Detection and characterization of ESBL positive *Klebsiella pneumoniae* clinical isolates

by

Gumanzi Isaac Manenzhe

Detection and characterization of ESBL positive *Klebsiella pneumoniae* clinical isolates

by

Gumanzi Isaac Manenzhe

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

Department of Medical Microbiology
Faculty of Health Sciences
University of Pretoria
South Africa

January 2012

I declare that the dissertation, which I hereby submit for the MSc (Medical Microbiology) degree at the University of Pretoria, is my original work and has not previously been submitted to any other institution of higher learning. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.

Signed_____this_____day of_____2012

Good and bad things happen; most are beyond our control. But whatever happens, I know that I have the reliable constant of a God who is prepared to work with me and through me to accomplish something positive. I'm convinced that faith will always be rewarded through this process; even if the 'why's' are never explained- Philip Yancey

ACKNOWLEDGEMENTS

I would like to sincerely thank:

Dr MM Kock, Department of Medical Microbiology, University of Pretoria, for being the best supervisor, for guiding me into the right direction, for her encouragement, for helping me with molecular typing techniques and for always being there for me.

Prof MM Ehlers, Department of Medical Microbiology, University of Pretoria, for her encouragement, for supporting me during ethics meetings and for being a wonderful co-supervisor.

Prof AA Hoosen, Department of Medical Microbiology, University of Pretoria, for his encouragement, dedications and moral support he showed for my work and for believing in me.

Dr NA Ismail, Department of Medical Microbiology, University of Pretoria, for his useful advise and the moral support he showed for my work.

Dr S Manda, Medical Research Council (MRC), for his assistance with statistical calculations and sample size.

Fellow MSc Students (Matsidi and Nontuthuko), for boosting my confidence and for always being there for me when I needed them most.

Department of Medical Microbiology, UP/NHLS, for the financial support and advanced training I received from them.

Medical Research Council (MRC) of South Africa, for the financial support, without the MRC scholarship it would have been difficult to complete this study.

National Research Foundation (NRF), for the financial support, without NRF block grants I would not have completed this study.

DEDICATIONS

I dedicate this project to God almighty for giving me strength and without whom this project would have not been successful.

I would also like to dedicate this project to the most understanding people in my life, who had the most understanding minds, which I cherish.

I express my sincere gratitude to my father (Samuel Manenzhe), for his endless support and for always being there for me, dad you are my hero. I would also like to thank my brother (Amos) and sisters (Peggy, Eunice, Mashudu and Pinky) for their moral support and for paving my way.

Thanks to my mother (Sarah Manenzhe) for being the most valuable inducer throughout my life and for her endless prayers. Mom, I am proud of you.

This work is also dedicated to the most important person in my life (Tracy) for her encouragement, unconditional love, for giving me time to concentrate on my project and for always being there for me during hard times. Lastly, I dedicate this project to my child (Tshedza).

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

PAGE

LIST OF TABLES

ABBREVIATIONS

AES	Advanced expert system
bp	Base pairs
BHI	Brain Heart Infusion
CAZ	Ceftazidime
CLSI	Clinical Laboratory Standards Institute
CTX	Cefotaxime
CTX-M	Cefotaximase
CV	Clavulanic acid
ESBL	Extended spectrum beta-lactamases
ERIC	Enterobacterial repetitive intergenic consensus
FEMS	Federation of European Microbiological Societies
FIDSSA	Federation of Infectious Diseases Societies of Southern Africa
h	Hour
hrs	Hours
ICU	Intensive care unit
kb	Kilobases
LPS	Lipopolysaccharide
MBL	Metallo beta-lactamase
MHT	Modified Hodge Test
MIC	Minimum inhibitory concentration
Min	Minute
NCCLS	National Committee for Clinical Laboratory Standards
NDM-1	New Delhi Metallo-beta-lactamase-1
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
NICU	Neonatal intensive care unit
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PLA	Pyogenic liver abscess
RAPD	Random amplified polymorphic DNA
REP	Repetitive extragenic palindromic sequence
SHV	Sulphydryl Variable
TAD	Tshwane Academic Division
TEM	Temoniera
UP	University of Pretoria
UTI	Urinary tract infections
UPGMA	Unweighted pair group method with arithmetic mean

LIST OF ARTICLES SUBMITTED FOR PUBLICATION AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

Manenzhe GI, Ehlers MM, Ismail NA and Kock MM (2011) Detection and characterization of ESBL-positive *Klebsiella pneumoniae* clinical isolates. To be submitted for publication to: *FEMS Immunology and Medical Microbiology.*

CONFERENCE PRESENTATIONS

Manenzhe GI, Ehlers MM, Ismail NA, Hove P, Hoosen AA and Kock MM (2009) Determination of the effectiveness of Vitek2 AST N067 card, in detecting the ESBL-positive isolates of *Klebsiella pneumoniae* and *Enterobacter* species at Steve Biko Academic Hospital. Faculty day, Faculty of Health Sciences, University of Pretoria, 15 August 2009 (Poster)

Manenzhe GI, Ehlers MM, Ismail NA and Kock MM (2010) Detection and characterization of ESBL-positive *Klebsiella pneumoniae* clinical isolates. Faculty day, Faculty of Health Sciences, University of Pretoria, 20 August 2010 (Poster)

Manenzhe GI, Ehlers MM, Ismail NA and Kock MM (2010) Detection and characterization of ESBL-positive *Klebsiella pneumoniae* clinical isolates. Medical Research Council (MRC), Annual Research Day, MRC Conference Center, Cape Town, 14-15 October 2010 (Oral)

Manenzhe GI, Ehlers MM, Ismail NA and Kock MM (2011) Identification of ESBL-positive *Klebsiella pneumoniae* isolates in the Pretoria region, South Africa. *Federation of European Microbiological Societies*, Geneva, Switzerland, 26-30 July 2011 (Poster)

Detection and characterization of ESBL-positive *Klebsiella pneumoniae* clinical isolates

by

Gumanzi Isaac Manenzhe

SUPERVISOR: **Dr MM Kock**
CO-SUPERVISOR: **Prof MM Ehlers**
DEPARTMENT: **Medical Microbiology**
DEGREE: **MSc Medical Microbiology**

SUMMARY

Klebsiella pneumoniae is an important nosocomial pathogen that has the potential to cause severe morbidity and mortality, particularly in intensive care units, as well as in medical and surgical wards. In recent years, following extensive use of the expanded-spectrum cephalosporins, outbreaks of infections caused by extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* isolates have been reported throughout the world. Laboratory detection of ESBL-producing bacteria is important, if left undetected it can result in treatment failure, leading to serious consequences, such as an increased mortality rate. It is essential for diagnostic laboratories to use rapid and reliable methods for the detection of ESBL-producing bacteria.

In this study, 150 *K. pneumoniae* clinical isolates were collected from the diagnostic laboratory of the Department of Medical Microbiology, National Health Laboratory Service (NHLS). The isolates were collected within a four month period (September 2009 to December 2009). The isolates were analyzed and characterized using phenotypic and genotypic methods. The aim of the study was to investigate the prevalence of ESBL-producing *K. pneumoniae* isolates. Secondly, to compare the sensitivity and specificity of the Vitek2 advanced expert system (AES) (bioMérieux, France) and Multiplex PCR assay (targeting bla_{SHV}, bla_{TEM} and $bla_{CTX\text{-}M}$ genes) against the combination disc method (Gold standard) in detecting ESBL-production. Lastly, the study investigated the clonal relatedness of the collected isolates using ERIC, REP and BOX PCR fingerprinting assays.

The combination disc method was performed and interpreted according to the Clinical and Laboratory Standards Institute 2009 guidelines. The prevalence of ESBL-positive *K. pneumoniae* isolates according to the combination disc method was 57.4% (85/148). The sensitivity and specificity of the Vitek2 AES in detecting ESBL production was 99% (84/85) and 98% (62/63) respectively when compared to the combination disc method. The sensitivity and specificity of the multiplex PCR assay (using $bla_{\text{CTX-M}}$ gene as a true marker for ESBL-production) were 96% (82/85) and 98% (62/63) respectively.

A good correlation was obtained between the three assays (combination disc method, Vitek2 AES and multiplex PCR assay) evaluated in this study. There is a high prevalence of ESBL-producing isolates in the Tshwane area. The high prevalence of ESBL-producing isolates is of great concern; proper infection control measures to reduce the spread of ESBL-producing *K. pneumoniae* isolates is recommended.

Genotyping revealed a high level of similarity among ESBL and non ESBL-producing *K. pneumoniae* isolates. However, there was no sign of an outbreak among the ESBL-producing *K. pneumoniae* isolates because ESBL and non-ESBL producing isolates were uniformly distributed in the groups obtained with the ERIC and BOX PCR dendrogrammes.

Key words: Extended spectrum beta-lactamase, *Klebsiella pneumoniae,* ESBL detection methods, genotyping.

CHAPTER 1

Introduction

Klebsiella pneumoniae is a Gram-negative rod, belonging to the bacterial family, *Enterobacteriaceae* (Podschun and Ullmann, 1998). *Klebsiella pneumoniae* is a common cause of community and hospital-acquired infections (Fung *et al*., 2000). It is among the leading hospital-acquired pathogens that commonly cause pneumonia and blood stream infections (BSI) (Marchaim *et al*., 2008). The rate of *K. pneumoniae* infections is increased in individuals with impaired host defences, such as malignancy, renal failure, diabetes mellitus and alcoholism (Murray *et al*., 2005).

Klebsiella pneumoniae remains the major ESBL-producing pathogen isolated worldwide (Pitout and Laupland, 2008). The ESBL enzymes render the bacteria resistant to most beta-lactam antibiotics (eg ceftazidime, cefpodoxime and aztreonam) (Bradford, 2001; Dubois *et al*., 2002). Furthermore, the ESBLs are known to be a large, rapidly evolving group of plasmid-mediated enzymes conferring resistance to the oxyimino-cephalosporins and monobactams (Dashti *et al*., 2009). Prior to the year 2001, there were more than 200 different ESBL enzymes classified in several groups (TEM, SHV, CTX-M and OXA) (Bradford, 2001). Now, more than 400 different ESBL enzymes have been identified (http://www.lahey.org/studies).

The increasing incidence of ESBL-producing *Enterobacteriaceae* worldwide has created a great need for accurate techniques that can be used to detect ESBL-producing isolates (Giamarellou, 2005). The CLSI (2009) recommends the use of the combination disc method and micro-dilution methods as confirmatory tests for detecting ESBL-production by *K. pneumoniae* isolates. However, other commercial assays, such as the ESBL E-test (AB Biodisc, Solna, Sweden), Vitek2 advanced expert system (AES) (bioMérieux, France) and the BD Phoenix system (Becton Dickinson Biosciences, Sparks, MD) have the ability to detect ESBL-producing bacteria (Stürenburg *et al*., 2004; Spanu *et al*., 2006; Drieux *et al*., 2008). Molecular methods, such as PCR and DNA sequencing can be used to detect ESBLs (Nyberg *et al*., 2008). However, DNA sequencing is not yet suitable for use in the routine diagnostic setting (Nyberg *et al*., 2008).

Outbreaks of ESBL-producing *K. pneumoniae* strains have been reported worldwide, these outbreaks often start in the intensive care units and then spread to other parts of the hospital by the usual transmission routes (eg contaminated hands of health care workers) (Bermudes *et al*., 1997). Epidemiological studies on the spread of *K. pneumoniae* isolates depend on the availability of sensitive and discriminative tests that permit differentiation between individual and epidemic strains (Hamouda *et al*., 2003). Traditionally used phenotypic typing methods lack reproducibility and discriminatory power and are not always useful in epidemiological typing studies (Podschun and Ullmann, 1998). Many investigators prefer using pulsed field gel electrophoresis (PFGE) to determine the clonality of strains involved in outbreaks of infections (Shah *et al*., 2004). However, other molecular typing techniques, such as ribotyping and rep-PCR also have the capacity to determine the clonality of *K. pneumoniae* isolates and have proven to be useful in epidemiological studies (Ahmad *et al*., 1999; Galani *et al.,* 2002; Paterson *et al*., 2004).

The use of a rapid and accurate method in detecting ESBL-producing isolates is crucial (Bush, 1996). As long as there is no rapid and reliable test to detect ESBL-producing isolates, patients will continue to be at risk of being treated with beta-lactam antibiotics that are not effective against ESBL-producing bacteria. The aim of this study was to compare the sensitivity and specificity of the Vitek2 AES (bioMérieux, France) and multiplex PCR assay against the combination disc method (gold standard) in the detection of ESBL-positive *K. pneumoniae* isolates. Secondly, the study investigated the prevalence of ESBL-producing *K. pneumoniae* isolates and determined the clonal relatedness of the *K. pneumoniae* clinical isolates.

The objectives of this study were

a) To collect 150 consecutive *K. pneumoniae* clinical isolates identified as ESBL-positive or negative by Vitek2 AES (bioMérieux, France)
b) To confirm the presence of ESBLs using the combination disc method
c) To extract DNA from *K. pneumoniae* isolates using a commercial DNA extraction kit (Zymogen, USA)
d) To perform a multiplex PCR reaction using total DNA extracted from *K. pneumoniae* isolates
e) To determine the clonal relatedness of the *K. pneumoniae* clinical isolates

f) To analyze the data and compare the sensitivity, specificity and accuracy of the three ESBL detection methods

References

Ahmad M, Urban C, Mariano N, Bradford PA, Calcagni E, Projan SJ, Bush K and Rahal JJ (1999) Clinical characteristics and molecular epidemiology associated with imipenem-resistant *Klebsiella pneumoniae*. *Clinical Infectious Diseases* **29**: 352-355.

Bermudes H, Arpin C, Jude F, El-Harrif Z, Bebear C and Quentin C (1997) Molecular epidemiology of an outbreak due to extended spectrum beta-lactamase producing *Enterobacteriaceae* in a French Hospital. *European Journal of Clinical Microbiology and Infectious Diseases* **16**: 523-529.

Bradford PA (2001) Extended-Spectrum beta-lactamases in the 21st Century: Characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* **14**: 933–951.

Bush K (1996) It is important to identify extended spectrum beta-lactamase producing isolates. *European Journal of Clinical Microbiology and Infectious Diseases* **15**: 361-364.

Clinical and Laboratory Standards Institute (2009) Performance standards for antimicrobial susceptibility testing: *Nineteenth Informational Supplement CLSI*, Wayne, PA, USA.

Dashti AA, Jadaon MM and Habeeb (2009) Can we rely on one laboratory test in detection of extended-spectrum beta-lactamase among *Enterobacteriaceae*? An evaluation of the Vitek2 system and comparison with four other detection methods in Kuwait. *Journal of Clinical Pathology* **62**: 739-742.

Drieux L, Brossier F, Sougakoff W and Jarlier V (2008) Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae*: review and bench guide. *Clinical Microbiology and Infection* **14**: 90-103.

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P and Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{GES-1} and a fused product of acc (3)-Ib/aac (6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **46**: 638-645.

Fung CP, Hu BR, Chang FY, Lee SC, Kuo BIT, Ho M, Siu LK and Liu CY (2000) A 5-year study of the seroepidemiology of *Klebsiella pneumoniae:* High prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy. *Journal of Infectious Diseases* **181**: 2075-2079.

Galani I, Xirouchaki E, Kanellakopoulou K, Petrikkos G and Giamarellou H (2002) Transferable plasmid mediating resistance to multiple antimicrobial agents in *Klebsiella pneumoniae* isolates in Greece. *Clinical Microbiology and Infection* **8**: 579-588.

Giamarellou H (2005) Multidrug resistance in Gram-negative bacteria that produce extended-spectrum beta-lactamases (ESBLs). *Clinical Microbiology Infections* **11**: 1-16.

Hamouda TB, Foulon T, Masmoudi ABC, Fendri C, Belhadi O and Mahrez KB (2003) Molecular epidemiology of an outbreak of multiresistant *Klebsiella pneumoniae* in a Tunisian neonatal ward. *Journal of Medical Microbiology* **52**: 427-433.

http://www.lahey.org/studies, accessed 24 September 2011.

Marchaim D, Navon-Venezia S, Schwaber MJ and Carmeli (2008) Isolation of imipenem-resistant *Enterobacter* species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. *Antimicrobial Agents and Chemotherapy* **52**: 1413-1418.

Murray PR, Rosenthal KS and Pfaller MA (2005) Medical Microbiology. 5th ed. Philadelphia, *Elsevier Mosby* pp: 335-336.

Nyberg SD, Meurman O, Jalava J and Jalava KR (2008) Evaluation of detection of ESBLs among *Escherichia coli* and *Klebsiella* spp isolates by Vitek2 AST-NO29 compared to the agar dilution and disc diffusion methods. *Scandinavian Journal of Infectious Diseases* **40**: 355-362.

Paterson DL, Ko WC, Gottberg AV, Mohapatra S, Casellas J M, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG and Yu VL (2004) Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of beta-lactamases. *Clinical Infectious Diseases* **39**: 31-39.

Pitout JDD and Laupland KB (2008) Extended spectrum beta-lactamase producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infectious Diseases* **8**:159-166.

Podschun R and Ullmann U (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews* **11**: 589–603.

Shah AA, Hasan F, Ahmed S and Hameed A (2004) Characteristics, epidemiology and clinical importance of emerging strains of gram-negative bacilli producing extended-spectrum beta-lactamases. *Research in Microbiology* **155**: 409-421.

Spanu T, Sanguinetti M, Tumbarello M, Dinzeo T, Fiori B and Posteraro B (2006) Evaluation of the new Vitek2 ESBL test for rapid detection of ESBL production in *Enterobacteriaceae* isolates. *Journal of Clinical Microbiology* **44**: 3257-3262.

Stürenburg E, Sobottka I, Noor D, Laufs R and Mack D (2004) Evaluation of new cefepine-clavulanate ESBL E-test to detect extended spectrum beta-lactamase in an *Enterobacteriaceae* strain collection. *Journal of Antimicrobial Chemotherapy* **54**: 134-138.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Klebsiella pneumoniae is an important pathogen, which commonly infect immunocompromised patients and cause severe infections, particularly in the neonatal intensive care units (NICUs) (Hart, 1993). It is one of the most common Gram-negative bacteria encountered by physicians worldwide and is among the top ten pathogens that commonly cause bloodstream infections (BSI) in the United States and Canada (Ko *et al*, 2002; Marra *et al*., 2006). The prevalence of *K. pneumoniae* ranges from 3% to 17% of all nosocomial bacterial infections (Sahly *et al*., 2004). *Klebsiella pneumoniae* has been described as a common community-acquired and hospital-acquired bacteria; causing urinary tract infections, hospital acquired pneumonia, intra-abdominal infections, bacteraemia and meningitis (Ko *et al*., 2002).

Most *K. pneumoniae* isolates are multidrug resistant, this resistance occurs as a result of extended spectrum beta-lactamase (ESBL) enzymes (Bonnet, 2004). Following the first report of an ESBL-producing *K. pneumoniae* isolate in 1983 in Germany, ESBL-producing *K. pneumoniae* isolates have been detected with increasing frequency (Hart, 1993; Gupta *et al*., 2004). Outbreaks of ESBL-producing *K. pneumoniae* isolates occur mainly in intensive care units and have been associated with a high mortality rate (Leroyer *et al*., 1997; Paterson *et al*., 2004).

In South Africa during the year 2010, ESBL-producing *K. pneumoniae* isolates were implicated in an outbreak that resulted in the death of six neonates at the Charlotte Maxeke Johannesburg Academic Hospital (Report of the Portfolio Committee on Health, 2010). The high rate of mortality associated with *K. pneumoniae* infections is mainly due to the antibiotic resistance of the ESBL-producing strains (Shah *et al*., 2004a). Extended spectrum beta-lactamase producing *K. pneumoniae* isolates are generally resistant to penicillins, first and second generation cephalosporins, including the third generation oxyimino-cephalosporins and monobactams (Livermore, 1995; Bradford, 2001; Bush, 2001).

The production of ESBLs usually occurs as a result of a point mutation in the $bla_{\text{sulfhydryl variable}}$ and/or $bla_{\text{temoneira}}$ genes, which alters the primary amino sequences of the respective beta-lactamase enzymes (Livermore, 1995; Bradford, 2001; Gniadkowski, 2008). However, other types of ESBLs have been identified, in particular, the cefotaximase (CTX-M) ESBLs (Bush, 2001). The CTX-M enzymes are now reported with increasing frequency throughout the world (Edelstein *et al*., 2003; Paterson *et al*., 2003; Bonnet, 2004). The CTX-M family consists of more than 100 beta-lactamases (http://www.lahey.org/studies), which are grouped on the basis of sequence similarity into five distinct clusters (subtypes), namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Paterson and Bonomo, 2005).

In addition to the ESBL-producing *K. pneumoniae* isolates, carbapenemase (KPC)-producing *K. pneumoniae* isolates have been reported in countries, such as America (Bradford *et al*., 2004; Naas *et al*., 2005) and South Africa (Coetzee *et al*., 2011). The carbapenemase enzyme renders the *K. pneumoniae* bacteria resistant to all beta-lactam agents, including penicillins, cephalosporins, monobactams and carbapenems (eg ertapenem and meropenem) (Moland *et al*., 2003). Yong and colleagues (2009) identified a novel class B carbapenemase called the New Delhi Metallo beta-lactamase-1 (NDM-1) in *K. pneumoniae* and *Escherichia coli* isolates recovered from a Swedish patient admitted to a hospital in New Delhi, India. The emergence of NDM-1 producing *K. pneumoniae* isolates is a great concern. Similar to other metallo beta-lactamases, the NDM-1 enzyme has the ability to make the bacteria resistant to beta-lactam antibiotics including carbapenem antibiotics that are normally used to treat infections caused by multidrug resistant strains (Poirel *et al*., 2010; Robert and Moellering, 2010). In Kenya, Poirel and colleagues (2011a) detected seven NDM-1-producing *K. pneumoniae* isolates from patients hospitalized between 2007 and 2009 in different wards at a referral and tertiary care center situated in Nairobi. Poirel and colleagues (2011a) have traced the NDM-1-producing *K. pneumoniae* detected in Kenya back to 2007, which is prior to the first report of NDM-1 in 2009 by Yong and colleagues. The NDM-1 producing isolates have also been detected in Morocco (Poirel *et al*., 2011b) and South Africa (Coetzee *et al*., 2011).

The detection of ESBL-producing *K. pneumoniae* isolates can be done using phenotypic and/or molecular methods (Falagas and Karageorgopoulos, 2009). Phenotypic detection methods (eg combination disc method, disc approximation test and ESBL E-test) are based on the resistance that ESBLs confer to oxyimino-beta-lactam antibiotics (eg ceftazidime,

cefotaxime and cefpodoxime) and the ability of the beta-lactamase inhibitor to block this resistance (CLSI, 2009; Falagas and Karageorgopoulos, 2009). The combination disc test is regarded as a confirmatory test for detecting ESBL-production (CLSI, 2009). However, the automated identification and antimicrobial test systems, such as the Vitek2 advanced expert system (AES) and BD Phoenix are used to detect ESBL-producing bacteria (Stürenburg and Mack, 2003).

The automated systems have an ESBL prediction test integrated into the automated procedure (Stürenburg and Mack, 2003). Studies evaluating these automated systems reported comparable results between these systems and conventional methods (Leverstein-van Hall *et al*., 2002; Spanu *et al*., 2006; Nyberg *et al*., 2007). The molecular methods, which can be used to detect ESBL-producing bacteria, include polymerase chain reaction (PCR), DNA probing, restriction fragment length polymorphism (RFLP), isoelectric focusing (IEF) and DNA sequencing (Stürenburg and Mack, 2003).

Transmission of *Klebsiella* strains between patients and contamination from hospital environmental sources are common (Jonas *et al*., 2004). As a result, typing of *Klebsiella* strains is needed in order to track isolates and control the transmission of these bacteria (Jonas *et al*., 2004). Several phenotypic and molecular typing techniques of *K. pneumoniae* isolates have been proposed (Brisse and Verhoef, 2001; Brisse *et al*., 2004). The phenotypic typing methods (eg serotyping and phage typing) are normally considered to have poor reproducibility and low discriminatory power (Podschun and Ullmann, 1998). Molecular typing methods (eg pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) and ribotyping) have successfully been used to type *Klebsiella* species (Paterson and Bonomo, 2005).

