8	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	128	<=0.5	>=320
35	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	16	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	1	256	<=0.5	>=320
36	>=32	>=32	>=128	>=64	>=64	8	>=64	>=64	8	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	256	<=0.5	>=320
37	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	4	<=0.25	1	128	<=0.5	>=320
38	>=32	16	32	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	4	<=0.25	<=0.5	128	<=0.5	<=20
39	>=32	8	<=4	>=64	>=64	<=4	8	<=1	<=1	<=0.5	<=1	<=0.25	<=2	>=16	8	0.5	2	64	<=0.5	>=320
40	>=32	8	>=4	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	<=1	<=32	>=4	4	256	2	80
41	>=32	16	32	>=64	>=64	<=4	>=64	>=16	2	<=0.5	<=1	<=0.25	4	>=16	4	<=0.25	1	128	1	>=320
42	>=32	16	16	>=64	>=64	<=4	>=64	8	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	1	128	1	>=320
43	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	>=512	2	>=320
44	>=32	16	64	>=64	>=64	8	>=64	>=64	>=64	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	2	>=512	1	>=320
45	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	8	>=16	>=32	>=4	1	64	1	>=320
46	>=32	8	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	4	256	2	160
47	>=32	16	64	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	4	<=1	<=2	<=0.25	<=0.5	128	2	<=20
48	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	16	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	2	64	1	>=320
49	>=32	>=32	8	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	16	0.5	<=0.5	64	2	>=320
50	>=32	8	16	>=64	>=64	<=4	8	16	<=1	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	2	256	<=0.5	<=20

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CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
A: Information absent

Table C3: VITEK®2 automated system (bioMérieux, France) antibiotic MICs for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
51	>=32	16	16	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	4	<=0.25	1	128	<=0.5	<=20
52	>=32	>=32	>=128	>=64	>=64	16	>=64	>=64	16	<=0.5	<=1	<=0.25	4	>=16	>=32	>=4	>=8	256	2	>=320
53	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	2	>=512	<=0.5	>=320
54	>=32	16	64	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	4	>=16	>=32	>=4	<=0.5	128	<=0.5	>=320
55	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	32	<=1	<=2	<=0.25	<=0.25	64	<=0.5	<=20
56	>=32	>=32	>=128	>=64	>=64	>=64	32	>=64	2	2	<=1	<=0.25	16	<=1	>=32	>=4	4	64	8	>=320
57	>=32	8	8	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	<=1	<=2	>=4	4	256	<=0.5	80
58	>=32	16	64	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	4	>=16	>=32	>=4	1	128	2	>=320
59	>=32	16	>=128	>=64	>=64	<=4	>=64	16	32	<=0.5	<=1	<=0.25	4	<=1	>=32	0.5	<=0.5	64	2	>=320
60	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	1	64	<=0.5	>=320
61	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	4	>=16	<=2	<=0.25	<=0.5	64	1	>=320
62	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	1	128	<=0.5	>=320
63	>=32	4	<=4	>=64	>=64	<=4	4	<=1	<=1	<=0.5	<=1	<=0.25	<=2	>=16	16	0.5	1	128	<=0.5	40
64	>=32	>=32	>=128	>=64	>=64	>=64	32	>=64	2	2	<=1	<=0.25	16	<=1	>=32	>=4	>=8	128	<=0.5	>=320
65	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	16	>=16	>=32	>=4	1	128	<=0.5	>=320
66	>=32	16	16	>=64	>=64	<=4	>=64	8	2	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	1	64	<=0.5	>=320
67	>=32	16	64	>=64	>=64	8	>=64	16	4	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	2	>=512	<=0.6	>=320
68	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	<=16	>=132	>=4	1	64	4	>=320
69	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	4	>=16	4	<=0.25	1	128	<=0.5	>=320
70	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	16	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	1	64	<=0.5	>=320
71	>=32	>=32	>=128	>=64	>=64	>=64	>=64	>=64	>=64	>=8	<=1	1	16	>=16	>=32	>=4	2	>=512	<=0.5	>=320
72	>=32	16	64	>=64	>=64	<=4	>=64	16	>=64	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	1	>=512	<=0.5	40
73	>=32	4	<=4	>=64	>=64	<=4	>=64	>=64	16	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	1	128	<=0.5	>=320
74	>=32	16	32	>=64	>=64	<=4	>=64	>=64	16	<=0.5	<=1	<=0.25	<=2	<=1	>=32	>=4	2	256	<=0.5	>=320
75	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	16	<=0.5	<=1	<=0.25	<=2	>=16	16	<=0.25	1	32	<=0.5	>=320

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FEP: Cefepime
ETP: Ertapenem
IPM: Imipenem
MEM: Meropenem
AMK: Amikacin
GEN: Gentamycin
NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
A: Information absent