2.2 Characteristics of *Klebsiella pneumoniae*

Klebsiella pneumoniae has been a known human pathogen, since the late nineteenth century when it was first isolated by Edwin Klebs (Martínez *et al*., 2004). The bacterium is often called “Friedländers pneumonia” in honour of the first man (Carl Friedländer) who identified it as a significant respiratory pathogen in 1882 (Ristuccia and Cunha, 1984).

2.2.1 Scientific classification of *Klebsiella pneumoniae*

Klebsiella pneumoniae belongs to the family *Enterobacteriaceae* and the genus *Klebsiella* (Table 2.1) (Garrity *et al*., 2001). Historically, the classification of *Klebsiella* species was based on the origin of the bacterium or the pathogenic features of the bacteria (Martínez *et al*., 2004). Phenotypic features, such as substrate utilization and enzymatic activities were later proposed to be included in the classification of *Klebsiella* species (Martínez *et al*., 2004). In the Bergey's Manual of Systematic Bacteriology, the genus *Klebsiella* is classified into five species, namely *K. pneumoniae, K. oxytoca, K. planticola, K. terrigena* and *K. ornithinolytica.* (Ørskov and Ørskov, 1984; Drancourt *et al*., 2001). The taxonomic classification in Bergey's Manual of Systematic Bacteriology was based on numerical taxonomy and relied on the phenotypic and biochemical characters as well as the data derived from DNA-DNA hybridization studies (Drancourt *et al*., 2001).

Table 2.1 **Summary of the scientific classification of *K. pneumoniae* (Garrity *et al*., 2001; Euzéby, 2011)**

Rank	Scientific name
Kingdom	*Bacteria*
Phylum:	*Proteobacteria*
Class:	*Gamma Proteobacteria*
Order:	*Enterobacteriales*
Family:	*Enterobacteriaceae*
Genus:	*Klebsiella*
Species:	*K. pneumoniae*
Subspecies:	*K. pneumoniae* subsp *pneumoniae* *K. pneumoniae* subsp *ozaenae* *K. pneumoniae* subsp *rhinoscleromatis*

The sequencing of the 16S rDNA gene has been used to study the phylogenetic relationship between microorganisms and in species identification (Boye and Hansen, 2003). A phylogenetic study by Drancourt *et al*. (2001) demonstrated heterogeneity within the genus *Klebsiella* and suggested that the genus must be divided into two genera, with the *Raoultella* genus being proposed to replace *Klebsiella planticola, Klebsiella terrigena* and *Klebsiella ornithinolytica* species (Drancourt *et al*., 2001). In a study conducted by Boye and Hansen, (2003), the sequencing of the *Klebsiella* 16S rDNA revealed that there is five closely related clusters among *Klebsiella*.

Clinically, *K. pneumoniae* is the most important species of the genus *Klebsiella* (Brisse and Verhoef, 2001) and is further divided into three subspecies; namely, *Klebsiella pneumoniae* subsp *pneumoniae, Klebsiella pneumoniae* subsp *ozaenae* and *Klebsiella pneumoniae* subsp *rhinoscleromatis* (Table 2.1) (Podschun and Ullmann, 1998; Garrity *et al*., 2001). The division of *K. pneumoniae* into three subspecies suggested that this species consists of at least three different lineages as determined by tDNA-PCR (Lopes *et al*., 2007). This is in agreement with the ealier report in Europe by Brisse and Verhoef (2001).

2.2.2 Morphology, growth and metabolism of *K. pneumoniae*

Klebsiella pneumoniae is a non-motile, oxidase-negative, catalase-positive, Gram-negative rod-shaped bacterium (Ryan *et al*., 2004). The rods are straight, vary from 0.6 to 6.0 µm in length and are arranged singly, in pairs or in short chains (Figure 2.1).

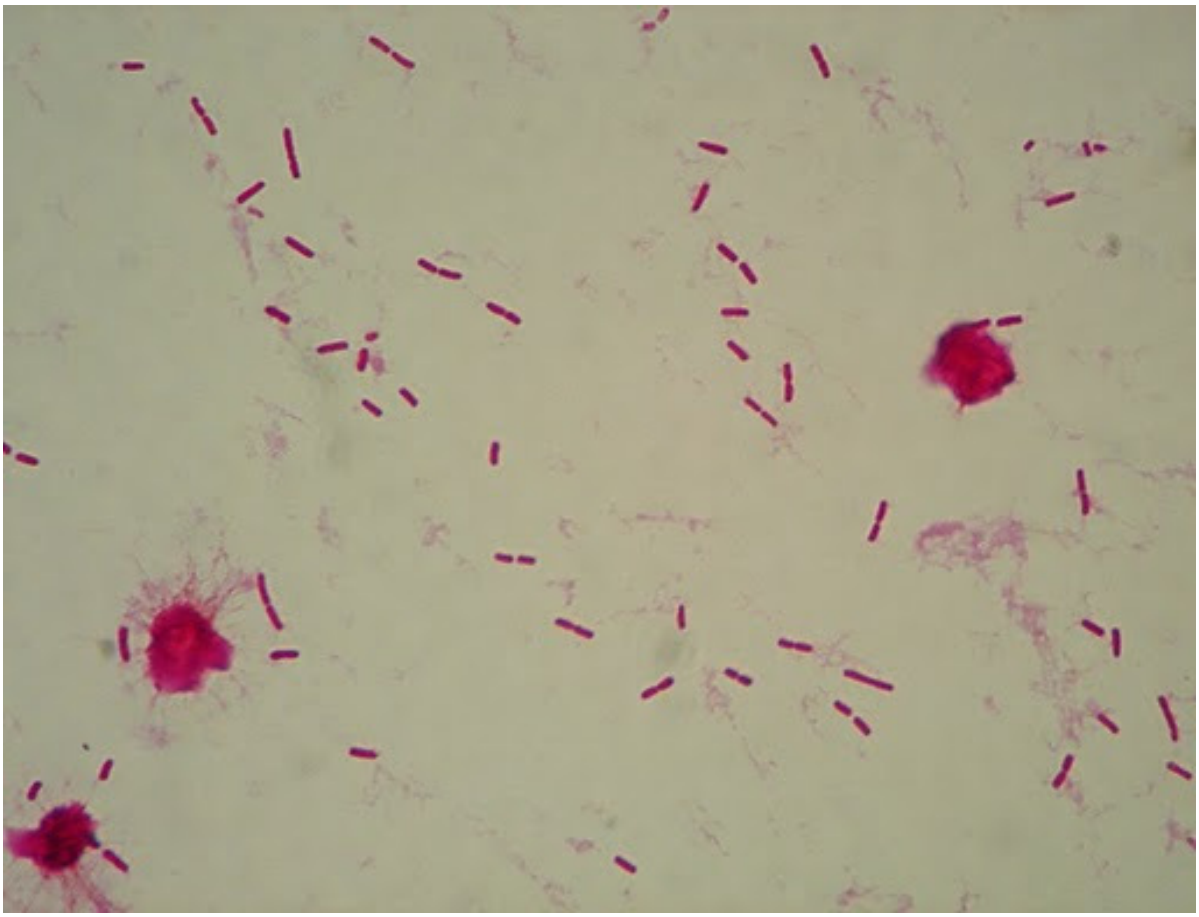

Figure 2.1: **Image of Gram-negative *Klebsiella pneumoniae* straight rods, visualized using the 100X objective under a light microscope. (http://www.picasaweb.google.com/taniyan260/Gramstaingallery#5547, accessed, 6 February 2011).**

The bacterium contains a large polysaccharide capsule, which gives rise to a large mucoid colony, especially on a carbohydrate-rich medium (Ryan *et al.*, 2004). It is a facultative anaerobic bacterium, capable of both aerobic and anaerobic respiration (Ryan *et al*., 2004). The optimal temperature for *K. pneumoniae* growth is 35˚C to 37˚C and the optimal pH is about 7.2 (Ristuccia and Cunha, 1984). *Klebsiella pneumoniae* is a lactose-fermenting bacterium and appears as large pink mucoid colonies on blood agar medium and MacConkey agar medium (Ristuccia and Cunha, 1984; Ryan *et al*., 2004). The bacterium is able to catabolize D-glucose and other carbohydrates with the production of acid and gas (Drancourt *et al*, 2001).

2.2.3 Pathogenesis and virulence factors of *K. pneumoniae*

Klebsiella pneumoniae has numerous virulence factors (Figure 2.2) that contribute to its pathogenesis (Podschun and Ullmann, 1998). The virulence factors produced may vary depending upon the site of the infection (Podschun and Ullmann, 1998).

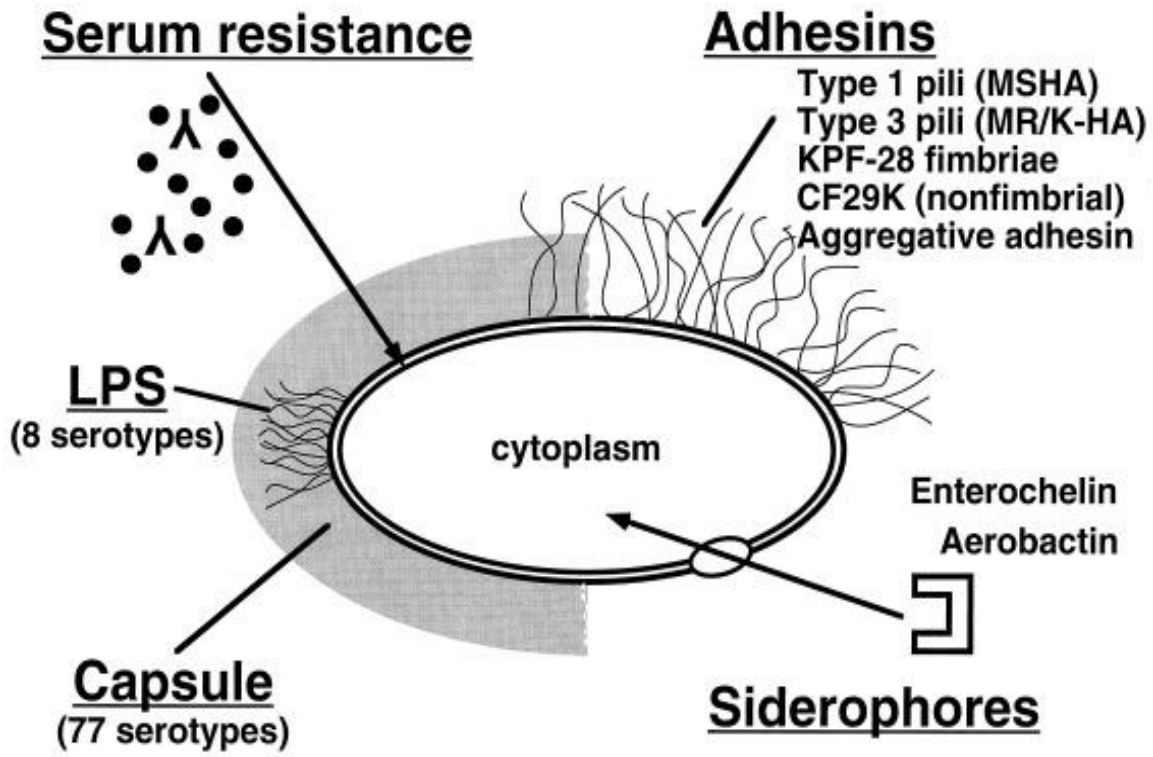

Figure 2.2: Schematic representation of the virulence factors of *K. pneumoniae* (Podschun and Ullmann, 1998).

These virulence factors include: fimbrial adhesins, siderophores, O-antigens, lipopolysaccharides (LPS) and capsular polysaccharides (CPS) (Figure 2.2) (Podschun and Ullmann, 1998). The adhesins allow the bacteria to attach to respiratory, gastrointestinal and urinary tract mucosal cells (Sharon and Ofek, 2002). Once the bacteria is inside the host cells, the CPS and cell surface LPS helps the bacteria to elude the bactericidal effect of serum and phagocytosis by polymorphonuclear leucocytes (toxins) (Podschun and Ullmann, 1998).

The siderophores supply the bacteria with iron and subsequently permit the multiplication of the bacteria within the iron-restricted environment of the host cells (Griffiths, 1980; Payne and Neilands, 1988). The major pathogenic factor of *K. pneumoniae* is its polysaccharide capsule (Blanchette and Rubin, 1980). Currently, about 80 different capsular (K) antigens of *K. pneumoniae* are known (Gupta *et al*., 2003).

The frequency and expression of *K. pneumoniae* virulence factors has been reported to differ between community-acquired bacterial strains and hospital-acquired bacterial strains in a Taiwanese study by Yu *et al*. (2006). The study noted that the capsular serotype K1 is more common among community-acquired bacterial strains (30%) versus hospital-acquired bacterial strains (14%) (Yu *et al*., 2006). In a series of patients from Taiwan presenting with *K. pneumoniae* bacteraemia, the hyperviscosity phenotype was found to be more common among community-acquired strains (49%) than in hospital-acquired strains (15%) (Yu *et al*., 2006).

2.2.4 Epidemiology of *K. pneumoniae*

Although *K. pneumoniae* can be found in soil and water, humans are the primary reservoir for this pathogenic bacterium (Cryz *et al*., 1986). The common sites of colonization in healthy humans are the gastrointestinal tract, eyes, respiratory tract and genitourinary tract (Podschun and Ullman 1998). The carrier rate of *K. pneumoniae* in humans has been reported to range from 5% to 38% in stool specimens and 1% to 6% in the nasopharynx (Yeh *et al*., 2007). The carrier rates are markedly increased in hospitalized patients; in whom the reported cases are 42% on hands, 19% in the pharynx and as high as 77% in the stool (Chung *et al.,* 2007). This high rate of colonization is primarily related to the use of antibiotics (Jenney *et al.,* 2006; Chung *et al.,* 2007).

During the 1980s, animal studies demonstrated that *K. pneumoniae* serotype K1 strains are highly virulent (Domenico *et al*., 1982; Ofek *et al*., 1993). In addition, Lin *et al*. (2004) reported that the K1/K2 isolates are more resistant to phagocytosis than non-K1/K2 isolates. A study conducted by Fang *et al*. (2007), reported that most primary liver abscesses (81%) were caused by K1 and most secondary liver abscesses (58%) were caused by non-K1 strains (P<0.001). The same study reported that patients infected with genotype K1 strains had a significantly higher risk for developing septic ocular or CNS complications than those infected with non-K1 strains (19% vs 5%; P= 0.007) (Fang *et al*., 2007).

Klebsiella pneumoniae has become an important cause of hospital-acquired infections, especially among patients in the neonatal intensive care unit (Hart, 1993; Podschun and Ullman 1998; Kim *et al*., 2002). The frequency of infections caused by ESBL-producing *K. pneumoniae* bacterial strains has increased drastically and it is clear that the ESBL-producing *K. pneumoniae* strains are distributed worldwide (Tofteland *et al*., 2007).

In several parts of the world the frequency of ESBL-producing *K. pneumoniae* ranges from 5% to 25% (Winokur *et al*., 2001; Paterson *et al*., 2003b). In Brazilian hospitals, the frequency of ESBL-producing *K. pneumoniae* is 45%, which is higher than that observed in many European and American hospitals (Gales *et al*., 2002). In Israel, the prevalence of ESBL-producing *K. pneumoniae* has been reported to be 32% (Bishara *et al*., 2005). Whereas, a study conducted in Southern Thailand reported an ESBL prevalence of 44% (16/36) in *K. pneumoniae* bacteria isolated from blood specimens (Jitsurong and Yodsawat, 2006). In Pakistan, the prevalence of ESBL-producing *K. pneumoniae* is very high, in a study done by Shah *et al.* (2004b), a prevalence of 70% was reported. In Asia and South Africa, the prevalence of ESBL-producing bacteria is reported to be more than 20% (Hirakata *et al*., 2005). Bell *et al*. (2002) reported that 36% of (13/36) of *K. pneumoniae* isolates collected in a single South-African hospital between 1998 and 1999 were ESBL-producers.

2.2.5 Clinical manifestations of *K. pneumoniae*

Klebsiella pneumoniae has been reported to cause community-acquired pneumonia and various hospital-acquired diseases, such as urinary tract infections, pneumonia, bacteraemia, cholangitis, meningitis, endocarditis and bacterial endophthalmitis (Paterson *et al*., 2003). The likelihood of *K. pneumoniae* hospital-acquired infections is greatly increased by the

presence of invasive devices, such as catheters in hospitalized patients (Menashe *et al*., 2001; Bisson *et al*., 2002).

2.2.5.1 Pneumonia

Patients infected with community-acquired pneumonia typically present with an acute onset of high fever, chills, flu-like symptoms and a productive cough with abundant, thick, tenacious, and blood-tinged sputum that is sometimes referred to as "currant jelly" sputum (Paterson *et al*., 2003; Kawai *et al*., 2006). Most of the pulmonary diseases caused by *K. pneumoniae* are in the form of bronchopneumonia or bronchitis (Paterson *et al*., 2003). Alcoholics and individuals with compromised pulmonary function are at an increased risk of developing pneumonia, this is due to the inability of these persons to clear aspirated oral secretions from the lower respiratory tract (Murray *et al*., 2005).

2.2.5.2 Urinary tract infections (UTIs)

Klebsiella is a frequent cause of UTIs (Brisse and Verhoef, 2001). In hospital-acquired infections, catheterization is believed to be an important factor in the incidence and spread of *Klebsiella* strains (Janda and Abbott, 2006). Furthermore, patients with diabetes, poor immune status or structural abnormalities are more prone to *K. pneumoniae* UTIs (Ristuccia and Cunha, 1984). Clinical features of UTI include frequency, dysuria, low back pain and suprapubic discomfort (Janda and Abbott, 2006). Systemic symptoms, such as fever and chills are usually indicative of a concomitant pyelonephritis (Ko *et al*., 2002).

2.2.5.3 Bacteraemia

Bacteraemia caused by ESBL-producing *K. pneumoniae* isolates is a major concern for clinicians due to the increased rate of treatment failure as well as the high mortality rate (Tumbarello *et al*., 2006). The frequent underlying conditions of *K. pneumoniae* bacteraemia include alcoholism, cirrhosis, diabetes mellitus, malignancies as well as ageing (Janda and Abbott, 2006). Patients with indwelling catheters, patients receiving antibiotic therapy and those undergoing invasive procedures are at the highest risk for *K. pneumoniae* bacteraemia (Korvick *et al*., 1992).

2.2.5.4 Liver abscess

Pyogenic liver abscess (PLA) has been described as a potentially life threatening disease with a reported mortality of up to 31% (Seeto and Rockey, 1996; Farges *et al*., 1998; Alvarez *et al*., 2001). Initially, *Escherichia coli* was regarded as the most common causative agent of PLA (Seeto and Rockey, 1996; Alvarez *et al*., 2001). However, Chan *et al.* (2007) reported in 2007 that *K. pneumoniae* has now become the most frequent causative agent for PLA. Chan and colleagues (2007) reported that 80% of PLA were caused by *K. pneumoniae* in a study conducted in China that included 84 patients. Pyogenic liver abscesses caused by *K. pneumoniae* isolates are regarded as an emerging disease (Keynan and Rubinstein, 2007). Most cases of *K. pneumoniae* liver abscesses have been reported in Taiwan (Keynan and Rubinstein, 2007). Chuang *et al*. (2006) reported that *K. pneumoniae* K1 serotype is the predominant serotype that causes primary pyogenic liver abscess (Chuang *et al*., 2006).

2.2.6 Treatment of *K. pneumoniae* infections

Management and treatment of ESBL-producing *K. pneumoniae* infections can be challenging (Gupta *et al*., 2003). The therapeutic choices for infections caused by ESBL-producing *K. pneumoniae* isolates remain extremely limited; this is largely due to the broad spectrum of the beta-lactamases commonly produced by *K. pneumoniae* strains (Paterson, 2000; Paterson *et al*., 2003b). In order to guide the selection of an effective treatment regimen, antibiotic susceptibility tests are done on all *K. pneumoniae* isolates (Brook *et al*., 2010).

Klebsiella pneumoniae has been reported to be naturally resistant to aminopenicillins and carboxypenicillins (carbenicillin and ticarcillin); this natural resistance is mainly due to the production of class A (low-level) beta-lactamases (Livermore, 1995). To overcome this low-level resistance, the extended spectrum antibiotics (third generation cephalosporins) were developed and used as the drug of choice for treating infections caused by *K. pneumoniae* isolates (Medeiros, 1997). However, since 1983 the occurrence of *K. pneumoniae* isolates that are resistant to the third generation cephalosporins have been reported with increasing frequency (Paterson *et al*., 2003).

The production of ESBLs by *K. pneumoniae* isolates resulted in resistance to many beta-lactam antibiotics (eg cefotaxime, ceftazidime and aztreonam) (Moland *et al*., 2008; Gröbner

et al., 2009). After the emergence of ESBL-production by *K. pneumoniae* isolates, carbapenem antibiotics (imipenem and meropenem) were considered to be the most reliable agents to treat infections caused by ESBL-producing *K. pneumoniae* isolates (Gröbner *et al*., 2009). The effectiveness and usefulness of carbapenem antibiotics was supported by the findings of a study conducted by Paterson *et al*. (2004).

However, the occurrence and spread of *K. pneumoniae* strains resistant to carbapenems (KPCs) have been reported in New York and other parts of the world (Galani *et al*., 2004; Hirch and Tam, 2010). The carbapenemases are able to hydrolyze the carbapenem antibiotics and confer resistance to a broad spectrum of antibiotics, including the penicillins, cephalosporins, carbapenems and monobactams (Hirsch and Tam, 2010). The optimal treatment of infection caused by carbapenemase-producing *K. pneumoniae* bacteria is yet to be determined (Hirsch and Tam, 2010). Colistin was once considered as being a toxic antibiotic; however, clinicians are now using it as a last resort antibiotic agent for treating infections caused by multidrug-resistant *K. pneumoniae* (Perez *et al*., 2007).

2.3 Mechanisms of antibiotic resistance in *K. pneumoniae*

Antibiotic resistance in *K. pneumoniae* can either be natural or acquired (Hogan and Kolter, 2002). Acquired resistance is caused by the acquisition of a plasmid and/or transposon that harbours determinants for resistance or by chromosome mutation (Hogan and Kolter, 2002). As a Gram-negative bacterium, *K. pneumoniae* becomes resistant to antibiotics by using either of the following major mechanisms of resistance: (i) modification of the target site, (ii) drug inactivation by the enzymes, (iii) reduced membrane permeability and (iv) efflux pumps (Hogan and Kolter, 2002).

2.3.1 Modification of target site

The most common mechanism of target alteration is the acquisition of new genes, carried on plasmids or transposons (Figure 2.3). These genes result in the enzymatic modification of the normal target so that it does not bind the antibiotics (Walsh, 2000).

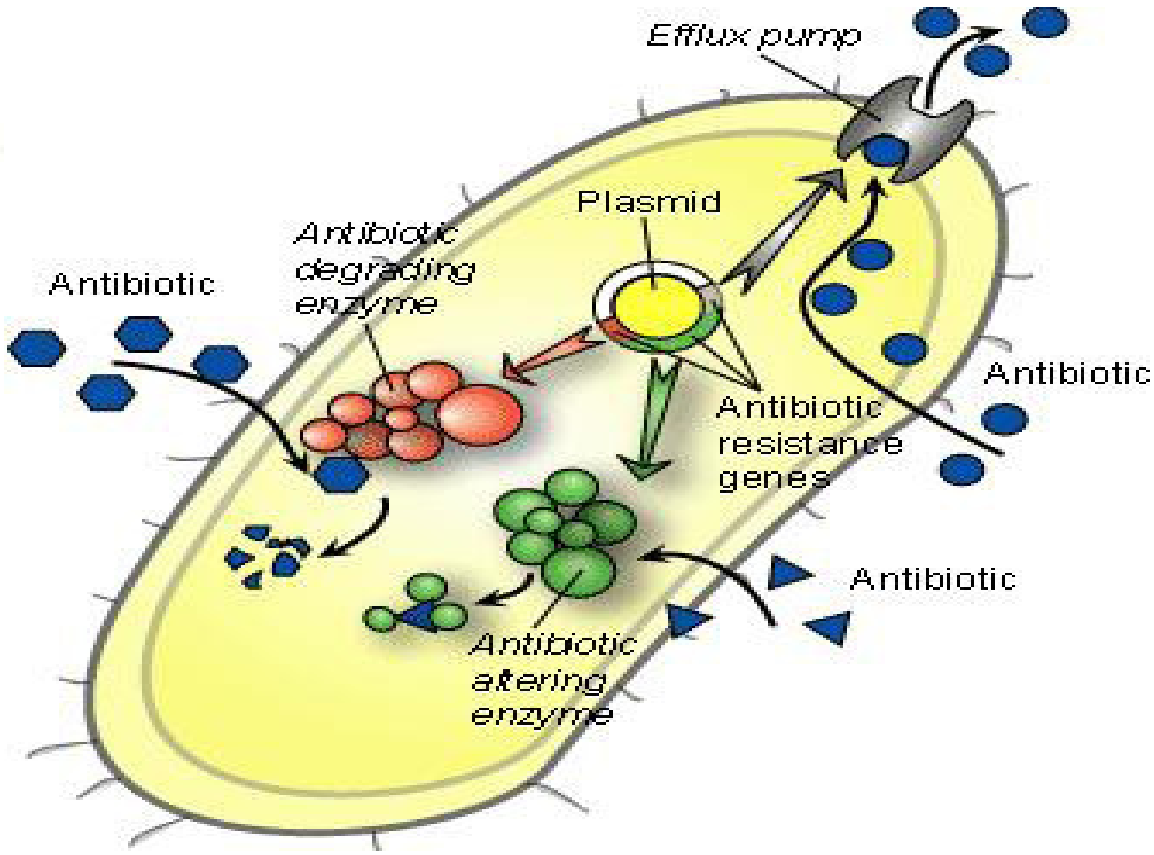

Figure 2.3: **A schematic representation of the mechanisms of bacterial resistance to antibiotics (www.textbookofbacteriology.net/resantimicrobiology, accesed 13 September 2009).**

After modification, the target site still retains its essential cellular function but is inaccessible by antibiotic inhibition (Chroma and Kolar, 2010). Beta-lactam antibiotic resistance in bacteria can be caused not only by beta-lactamase production, but also by alteration or mutation of the penicillin binding proteins (PBPs) that have a low affinity for beta-lactam antibiotics (Spratt, 1994).

2.3.2 Efflux pumps

Klebsiella pneumoniae can become resistant to antibiotics by preventing the access of antibiotics to their targets, this is done by either decreasing the influx or increasing the efflux of antibiotics across the biological membrane (Figure 2.3) (Nikaido, 1994). Mutants lacking an outer membrane porin channel (OprD2) decrease the uptake of a beta-lactam antibiotic and increase resistance to this antibiotic as a result of low accessibility of the drug to its target (Yoneyama and Nakae, 1991).

2.3.3 Inactivation of antibiotics

Enzymatic inactivation of antibiotics either by hydrolysis or by modification is one of the major natural mechanisms of resistance used by *K. pneumoniae* bacteria against antibiotics, such as beta-lactams (penicillins and cephalosporins) (Davies, 1994; Walsh, 2000). In most cases, the antibiotic-resistant *K. pneumoniae* isolates acquire the antibiotic resistance genes carried on plasmids (Davies, 1994).

2.4 The extended spectrum beta-lactamases of *K. pneumoniae*

Extended-spectrum beta-lactamases (ESBLs) are beta-lactamases produced by a variety of Gram-negative bacilli (Canton *et al*., 2002). The distinguishing feature of these enzymes is that compared with the broad-spectrum beta-lactamases, such as temoneira (TEM)-1, temoneira (TEM)-2 and sulfhydryl variable (SHV)-1, the ESBLs have extended substrate profiles that confer resistance to the expanded-spectrum cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime and others) and aztreonam (Gheldre *et al*., 2003). In summary, ESBLs are enzymes capable of conferring bacterial resistance to the penicillins, first, second and third generation cephalosporins as well as aztreonam by hydrolysing these antibiotics (Paterson and Bonomo, 2005).

2.4.1 Evolution of ESBLs

The ESBL enzymes arose as a result of amino-acid substitutions in Temoneira (TEM) or sulfyhydryl (SHV) or oxacillinases (OXA) of more limited hydrolytic activity (Stürenburg and Mack, 2003). The amino-acid substitution resulted in serious structural alterations within the active site of the protein and increased the beta-lactamase activity towards the third generation cephalosporins (Stürenburg and Mack, 2003).

2.4.2 Classification of beta-lactamases

Two systems (Ambler and Bush-Jacoby-Medeiros) are generally used to classify beta-lactamases (Perez *et al*., 2007). The Ambler system classify beta-lactamases according to their primary structure into four molecular classes designated A to D (Ambler, 1980; Ambler *et al*., 1991) whereas, the Bush-Jacoby-Medeiros system classifies beta-lactamases into four

groups (1 to 4) and six subgroups (a to f) (Table 2.2) based on their substrate spectrum and their susceptibility to inhibition by clavulanate (Bush *et al.*, 1995).

Table 2.2: Classification of beta-lactamases (Ambler *et al.*, 1991; Bush *et al*., 1995; Perez *et al*, 2007)

Bush-Jacoby-Medeiros system	Major	Ambler system	Main attributes
Group 1 cephalosporinase	-	C (cephalosporinase)	Usually chromosomal; resistant to all beta-lactams, except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (clavulanic acid susceptible)	2a	A (Serine beta-lactamases)	Staphylococcal penicillinases
	2b	A	Broad-spectrum-TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum-TEM: 3-160, SHV: 2-101
	2br	A	Inhibitor resistant TEM (IRT)
	2c	A	Carbenicillin-hydrolysing
	2e	A	Cephalosporinase inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D (oxacillin-hydrolyzing)	Cloxacillin-hydrolysing (OXA)
Group 3 metallo-beta-lactamase	3a	B (metallo-beta-lactamases)	Zinc-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

The Bush-Jacoby-Medeiros classification scheme (Table 2.2) is of more immediate relevance to the physician or microbiologist in a diagnostic laboratory because it considers the beta-lactamase and beta-lactam substrates that are of clinical relevance (Bush *et al*., 1995). In this classification, the ESBLs are classified into group 2be or group 2d (OXA-type) (Table 2.2) (Bush *et al*., 1995). Group 2d shares most of the fundamental properties of group 2be enzymes though differing in being inhibitor resistant (Bush *et al*., 1995). The 2be designation shows that these enzymes are derived from group 2b β-lactamases (eg TEM-1, TEM-2 and SHV-1); the ‘e’ of 2be denotes the extended spectrum of the beta-lactamases (Stürenburg and Mack, 2003). The ESBLs derived from TEM-1, TEM-2 or SHV-1 differs from their progenitors by as few as one amino acid (Stürenburg and Mack, 2003).

2.4.3 Diversity of ESBLs type

Extended spectrum beta-lactamases have been classified into different types (Canton and Conque, 2006). Initially, the two most frequent types of ESBL were the TEM types and the SHV types (Canton and Conque, 2006). However, another type referred to as the

cefotaximase (CTX-M) beta-lactamases are detected with increasing frequency (Rodrĭguez-Banŏ *et al*., 2006; Perez *et al*., 2007).

2.4.3.1 Cefotaximases type

The cefotaximase (CTX-M) enzyme is a described family of the ESBLs (Tzouvelekis *et al*., 2000). The enzyme has a potent hydrolytic activity against cefotaxime (Tzouvelekis *et al*., 2000; Bonnet, 2004). The CTX-M-type enzymes are typical ESBLs and have been classified in the functional group 2be of the Bush-Jacoby-Medeiros classification scheme (Tzouvelekis *et al*., 2000). The CTX-M enzymes can be classified into five major groups (Group I to V) on the basis of their amino acid sequence homologies (Parveen *et al*., 2011). Group I includes CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-11, CTX-M-12, CTX-M-15, CTX-M-22, CTX-M 23, CTX-M-28, CTX-M-29 and CTX-M 30 (Parveen *et al*., 2011). Group II includes CTX-M-2, CTX-M-4, CTX-M-7, Toho-1 and CTX-M-20 (Parveen *et al*., 2011). Group III includes CTX-M-8 (Parveen *et al*., 2011). Group IV includes CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-19, CTX-M-21 and CTX-M-27 (Parveen *et al*., 2011). Lastly, Group V consists of CTX-M-25 and CTX-M-26 (Parveen *et al*., 2011). The CTX-M-2 was shown to be 95% similar to the chromosomally encoded beta-lactamase of *Kluyvera ascorbata* and the CTX-M-8 was shown to be 100% similar to the chromosomally encoded beta-lactamase of *Kluyvera georgiana*, respectively (Humeniuk *et al*., 2002).

The first two CTX-M enzymes (CTX-M-1 and CTX-M-2) were reported in Europe in 1989 (Barthélémy *et al*., 1992). Since then the number of CTX-M beta-lactamases has rapidly increased (Tzouvelekis *et al*., 2000). The CTX-M enzymes have been identified in various countries of North America, Asia, Europe, South America and Africa (Cao *et al*., 2002; Radice *et al*., 2002). Currently, more than 100 variants of CTX-M-type beta-lactamases have been identified mostly in enterobacterial species, such as *E. coli*, *K. pneumoniae* and *Salmonella* species (http://www.lahey.org/studies). *Klebsiella pneumoniae* bacteria producing CTX-M beta-lactamases have been reported to have a cefotaxime MIC range of >64 µg/ml (Perez *et al*., 2007). The ceftazidime MICs for *K. pneumoniae* producing CTX-M enzymes are usually in the susceptible range of 2 to 8 µg/ml (Perez *et al*., 2007).

The CTX-M 15 is the most dominant CTX-M enzyme in the world and the primary cause of acquired resistance to third generation cephalosporins in *Enterobacteriaceae* (Canton and Conque, 2006; Livermore *et al*., 2007). The CTX-M-15 enzyme was first reported in India in the mid 1990s (Walsh *et al*, 2003).

2.4.3.2 Temoneira type

The word Temoneira (TEM) refers to the name of the patient in which the first TEM-1 was isolated from in the early 1960s (Giamarellou, 2005). The TEM-1 beta-lactamase is one of the most commonly encountered beta-lactamases in Gram-negative bacteria (Giamarellou *et al*., 2005). Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Livermore, 1995). Although the TEM-type ESBLs are most commonly found in *K. pneumoniae* and *E. coli*, these enzymes have been reported in other Gram-negative bacteria, such as *Enterobacter cloacea*, *Salmonella* species and *Proteus mirabilis* (Livermore, 1995). The TEM-3, originally reported in 1989, was the first TEM-type beta-lactamase that displayed the ESBL phenotype (Shah *et al*., 2004a). Since that first report over 130 additional TEM derivatives have been described (http://www.lahey.org/studies). Some of these beta-lactamases are inhibitor resistant enzymes, but the majority of the new derivatives are ESBLs (Bradford, 2001).

2.4.3.3 Sulfhydryl variable type

The Sulfhydryl variable (SHV) enzyme, named after the "sulfhydryl variable" active site, is commonly associated with *K. pneumoniae* (Shah *et al*., 2004a). This enzyme is responsible for up to 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae* (Shah *et al*., 2004a). At first the *K. pneumoniae* bacteria contained a single ESBL gene, but now multiple ESBL genes are commonly present in a single strain (Samaha-Kfoury and Araj, 2003). Recently, more than 120 SHV-type beta-lactamases have been identified worldwide (http://www.lahey.org/studies). However, unlike the TEM-type beta-lactamases, there are relatively few derivatives of SHV (Bradford, 2001). The majority of SHV-type derivatives possess the ESBL phenotype (Bradford, 2001). The inhibitor-resistant SHV phenotype has been reported (Shah *et al*., 2004a).

2.4.3.4 Oxacillin-hydrolyzing type

The oxacillin-hydrolyzing (OXA) type enzymes are another widespread family of beta-lactamases and have been found mainly in *Pseudomonas aeruginosa* (Hall *et al*., 1993; Philippon *et al*., 1997). The OXA-type beta-lactamases differ from the TEM and SHV enzymes in that they belong to molecular class D and functional group 2d (Shah *et al*., 2004a). While some oxacillinases present a significant degree of amino acid identity, the overall amino acid identity within the OXA class is only 20% to 30%. Thus, the OXA family represents a phenotypic rather than a genotypic group (Bradford, 2001). The OXA beta-lactamases contain active site serine groups, as do class A and class C beta-lactamases. The OXA beta-lactamases usually confer resistance to penicillins and possess a high-level hydrolytic activity against cloxacillin, oxacillin and methicillin (Bradford, 2001). The activities of the OXA enzymes are poorly inhibited by clavulanate, except for OXA-18 (Strürenburg and Mark, 2003).

The OXA-48 enzyme is one of the few members of the OXA family with the ability to hydrolyze carbapenem antibiotics (Poirel *et al*., 2004). The OXA-48 enzyme was first described in *K. pneumoniae* bacteria isolated from Turkey in 2004 (Carrër *et al*., 2008). Since then the gene ($bla_{\text{OXA-48}}$) that code for the OXA-48 enzyme is continuously spreading, particularly in Europe and the Middle East (Carrër *et al*., 2010). Recently, O'Brien and colleagues (2011) detected five OXA-48-producing *K. pneumoniae* isolates in an Ireland tertiary hospital.

Most of the OXA beta-lactamase genes are plasmid, transposon or integron located, thus providing them a means for a wide distribution (Shah *et al*., 2004a). In addition to the OXA-type ESBLs, a number of OXA derivatives that are not ESBLs have also been described (Shah *et al*., 2004). These include OXA-22, OXA-24, OXA-25 and OXA-30 families of beta-lactamases (Shah *et al*., 2004a).

2.4.3.5 Carbapenemases and other ESBL types

Carbapenemases are beta-lactamases, which have the ability to hydrolyze penicillin, cephalosporins, monobactams and carbapenems (Nordmann *et al*., 2009). According to the Ambler classification, carbapenemases may belong to class A, B or D (Ambler *et al*., 1991).

Class A and D carbapenemases are similar to ESBLs in that they have a serine group at the active site (Queenan and Bush, 2007). The class B carbapenemases are known as metallo-beta-lactamases because they use zinc as a co-factor (Queenan and Bush, 2007). *Klebsiella pneumoniae* carbapenemase (KPC) of molecular class A was first isolated in 1996 from a patient in North Carolina (Yigit *et al*., 2001). Since then the KPC has become the most prevalent enzyme in the United States and is now considered endemic in certain parts of New York and New Jersey (Landman *et al*., 2007; Nordmann *et al*., 2009). South Africa has recently become the first country on the African continent to report the KPC-production among *Enterobacter cloacae* and *K. pneumoniae* isolates (Coetzee *et al*., 2011).

A novel metallo beta-lactamase called the New Delhi metallo beta-lactamase (NDM-1) has been identified in 2008 from *K. pneumoniae* bacteria isolated from a Swedish woman admitted to a hospital in New Dehli (Yong *et al*., 2009). Yong *et al*. (2009) postulated that the NDM-1 appears to have originated in India (Yong *et al*., 2009). However, since the first report of NDM-1 enzyme by Yong and colleagues (2009), *Enterobacteriaceae* isolates harbouring NDM-1 enzymes have been reported in other parts of the world, such as Pakistan, United States, France, China, Israel, Turkey, Australia and Africa (Robert and Moellering, 2010; Poirel *et al*., 2011). Following reports from Kenya (Poirel *et al*., 2011) and Morocco, South Africa became the third African country to report the emergence of the NDM-1-producing *K. pneumoniae* isolate (Coetzee *et al*., 2011). Recently, an outbreak of NDM-1-producing bacterial isolates has been reported in a Gauteng Provincial Hospital (National Institute for Communicable Diseases, 2011). This outbreak resulted in the death of two patients; however, both patients had an underlying chronic illness (National Institute for Communicable Diseases, 2011).

The discovery of *Enterobacteriaceae* isolates harbouring NDM-1 is of great concern because these isolates possess resistance to most of the commonly used antibiotics, such as beta-lactams, fluoroquinolones and aminoglycosides (Mulvey *et al*., 2011). Furthermore, the NDM-1-producing isolates have the ability to spread rapidly because the NDM-1 encoding gene is located on a plasmid (a 180-kb plasmid for *K. pneumoniae*) that is easily transferable to susceptible bacteria (Yong *et al*., 2009; Centers for Disease Control and Prevention, 2010).

In addition to the KPC and NDM-1 enzymes, other unusual beta-lactamase enzymes (eg PER and VEB) that have extended-spectrum activity have been discovered (Bradford, 2001; Lu

et al., 2010; Bush and Fisher, 2011). The emergence of these novel groups of beta-lactamases indicates the abundance of beta-lactamase genes that are available in the bacterial gene pool (Bradford, 2001; Stürenburg and Mack, 2003; Lu *et al*., 2010).

2.5 Risk factors for colonization and infection with ESBL-producing *K. pneumoniae*

Patients at risk of being colonized and infected with ESBL-producing *K. pneumoniae* bacteria are those who are in the intensive care units (Paterson and Bonomo, 2005). In addition, other risk factors associated with infection and colonization with ESBL-producing *K. pneumoniae* bacteria include: previous hospitalization, prolonged hospital stay, increased severity of illness, mechanical ventilation, urinary catheters and prior exposure to antimicrobial agents, especially the oxyimino beta-lactam antibiotics (Lautenbach *et al*., 2001; Menashe *et al*., 2001; Bisson *et al*., 2002; Kim *et al*., 2002; Stürenburg and Mack, 2003). In a case-control study conducted by Graffunder *et al*. (2005) it was concluded that prolonged hospital stay, poor ventilation and prior-exposure to third generation cephalosporins increased the risk of infection with ESBL-producing bacteria.

2.6 Laboratory detection of ESBL-producing *K. pneumoniae*

The detection of ESBL-producing *K. pneumoniae* strains can be done using phenotypic or genotypic tests (Falagas and Karageorgopoulos, 2009). Phenotypic tests are mainly used in the routine clinical laboratories, whilst the genotypic tests are mainly used in reference or research laboratories (Falagas and Karageorgopoulos, 2009). The CLSI (2009) recommends a variety of phenotypic screening and confirmatory tests to detect the presence of ESBLs in *K. pneumoniae* isolates. These tests include the combination disc assay and the micro-dilution method, where the growth of bacteria is measured in the presence of five different advance-generation cephalosporins together with or in the absence of a beta-lactamase inhibitor (Helfand and Bonomo, 2005).

2.6.1 Phenotypic detection of ESBL-producing *K. pneumoniae*

The detection of ESBL-producing *K. pneumoniae* bacteria by phenotypic methods usually includes screening for reduced susceptibility to third-generation cephalosporins followed by confirmatory testing (Falagas and Karageorgopoulos, 2009). The screening step involves

testing for reduced susceptibility to cefpodoxime (≤ 17 mm), cefotaxime (≤ 27 mm), ceftriaxone (≤ 25 mm) and ceftazidime (≤ 22 mm) by the bacterial isolate (CLSI, 2009). The screening test can be performed using broth dilution and / or disc diffusion methods as recommended by the CLSI guidelines (CLSI, 2009). The sensitivity of the screening methods can vary depending on the type of antimicrobial agent used for screening (Ho *et al*., 2000).

The confirmatory step for ESBL detection is based on the demonstration of synergy between third-generation cephalosporins and clavulanic acid (Drieux *et al*., 2008; CLSI, 2009). The tests that can be used to confirm ESBL-production by *K. pneumoniae* strains include the combination disc method, double disc approximation test, minimum inhibitory concentration (MIC) test and the E-test ESBL strips (CLSI, 2009; Falagas and Karageorgopoulos, 2009).

Semi-automated systems, such as the Vitek2 AES (bioMérieux, France) and the BD Phoenix automated system (Becton Dickinson Diagnostic Systems, Sparks, MD) can also be used to detect ESBL-production by the bacterial isolates (Spanu *et al*., 2006; Nyberg *et al*., 2008). These automated systems have an ESBL prediction test integrated into the automated procedure (Stürenburg and Mark, 2003). The performance of these automated systems is variable (Wiegand *et al*., 2007). However, many studies have reported a good correlation between the conventional methods and the automated systems in detecting ESBL-producing *K. pneumoniae* bacterial isolates (Spanu *et al*., 2006; Nyberg *et al*., 2008).

2.6.1.1 Double disc approximation method

The double disc approximation method is one of the first methods used to detect ESBL-production by bacterial isolates (Giamarellou, 2005). The double disc approximation test was initially designed to differentiate between the cefotaxime resistant strains (eg those over-producing cephalosporinase) and those producing ESBLs (Drieux *et al*., 2008). In the double disc approximation test, an antibiotic disc containing amoxycillin-clavulanate is placed in the center of the plate and two discs containing one of the oxyimino-beta-lactam antibiotics are placed 30 mm apart from the amoxicillin-clavulanic disc (Figure 2.4) and incubated overnight at 37ºC (Shah *et al*., 2004b).

Figure 2.4: **Detection of ESBL-production using the double disc method. The disc on the left is cefotaxime (30 mg): the disc in the centre is amoxicillin-clavulanate (20+10 mg), the disc on the right is ceftazidime (30 mg). Note the expansion of the zones around the cefotaxime and ceftazidime discs adjacent to the amoxicillin-clavulanate (BSAC: British Society for Antimicrobial Chemotherapy, 2003)**

After the overnight incubation, the enhancement of the zone of growth inhibition of the oxyimino-beta-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disc indicates a positive result for ESBL-production (Shah *et al*., 2004a). The double disc approximation test has been reported to work well with a broad range of enterobacterial species and ESBL types and it is regarded as a reliable method to detect ESBL-production by bacteria (Drieux *et al*., 2008).

2.6.1.2 Combination disc methods

The combination disc method is one of the methods recommended by the CLSI guidelines as a confirmatory test for detecting ESBL-production by *K. pneumoniae* strains (CLSI, 2009). The principle of the combination disc method is to measure the zone of inhibition around a cephalosporin disc alone (e.g. cefotaxime or ceftazidime) and around a disc of the same cephalosporin plus clavulanate (Drieux *et al*., 2008). The Mueller-Hinton agar plate is inoculated with the bacterial isolates using the Kirby-Bauer method (CLSI, 2009). After inoculation, the pairs of discs containing an extended-spectrum cephalosporin (cefotaxime, ceftazidime or cefpodoxime) with and without clavulanic acid are placed on opposite sides of the inoculated plate (Figure 2.5) (CLSI, 2009; Haque and Salam, 2010).

Figure 2.5: **A Mueller-Hinton agar plate showing an ESBL-positive bacterial isolate detected using the combination disc method. Note the expansion of the zone diameters around the cefotaxime (CTX) and ceftazidime (CAZ) discs when tested in the presence of clavulanic acid (CV) versus the zone diameters for the agents tested alone (B). A ≥ 5 mm in zone diameter for at least one combination disc indicates ESBL-production (Haque and Salam, 2010).**

The zones of growth inhibition around the discs are measured following overnight incubation in ambient air at 37ºC (CLSI, 2009). Depending on the particular product used, the tested bacterial isolate is regarded as ESBL-positive if the zone of inhibition around the combination disc is at least 5 mm larger than that of the cephalosporin disc alone (CLSI, 2009), or if the zone diameter is expanded by 50% in the presence of the clavulanic acid (Figure 2.5) (Carter *et al*., 2000; Haque and Salam, 2010). Linscott and Brown (2005) reported a sensitivity and specificity of 96% and 100% respectively for the combination disc method in detecting ESBL-production by *Enterobacteriaceae* when compared to the broth microdilution method (Linscott and Brown, 2005).