Table C3: VITEK®2 automated system (bioMérieux, France) antibiotic MICs for *Klebsiella pneumoniae* isolates (Continued)

Isolate nr	Antimicrobial susceptibility profile																		
	AMP	AMC	TZP	CXM	CXM-AX	FOX	CTX	CAZ	FEP	ETP	IPM	MEM	AMK	GEN	NAL	CIP	TGC	NIT	CST	SXT
76	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	>=512	<=0.5	>=320
77	>=32	>=32	>=128	>=64	>=64	8	>=64	>=64	>=64	<=0.5	<=1	<=0.25	8	>=16	>=32	>=4	2	256	<=0.5	>=320
78	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	16	>=16	>=32	>=4	2	128	<=0.5	>=320
79	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	128	<=0.5	>=320
80	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	<=2	<=0.25	1	128	<=0.5	>=320
81	>=32	16	16	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	4	>=16	16	2	1	64	<=0.5	>=320
82	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	8	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	<=0.5	128	<=0.5	>=320
83	>=32	16	16	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	>=32	>=4	1	64	<=0.5	>=320
84	>=32	16	64	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	8	>=16	16	2	1	64	<=0.5	>=320
85	>=32	>=32	>=128	>=64	>=64	<=4	>=64	16	>=64	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	256	<=0.5	>=320
86	>=32	<=2	<=4	16	16	8	<=1	2	<=1	1	<=1	<=0.25	<=2	<=1	<=2	<=0.25	<=0.5	128	<=0.5	<=20
87	>=32	8	<=4	>=64	>=64	<=4	8	<=1	2	<=0.5	<=1	<=0.25	<=2	<=1	16	1	<=0.5	128	<=0.5	<=20
88	>=32	16	>=128	>=64	>=64	<=4	>=64	16	4	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	<=0.5	256	<=0.5	>=320
89	>=32	16	32	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	8	<=0.25	1	128	2	<=20
90	>=32	>=32	>=128	>=64	>=64	16	>=64	>=64	>=64	<=0.5	<=1	<=0.25	8	>=16	<=2	<=0.25	1	256	2	<=320
91	>=32	16	32	>=64	>=64	<=4	>=64	8	2	<=0.5	<=1	<=0.25	<=2	>=16	16	2	1	128	2	>=320
92	>=32	>=32	>=128	>=64	>=64	<=4	>=64	>=64	32	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	<=0.5	>=512	1	>=320
93	>=32	16	16	>=64	>=64	<=4	>=64	8	2	<=0.5	<=1	<=0.25	<=2	>=16	<=2	<=0.25	<=0.5	64	2	>=320
94	>=32	>=32	>=128	>=64	>=64	8	>=64	>=64	>=64	<=0.5	<=1	<=0.25	4	>=16	16	<=0.25	4	128	<=0.5	>=320
95	>=32	16	32	>=64	>=64	<=4	>=64	>=64	>=64	<=0.5	<=1	<=0.25	16	>=16	>=32	>=4	1	256	<=0.5	>=320
96	>=32	16	64	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	4	>=16	16	2	1	64	<=0.5	>=320
97	>=32	16	16	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	16	2	1	64	<=0.5	>=320
98	>=32	16	>=128	>=64	>=64	<=4	>=64	16	2	<=0.5	<=1	<=0.25	<=2	>=16	>=32	>=4	4	64	<=0.5	>=320
99	>=32	16	8	>=64	>=64	<=4	>=64	4	2	<=0.5	<=1	<=0.25	<=2	>=16	8	1	<=0.5	64	<=0.5	<=20
100	>=32	8	<=4	4	4	<=4	2	2	<=1	<=0.5	<=1	>=0.25	<=2	>=16	<=2	<=0.25	<=0.5	64	<=0.5	<=20

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NAL: Nalidixic Acid
CIP: Ciprofloxacin
TGC: Tigecycline
NIT: Nitrofurantoin
CST: Colisitin
SXT: Trimethoprim/Sulphamethoxazole
A: Information absent

Table C4: The overall results for the PCR detection of β-lactamase genes in ESBL-producing *Klebsiella pneumoniae* isolates

Isolate number	B-lactamase genes												
	CTX-M Degen 1	CTX-M Degen 2	CTX-M Universal	TEM	SHV	OXA-1-Like	OXA-48-Like	NDM-1	KPC	VIM	IMP	IMI	VEB
1	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
2	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
3	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
4	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
5	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
6	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
7	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
8	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
9	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
10	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
11	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
12	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
13	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
14	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
15	-	-	L	+	+	+	+	-	NT	NT	NT	NT	NT
16	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
17	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
18	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
19	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
20	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
21	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
22	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
23	-	-	L	-	+	+	+	-	NT	NT	NT	NT	NT
24	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
25	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT

NT: Not tested L: Amplicon size larger than expected, re-tested negative F: Faint positive