2.6.1.3 Broth microdilution

The confirmatory testing for ESBL-production in *K. pneumoniae* isolates can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 μg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 μg/ml), cefotaxime (0.25 to 64 μg/ml) or cefotaxime plus clavulanic acid (0.25/4 to 64/4 μg/ml) (Queenan *et al*., 2004). In a broth microdilution method, the tested isolate is considered ESBL-producing if there is a $\geq$ 3 twofold serial-dilution decrease in the minimum inhibitory concentration (MIC) of either cephalosporin in the presence of clavulanic acid compared to its MIC when tested alone (CLSI, 2009).

2.6.1.4 ESBL E-test strip method

The E-test ESBL strip (AB Biodisc, Solna, Sweden) is a two-sided strip that contains a gradient of ceftazidime (TZ) at one end and a gradient of ceftazidime plus clavulanic acid (TZL) at the other end (Figure 2.6). The detection of ESBL-producing *K. pneumoniae* strains by E-test is based on a similar principle to that of the combination disc method and has been shown to compare well with the disc methods (Bradford, 2001).

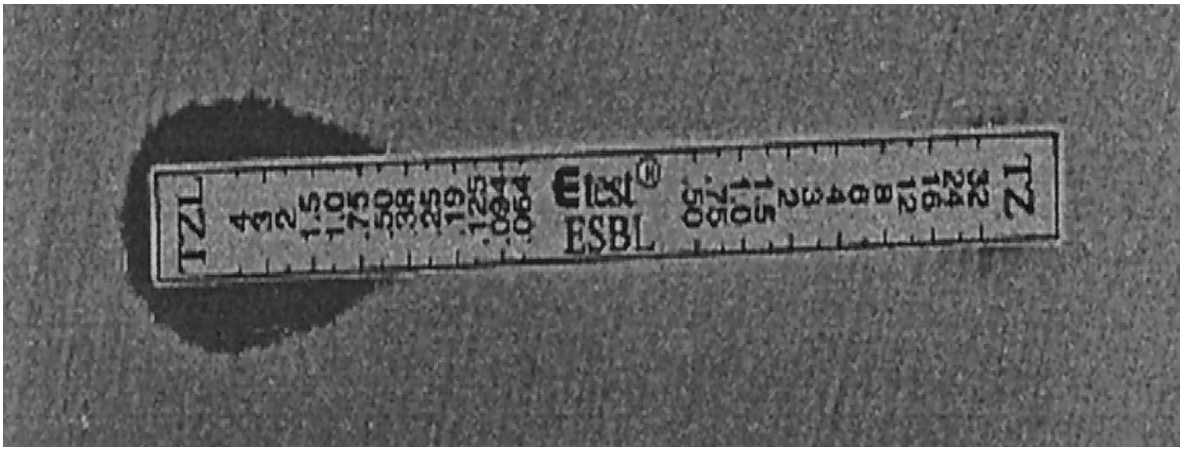

Figure 2.6: **Diagram showing an ESBL-positive bacterial isolate as confirmed with an ESBL E-test strip. The ceftazidime (TZ) MIC is 32 in the absence of clavulanic acid and 0.5 in the presence of clavulanic acid (TZL). As there is a decrease in the MIC of 3 doubling dilutions in the presence of clavulanic acid, the bacterial isolate is inferred to be an ESBL producer (Bradford, 2001).**

The bacterial isolate is termed ESBL-positive if there is a ≥ three twofold-concentration decrease in the MIC of cefotaxime, ceftazidime or cefepime antibiotic combined with clavulanic acid versus its MIC when tested alone (Figure 2.6) (Bradford, 2001; CLSI, 2009). The ESBL E-test is considered positive when there is a phantom zone just below the lowest concentration of the TZL gradient or when there is a deformation of the TZ inhibition ellipse at the tapering end (Cormican *et al*., 1996).

Cormican *et al*. (1996) highlighted that the ESBL E-test is a convenient and easy to use test. However, Cormican and colleagues (1996) warned that the assay may sometimes be difficult to interpret, especially when the MICs of ceftazidime are low because the clavulanate may diffuse to the side that contains ceftazidime alone. Furthermore, a study conducted by Leverstein-van Hall *et al*. (2002) reported that laboratories may fail to correctly interpret the zone of inhibition in 30% of the cases.

2.6.1.5 The Vitek2 AES detection method

The Vitek2 AES is an automated system for rapid identification and antimicrobial susceptibility testing. It uses a manually prepared inoculum with a concentration of 0.5 to 0.6 McFarland standards (Dashti *et al*., 2006). The Vitek2 susceptibility test results are expressed

as MIC values and interpreted as susceptible, intermediate or resistant by reference to the CLSI 2009 guidelines (CLSI, 2009).

The Vitek2 system detects ESBL-production using two different ESBL identification tools (Valenza *et al*., 2011). The first one includes analysis and interpretation of minimal inhibitory concentration (MIC) of several beta-lactams using special software called the advanced expert system (AES) (Valenza *et al*., 2011). The second test (ESBL test) is based on simultaneous detection and assessment of antibacterial activity of cefepime, cefotaxime or ceftazidime, alone and in combination with the beta-lactamase inhibitor (clavulanic acid) (Drieux *et al*., 2008). In addition, the updated computer algorithms in the new Vitek2 system have been shown to categorize beta-lactamases based on the phenotype of susceptibility patterns with various beta-lactam antibiotics (Shah *et al*., 2004a).

In an evaluation study by Nyberg *et al*. (2008) involving 123 clinical isolates of *E. coli* and *Klebsiella* spp it was reported that the Vitek2 AES has an accuracy of 91.1% (113/123) in detecting ESBL-producing *E. coli* and *Klebsiella* species when compared to a molecular assay (PCR followed by DNA sequencing). Nyberg *et al*. (2008) concluded that the Vitek2 automated system is an accurate and time-saving tool for detection of ESBLs in *E. coli* and *Klebsiella* spp. In another evaluation study of the Vitek2 ESBL test by Spanu *et al*. (2006) that included 1 129 *Enterobacteriaceae* clinical isolates, the authors reported that the Vitek2 automated system has a sensitivity and specificity of 97.7% (306/312) and 99.7% (815/817) respectively in detecting ESBL-producing isolates compared to a molecular method (PCR amplification of ESBL genes and sequencing) (Spanu *et al*., 2006).

2.6.1.6 BD Phoenix Automated Microbiology System

The BD Phoenix automated system is an automated system for bacterial identification and susceptibility testing introduced by Becton Dickinson. In this test, Phoenix ID broth is inoculated with bacterial colonies from MacConkey agar medium and adjusted to a 0.5 to 0.6 McFarland standard using the Crystal Spec Nephelometer (BD Diagnostic Systems, Sparks, MD). The BD Phoenix ESBL test relies on growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ESBLs by the *K. pneumoniae* strains (Drieux *et al*., 2008). In this system, the results are

interpreted through a computerized system and are usually available within 6 hours (AL-Muharrmi *et al*., 2008).

In an evaluation study of the BD Phoenix automated system conducted by Leverstein-van Hall *et al*. (2002) involving 17 control strains and 74 multiresistant *Enterobacteriaceae* clinical isolates (34 *E. coli*, 26 *K. pneumoniae* and 14 *K. oxytoca*), the sensitivity and specificity of the BD Phoenix automated system in detecting ESBL-production from the 74 clinical isolates were reported as 93% and 81% respectively when compared to the ESBL E-test, which was used as a reference method. The same study further concluded that the ESBL test results of the Phoenix system is better than that of the other automated systems (Vitek1 and Vitek2 systems) in detecting ESBL-production by multiresistant *E. coli* and *Klebsiella* species (Leverstein-van Hall *et al*., 2002). A study conducted by Sanguinneti *et al*. (2003) which included 510 clinical isolates of *Enterobacteriaceae* reported sensitivity and specificity values of 100% (319/319) and 98.9% (189/191) respectively by the BD Phoenix system compared to the double disc method in detecting ESBL-production by *Enterobacteriaceae*.

2.6.2 Detection of ESBL-producing *K. pneumoniae* by genotypic methods

The genotypic assays that can be used to detect ESBL-producing *K. pneumoniae* strains include DNA probing, polymerase chain reaction (conventional and real-time PCR), restriction fragment length polymorphism and isoelectric focusing (Stürenburg and Mack, 2003). Although technically challenging, the genotypic methods have the advantage of detecting low level resistance (Pitout and Laupland, 2008). Furthermore, genotypic methods have the ability to identify specific types of ESBLs present in the bacterial isolates (Pitout and Laupland, 2008).

In the early days of studying ESBLs, isoelectric focussing was sufficient to detect the production of ESBL by the bacterial isolates (D'Agata *et al*., 1998). However, due to the evolution of different variants of SHV, TEM, CTX-M and OXA ESBLs possessing identical isoelectric points, the use of the isoelectric point assay in detecting ESBL positive isolates is no longer reliable, as it can only give presumptive identification (D'Agata *et al*., 1998).

Another method which can be used to detect ESBL-production is the PCR hybridization assay using DNA probes that are specific for TEM and SHV ESBL enzyme (Huovinen *et al*., 1988;

Gallego *et al*., 1990). The oligotyping method can be used to discriminate between TEM-1 and TEM-2 (Tham *et al*., 1990). This method uses oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions (Tham *et al*., 1990). These probes are less sensitive for the detection of mutations, which are responsible for the extended substrate range (Tham *et al*., 1990). However, the use of DNA probes is labour intensive and therefore not an ideal assay to use in a diagnostic laboratory (Nyberg *et al*., 2008).

Thus far, the easiest and the most common genotypic assay used to detect the presence of ESBLs amongst *K. pneumoniae* strains is the use of PCR with oligonucleotide primers that are specific for a beta-lactamase gene (Bradford, 2001). The oligonucleotide primers can be designed and chosen from the sequences available in public databases, such as Genbank (http://www.ncbi.nlm.nih.gov/nuccore). However, the above PCR detection method does not discriminate among different variants of TEM or SHV (Fluit *et al*., 2001) and therefore requires an additional molecular technique, such as DNA sequencing in order to identify a specific point mutation that differentiate an ESBL enzyme from a none-ESBL enzyme (parent enzymes) (eg SHV-1) (Pitout and Laupland, 2008). Nucleotide sequencing remains the "gold standard" for determination of the ESBL gene present in a bacterial strain (Bradford, 1999).

The PCR-single-strand conformation polymorphism procedure (PCR-SSCP) has also been applied to study ESBLs (Chroma and Kolar, 2010). The PCR-SSCP is a simple and powerful method used to identify a mutation in an amplified DNA (Chroma and Kolar, 2010). The PCR-SSCP is based on the observation that the mobility of single-stranded DNA in nondenaturing polyacrylamide gel is very sensitive to primary sequence. Therefore, any difference in the base sequence of a single-stranded DNA sample due to a mutation will be detected as a mobility shift and will produce a different band pattern to the wild type (Chroma and Kolar, 2010). The assay has been used to detect a single base mutation at a specific location within a beta-lactamase gene (bla_{TEM}) (Edelstein and Stratchounski, 1998).

2.7 Clinical significance of detecting ESBL-producing *K. pneumoniae* strains

The accurate detection of ESBL-production by clinical bacterial isolates is crucial (Al-Jasser, 2006). The laboratory detection of ESBL positive *K. pneumoniae* bacteria is clinically important because if left undetected, it may result in treatment failure with serious consequences, such as a high mortality rate (Pitout and Laupland, 2008). Infections with

ESBL-producing bacterial isolates have an important impact on clinical outcomes and are associated with high rates of mortality, a prolonged hospital stay and higher hospital costs (Rawat and Nair, 2010).

Researchers have reported that patients infected with ESBL-producing *K. pneumoniae* tend to have less satisfactory treatment outcomes than those infected by non-ESBL-producing *K. pneumoniae* (Wong-Beringer *et al*., 2002). Furthermore, a prospective multinational study analyzing bloodstream infections due to ESBL-producing *K. pneumoniae* reported that cephalosporin mono-therapy was associated with a 40% 14-day mortality rate (Paterson *et al*., 2004).

2.8 Quality control when detecting ESBL-producing *K. pneumoniae* strains

The CLSI recommends the use of *K. pneumoniae* ATCC 700603 (ESBL positive) and *E. coli* ATCC 25922 (ESBL negative) strains when performing ESBL detection tests (CLSI, 2009; CLSI, 2011). On disc diffusion testing, the zone diameter ranges for *K. pneumoniae* ATCC 700603 strain are as follows: cefpodoxime 9 to 16 mm, ceftazidime 10 to 18 mm, aztreonam 9 to 17 mm, cefotaxime 17 to 25 mm and ceftriaxone 16 to 24 mm (CLSI, 2009, CLSI 2011). In the disc diffusion phenotypic testing, the *K. pneumoniae* ATCC 700603 strain shows an equal or a more than 5 mm increase in ceftazidime-clavulanic acid and a more than 3 mm increase in the cefotaxime-clavulanic acid zone diameter (CLSI, 2009; CLSI, 2011). On disc diffusion testing, the zone diameter of *E. coli* ATCC 25922 strain shows a $\leq$ 2 mm increase in zone diameter for an antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid (CLSI, 2009; CLSI, 2011).

2.9 Typing of *K. pneumoniae* isolates

Different phenotypic and genotypic methods have been used with various degrees of success in *Klebsiella* typing (Paterson and Bonomo, 2005; Mohamudha *et al*., 2010). From an epidemiological point of view, it is often necessary to determine the clonality of the strains (Mohamudha *et al*., 2010). This is particularly important in endemic and epidemic nosocomial outbreaks of *Klebsiella* infections in order to improve the management of such outbreaks (Mohamudha *et al*., 2010).

2.9.1 Typing of *K. pneumoniae* by phenotypic methods

The phenotypic methods traditionally used for the typing of *K. pneumoniae* isolates include phage typing, biotyping, bacteriocin typing and serotyping (Hall, 1971; Taylor, 1983; Arlet *et al*., 1990; Mohamudha *et al*., 2010). Unfortunately, because of inconsistently expressed phenotypic traits, these phenotypic typing approaches are often not able to discriminate between outbreaks of related *K. pneumoniae* strains (Hamouda *et al*., 2003). Phage typing of *Klebsiella* was first developed in the 1960s, although the phage reaction is easily read and the reproducibility of the method is acceptable; this technique has shown a relatively poor typing rate of 19% to 67% (Slopek, 1978).

Serotyping is potentially useful in discriminating ESBL-producing *K. pneumoniae* (Schembri *et al*, 2003). The drawback of the serotyping technique is the large number of serological cross-reactions that occur among the 77 capsule types (Podschun and Ullmann, 1998). Secondly, the individual sera have to be absorbed with the cross-reacting K antigens (Podschun and Ullmann, 1998). The antisera are not commercially available and the typing procedure is time consuming (Podschun and Ullmann, 1998). Finally, the test is susceptible to subjectivity because of weak reactions that are not always easy to interpret (Podschun and Ullmann, 1998).

2.9.2 Typing of *K. pneumoniae* by molecular methods

Molecular typing methods, such as plasmid profiling, pulsed field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD) (Brisse and Verhoef, 2001; Paterson and Bonomo, 2005) and rep PCR assays have been used to determine the clonal relatedness of bacterial isolates (Cao *et al*., 2002; Galani *et al*., 2002). Primers used in PCR typing may be random or specific for a known sequence (Greenwood *et al*., 2007). Random primers are extensively used in the RAPD typing methods. Polymerase chain reaction genomic fingerprinting have been found to be extremely reliable, reproducible, rapid and highly discriminatory (Louws *et al.,* 1997; Sampaio *et al*., 2006).

2.9.2.1 Plasmid analysis

Plasmid profile analysis was the first and the simplest nucleic acid-based typing technique applied to epidemiological studies (Van Leeuwen, 2009). In this method, the extra-chromosomal genetic elements are isolated from each bacterium and then separated by agarose gel electrophoresis to determine their number and size (Brook *et al*., 2010). However, plasmids of identical size with different sequences can exist in many bacteria (Farber, 1996). Thus, digesting the plasmids with restriction endonuclease and then comparing the number and size of the resulting fragments often provides additional useful information (Van Leeuwen, 2009). Plasmid profile analysis is most useful in examining outbreaks that are restricted in time and place (Van Leeuwen, 2009).

2.9.2.2 Rep-PCR typing method

The rep-PCR genomic fingerprinting method has become a valuable tool for the identification and classification of bacteria and for molecular epidemiological studies of human and plant pathogens (Van Belkum, 1994; Louws *et al.,* 1996; Rademaker *et al*., 2000). This typing method makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Van Leeuwen, 2009).

One distinct advantage of the rep-PCR genomic fingerprinting method is that the primers used work in a variety of Gram-negative and Gram-positive bacteria (Versalovic *et al.,* 1991; Louws *et al.,* 1997; Rasschaert *et al*., 2005). This means that no previous knowledge of the genomic structure or nature of indigenous repeated sequences is necessary (Versalovic *et al.,* 1991). The assay also bypasses the need to identify suitable arbitrary primers by trial and error that is inherent to the RAPD protocol (Welsh and McClelland, 1990).

In rep-PCR genomic fingerprinting, primers are used to amplify specific fragments, which can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic *et al.* 1994; Cleland *et al*., 2008). Repetitive extragenic palindromic sequence based PCR (REP-PCR) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) have been successfully used for typing *K. pneumoniae* isolates (Gazouli *et al*., 1997; Cao *et al*., 2002; Galani *et al*., 2002). The ERIC-PCR is based on primers targeting the

highly conserved enterobacterial repetitive intergenic consensus and REP-PCR is based on primers targeting the repetitive extragenic palindromic sequence. The BOX-PCR is based on primers targeting the highly conserved repetitive DNA sequence of the BOXA subunit of the BOX element of the organism (Rasschaert *et al*., 2005). The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation of bacterial isolates to the species, subspecies and strain level. These fingerprints resemble "bar code" patterns (Cleland *et al*., 2008) analogous to universal product code (UPC) codes used in grocery stores (Figure 2.7) (Lupski, 1993).

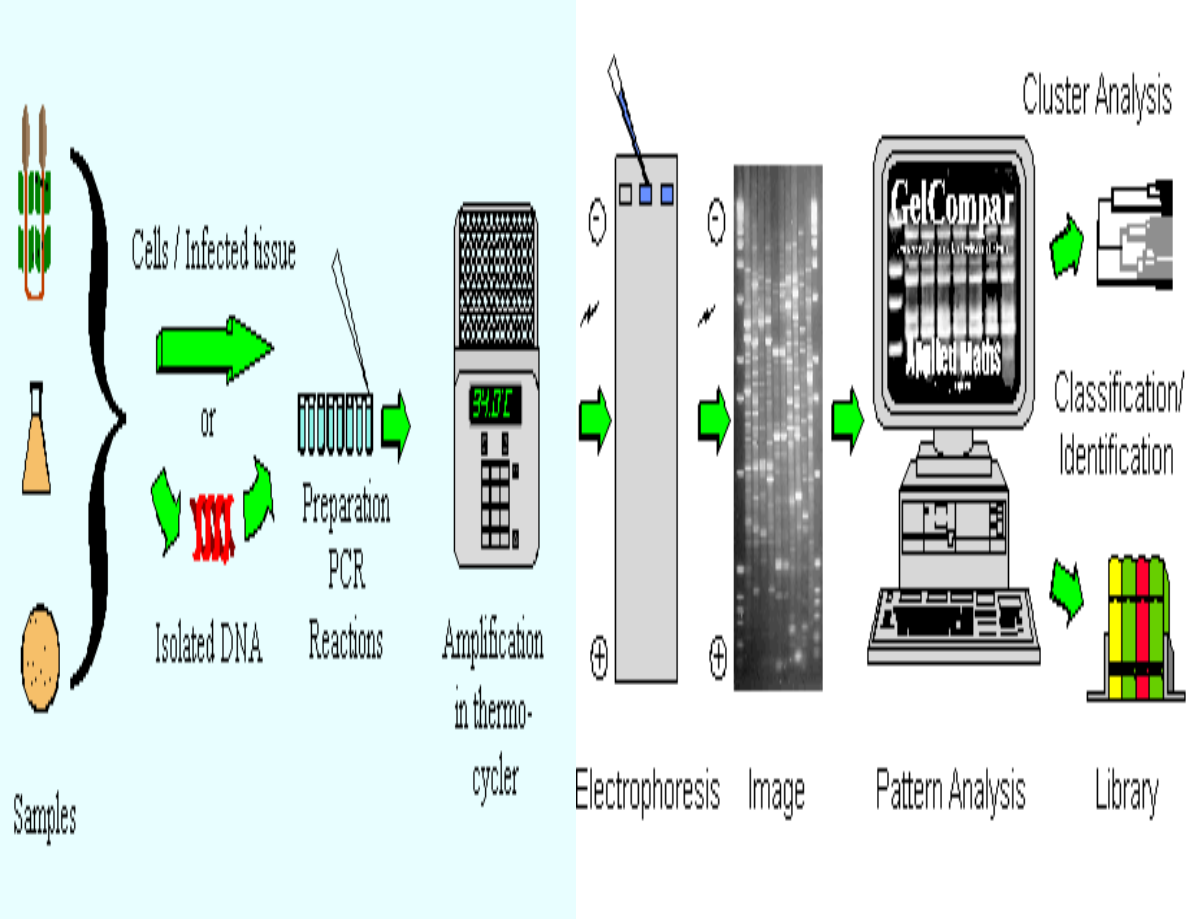

Figure 2.7: **Flow chart diagram showing a summary of steps used to perform REP, ERIC and BOX PCR (Rademaker and De Bruijn, 1997).**

The generated fragments can be analysed visually or with computer assisted analysis (Rademaker and De Bruijn, 1997). Recently, a semi-automated and highly standardized rep-PCR DNA fingerpriting system called DiversiLab (DL) (bioMérieux, France) has become commercially available (Fluit *et al*., 2010). The DL is a rapid bacterial typing system that can

easily be used by technologists with minimum molecular experience (Healy *et al*., 2005). A study conducted in the United States of America by Endimiani and colleagues (2009) which included 42 *K. pneumoniae* isolates concluded that the DiversiLab typing system can quickly type and discriminate between *K. pneumoniae* isolates.

2.9.2.3 Pulsed field gel electrophoresis typing

Due to its high discriminatory capacity and good reproducibility, pulsed field gel electrophoresis (PFGE) is widely considered the most powerful tool to study the hospital epidemiology of *K. pneumoniae* (Hansen *et al*., 2002). Bacterial isolates grown either in broth or on solid media are combined with molten agarose or poured into small molds, which forms agarose plugs containing the whole bacteria. The embedded bacteria are subjected to *in situ* detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme (Tang and Stratton, 2006). The digested bacterial plugs are inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals (Schwartz and Cantor, 1984; Tang and Stratton, 2006).

The pulsed field gel electrophoresis allows clear separation of very large molecular length DNA fragments ranging from 10 to 800 kb (Schwartz and Cantor, 1984). The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye, such as ethidium bromide. Gel results can be photographed and the data can be stored by using one of the commercially available digital systems, such as those manufactured by Alpha-Innotech, Bio-Rad, Hitachi, or Molecular Dynamics. Data analysis can be accomplished by using any of a number of commercially available software packages (Duck *et al*., 2003). The PFGE has proven to be superior to most other methods for biochemical and molecular typing (Lin *et al*., 2008).

2.9.2.4 Random amplified polymorphic DNA typing method

The random amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed PCR, was first described in the 1990s by Williams and colleagues (1990). The random amplified polymorphic DNA typing assay is based on short random primers that anneal at different locations on the bacterial genomic DNA (Welsh and McClelland, 1990; Van Leeuwen, 2009). When amplified, these primers generate PCR products of different lengths.

The generated PCR fragments are resolved on agarose or acrylamide gels (Ranjard *et al*., 2000). Thus, after separation of the amplification products by agarose gel electrophoresis, a pattern of bands, which in theory is characteristic of the particular bacterial strain is formed (Welsh and McClelland, 1990; Williams *et al*, 1990).

In most cases the sequences of the RAPD primers, which generate the best DNA pattern for differentiation must be determined empirically (Ranjard *et al*., 2000). Researchers have reported success in using the RAPD assay for bacterial typing, the assay was found to be rapid and reliable in revealing the differences between strains. A study conducted by Eisen and colleagues (1995) concluded that the RAPD assay is a useful tool in confirming outbreaks of multi-drug resistant *K. pneumoniae* strains.

2.9.2.5 Amplified fragment length polymorphism typing method

The amplified fragment length polymorphism (AFLP) assay is a genomic fingerprinting technique based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion (Dijkshoorn *et al*., 1996). Initially applied to the characterization of plant genomes, this assay has been applied to bacterial typing (Dijkshoorn *et al*., 1996). In the AFLP assay, bacterial DNA is extracted, purified and subjected to digestion with two different enzymes, such as *Eco*RI and *Mse*I (Blears *et al*., 1998). The restriction fragments are then ligated to linkers containing each restriction site and a sequence homologous to a PCR primer binding site (Blears *et al*., 1998). The PCR primers used for amplification contain DNA sequences homologous to the linker and contain one to two selective bases at their 3' ends. A selective primer directed against an *Eco*RI site might have the sequence 5'-GAATTCAA-3', where the first six bases are complementary to the *Eco*RI site while the two A residues at the 3' end are selective and allow amplification of only those *Eco*RI sites with the sequence 3'-CTTAAGTT-5'. Thus, the selective nucleotides allow amplification of only a subset of the genomic restriction fragments. The pattern of AFLP can be examined under UV light after staining a gel with ethidium bromide (Gibson *et al*., 1998). The AFLP assay has been reported to be reproducible and has a good ability to differentiate clonally derived strains (Dijkshoorn *et al*., 1996; Gibson *et al*., 1998). The differentiation power of AFLP appears to be greater than that of the PCR-based ribotyping method (Koeleman *et al*., 1998).

2.10 Summary

Klebsiella pneumoniae is a well-described pathogen capable of causing a range of infections including sepsis, urinary tract infections, pneumonia and soft tissue infections in patients in the intensive care unit including the neonatal intensive care unit (NICU) (Hart, 1993). The emergence of extended-spectrum beta-lactamase (ESBL) production in *K. pneumoniae* is of great concern because ESBL-producing bacteria may be difficult to detect and if detected must be reported as resistant to all penicillins, cephalosporins and aztreonam regardless of susceptibility testing results (Luzzaro *et al*., 2006).

Outbreaks of ESBL-producing strains have been reported worldwide and it is therefore essential for diagnostic microbiology laboratories to have the updated methods for the detection of ESBL-producing strains (Brown *et al*., 2000; Tofteland *et al*., 2007). The fact that patients most likely to become infected with ESBL-producing *K. pneumoniae* are those with prolonged stays in the ICU is of great concern, because any delay in detecting ESBL-producing strains may result in serious consequences, such as death (Stürenburg and Mack, 2006).

The aim of this study was to determine the prevalence of ESBL-producing *K. pneumoniae* clinical isolates; to investigate the sensitivity and specificity of multiplex PCR assay and the Vitek2 AES in detecting ESBL-production using the combination disc method as a gold standard. Lastly, the study investigated the clonal relatedness of *K. pneumoniae* clinical isolates using ERIC, BOX and REP-PCR genotypic assays.

This study will help in determining the prevalence of ESBL-producing *K. pneumoniae* isolates in the area of Tshwane. The results of this study will determine whether the Vitek2 AES and multiplex PCR assays are as good as the combination disc method in detecting ESBL-producing *K. pneumoniae* isolates. Lastly, the results of this study will help in determining whether the ESBL-producing *K. pneumoniae* isolates from the Steve Biko Academic Hospital are clonally related or not.

2.11 References

Al-Jasser AM (2006) Extended spectrum beta-lactamse (ESBL) A global problem. *Kuwait Medical Journal* **38**: 171-185.

Al-Muharrmi Z, Rafay AM, Balkhair A, Al-Tamemi S, Al-Mawali A and Al Sadir H (2008) Extended-spectrum beta-lactamase (ESBL) in Omani children study of prevalence, risk factors and clinical outcomes at Sultan Qaboos University Hospital, Sultanate of Oman. *Sultan Qaboos University Medical Journal* **8**: 171–177.

Alvarez JA, Gonzalez JJ, Baldonedo RF, Sanz L, Carreno G, Junco A, Rodriquez JI, Martinez MD and Jorge JI (2001) Clinical course, treatment and multivariate analysis of risk factors for pyogenic liver abscess. *American Journal of Surgery* **181**: 177-186.

Ambler RP (1980) The structure of beta-lactamases. *Philosophical Transactions of the Royal Society of London Biogical Sciences* **289**: 321-331.

Ambler RP, Coulson AF, Frère J M, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G and Waley SG (1991) A standard numbering scheme for the class A beta-lactamases. *Biochemical Journal* **276**: 269-270.

Arlet G, Sanson-le Pors MJ, Rouveau M, Fournier G, Marie O, Schlemmer B and Philippon A (1990) Outbreak of nosocomial infections due to *Klebsiella pneumoniae* producing SHV-4 beta-lactamase. *European Journal of Clinical Microbiology and Infectious Diseases* **9:** 797–803.

Barthélémy M, Péduzzi J, Bernard H, Tancrède C and Labia R (1992) Close amino-acid sequence relationship between the new plasmid-mediated extended-spectrum beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiellla oxytoca*. *Biochimica et Biophysica Acta* **1122**: 15–22.

Bell JM, Turnidge JD, Gales AC, Pfaller MA and Jones RN (2002) Prevalence of extended spectrum beta-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa: regional results from SENTRY Antimicrobial Surveillance Program (1998–99). *Diagnostic Microbiology and Infectious Diseases* **42:** 193–198.

Bishara JG, Livne S, Ashkenazi I, Levy S, Pitlik S and Lev B (2005) Antibacterial susceptibility of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. *Israel Medical Association Journal* **7:** 298–301.

Bisson G, Fishman NO, Patel JB, Edelstein PH and Lautenbach E (2002) Extended spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella* species: risk factors for colonization and impact of antimicrobial formulary interventions on colonization prevalence. *Infection Control and Hospital Epidemiology* **23**: 254-60.

Blanchette EA and Rubin SJ (1980) Seroepidemiology of clinical isolates of *Klebsiella* in Connecticut. *Journal of Clinical Microbiology* **11**: 474-478.

Blears MJ, De Grandis SA, Lee H and Trevors JT (1998) Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. *Journal of Industrial Microbiology and Biotechnology* **21**: 99-114.

Bonnet R (2004) Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy* **48**: 1-14.

Boye K and Hansen DS (2003) Sequencing of 16S rDNA of *Klebsiella*: taxonomic relations within the genus and to other *Enterobacteriaceae. International Journal of Medical Microbiology* **292**: 495-503.

Bradford PA (1999) Automated thermal cycling is superior to traditional methods for nucleotide sequencing of bla_{SHV} genes. *Journal of Antimicrobial Agents and Chemotherapy* **43**: 2960–2963.

Bradford PA (2001) Extended-Spectrum beta-lactamases in the 21st Century: Characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* **14**: 933–951.

Bradford PA, Bratu S, Urban C, Visalli M, Mariano N, Landman D, Rahal JJ, Brooks S, Cebular S and Quale J (2004) Emergence of carbapenem-resistance *Klebsiella* species possessing the class A carbapenem hydrolyzing KPC-2 and inhibitor-resistant TEM-30 beta-lactamases in New York City. *Clinical Infectious Diseases* **39**: 55-60.

Brisse S and Verhoef J (2001) Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyr*A and *par*C genes sequencing and automated ribotyping. *International Journal of Systematic and Evolutionary Microbiology* **51**: 915-924.

Brisse S, Issenhuth-Jeanjean S and Grimont PA (2004) Molecular serotyping of *Klebsiella* species isolates by restriction of the amplified capsular antigen gene cluster. *Journal of Clinical Microbiology* **42**: 3388–3398.

British Society for Antimicrobial Chemotherapy (BSAC) (2003) Detection of extended-spectrum beta-lactamases (ESBLs) in *E. coli* and *Klebsiella* species.

Brook GF, Carroll KC, Butol JS, Morse SA and Mietzner T (2010) Jawetz, Melnick and Adelberg Medical Microbiology 25th edition, *McGraw Hill Lange*, chapter 5, pp 219-219.

Brown DFJ, Andrews J, King A and Alasdair P (2000) Detection of extended-spectrum beta-lactamases with E test and double-disc potentiation methods. *Journal of Antimicrobial Chemotherapy* **146**: 323-342.

Bush K, Jacoby GA and Medeiros AA (1995) A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy* **39**: 1211-1233.

Bush K (2001) New beta-lactamases in Gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. *Clinical Infectious Diseases* **32:** 1085-1089.

Bush K and Fisher JF (2011) Epidemiological expansion, structural studies and clinical challenges of new beta-lactamase from gram negative bacteria. *Annual Review of Microbiology* **65**: 455-478.

Canton R, Perez-Vazquez M, Oliver A, Coque TM, Loza E, Ponz F and Baquero F (2001) Validation of the Vikek2 and the advance expert system with a collection of enterobacteriaceae harboring extended spectrum or inhibitor resistant beta-lactamases. *Diagnostic Microbiology and Infectious Disease* **41**: 65-70.

Canton R, Oliver A, Coque TM, Varela MC, Perez-Diaz JC and Baquero F (2002) Epidemiology of extended spectrum beta-lactamase-producing *Enterobacter* isolates in a Spanish hospital during a 12-year period. *Journal of Clinical Microbiology* **40**: 1237-1243.

Canton R and Coque TM (2006) The CTX-M beta-lactamase pandemic. *Current Opinion in Microbiology* **9**: 466-475.

Cao V, Lambert T, Nhu DQ, Loan HK , Hoang NK, Arlet K and Courvalin P (2002) Distribution of extended-spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae* in Vietnam. *Antimicrobial Agents and Chemotherapy* **46:** 3739–3743.

Carrër A, Poirel L, Eraksoy H, Cagatay AA, Badur S and Nordmann P (2008) Spread of OXA-48-positive carbapenem resistant *Klebsiella pneumoniae* isolates in Istanbul, Turkey. *Antimicrobial Agents and Chemotherapy* **52**: 2950-2954.

Carter MW, Oakton KJ, Warner M and Livermore DM (2000) Detection of extended spectrum beta-lactamase in *Klebsiellae* with Oxoid combination disc method. *Journal of Clinical Microbiology* **38**: 4228-4232.

Centers for Disease Control and Prevention (2010) Detection of *Enterobacteriaceae* isolates carrying metallo beta-lactamase. *Morbidity and Mortality Weekly Report* **59**: 750-750.

Chan K, Yu W, Tsai C, Cheng K, Hou C, Lee M and Tan C (2007) Pyogenic liver abscess caused by *Klebsiella pneumoniae*: analysis of the clinical characteristics and outcomes of 84 patients. *Chinese Medical Journal* **120**: 136-139.

Chuang YP, Fang CT, Lai SY, Chang SC and Wang JT (2006) Genetic determinants of capsular serotype K1 of *K. pneumoniae* causing primary pyogenic liver abscess. *Journal of Infectious Diseases* **193**: 645-654.

Chroma M and Kolar M (2010) Genetic methods for detection of antibiotic resistance: focus on extended spectrum beta-lactamases. *Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czech Republic* **154**: 289-296.

Chung DR, Lee SS, Lee HR, Kim HB, Choi HJ, Eom JS, Kim JS, Choi YH, Lee JS, Chung MH, Kim YS, Lee H, Lee MS and Park CK (2007) Emerging invasive liver abscess caused by K1 serotype *Klebsiella pneumoniae* in Korea. *Journal of Infectious Diseases* **54:** 578-586.

Cleland D, Kradar P and Emerson D (2008) Use of the Diversilab repetitive sequence-based PCR system for genotyping and identification of *Archaea*. *Journal of Microbiological Methods* **73**: 172-178.

Clinical and Laboratory Standards Institute (2009) Performance standards for antimicrobial susceptibility testing: *Nineteenth Informational Supplement CLSI*, Wayne, PA, USA.

Clinical and Laboratory Standard Institute (2011) Performance standards for antimicrobial susceptibility testing; *Twenty-First Information Supplement CLSI* Wayne. PA, USA.

Coetzee J, Clay CG and Brink A (2011) Clinical microbiology considerations related to the emergence of New Delhi metallo beta-lactamase (NDM-1) and *Klebsiella pneumoniae* carbapenemases (KPC) among hospitalized patients in South Africa. *Federation of Infectious Diseases Societies of Southern Africa* (Special report)

Cormican MG, Marshall SA and Jones RN (1996) Detection of extended-spectrum beta-lactamase (ESBL)-producing strains by the Etest ESBL screen. *Journal of Clinical Microbiology* **43**: 1880-1884.

Cryz S J, Mortimer P M, Mansfield V and Germanier R (1986) Seroepidemiology of *Klebsiella* bacteremic isolates and implications for vaccine development. *Journal of Clinical Microbiology* **23:** 687-690.

D'Agata E, Venkataraman L, DeGirolami P, Weigel L, Samore M and Tenover F (1998) The molecular and clinical epidemiology of *Enterobacteriaceae* producing-extended spectrum beta-lactamases in a tertiary care hospital. *Journal of Infection* **36**: 279-285.

Dashti AA, West P, Paton R and Amyes SGB (2006) Characterization of ESBL-producing Kuwait and UK strains identified by the Vitek2 system, and subsequent comparison of the Vitek system with other commercial ESBL-testing systems using these strains. *Journal of Medical Microbiology* **55**: 417-421.

Davies J (1994) Inactivation of antibiotics and dissemination of resistance genes. *Science* **264**: 375-382.

Dijkshoorn LH Aucken M, Gerner-Smidt P, Janssen P, Kaufman ME, Garaizar J, Ursing J and Pitt TL (1996) Comparison of outbreak and non-outbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *Journal of Clinical Microbiology* **34**: 1519–1525.

Domenico P, Johanson WG and Straus DC (1982) Lobar pneumonia in rats produced by clinical isolates of *Klebsiella pneumoniae*. *Infection and Immunity* **37**: 327-335.

Drancourt M, Bollet C, Carta A and Rousselier P (2001) Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella ornithinolytica* comb.nov., *Raoultella terrigena* comb.nov. and *Raoultella planticola* comb.nov. *International Journal of Systematic and Evolutionary Microbiology* **51**: 925-932.

Drieux L, Brossier F, Sougakoff W and Jarlier V (2008) Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae*: review and bench guide. *Clinical Microbiology and Infection* **14**: 90-103.

Duck WM, Steward CD, Banerjee SN, McGowan JE and Tenover FC (2003) Optimization of computer software settings improves accuracy of pulsed-field gel electrophoresis macrorestriction fragment pattern analysis. *Journal of Clinical Microbiology* **41**: 3035–3042.

Edelstein M and Stratchounski L (1998) Development of single strand conformational polymorphism (SSCP) PCR method for discriminatory detection of genes coding for TEM-family beta-lactamases. *38th Interscience Conference on Antimicrobial Agents and Chemotherapy* Poster No: E096.

Edelstein M, Pimkin M, Palagin I, Edelstein I and Stratchounski L (2003) Prevalence and molecular epidemiology of CTX-M extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Russian Hospital. *Antimicrobial Agents and Chemotherapy* **47**: 3724-3732.

Eisen D, Russell EG, Tymms M, Roper EJ, Grayson ML and Turnidge J (1995) Random amplified polymorphic DNA and plasmid analyses used in investigation of an outbreak of multi drug resistant *Klebsiella pneumoniae. Journal of Clinical Microbiology* **33**: 713-717.

Endimiani A, Huler AM, Perez F, Bethel CR, Hujer KM, Kroeger J, Oethinger M, Paterson DL, Adams MD, Jacobs MR, Diekema DJ, Hall GS, Jenkins SG, Rice LB, Tenover FC and Bonomo RA (2009) Characterization of *bla* KPC-containing *Klebsiella pneumoniae* isolates detected in different institutions in the Eastern USA. *Journal of Antimicrobial Chemotherapy* **63**: 427-437.

Euzéby JP (2011) List of prokaryotic names with standing in nomenclature.last updated: 07 Octomber 2011, accessed 18 October 2011, from the cite: http://bacteria.cict.fr/classifphyla.html.

Falagas ME and Karageorgopoulos DE (2009) Extended-spectrum beta-lactamase producing organisms. *Journal of Hospital Infection* **73**: 345-354

Fang CT, Lai SY, Yi WC, Hsueh PR, Liu KL and Chang SC (2007) *Klebsiella pneumoniae* genotype K1: An emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. *Clinical Infectious Diseases* **45**: 284-293.

Farber JM (1996) An introduction to hows and whys of molecular typing. *Journal of Food Protection* **59**: 1091-1101.

Farges O, Leese T and Bismuth H (1998) Pyogenic liver abscess: an improvement in prognosis. *British Journal of Surgery* **75**: 862-865.

Fluit AC, Visser MR and Schmitz FJ (2001) Molecular detection of antimicrobial resistance. *Clinical Microbiology Reviews* **14**: 836-871.

Fluit AC, Terlingen AM, Adriessen L, Ikawaty R, Mansfeld R, Top J, Stuart JWC, Leversstein-van Hall MA and Boel CHE (2010) Evaluation of the DiversiLab system for detection of hospital outbreaks of infections by different bacterial species. *Journal of Clinical Microbiology* **48:** 3979-3989.

Galani I, Xirouchaki E, Kanellakopoulou K, Petrikkos G and Giamarellou H (2002) Transferable plasmid mediating resistance to multiple antimicrobial agents in *Klebsiella pneumoniae* isolates in Greece. *Clinical Microbiology and Infection* **8:** 579–588.

Galani I, Souli M, Chryssouli Z, Katsala D and Giamarellou H (2004) First identification of an *Escherichia coli* clinical isolate producing both metallo-beta-lactamase VIM-2 and extended-spectrum beta-lactamase IBC-1. *Clinical Microbiology and Infection* **10**: 757-760.

Gales AC, Sander HS and Jones RN (2002) Respiratory tract pathogens isolated from patients hospitalized with suspected pneumonia in Latin America: frequency of occurrence and antimicrobial susceptibility profile: results from the SENTRY Antimicrobial Surveillance Program (1997-2000). *Diagnostic Microbiology and Infectious Disease* **44**: 301-311.

Gallego LA, Umaran J, Garaizar K, Colom and Cisterna R (1990) Digoxigenin-labeled DNA probe to detect TEM type beta-lactamases. *Journal of Microbiological Methods* **11**: 261–267.

Garrity GM, Winters M and Searles DB (2001) Taxonomic outline of the prokaryotes, Bergey's Manual of Systematic Bacteriology 2nd edition. *Springer Verlag New York*: 1-87.

Gazouli M, Kaufmann ME, Tzelepi E, Dimopoulou H, Paniara O and Tzouvelekis LS (1997) Study of an outbreak of cefoxitin-resistant *Klebsiella pneumoniae* in a general hospital. *Journal of Clinical Microbiology* **35:** 508–510.

Gheldre YD, Avesani V, Berhin C, Delmée M and Glupczynski Y (2003) Evaluation of Oxoid combination discs for detection of extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy* **52**: 591–597.

Giamarellou H (2005) Multidrug resistance in Gram-negative bacteria that produce extended spectrum beta-lactamases (ESBLs). *Clinical Microbiology and Infection* **11**: 1–16.

Gibson JR, Slater E, Xery J, Tompkins DS and Owen RJ (1998) Use of an amplified-fragment length polymorphism technique to fingerprint and differentiate isolates of *Helicobacter pylori*. *Journal of Clinical Microbiology* **36**: 2580–2585.

Gniadkowski (2008) Evolution of extended-spectrum beta-lactamases by mutation. *Clinical Microbiology and Infection* **14**: 11-32.

Graffunder EM, Preston KE , Evans AM and Venezia RA (2005) Risk factors associated with extended spectrum beta-lactamase-producing organisms at a tertiary care hospital. *Journal of Antimicrobial Chemotherapy* **56**: 139-145.

Greenwood D, Richard C, Slack CB, Peutherer JF and Barer MR (2007) Medical Microbiology e-book: a guide to microbial infections: pathogenesis, immunity, laboratory diagnosis and control. 16th edition. *Churchill Livingstone* pp: 1-752

Griffiths E, Rogers HJ and Bullen JJ (1980) Iron, plasmids and infection. *Nature* **284**: 508-509.

Gröbner S, Linke D, Schütz W, Fladerer C, Madlung J, Autenrieth JB, Witte W and Pfeifer Y (2009) Emergence of carbapenem-non-susceptible extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* isolates at the university hospital of Tübingen, Germany. *Journal of Medical Microbiology* **58**: 912-922.

Gupta A, Ampofo, K, Rubenstein D and Saiman L (2003) Extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* infections: a review of the literature. *Journal of Perinatology* **23**: 439-443.

Gupta A, Della-Latta P, Todd BRN, Gabriel PS, Haas J, Wu RF, Rubenstein D and Saiman L (2004) Outbreak of ESBL producing *Klebsiella pneumoniae* in a neonatal intensive care unit linked to artificial nails. *Infection Control and Hospital Epidemiology* **25**: 210-215.

Hall FA (1971) Bacteriocin typing of *Klebsiella* spp. *Journal of Clinical Pathology* **24:** 712–716.

Hall LM, Livermore DM, Gur D, Akova M and Akalin HE (1993) OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from *Pseudomonas aeroginosa*. *Antimicrobial Agents and Chemotherapy* **37**: 1637-1644.

Hamouda TB, Foulon T, Masmoudi ABC, Fendri C, Belhadj O and Mahrez KB (2003) Molecular epidemiology of an outbreak of multiresistant *Klebsiella pneumoniae* in a Tunisian neonatal ward. *Journal of Medical Microbiology* **52**: 427-433.

Hansen DS, Benedi SR, Sperling V and Kolmos HJ (2002) *Klebsiella* typing: pulsed field gel electrophoresis (PFGE) in comparison with O: K-serotyping. *Clinical Microbiology and Infections* **8**: 397-404.

Haque R and Salam MA (2010) Detection of ESBL producing nosocomial gram negative bacteria from tertiary care hospital in Bangladesh. *Pakistan Journal of Medical Sciences* **26**: 887-891.

Hart C (1993) *Klebsiella* and the neonate. *Journal of Hospital Infections* **23**: 83-86.

Healy M, Huong J, Bittner T, Lising M, Frye S and Raza S (2005) Microbial DNA typing by automated repetitive sequence based PCR. *Journal of Clinical Microbiology* **43:** 199-207.

Helfand MS and Bonomo RA (2005) Current challenges in antimicrobial chemotherapy: the impact of extended-spectrum beta-lactamases and metallo-beta-lactamases. *Current Opinion in Pharmacology* **5**: 452-458.

Hirakata Y, Matsuda J, Miyazaki Y, Kamihira S, Kawakami S, Miyazawa Y, Ono Y, Nakazaki N, Hirata Y, Inoue M, Turnidge JD, Bell JM, Jones RN and Kohno S (2005) Regional variation in the prevalence of extended-spectrum beta-lactamase-producing clinical isolates in the Asia-Pacific region (SENTRY 1998–2002). *Diagnostic Microbiology and Infectious Diseases* **52**: 323-329.

Hirsch EB and Tam VH (2010) Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *Journal of Antimicrobial Chemotherapy* **65**: 1119-1125.

Ho PL, Tsang DN, Que TL, Ho M and Yuen KY (2000) Comparison of screening methods for detection of ESBLs and their prevalence among *Escherichia coli* and *Klebsiella* species in Hong Kong. *Acta Pathologica, Microbiologica Immunologica Scandinavica (APMIS)* **108**: 237-240.

Hogan D and Kolter R (2002) Why are bacteria refractory to antimicrobials? *Current Opinion in Microbiology* **5**: 472- 477.

http://www.lahey.org/studies, accessed 24 September 2011

http://www.ncbi.nlm.nih.gov/nuccore

http//www.picasaweb.google.com/taniyan260/Gramstaingallery#5547, accessed 16 February 2011

http//www.textbookofbacteriology.net/resantimicrobiology, accessed 13 September 2009

Humeniuk C, Arlet G , Gautier V, Grimont P, Labia R and Philippon A (2002) Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. *Antimicrobial Agents and Chemotherapy* **46**: 3045-3049.