Table C4: The overall results for the PCR detection of β-lactamase genes in ESBL-producing *Klebsiella pneumoniae* isolates (Continued)

Isolate number	B-lactamase genes												
	CTX-M Degen 1	CTX-M Degen 2	CTX-M Universal	TEM	SHV	OXA-1-Like	OXA-48-Like	NDM-1	KPC	VIM	IMP	IMI	VEB
26	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
27	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
28	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
29	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
30	+	-	+	+	+	+	-	-	NT	NT	NT	NT	NT
31	+	-	+	+	+	+	-	-	NT	NT	NT	NT	NT
32	+	-	+	+	+	+	-	-	NT	NT	NT	NT	NT
33	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
34	-	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
35	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
36	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
37	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
38	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
39	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
40	+	-	+	-	+	-	+	-	NT	NT	NT	NT	NT
41	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
42	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
43	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
44	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
45	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
46	+	-	+	-	+	-	+	-	NT	NT	NT	NT	NT
47	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
48	+	-	+	+	+	-	+	-	NT	NT	NT	NT	NT
49	+	-	+	+	+	F	+	-	NT	NT	NT	NT	NT
50	+	-	+	-	+	F	+	-	NT	NT	NT	NT	NT

NT: Not tested L: Amplicon size larger than expected, re-tested negative F: Faint positive

Table C4: The overall results for the PCR detection of β-lactamase genes in ESBL-producing *Klebsiella pneumoniae* isolates (Continued)

Isolate number	B-lactamase genes												
	CTX-M Degen 1	CTX-M Degen 2	CTX-M Universal	TEM	SHV	OXA-1-Like	OXA-48-Like	NDM-1	KPC	VIM	IMP	IMI	VEB
51	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
52	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
53	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
54	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
55	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
56	-	-	-	+	+	+	+	-	-	-	-	-	-
57	+	-	+	-	+	-	+	-	NT	NT	NT	NT	NT
58	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
59	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
60	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
61	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
62	+	-	+	+	+	+	-	-	NT	NT	NT	NT	NT
63	+	-	+	-	+	F	-	-	NT	NT	NT	NT	NT
64	-	-	-	+	+	F	+	-	NT	NT	NT	NT	NT
65	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
66	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
67	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
68	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
69	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
70	+	-	+	+	+	F	+	-	NT	NT	NT	NT	NT
71	+	-	+	-	+	+	+	-	-	-	-	-	-
72	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
73	+	-	+	-	+	-	+	-	NT	NT	NT	NT	NT
74	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
75	+	-	+	+	+	F	+	-	NT	NT	NT	NT	NT

NT: Not tested L: Amplicon size larger than expected, re-tested negative F: Faint positive

Table C4: The overall results for the PCR detection of β-lactamase genes in ESBL-producing *Klebsiella pneumoniae* isolates (Continued)

Isolate number	B-lactamase genes												
	CTX-M Degen 1	CTX-M Degen 2	CTX-M Universal	TEM	SHV	OXA-1-Like	OXA-48-Like	NDM-1	KPC	VIM	IMP	IMI	VEB
76	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
77	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
78	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
79	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
80	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
81	+	-	+	+	+	+	-	-	NT	NT	NT	NT	NT
82	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
83	+	-	L	-	+	+	+	-	NT	NT	NT	NT	NT
84	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
85	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
86	-	-	L	-	+	+	+	-	-	-	-	-	-
87	+	-	+	-	+	+	+	-	NT	NT	NT	NT	NT
88	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
89	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
90	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
91	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
92	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
93	+	-	+	+	+	F	+	-	NT	NT	NT	NT	NT
94	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
95	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
96	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
97	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
98	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
99	+	-	+	+	+	+	+	-	NT	NT	NT	NT	NT
100	-	-	L	+	+	+	+	-	NT	NT	NT	NT	NT

NT: Not tested L: Amplicon size larger than expected, re-tested negative F: Faint positive

Table C5: **The PCR results for β-lactamase detection in *K. pneumoniae* plasmid DNA for 15 isolates representing variable β-lactamase gene profiles**

Isolates	β-lactamase genes						
	CTX-M Degen 1	CTX-M Degen 2	CTX-M universal	TEM	SHV	OXA-1-Like	OXA-48-Like
1	-	-	+	+	+	+	-
2	+	-	+	+	+	+	-
5	+	-	+	+	+	+	-
15	-	-	-	+	+	-	-
20	+	-	+	-	-	+	-
23	-	-	-	-	+	+	-
48	+	-	+	+	+	+	-
52	+	-	+	+	+	+	-
56	-	-	-	+	+	+	-
63	+	-	+	-	+	+	-
64	+	-	+	+	+	+	-
71	-	-	-	-	+	+	-
86	-	-	-	-	+	+	-
87	+	-	+	+	+	-	-
98	+	-	+	-	+	+	-