Huovinen S, Huovinen P and Jacoby GA (1988) Detection of plasmid mediated beta-lactamases with DNA probes. *Antimicrobial Agents and Chemotherapy* **32**: 175–179.

Janda JM and Abbott SL (2006) The Enterobacteria. Second edition. *Microbiology Today* Chapter 9: pp 115-132.

Jenney AW, Clements A, Farn JL, Wijburg OL, McGlinchey A, Spelman DW, Pitt TL, Kaufmann ME, Liolios L, Moloney MB, Wesselingh SL and Strugnell RA (2006) Seroepidemiology of *K. pneumoniae* in an Australian tertiary hospital and its implications for vaccine development. *Journal of Clinical Microbiology* **44**: 102-107.

Jitsurong S and Yodsawat J (2006) Prevalence of ESBLs produced in blood isolates of gram negative bacteria in a teaching hospital in Southern Thailand. *The South East Asian Journal of Tropical Medicine and Public Health* **37:** 131-135.

Kawai T (2006) Hypermucoviscosity: An extremely sticky phenotype of *Klebsiella pneumoniae* associated with emerging destructive tissue abscess syndrome. *Clinical Infectious Diseases* **42**: 1359-1361.

Keynan Y and Rubinstein E (2007) The changing face of *Klebsiella pneumoniae* infections in the community. *International Journal of Antimicrobial Agents* **30**: 385-389.

Kim YK, Pai H and Lee HJ (2002) Bloodstream infection by ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* in children: epidemiology and clinical outcome. *Antimicrobial Agents and Chemotherapy* **46**: 1481-1491.

Ko WC, Paterson DL, Sagnimeni AJ, Hansen DS and Gottberg AV (2002) Community-Acquired *Klebsiella pneumoniae* bacteremia: Global differences in clinical patterns. *Emerging Infectious Diseases* **8**: 160-166.

Koeleman JGM, Stoof J, Biesmans DJ, Savelkoul PHM and Vandenbrouke-Grauls CMJE (1998) Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. *Journal of Clinical Microbiology* **36**: 2522–2529.

Korvick JA, Bryan CS, Farber B, Beam TR, Schenfeld Jr I, Muder RR, Weinbaum D, Lumish R and Gerding DN (1992) Prospective observational study of *Klebsiella* bacteremia in 230 patients: outcome for antibiotic combination versus monotherapy. *Antimicrobial Agents and Chemotherapy* **36**: 2639-2644.

Lautenbach E, Patel JB, Bilker WB, Edelstain PH and Fishman NO (2001) Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*: risk factors for infection and impact on resistance of outcomes. *Clinical and Infectious Diseases* **32**: 1162-1171.

Leroyer A, Bedu A, Lombrail P, Desplanques L, Diakite B, Bingen E, Aujard Y and Brodin M (1997) Prolongation of hospital stay and extra costs due to hospital-acquired infection in a neonatal unit. *Journal of Hospital Infections* **35**: 37-45.

Leverstein-van Hall MA, Fluit AC, Paauw A, Box AT, Brisse S and Verhoef J (2002) Evaluation of the E-test ESBL and the BD Phoenix, Vitek1 and Vitek2 automated instruments for detection of extended-spectrum beta-lactamases in multi resistant *Escherichia coli* and *Klebsiella* spp. *Journal of Clinical Microbiology* **40**: 3703-3711.

Lin JC, Chang FY, Fung CP, Xu JZ, Cheng HP, Wang JJ, Huang LY and Siu LK (2004) High prevalence of phagocytic-resistance capsular serotypes of *Klebsiella pneumoniae* in liver abscess. *Microbes and Infection* **6**: 1191-1198.

Lin CW, Chiou CS, Chang YC and Yang TC (2008) Comparison of pulsed field gel electrophoresis and three REP-PCR methods for evaluating the genetic relatedness of *Stenotrophomonas maltophilia* isolates. *Letters in Applied Microbiology* **47**: 393-398.

Linscott AJ and Brown WJ (2005) Evaluation of four commercially available extended spectrum beta-lactamase phenotypic confirmation tests. *Journal of Clinical Microbiology* **43**: 1081-1085.

Livermore DM (1995) Beta-lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews* **8**: 557-584.

Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz IK, Luzzaro F, Poirel L and Woodford N (2007) CTX-M: changing the face of ESBLs in Europe. *Journal of Antimicrobial Chemotherapy* **59**: 165–174.

Lopes ACS, Rodrigues JF, Clementino MBM, Miranda CAC, Nascimento APA and Antonio de Morais Júnior M (2007) Application of PCR ribotyping and tDNA-PCR for *Klebsiella pneumoniae* identification. *Memórias do Instituto Oswaldo Cruzol*. **102**: 827-832.

Louws F, Schneider J and De Bruijn FJ (1997) Assessing genetic diversity of microbes using repetitive sequence-based PCR (REP-PCR). *Nucleic Acid Amplification Methods for the Analysis of Environmental Microbes*: 63-94.

Lu SH, Zhang YL, Geng SN, Li TY, Ye ZM, Zhang DS, Zou F and Zhou HW (2010) High diversity of extended spectrum beta-lactamase producing bacteria in an urban river sediment habitat. *Applied and Environmental Microbiology* **76**: 5972-5976.

Lupski JR (1993) Molecular epidemiology and its clinical application. *Journal of American Medical Association* **270**: 1363-1364.

Luzzaro F, Gesu G, Endimiani A, Ortisi G, Malandrin S, Pagani L and Rossolini GM (2006) Performance in detection and reporting beta-lactam resistance phenotypes in *Enterobacteriaceae*: a nationwide proficiency study in Italian laboratories. *Journal of Diagnostic Microbiology and Infections* **55**: 311-318

Marra A R, Wey SB, Castelo A, Gales AC, Cal RC, Filho JR, Edmond MB and Pereira CA (2006) Nosocomial bloodstream infections caused by *Klebsiella pneumoniae*: impact of extended-spectrum β-lactamase (ESBL) production on clinical outcome in a hospital with high ESBL prevalence. *BMC Infectious Diseases* **6**: 24-32.

Martínez J, Martínez L, Rosenblueth M, Silva J and Romero EM (2004) How are gene sequence analyses modifying bacterial taxonomy? The case of *Klebsiella*. *International Microbiology* **7**: 261-268.

Medeiros AA (1997) Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. *Clinical Infectious Diseases* **24**: 19-45.

Menashe G, Borer A, Yagupsky P, Peled N, Gilad J, Fraser D, Riesenberg K and Schlaeffer (2001) Clinical significance and impact on mortality of extended-spectrum beta lactamase-producing *Enterobacteriaceae* isolates in nosocomial bacteremia. *Journal of Infectious Diseases* **33**: 188-193.

Mohamudha PV, Srinivas AN, Rahul D, Harish BN and Parija SC (2010) Molecular epidemiology of multidrug resistant extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* outbreak in a neonatal intensive care unit. *Journal of Collaborative Research on Internal Medicine and Public Health* **2**: 226-237.

Moland SE, Hanson ND, Herrera VL, Black JA, Lockhart, TJ, Hossain A, Johnson JA, Goering RV and Thomson KS (2003) Plasmid-mediated, carbapenem hydrolyzing beta-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. *Journal of Antimicrobial Chemotherapy* **51**: 711-714.

Moland ES, Kim SY, Hong SG and Thomson KS (2008) Newer beta-lactamases: clinical and laboratory implications, part1. *Clinical Microbiology* **10**: 71-77.

Mulvey MR, Grant JM, Plewes K, Roscoe D and Boyd DA (2011) New Delhi metallo beta-lactamase in *Klebsiella pneumoniae* and *Eschericia coli*. *Emerging Infectious Diseases* **17**: 103-106.

Murray PR, Rosenthal KS and Pfaller MA (2005). Medical Microbiology. 5th ed. Philadelphia. *Elsevier Mosby*. Chapter **17**: 335-336.

Naas T, Nordmann P, Vedel G and Poyart C (2005) Plasmid-mediated carbapenem-hydrolysing beta-lactamase KPC in a *Klebsiella pneumoniae* isolate from France. *Antimicrobial Agents and Chemotherapy* **49**: 4423-4424.

National Institute for Communicable Diseases (2011) Outbreak of newly emerged, highly antibiotic resistant bacteria in hospitalized patients in Gauteng Province. (special report)

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264:** 382-388.

Nordmann P, Cuzon G and Naas T (2009) The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infectious Diseases* **9**: 228-236

Nyberg SD, Meurman O, Jalava J and Rantakokko-Jalava K (2008) Evaluation of detection of extended-spectrum beta-lactamases among *Escherichia coli* and *Klebsiella* spp. isolates by VITEK 2 AST-NO29 compared to the agar dilution and disk diffusion methods. *Scandinavian Journal of Infectious Diseases* **40**: 355-362.

O'Brien DJ, Wrenn C, Roche C, Rose L, Fenelon C, Flynn A, Murphy V, Fitzgerals SF, Fenelon LE, Crowley B and Schaffer (2011) First isolation and outbreak of OXA-48-producing *Klebsiella pneumoniae* in an Irish hospital, March to June 2011. *European Surveillance* **16**: 1-3.

Ofek I, Kabha K, Athamna A, Frankel G, Wozniak DJ, Hasty DL and Ohman DE (1993) Genetic exchange of determinants for capsular polysaccharides biosynthesis between *Klebsiella pneumoniae* strains expressing serotypes K2 and K21a. *Infection and Immunity* **61**: 4208-4216.

Ørskov I and Ørskov F (1984) Serotyping of *Klebsiella pneumoniae*. *Methods in Microbiology* **14**: 143-164.

Parveen MR, Manivannan S, Harish BN and Parija (2011) Study of CTX-M type of extended spectrum beta-lactamase among nosocomial isolates of *Escherichia coli* and *Klebsiella pneumoniae* in South India. *Indian Journal of Microbiology* : 1-6.

Paterson DL (2000) Recommendation for treatment of severe infections caused by *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBLs). *Clinical Microbiology and Infections* **6**: 460-463.

Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, Rice LB and Bonomo RA (2003) Extended-spectrum beta-lactamase in *Klebsiella pneumoniae* blood stream isolates from seven countries: dominance and widespread prevalence of SHV and CTX-M-type beta-lactanases. *Antimicrobial Agents and Chemotherapy* **47**: 3554-3560.

Paterson DL, Ko WC, Gottberg AV, Mohapatra S, Casellas J M, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG and Yu VL (2004) Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of beta-lactamases. *Clinical Infectious Diseases* **39**: 31-37.

Paterson DL and Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Review*s **18**: 657-686.

Payne SM and Neilands IB (1988) Iron and virulence in the family *Enterobacteriaceae*. *Critical Reviews in Microbiology* **16**: 81-111.

Perez F, Endimiani A, Hujer KM and Bonomo RA (2007) The continuing challenge of ESBLs. *Current Opinion in Pharmacology* **7**: 459-469.

Philippon LN, Nass T, Bouthor AT, Barakett V and Nordmann P (1997) OXA-18, a class D clavulanic acid-inhibited extended spectrum beta-lactamase from *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **41**: 2188-2195.

Pitout JD and Laupland KB (2008) Extended spectrum beta-lactamase producing *Enterobactericeae*: an emerging public health concern. *Lancet Infectious Diseases* **8**: 159-166.

Podschun R and Ullmann U (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews* **11**: 589–603.

Poirel L, Heritier C, Tolu V and Nordmann P (2004) Emergence of oxacillinase-Mediated resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **48**: 15-22.

Poirel L, Lagrutta E, Taylor P, Pham J and Nordmann P (2010) Emergence of metallo bela-lactamase NDM-1 producing multidrug resistant *Escherichia coli* in Australia. *Antimicrobial Agents and Chemotherapy* **54**: 4914-4916.

Poirel L, Revathi G, Bernabeu S and Nordmann P (2011a) Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. *Antimicrobial Agents and Chemotherapy* **55**: 934-936.

Poirel L, Benouda A, Hays C and Nordmann P (2011b) Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco. *Journal of Antimicrobial Chemotherapy* Advance access published on 19 September 2011.

Queenan AM, Foleno B, Gownley C, Wira E and Bush K (2004) Effects of inoculum and beta-lactamase activity in AmpC and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using National Committee for Clinical Laboratory Standards ESBL methodology. *Journal of Clinical Microbiology* **42**: 269–275.

Queenan AM and Bush K (2007) Carbapenemases: the versatile beta-lactamase. *Clinical Microbiology Reviews* **20**: 440-458.

Rademaker JLW and De Bruijn FJ (1997) Charaterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. In: DNA markers, protocols, applications and overviews. *WilleyLiss, Inc,* New York pp 151-171.

Rademaker JL, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P and De Bruijn FJ (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* **50**: 665-667.

Radice M, Power P, DiConza J and Gutkind G (2002) Early dissemination of CTX-M derived enzymes in South America. *Antimicrobial Agents and Chemotherapy* **46**: 602-604.

Ranjard L, Poly F and Nazaret S (2000) Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Research in Microbiology* **151**: 167-177.

Rasschaert G, Houf K and Imberechts H (2005) Comparison of five repetitive-sequence-based PCR typing methods for molecular discrimination of *Salmonella enterica* isolates. *Journal of Clinical Microbiology* **43**: 3615-3623.

Rawat D and Nair D (2010) Extended-spectrum beta-lactamases in Gram-negative bacteria. *Journal of Global Infectious Diseases* **2**: 263-274.

Report of the portfolio committee on health on an oversight visit to Charlotte Maxeke Johannesburg Academic Hospital on 21 May 2010 (2010). (www.pmg.org.za/2010/comreports/100721pchealthreport.htm).

Ristuccia PA and Cunha BA (1984) *Klebsiella*. *Infection Control* **5**: 343-348.

Robert C and Moellering MD (2010) NDM-1 A cause for worldwide concern. *New England Journal of Medicine* **363**: 2377-2379.

Rodrĭguez-Banŏ J, Navarro MD, Romero L, Muniain MA, Perea JE, RamónPérez-Cano, Hernández JR and Pascual A (2006) Clinical and molecular epidemiology of extended- spectrum beta-lactamase producing *Escherichia coli* as a cause of nosocomial infection or colonization: implications for control. *Clinical Infectious Diseases* **42**:37–45.

Ryan KJ, Ray CG and Sherris JC (2004) *Sherris* Medical Microbiology: an introduction to infectious diseases. *McGraw-Hill Professional,* pp: 343-370.

Sahly H, Auken H, Benedi VJ, Forestier C, Fussing V, Hansen DS, Ofek I, Podschun R, Sirot D, Tomas JM, Sandvang D and Ullmann U (2004) Increased serum resistance in *Klebsiella pneumoniae* strains producing extended spectrum beta-lactamases. *Antimicrobial Agents and Chemotherapy* **48**: 3477-3482.

Samaha-Kfoury JN and Araj GF (2003) Recent developments in beta-lactamases and extended spectrum beta-lactamases. *British Medical Journal* **327**: 1209-1213.

Sampaio JLM, Viana-Niero C, Freitas D, Hofling-Lima AS and Leao SC (2006) Enterobacterial repetitive intergenic consensus PCR is useful tool for typing *Mycobacterium chelonae* and *Mycobacterium abscessus* isolates. *Diagnostic Microbiology and Infectious Disease* **55**: 107-118.

Sanguinetti M, Posteraro B, Spanu T, Ciccaglione D, Romano L, Fiori B, Nicoletti G, Zanetti S and Fadda G (2003) Characterization of clinical isolates of *Enterobacteriaceae* from Italy by the BD Phoenix extended-spectrum beta-lactamase detection method. *Journal of Clinical Microbiology* **41**: 1463-1468.

Schembri MA, Blom J, Krogfelt KA and Klemm P (2003) Capsule and fimbria interaction in *Klebsiella pneumoniae*. *Journal of Infection and Immunity* **73**: 4626–4633.

Schwartz DC and Cantor CR (1984) Separation of yeast chromosome sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**: 67–75.

Seeto RK and Rockey DC (1996) Pyogenic liver abscess: changes in etiology, management and outcome. *Medicine* **75**: 99-113.

Shah AA, Hasan F, Ahmed S and Hameed A (2004a) Characteristics, epidemiology and clinical importance of emerging strains of gram-negative bacilli producing extended-spectrum beta-lactamases. *Research in Microbiology* **155**: 409-421.

Shah AA, Hasan F, Ahmed S and Hameed A (2004b) Prevalence of extended spectrum beta-lactamases in nosocomial and outpatients (ambulatory). *Pakistan Journal of Medical Science* **19**: 187-191.

Sharon N and Ofek I (2002) Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. *Critical Reviews in Food Science and Nutrition* **42**: 267-272.

Slopek S (1978) Phage typing of *Klebsiella*. *Academic Press* pp: 193–222.

Spanu T, Sanguinetti M, Tumbarello M, D'Inzeo T, Fiori B and Posteraro B (2006) Evaluation of the new Vitek2 ESBL test for rapid detection of ESBL production in *Enterobacteriaceae* isolates. *Journal of Clinical Microbiology* **44**: 3257-3262.

Spratt B (1994) Resistance to antibiotics mediated by target alterations. *Science* **264**: 388-393.

Stürenburg E and Mack D (2003) Extended-spectrum beta lactamases: implications for the clinical microbiology laboratory, therapy and infection control. *Journal of Infection* **47**: 273-295.

Tang YW and Stratton CW (2006) Advanced techniques in diagnostic microbiology. *Springer Science Business Media*, New York pp: 143-163.

Taylor P W (1983) Bactericidal and bacteriolytic activity of serum against gram negative bacteria. *Microbiology Reviews* **47**: 46–83.

Tham TN, Mabilat C, Courvalin P and Guesdon JL (1990) Biotinylated oligonucleotide probes for the detection and the characterization of TEM-type extended broad spectrum beta-lactamases in *Enterobacteriaceae*. *FEMS Microbiology Letters* **69**: 109-116.

Tofteland S, Haldorsen B, Dahl KH, Simonsensen GS, Steinbakk M, Walsh TR, Sundsfjord A and Norwegian ESBL Study group (2007) Effects of phenotype and genotype on methods for detection of extended-spectrum beta-lactamase producing clinical isolates of *Eshcerichia coli* and *Klebsiella pneumoniae* in Norway. *Journal of Clinical Microbiology* **45**: 199-205.

Tumbarello M, Spanu T, Sanguinetti M, Citton R, Montuori E, Leone F, Fadda G and Cauda R (2006) Bloodstream infections caused by extended spectrum beta-lactamase-producing *Klebsiella pneumoniae*: risk factors, molecular epidemiology and clinical outcome. *Antimicrobial Agents and Chemotherapy* **50**: 498-504.

Tzouvelekis LS, Tzelepi E, Tassios PT and Legakis NJ (2000) CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. *International Journal of Antimicrobial Agents* **14**: 137-142.

Valenza G, Müller S, Schmitt C, Turnwald D, Lam TT, Frosch M, Abele-Horn M and Pfeifer Y (2011) Evaluation of the Vitek2 AST-N111 card for detection of extended-spectrum beta-lactamases (ESBLs) in *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* compared to ESBL E-tests and combination disc methods. *European Journal of Clinical Microbiology and Infectious Diseases* **10**: 1169-1172.

Van Belkum A (1994) DNA fingerprinting of medically important microorganisms by use of PCR. *Clinical Microbiology Review* **7**: 174-184.

Van Leeuwen WB (2009) Molecular Diagnostics in Clinical Microbiology. *Iranian Journal of Microbiology* **1**: 5-20.

Versalovic J, Koeuth T, and Lupski JR (1991) Distribution of repetitive DNA sequences in Eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**: 6823-6831.

Versalovic J, Schneider M, Bruijn FJ and Lupski JR (1994) Genomic fingerprinting of bacteria using the repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cell Biology* **5**: 25-40.

Walsh C (2000) Molecular mechanisms that confer anti-bacterial drug resistance. *Nature* **406**: 775-781.

Walsh TR, Toleman MA and Jones RN (2003). Comment on occurrence, prevalence and genetic environment of CTX-M beta-lactamases in *Enterobacteriaceae* from Indian hospitals. *Journal of Antimicrobial Chemotherapy* **59**: 799–800.

Welsh J and McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* **18**: 7213-7218.

Wiegand I, Geiss HK, Mack D, Sturenburg E and Seifert H (2007) Detection of extended-spectrum beta-lactamases among *Enterobacteriaceae* by use of semiautomated microbiology systems and manual detection procedures. *Journal of Clinical Microbiology* **45**: 1167-1174.

Williams JG, Kubelik AR, Livak KJ, Rafalsky JA and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Research* **18**: 6531–6535.

Winokur PL, Canton R, Casellas JM and Legakis N (2001) Variations in the prevalence of strains expressing an extended-spectrum beta-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. *Clinical Infectious Diseases* **32**: 94–103.

Wong-Beringer A, Hindler J, Loeloff M, Queenan AM, Lee N, Pegues A, Quinn JP and Bush K (2002). Molecular correlation for the treatment outcomes in bloodstream infections caused by *Escherichia coli* and *Klebsiella pneumoniae* with reduced susceptibility to ceftazidime. *Clinical Infectious Diseases* **34**: 135-146.

Yeh KM, Kurup A, Siu LK, Koh YL, Fung CP, Lin JC, Chen TL, Chang FY and Koh TH (2007) Capsular serotype K1 or K2, rather than *magA* and *rmpA*, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. *Journal of Clinical Microbiology* **45:** 466-471.

Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K and Tenover FC (2001) Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **45:** 1151–1161

Yoneyama H and Nakae T (1991) Cloning of the protein D2 gene of *Pseudomonas aeruginosa* and its functional expression in the imipenem-resistant host. *Federation of European Biochemical Society Letters* **283**: 177-179.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K and Walsh TR (2009) Characterization of a new metallo beta-lactamase gene, $bla_{\text{NDM-1}}$ and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrobial Agents and Chemotherapy* **53**: 5046-5054.

Yu W, Chuang Y and Rasmussen W (2006) Extended-spectrum beta-lactamases in Taiwan: epidemiology, detection, treatment and infection control. *Journal of Microbiology, Immunology and Infection* **39**: 264-277.

Detection and characterization of ESBL positive *Klebsiella pneumoniae* clinical isolates

The editorial style of the journal, FEMS Immunology and Medical Microbiology, was followed in the following chapter

CHAPTER 3

3.1 ABSTRACT

Klebsiella pneumoniae is an opportunistic pathogen that can cause infection under certain conditions. The bacterium tends to cause infection in people with underlying diseases. Treatment of *K. pneumoniae* infection can be challenging, this is due to its ability to produce extended spectrum beta-lactamases. This study investigated the prevalence of ESBL-producing *K. pneumoniae* clinical isolates and determined the sensitivity and specificity of the Vitek2 advanced expert system (AES) (bioMérieux, France) and a multiplex PCR assay (targeting the bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes) against the combination disc method in detecting ESBL-production. A total of 150 consecutive clinical isolates of *K. pneumoniae* were collected and analysed for ESBL-production. The combination disc method was considered the gold standard; this method was performed and interpreted according to the Clinical and Laboratory Standards Institute's 2009 guidelines. The prevalence of ESBL positive *K. pneumoniae* according to the combination disc method was 57.4% (85/148). Compared to the combination disc method, which was used as a confirmatory test, the sensitivity and specificity of the Vitek2 AES in detecting ESBL-production were 99% (84/85) and 98% (62/63) respectively. The sensitivity and specificity of the multiplex PCR assay (using bla_{CTX-M} as a true marker for ESBL-production) were found to be 96% (82/85) and 98% (62/63) respectively. A good correlation was obtained between the three assays (multiplex PCR assay, Vitek2 AES and the combination disc method) evaluated in this study. According to the results, there is a high prevalence of ESBL-producing *K. pneumoniae* isolates in the Tshwane area. Infection control measures to reduce the spread of ESBL-producing isolates are recommended.

Key words: *Klebsiella pneumoniae*, $bla_{CTX-M,}$ extended spectrum beta-lactamases, prevalence, sensitivity and specificity

3.2 INTRODUCTION

Extended-spectrum beta-lactamase (ESBL)-producing bacteria were first isolated in Germany in 1983 (Huang *et al*, 2007). Extended-spectrum beta-lactamases are plasmid-mediated enzymes capable of hydrolyzing a beta-lactam bond in commonly prescribed beta-lactam antibiotics, such as penicillins, broad spectrum cephalosporins and aztreonam (Moland *et al*., 2008). Once the antibiotics are hydrolyzed they become ineffective (Moland *et al*., 2008). During the 1990s, the temoniera (TEM) and sulphydryl (SHV) ESBLs were the most dominant ESBLs all over the world, with cefotaximase (CTX-M) enzymes being rarely detected (Canton and Coque, 2006). The CTX-M enzymes are increasingly detected throughout the world and are becoming the most prevalent ESBLs found in bacterial isolates (Perez *et al*., 2007).

The marked increase in the incidence of infections caused by ESBL-producing bacteria is of great concern because any delay in detecting ESBL-producing strains may result in serious consequences, such as treatment failure and death (Paterson *et al*., 2003). Currently, the Clinical Laboratory Standards Institute (CLSI, 2009; CLSI, 2011) recommends screening for ESBL-production among *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca* and *Proteus mirabilis* (Pitout *et al*., 2005; Jeong *et al.,* 2008).

Klebsiella pneumoniae is among the leading nosocomial pathogens that cause pneumonia and bloodstream infections in intensive care units (Paterson, 2006; Marchaim *et al*., 2008). However, most of the *K. pneumoniae* strains have the tendency to produce ESBLs, which as described above renders these strains to be resistant to beta-lactam antibiotics (Luzzaro *et al*., 2006). Although ESBLs have been detected in a wide variety of Gram-negative bacteria, *K. pneumoniae* has been found to be the most common bacterial species to produce ESBLs (Paterson *et al*., 2004).

In the Nordic countries, the disc diffusion method is commonly used in clinical settings for ESBL detection and has been shown to have a specificity of 100% and sensitivity of 93.6% (Robin *et al*., 2007). Molecular methods, such as PCR and DNA sequencing can increase the detection of ESBL-production; however, these methods are not suitable for most routine diagnostic laboratories (Nyberg *et al*., 2008). The Vitek2 automated expert system (AES) (bioMérieux, France) detects ESBLs by giving an estimation of the minimum inhibitory

concentration (MIC), by comparing the growth curves to those of reference strains with known MICs and / or comparing the MIC of an antimicrobial agent when tested alone versus the MIC of antimicrobial agents when tested in combination with beta-lactamase inhibitors (clavulanic acid) (Dashti *et al*., 2006). In a study done in Italy the Vitek2 ESBL AES test system correctly identified 306 of the 312 ESBL-producing bacteria, yielding a sensitivity of 98.1% and a specificity of 99.7% (Spanu *et al*., 2006).

The prevalence of ESBL producers among enterobacterial isolates differs at institutions and varies between geographic regions (Yu *et al*., 2006). In some parts of the world, the prevalence of ESBL-producing *K. pneumoniae* strains in hospitals ranges from 5% to 25% (Marra *et al*., 2006). In the United States, the prevalence of ESBL-producing *K. pneumoniae* isolates is usually less than 9% (Dandekar *et al*., 2004). In Europe, the prevalence is around 15% to 20% (Luzzaro *et al*., 2006). In South Africa, several outbreaks of infections caused by ESBL-producing *Klebsiella* have been reported, but no national surveillance figures have been published (Bell *et al*., 2002). However, Bell *et al*. (2002) reported that 36% (13/36) of the *K. pneumoniae* isolates collected in a single South African hospital in 1998 and 1999 were ESBL producers.

The fact that patients most likely to become infected with ESBL-producing *Enterobacteriaceae* are those with prolonged stays in the ICU is of great concern (Paterson and Bonomo, 2005). Failure to rapidly detect ESBL-producing strains may result in serious consequences, such as treatment failure and death (Stürenburg and Mack, 2003; Pitout and Laupland, 2008). It is essential for diagnostic laboratories to use rapid and reliable methods for the detection of ESBL-producing bacteria (Brown *et al*., 2000; Tofteland *et al*., 2007). As long as there is no rapid and reliable test to detect ESBL-producing strains, patients will continue to be at risk of becoming infected with strains that will not respond to seemingly appropriate therapy, let alone the high mortality rate especially in ICUs.

The aim of this study was to determine the prevalence of ESBL-positive clinical isolates of *K. pneumoniae.* The study compared the sensitivity and specificity of the Vitek2 AES (bioMérieux, France) and a multiplex PCR assay against the combination disc method in detecting ESBL-producing *K. pneumoniae* isolates.

3.3 MATERIALS AND METHODS

3.3.1 Study setting, sample collection and storage

The study was conducted at the University of Pretoria, Department of Medical Microbiology/ National Health Laboratory Service (NHLS), Pretoria, South Africa. This study was approved by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (protocol number S21/2010).

A total of 150 consecutive clinical isolates of *K. pneumoniae* identified by the Vitek2 AES (bioMérieux, France) were obtained from clinical specimens sent for analysis from the Steve Biko Academic Hospital to the Department of Medical Microbiology, Tshwane Academic Division, National Health Laboratory Service (NHLS). The sample size was calculated for the conservative series where a prevalence of 60% is assumed. The collected isolates were temporarily stored in a cold room (for less than two weeks); the isolates were subcultured on sheep blood agar and incubated for 24 hrs at 37˚C (HF 212 UV, Shangai Lishen Scientific Equipment, China) in order to obtain a fresh culture. The fresh culture was used to prepare 0.5 McFarland standard suspensions (for ESBL combination disc detection method) and to inoculate 3 ml of Brain Heart Infusion (BHI) broth (Oxoid, England), which was subsequently incubated for 18 to 24 hrs at 37˚C (HF 212 UV, Shangai Lishen Scientific Equipment, China). After incubation, 200 µl of the cultured broth was used for DNA extraction and the remaining broth culture was mixed with an equal volume of 50% sterile glycerol and stored at -70˚C (New Brunswick Scientific, Germany) for future use.

3.3.2 ESBL detection methods

The Vitek2 AES (bioMérieux, France), the combination disc method (Mast Diagnostics) and a multiplex PCR assay were used to detect ESBL-producing *K. pneumoniae* isolates. *Klebsiella pneumoniae* ATCC 700603 (ESBL-positive) and *Escherichia coli* ATCC 25922 (ESBL-negative) quality control strains were included in all the ESBL detection methods. All isolates showing discordant results were re-tested with all three assays. All isolates with discordant results after re-testing were sequenced by Inqaba biotec (Pretoria). The sequences were edited with CLC DNA workbench 6.0 (CLC bio, Denmark) and analyzed using the basic local alignment tool software (BLAST) programme (http://www.ncbi.nlm.nih.gov/BLAST).

3.3.2.1 Vitek2 AES ESBL detection method

The Vitek2 AES (bioMérieux, France) ESBL detection method was performed by a qualified medical technologist as part of the routine laboratory diagnostic testing at the Department of Medical Microbiology (NHLS/TAD). In brief, a fresh culture of the bacterial isolate to be tested was diluted to a standard concentration (0.5 to 0.6 McFarland standard) and inoculated into the Vitek2 AST NO64 and/or AST N133 card (bioMérieux, France) containing antibiotics in the wells at different concentrations. The system monitors the growth of each well in the card over a defined period of time (Nyberg *et al*., 2008). After incubation, the MIC values were measured using the Vitek2 software. After determining the MIC values, a report classifying the tested bacterial isolate as ESBL-positive or negative was generated (Dashti *et al*., 2006).

3.3.2.2 Combination disc ESBL detection method

The combination disc method (CLSI, 2009; CLSI, 2011) was used as the gold standard and it was used for the confirmation of all ESBL-positive strains as recommended by the CLSI guidelines (2009, 2011). Briefly, a 0.5 McFarland standard of the tested bacterium was prepared according to the standard disc diffusion recommendations. The 0.5 McFarland standard suspension was then spread in three directions onto the Mueller-Hinton (Oxoid, England) media plate. Two sets of discs (Mast diagnostic, Merseyside, UK), ceftazidime (30 µg) and cefotaxime (30 µg) discs alone and in combination with beta-lactamase inhibitor (clavulanic acid 10 µg) were applied on the agar using a sterile disc dispenser. The media plates were incubated at +/- 37˚C (Shangai Lishen Scientific Equipment, China) for 16 to 18 hours. After incubation, the plates were examined for growth and the zone of inhibition was measured. The isolates were termed ESBL producers if there was an equal or more than 5 mm increase in the zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone (CLSI, 2009; CLSI, 2011).

3.3.2.3 Multiplex PCR ESBL detection method

The multiplex PCR assay (Monstein *et al*., 2007) was performed using the Qiagen Multiplex PCR Kit (Qiagen, Germany). This assay was used to simultaneously detect the presence of three sets of genes ($bla_{\text{CTX-M}}$ =593 bp, bla_{SHV} =747 bp, and bla_{TEM} = 445 bp) associated with

ESBL-production. The product size and primer sequences of these genes are shown in Table 3.1. The DNA was manually extracted using a commercial DNA extraction kit (Zymo Research, USA), according to the manufacturer's protocol. The extracted DNA samples were stored at -20ºC (Whirlpool, USA) until further use.

The multiplex PCR reaction mix was prepared according to the manufacturer's instructions (Qiagen, Germany). The total reaction volume was 50 µl and it consisted of the following multiplex PCR components: 25 µl of 2X Qiagen multiplex PCR master mix (Hotstar*Taq* DNA polymerase, multiplex PCR buffer and dNTP mix), 5 µl of 20 pmol/µl primer mix, 5 µl of 5X Q-Solution, 10 µl of RNase- free water (Qiagen, Germany) and 5 µl of template DNA. The amplification of DNA was done using a Mastercycler (Eppendorf, Germany) with the following cycling conditions: initial activation step (HotStar*Taq* DNA Polymerase) at 95˚C for 15 minutes, 40 cycles of denaturation at 94˚C for 30 seconds, annealing at 59˚C for 90 seconds, extension at 72˚C for 90 seconds, followed by a final extension step at 72˚C for 10 minutes.

The separation of the amplified products was done using 1% (m/v) agarose (Seakem, USA) gel electrophoresis in a 1X TBE buffer [1 mM EDTA (Promega, Madison, USA), 10 mM Boric acid (Analar, Merck, Canada) and 10 mM Tris-HCl (Calbiochem, Merck, Canada), pH 8.0] at 80 V for 120 minutes. On each gel a standard molecular size marker (1 Kb ladder) (Fermentas, Lithuania) was included to estimate the sizes of the DNA fragments. The DNA fragments were visualized using the Ultraviolet Light Box (UVP products, USA). Visualization of the DNA fragments was made possible by the addition of 5 µl ethidium bromide (10 µg/ml) (Promega, Madison, USA) in the gel before it was completely solidified.

3.3.3 Statistical analysis

In this study, descriptive statistics (percentage frequencies) was used do determine the prevalence rates of ESBL-positive bacterial isolates using the combination disc method recommended by the CLSI. The two ESBL detection methods (Vitek2 AES and multiplex PCR assay) were investigated and compared using specificity and sensitivity with the combination disc method used as the confirmatory test (gold standard).

The sensitivity (%) was calculated as follows: (number of isolates positive for ESBL-production by multiplex PCR or Vitek2 AES) X 100/ (number of isolates positive for ESBL-production by the combination disc method). The specificity (%) was calculated as follows: (number of isolates showing a negative result for ESBL-production by multiplex PCR or Vitek2 AES X100/ (number of isolates that failed to produce any ESBLs by the combination disc method).

3.4 RESULTS

The results of the quality control strains were all within the reference range; *K. pneumoniae* ATCC 700603 showed a more than 5 mm increase in zone diameter of ceftazidime and cefotaxime disc tested alone vs its zone when tested in combination with clavulanic acid. The *E. coli* ATCC 25922 strain showed an equal or less than 2 mm increase in zone diameter for an antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid). Two isolates were contaminated and excluded from the study, thus, a total of 148 *K. pneumoniae* isolates were analysed.

The combination disc method was used as a confirmatory test for detecting ESBL-production and it was performed and interpreted according to the CLSI, 2009 guidelines. The combination disc containing cefotaxime detected the presence of ESBL activity in 57% (84/148) *K. pneumoniae* clinical isolates, while the ceftazidime combination disc detected ESBL activity in 57% (85/148) *K. pneumoniae* isolates (Table 3.4). The overall prevalence of ESBL-producing *K. pneumoniae* isolates was 57% (85/148) (Table 3.2) when the results of both the two cephalosporin combination discs were taken into consideration. The results from the Vitek2 AES (bioMérieux, France) were reported as either ESBL-positive or ESBL-negative. The overall sensitivity and specificity of the Vitek2 AES in this study were 99% (84/85) and 98% (62/63) respectively when compared to the combination disc method (confirmatory test) (Table 3.3).

The multiplex PCR assay was successfully performed on all 148 *K. pneumoniae* clinical isolates. The 1% agarose gel electrophoresis revealed a distinct separation of the targeted genes associated with ESBL-production ($bla_{\text{CTX-M}}$ =593 bp, bla_{SHV} =747 bp, and bla_{TEM} = 445 bp) (Figure 3.1). The bla_{SHV} gene was detected in 79% (117/148) of the bacterial isolates, bla_{TEM} gene was detected in 51% (76/148) of the bacterial isolates and the $bla_{\text{CTX-M}}$ gene was

detected in 57% (84/148) bacterial isolates. The combination of the bla_{SHV} and bla_{TEM} genes were detected in 13% (6/148) of the *K. pneumoniae* isolates, whilst the combination of the bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes was detected in 41% (61/148) of the *K. pneumoniae* clinical isolates. In this study the bla_{SHV} gene alone was detected in 18% (27/148) of the bacterial isolate. The multiplex PCR assay in this study revealed the absence of bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes in 11% (16/148) of the *K. pneumoniae* clinical isolates. The sensitivity and specificity of the multiplex PCR assay in detecting ESBL-positive *K. pneumoniae* isolates (using CTX-M gene as a true marker for ESBL-production) were reported as 96% (82/85) and 98% (62/63) respectively (Table 3.3) when compared to the combination disc method. Sequencing of discordant results were as follows; isolate number 44 had bla_{SHV2} and bla_{TEM} genes, isolate number 93 had bla_{SHV-1} and bla_{TEM-1} genes, isolate number 111 had bla_{SHV-1} and bla_{TEM-1} genes. Sequencing of the bla_{CTX-M} gene was unsuccessful.

3.5 DISCUSSION

In this study, the prevalence of ESBL-producing *K. pneumoniae* isolates was successfully determined using several different methods. The sensitivity and specificity of the Vitek2 AES and multiplex PCR assay in detecting ESBL-positive *K. pneumoniae* isolates when compared to the combination disc method (gold standard) were determined. The prevalence of ESBL-producing *K. pneumoniae* clinical isolates observed in this study was 57% (85/148), which is higher than that described in similar studies. Bell and colleagues (2002) reported a prevalence of 36% (13/36) among *K. pneumoniae* isolates collected between 1998 and 1999 in a South African medical centre. Another study conducted in a South African medical center by Hirakata *et al*. (2005) which included 135 *K. pneumoniae* isolates collected between 1998 and 2002, reported a prevalence of 31% (38/135) ESBL-producing *K. pneumoniae* isolates. The results of this study when compared to the study by Hirakata *et al*. (2005) shows that the prevalence of ESBL-producing isolates in South Africa have increased in the past 10 years.

In Tanzania, Mshana and colleagues (2009) reported a prevalence of 63% (58/91) ESBL-producing *K. pneumoniae* isolates, which is slightly higher than the 57% (85/148) prevalence observed in this study. Similar studies conducted in Southern Thailand by Jitsurong and Yodsawat (2006) reported an ESBL prevalence of 44% (16/36) in *K. pneumoniae* isolates obtained from blood culture specimens. A study done in South Arabia by Tawfik *et al.* (2011)

which included 430 *K. pneumoniae* isolates collected over a six months period from January to June 2008, reported a prevalence of 25.6% (110/430) ESBL-producing *K. pneumoniae*.

The prevalence of ESBL-production observed in this study is high and poses a threat to the treatment of serious infections due to *K. pneumoniae* isolates. All the ESBL isolates from this study were reported as being sensitive to meropenem and imipenem by the Vitek2 AES. However, this does not necessarily mean that all isolates were sensitive to meropenem and imipenem because the Vitek2 AES might have misinterpreted the susceptibility results. The sensitivity and specificity of the Vitek2 AES system in the identification of carbapenemase is 78% and 38% respectively (Coetzee *et al*., 2011). A multiplex PCR assay targeting genes associated with carbapenemase-production (OXA-23, OXA-24, OXA-51 and OXA 58) corroborated the results obtained with the Vitek2 AES by yielding negative results for all the isolates tested (data not shown). The results showed that in the Tshwane region (South Africa), it is rare to find *K. pneumoniae* isolates that are resistant to carbapenem antibiotics. Thus making these carbapenems a drug of choice in treating infections caused by ESBL-producing isolates. However, in the near future the carbapenem antibiotics will become useless due to the emergence of carbapenem resistant isolates, such as the KPC and NDM-1-producing isolates, which have recently been reported in the Gauteng region of South Africa (Coetzee *et al*., 2011).

In other settings, such as New York, the production of carbapenemases by *K. pneumoniae* isolates have been reported (Woodford *et al*., 2004). Yong and colleagues (2009) identified a new type of carbapenem-resistant *K. pneumoniae* isolate designated New Delhi Metallo-beta-lactamase-1 strain (NDM-1). Payal *et al*. (2010) have recently isolated NDM-1 isolates in patients admitted at a tertiary hospital centre in Mumbai. In South Africa, an outbreak of NDM-1 producing isolates have recently been reported (National Institute for Communicable Diseases, 2011). The emergence of the NDM-1 strain is a great concern in the clinical setting; this strain has been reported to be resistant to almost all beta-lactam antibiotics and other classes of antibiotics (Raghunath, 2010). Colistin and tigecycline are some of the few antibiotics that can be used to treat infections caused by NDM-1-producing isolates (National Institute for Communicable Diseases, 2011). However, colistin is highly toxic and has significant side effects, such as nephrotoxicity and neurotoxicity (Perez *et al*., 2007).

This study revealed that the ceftazidime combination disc is more specific than the cefotaxime combination disc in detecting ESBL activity (Table 3.4). This is supported by the fact that the cefotaxime disc failed to detect one *K. pneumoniae* isolate (isolate number 44) as ESBL positive, instead it reported the isolate as ESBL-negative. This observation has been reported in a similar study (Gheldre *et al*., 2003). A study done by Gheldre *et al*. (2003) concluded that the detection rate of ESBL-production is increased when more than one cephalosporin combination disc are used. In line with results by Gheldre *et al*. (2003), this study confirmed the higher sensitivity of the ceftazidime/clavulanate discs compared with the cefotaxime/clavulanate discs in detecting ESBL-producing *K. pneumoniae* isolates.

The Vitek2 AES was able to detect the presence of ESBL in 57% (84/148) of *K. pneumoniae* isolates (Table 3.2). When the Vitek2 AES was compared to the combination disc assay (gold standard), it revealed a high level of agreement, with the exception of one isolate, which was misidentified (isolate no 44) as ESBL-negative by the Vitek2 AES. In comparison with the combination disc method the overall sensitivity and specificity of the Vitek2 AES were 99% and 98%, respectively (Table 3.3). Sorlózano *et al*. (2005) reported that the Vitek2 AES is as good as phenotypic methods, such as disc diffusion and ESBL E-test in detecting ESBL-producing isolates. Furthermore, the high sensitivity and specificity of the Vitek2 AES observed in this study are consistent with that observed by Spanu *et al.* (2006), where a sensitivity of 97.7% (306/312) and a specificity of 99.7% (815/817) by the Vitek2 AES in detecting ESBL-production among a total of 1 129 *Enterobacteriaceae* clinical isolates were reported. In a study by Nyberg *et al.* (2008), the Vitek2 AES reported an accuracy of 91.1% (113/123) in detecting ESBL-production. The sensitivity and specificity were reported as 89.7% and 85.7% respectively, which is much lower than that observed in this study. The results of this study showed that the Vitek2 AES is a reliable and easy to perform assay in detecting ESBL-producing *K. pneumoniae* isolates.

The prevalence rates of the bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes in *K. pneumoniae* isolates were found to be 79% (117/148), 57% (85/148) and 51% (76/148) respectively. In this study, the bla_{CTX-M} gene was detected in 96% (82/85) of the ESBL-producing *K. pneumoniae* clinical isolates. The widespread distribution of the bla_{CTX-M} enzymes in hospital settings is likely to be caused by the spread of mobile genes through plasmids and transposons among unrelated strains (Canton and Coque, 2006).

The multiplex PCR assay (Monstein *et al*., 2007) employed in this study revealed the absence of the *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes in 11% (16/148) of the *K. pneumoniae* clinical isolates, as anticipated, all of these 16 isolates were reported as none ESBL-producers by the combination disc method. Interestingly, some of the strains harbouring the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes were found to be none ESBL-producers by the combination disc method (gold standard) as well as by the Vitek2 AES. These discrepancies are; however, not surprising, because there are various variants of SHV and TEM genes not associated with ESBL-production. Such variants include the SHV-1 subtype that is not associated with ESBL-production (Chaves *et al*., 2001), instead this gene has been reported to be chromosomally located in the *K. pneumoniae* genome (Chaves *et al*., 2001; Ford and Avison, 2004).

Discordant results were encountered in six (44, 59, 61, 88, 93 and 111) of the 148 isolates. The discordant results obtained for isolates number 88 and 61 were resolved by repeating the multiplex PCR assay. Isolate number 44 was ESBL-positive by the combination disc method but was reported as ESBL-negative by the Vitek2 AES. However, this isolate was found to harbour the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes according to the multiplex PCR assay. The sequencing of these genes revealed that this isolate was misidentified as ESBL-negative by the Vitek2 AES, since it was found to possess the *bla*$_{SHV-2}$ subtype, which is consistent with ESBL-production as reported by Nyberg and colleagues (2008). Isolate number 59 was found to be ESBL-negative by the combination disc method and it was found to harbour no ESBL gene by the multiplex PCR assay; surprisingly the Vitek2 AES falsely reported this isolates as an ESBL-producing isolate. There is no perfect explanation with regard to the discordant results showed by isolate number 59. However, similar results were reported by Nyberg and colleagues (2008) in which one *E. coli* isolate tested negative for ESBL genes (*bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{TEM}$) but was reported as an ESBL-producing isolate by the Vitek2 AES. However, the same isolate was reported as an ESBL-negative by the disc diffusion and agar dilution ESBL tests (Neyberg *et al*., 2008).

Isolate number 93 was reported as being a non-ESBL-producing isolate by the combination disc method, in the Vitek2 AES this isolate was reported as being an inhibitor resistant TEM (IRT) isolate. In the multiplex PCR assay, the isolate was found to harbour the *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes. Sequencing of the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes revealed that these genes did not code for ESBL-production since they were of the *bla*$_{SHV-1}$ and *bla*$_{TEM-1}$ subtypes (Chaves *et al*., 2001; Nyberg *et al*., 2008). However, isolate number 93 was found to harbour the

bla$_{CTX-M}$ gene but was still reported as ESBL-negative by the Vitek2 AES and the combination disc method. Discordant results showed by isolate number 93 might have been caused by the failure of the phenotypic assays to detect ESBL-production in an inhibitor resistant isolate coexisting with ESBL enzymes. A similar observation has been reported in a study by Bell and colleagues (2007) in which a *K. pneumoniae* isolate was found to harbour *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes but yet reported as ESBL-negative by combination disc method. The sequencing of the *bla*$_{CTX-M}$ gene failed because of the degeneracy of the primers that were used.

Isolate number 111 was reported as ESBL-positive by the Vitek2 AES and the combination disc method. This isolate was found to harbour a *bla*$_{SHV}$ gene by the multiplex PCR assay, sequencing of this gene showed that it was a *bla*$_{SHV-1}$ subtype which is mostly a non-ESBL subtype (Chaves *et al*., 2001). The discordant results in isolate number 111 might be due to hyperproduction of the SHV-1 enzyme. Hyperproduction of the SHV-1 enzyme has been shown to give false positive ESBL results in phenotypic methods (Rice *et al*., 2000). Similar results were reported by Wu and colleagues (2001) in a study which included nine isolates of *K. pneumoniae* collected during an outbreak in a neonatal ward from June 1997 to April 1998.

Most of the SHV-1, TEM-1 and some other variants of the SHV and TEM genes code for non-ESBL phenotypes, therefore it is essential to sequence and analyze the SHV and TEM amplicons detected by the multiplex PCR assay using Bioinformatics. Further analysis will aid to reveal if the detected genes are of ESBL genotype or non ESBL genotype. If these genes are not sequenced, it will be difficult to conclude whether these genes are associated with ESBL-production or not, unless compared with other detection methods, such as the combination disc method, as was done in this study.

This study showed that the presence of the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes in the bacterial genome does not always correlate with the level of ESBL-production by the bacterial isolate (poor comparison with combination disc method). Nevertheless, in contrast with the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes, the results of this study showed that the detection of the *bla*$_{CTX-M}$ gene is usually associated with ESBL-production. Pitout and Laupland (2008) outlined that the PCR amplification of *bla*$_{CTX-M}$ specific products in bacterial isolates suspected of producing ESBLs without sequencing usually provides sufficient evidence that a *bla*$_{CTX-M}$ gene is responsible for the ESBL-production (Pitout and Laupland, 2008).

3.6 CONCLUSIONS

There is a high prevalence of ESBL-producing *K. pneumoniae* clinical isolates in the Tshwane area. The high prevalence of ESBL-producing *K. pneumoniae* in this clinical setting is a great concern; infections with these bacteria have serious implications, such as high mortality rate, because ESBL-production is one of the important causes of treatment failure. Strict infection control measures, such as the appropriate use of antibiotics and isolation of patients infected with ESBL-producing isolates are needed.

The Vitek2 AES results were found to be comparable to the results of the combination disc method recommended by the CLSI in detecting ESBL-positive *K. pneumoniae* clinical isolates. Detection of the bla_{SHV} and bla_{TEM} genes in a bacterial isolate does not always correlate with ESBL-production by a bacterial isolate. The multiplex PCR assay (using the $bla_{CTX\text{-}M}$ gene as a true marker for ESBL production) evaluated in this study was also comparable to the combination disc method. However, the assay is not as rapid as the Vitek2 AES in detecting ESBL-production among *K. pneumoniae* isolates. Nevertheless, the multiplex PCR assay may be useful, if used as a screening assay for the detection of circulating *bla* genes in the Tshwane region. Future studies with an additional panel of various bacteria (eg *E. coli*) is recommended in order to assess the overall usefulness of the Vitek2 AES (bioMérieux, France) in detecting ESBL-producing bacterial isolates.

3.7 ACKNOWLEDGEMENTS

The authors would like to thank the Department of Medical Microbiology, UP/NHLS for the financial support provided for this research project. Mr GI Manenzhe would like to thank the South African Medical Research Council (MRC) and National Research Foundation (NRF) for the financial support received. The authors would also like to thank the Diagnostic Division of the National Health Laboratory Service (NHLS), Pretoria, South Africa.

3.8 REFERENCES

Bell JM, Turnidge JD, Gales A C, Pfaller M and Jones RN (2002) Prevalence of extended spectrum beta-lactamase (ESBL)-producing clinical isolates in the Asia-Pacific region and South Africa: regional results from SENTRY Antimicrobial Surveillance Program (1998–99). *Diagnostic Microbiology and Infectious Disease* **42**: 193–198.

Bell JM, Chitsaz M, Turnidge JD, Barton M, Walters LJ and Jones RN (2007) Prevalence and significance of a negative extended-spectrum beta-lactamases (ESBL) confirmation test result after a positive ESBL screening test result for isolates of *Escherichia coli* and *Klebsiella pneumoniae*: results from the SENTRY Asia-Pacific surveillance program. *Journal of Clinical Microbiology* **45**: 1478-1482.

Brown DFJ, Andrews J, King A and Alasdair P (2000) Detection of extended-spectrum beta-lactamases with E-test and double-disc potentiation methods. *Antimicrobial Agents and Chemotherapy* **146**: 323-342.

Canton R and Coque TM (2006) The CTX-M beta-lactamase pandemic. *Current Opinion in Microbiology* **9**: 466-475.

Chaves J, Ladona MG, Segura C, Coira A, Reig R and Ampurdanes C (2001) SHV-1 beta-lactamase is mainly a chromosomal encoded species-specific enzyme in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **45**: 2856-2861.

Clinical and Laboratory Standards Institute (2009) Performance standards for antimicrobial susceptibility testing: *Nineteenth Informational Supplement CLSI*, Wayne, PA, USA.

Clinical and Laboratory Standard Institute (2011) Performance standards for antimicrobial susceptibility testing; *Twenty-First Information Supplement CLSI* Wayne. PA, USA.

Coetzee J, Clay CG and Brink A (2011) Clinical microbiology considerations related to the emergence of New Delhi metallo-beta-lactamase (NDM-1) and *Klebsiella pneumoniae* carbapenemases (KPC) amongst hospitalized patients in South Africa. *Federation of Infectious Diseases Societies of Southern Africa*. (Special report)

Dandekar PK, Tetreault J, Quinn JP, Nightingale CH and Nicolau DP (2004) Prevalence of extended spectrum beta-lactamase producing *Escherichia coli* and *Klebsiella* isolates in a large community teaching hospital in Connecticut. *Diagnostic Microbiology and Infectious Disease* **49**: 37-39.

Dashti AA, West P, Paton R and Amyes SGB (2006) Characterization of ESBL-producing Kuwait and UK strains identified by the Vitek2 system, and subsequent comparison of the Vitek2 system with other commercial ESBL-testing systems using these strains. *Journal of Medical Microbiology* **55**: 417-421.

Ford PJ and Avison MB (2004) Evolutionary mapping of the SHV beta-lactamase and evidence for two separate IS*26*-dependent bla_{SHV} mobilization events from *Klebsiella pneumoniae* chromosome. *Journal of Antimicrobial Chemotherapy* **54**: 69-75.

Gheldre YD, Avesani V, Berhin C, Delmée M and Glupczynski Y (2003) Evaluation of Oxoid combination discs for detection of extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy* **52**: 591–597.

Hikarata Y, Matsuda J, Miyazaki Y, Kamihira S, Kawakami S, Miyazawa Y, Ono Y, Nakazaki N, Hirata Y, Inoue M, Turnidge JD, Bell JM, Jones RN, Kohno S and Sentry Asia-Pacific participants (2005) Regional variation in the prevalence of entended spectrum beta-lactamase producing clinical isolates in the Asia-Pacific region (SENTRY 1998-2002). *Diagnostic Microbiology and Infectious Disease* **52**: 323-329.

http://www.ncbi.nlm.nih.gov/BLAST.

Huang Y, Zhuang S and DU S (2007) Risk factors of nosocomial infection with extended-spectrum beta-lactamase-producing bacteria in a neonatal intensive care unit in China. *Infection* **35**: 339-345.

Jeong SH, Song W, Park M, Kim J, Kim H, Bae IK and Lee KM (2008). Boronic acid disk tests for identification of extended-spectrum beta-lactamase production in clinical isolates of *Enterobacteriaceae* producing chromosomal AmpC beta-lactamases. *International Journal of Antimicrobial Agents* **31**: 467-471.

Jitsurong S and Yodsawat J (2006) Prevalence of ESBLs produced in blood isolates of gram negative bacteria in a teaching hospital in Southern Thailand. *The South East Asian Journal of Tropical Medicine and Public Health* **37:** 131-135.

Luzzaro F, Gesu G, Endimiani A, Ortisi G, Malandrin S, Pagani L and Rossolini GM (2006) Performance in detection and reporting beta-lactam resistance phenotypes in *Enterobacteriaceae*: a nationwide proficiency study in Italian laboratories. *Journal of Diagnostic Microbiology* **55**: 311-318.

Marchaim D, Navon-Venezia S, Schwaber MJ and Carmeli Y (2008) Isolation of imipenem-resistant *Enterobacter* species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. *Antimicrobial Agents and Chemotherapy* **52**: 1413-1418.

Marra AR, Pereira CAP, Castelo A, Do Carmo Filho JR, Cal RGR, Sader HS and Wey SB (2006) Health and economic outcomes of the detection of *Klebsiella pneumoniae* produced extended-spectrum beta-lactamase in a hospital with high prevalence of this infection. *International Journal of Infectious Diseases* **10**: 56-60.

Moland ES, Kim SY, Hong SG and Thomson KS (2008) Newer beta-lactamases: clinical and laboratory implications, part 1. Center for research in anti-infective and biotechnology. *Clinical Microbiology NewsLetter* **30**: 71-77.

Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K and Nilsson LE (2007) Multiplex PCR amplification assay for the detection of bla_{SHV}, bla_{TEM} and bla_{CTX-M} genes in *Enterobacteriaceae*. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **115**: 1400-1408.

Mshana SE, Kamugisha E, Mirambo M, Chakraborty T and Lyamuya EF (2009) Prevalence of multiresistant gram-negative organisms in a tertiary hospital in Mwanza, Tanzania. *BioMed Central Research Notes* **49**: 1-6.

National Institute for Communicable Diseases (2011) Outbreak of newly emerged, highly antibiotic resistant bacteria in hospitalized patients in Gauteng Province (special report).

Nyberg SD, Meurman O, Jalava J and Jalava KR (2008) Evaluation of detection of ESBLs among *E. coli* and *Klebsiella* spp isolates by Vitek2 AST-NO29 compared to the agar dilution and disc diffusion methods. *Scandinavian Journal of Infectious Diseases* **40**: 355-362.

Paterson DL, Ko W, Gottberg AV, Mohapatra S, Casellas JM, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice BL, Wagener MM, McCormack JG and Yu VL (2004) Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. *Clinical Infectious Diseases* **39**: 31–37.

Paterson DL and Bonomo RA (2005) Extended-spectrum beta-lactamase: a clinical update. *Clinical Microbiology Reviews* **18:** 657-686.

Paterson DL (2006) Resistance in gram negative bacteria: *Enterobacteriaceae*. *The American Journal of Medicine* **6**: 20-28.

Payal D, Camilla R, Anjali S, Farhad K, Ashit H and Rajeev S (2010) New Delhi Metallo beta-lactamase (NDM-1) in *Enterobacteriaceae*: treatment options with carbapenems compromised. *Journal of Association of Physician of India* **58**: 147-150.

Perez F, Endimiani A, Hujer KM and Bonomo RA (2007) The continuing challenge of ESBLs. *Current Opinion in Pharmacology* **7**: 459-469.

Pitout JDD, Nordmann P, Laupland KB and Poirel L (2005) Emergence of *Enterobacteriaceae* producing extended-spectrum beta-lactamase (ESBLs) in the community. *Journal of Antimicrobial Chemotherapy* **10**: 1093.

Pitout JDD and Laupland KB (2008) Extended-spectrum beta-lactamase producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infectious Diseases* **8**: 159-166.

Raghunath D (2010) New metallo beta-lactamase NDM-1. *Indian Journal of Medical Research* **132**: 478-481.

Rice LB, Caries LL, Hujer AM, Bonafede M, Hutton R, Hoyen C and Bonomo RA (2000) High-level expression of chromosomaly encoded SHV-1 beta-lactamase and an outer membrane protein change confer resistance to ceftazidime and piperacillin-tazobactam in clinical isolate of *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **44**: 362-367.

Robin F, Delmas J, Schweitzer C and Bonnet R (2007) Evaluation of the Vitek-2 extended-spectrum beta-lactamase test against non-duplicate strains of *Enterobacteriaceae* producing a broad diversity of well-characterized beta-lactamases. *European Society of Clinical Microbiology and Infectious Diseases* **14**: 148-154.

Sorlózano A, Gutiérrez J, Piédrola G and José Soto M (2005) Acceptable performance of Vitek2 system to detect extended spectrum beta-lactamase in clinical isolates of *Escherichia coli*: a comparative study of phenotypic commercial methods and NCC guidelines. *Diagnostic Microbiology and Infectious Disease* **51**: 191-193.

Spanu T, Sanguinetti M, Tumbarello M, D'Inzeo T, Fiori B and Posteraro B (2006) Evaluation of the new Vitek2 ESBL test for rapid detection of ESBL production in *Enterobacteriaceae* isolates. *Journal of Clinical Microbiology* **44**: 3257-3262.

Stürenburg E and Mack D (2003) Extended-spectrum beta lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. *Journal of Infection* **47**: 273-295.

Tawfik AF, Alswailem AM, Shib AM and AL-Agamy MH (2011) Prevalence and genetic characteristics of TEM, SHV and TEM in clinical *Klebsiella pneumoniae* isolates from Saudi Arabia. *Microbial Drug Resistanc*e **3**: 383-388.

Tofteland S, Haldorsen B, Dahl KH, Simonsensen GS, Steinbakk M, Walsh TR, Sundsfjord A and Norwegian ESBL Study group (2007) Effects of phenotypic and genotypic on methods for detection of extended-spectrum beta-lactamase producing clinical isolates of *Eshcerichia coli* and *Klebsiella pneumoniae* in Norway. *Journal of Clinical Microbiology* **45**: 199-205.

Woodford N, Tierno Jr PM, Young K, Tysall L, Palepou MFI, Ward E, Painter RE, Suber DF, Shungu D, Silver LL, Inglima K, Kornblum J and Livermore DM (2004) Outbreak of *Klebsiella pneumoniae* producing a new carbapenem-hydrolyzing class A beta-lactamase, KPC-3, in a New York medical center. *Antimicrobial Agents and Chemotherapy* **48**: 4793-4799.

Wu TL, Siu LK, Su LH, Lauderdale TL, Lin FM, Leu HS, Lin TY and Ho M (2001) Outer membrane protein change combined with co-existing TEM-1 and SHV-1 beta-lactamases lead to false identification of ESBL-producing *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy* **47**: 755-761.

Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K and Walsh TR (2009) Characterization of a new metallo beta-lactamase gene, *bla*$_{\text{NDM-1}}$ and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrobial Agents and Chemotherapy* **53**: 5046-5054.

Yu W, Chuang Y and Rasmussen W (2006) Extended-spectrum beta-lactamases in Taiwan: epidemiology, detection, treatment and infection control. Review article. *Journal of Microbiology, Immunology and Infection* **39**: 264-277.

LIST OF TABLES

Table: 3.1 The nucleotide sequences of primers and expected product size used in the multiplex PCR assay reaction for the detection of ESBL genes (Monstein *et al*., 2007)

ESBL genes	Primer sequence 5'-3'	Size of amplicon (bp)
bla_{SHV}	Forward: ATGCGTTATATTCGCCTGTG Reverse: TGCTTTGTTCGGGCCAA	747
bla_{TEM}	Forward: TCGCCGCATACACTATTCTCAGAATGA Reverse: ACGCTCACCGGCTCCAGATTTAT	445
bla_{CTX-M}	Forward: ATGTGCAGYACCAGTAARGTKATGGC Reverse: TGGGTRAARTARGTSACCAGAAYCAGCGG	593

*All primers were manufactured by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) *

Table 3.2 The prevalence of ESBL-producing *K. pneumoniae* isolates by three detection methods: Vitek2 AES (bioMérieux, France), multiplex PCR assay (Monstein *et al*., 2007) and combination disc method (CLSI, 2009)

	ESBL status		
ESBL detection method	**Positive**	**Negative**	**Total No:**
Combination disc method	85 (57%)	63	148
Vitek2 AES	84 (57%)	62	148
Multiplex PCR	82 (55%)	62	148

Table 3.3: **Specificity and sensitivity of Vitek2 system (bioMérieux, France) and the multiplex PCR assay (Monstein *et al*., 2007) in detecting ESBL-production when compared to the combination disc method (CLSI, 2009)**

ESBL detection method	Sensitivity %	Specificity %
Vitek2 AES	99% (84/85)	98% (62/63)
Multiplex PCR	96% (82/85)	98% (62/63)

Table 3.4: **Phenotypic characterisation and detection of ESBL-production in *K. pneumoniae* clinical isolates**

	ESBL status		
ESBL detection method	**Positive**	**Negative**	**Total No:**
1. Vitek2 AES			
NO64 Card	32	21	54
N133 Card	52	41	94
2. Combination disc method			
Ceftazidime	85	63	148
Cefotaxime	84	64	148

LIST OF FIGURES

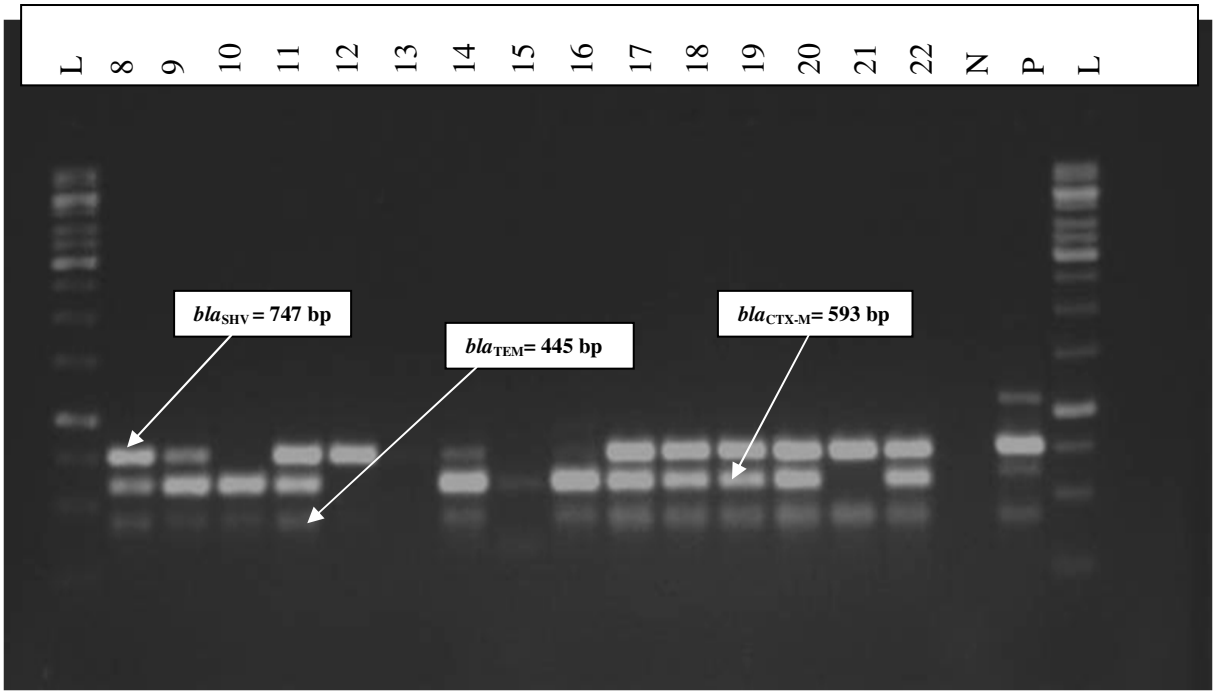

Figure 3.1: **Multiplex PCR agarose gel electrophoresis results showing ESBL genes visualized under UV light. Samples 8, 9, 11, 14, 17, 18, 19, 20 and 22 have the bla_{TEM} and bla_{CTX-M} genes. Sample 10 and 16 have the bla_{CTX-M} and bla_{TEM} genes. Sample 15 has the bla_{CTX-M} ESBL gene. Sample 13 posseses no ESBL gene. N= Negative control, P= positive control for ESBL-production and L= 1 Kb ladder.**

Hint: bla_{SHV}= 747 bp, bla_{CTX-M}= 593 bp and bla_{TEM}= 445 bp

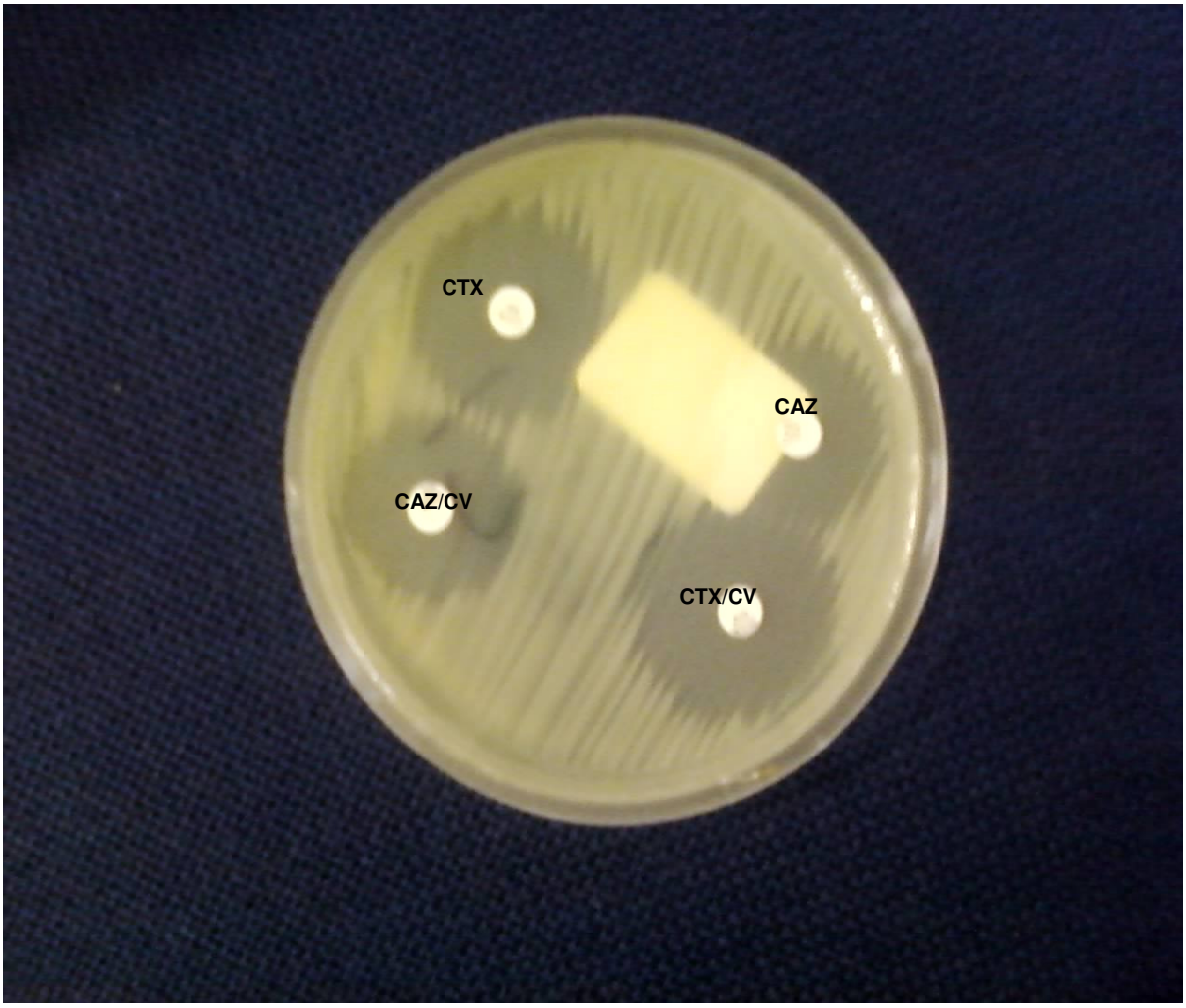

Figure 3.2 **The combination disc ESBL detection method showing a ESBL-negative isolate of *Klebsiella pneumoniae*. Note there is no expansion of the zone diameters around the cefotaxime (CTX) and ceftazidime (CAZ) discs when tested in the presence of clavulanic acid (CV) versus the zone diameters for the agents tested alone. A less than 5 mm increase in zone diameter for at least one combination disc indicates non-ESBL-production.**

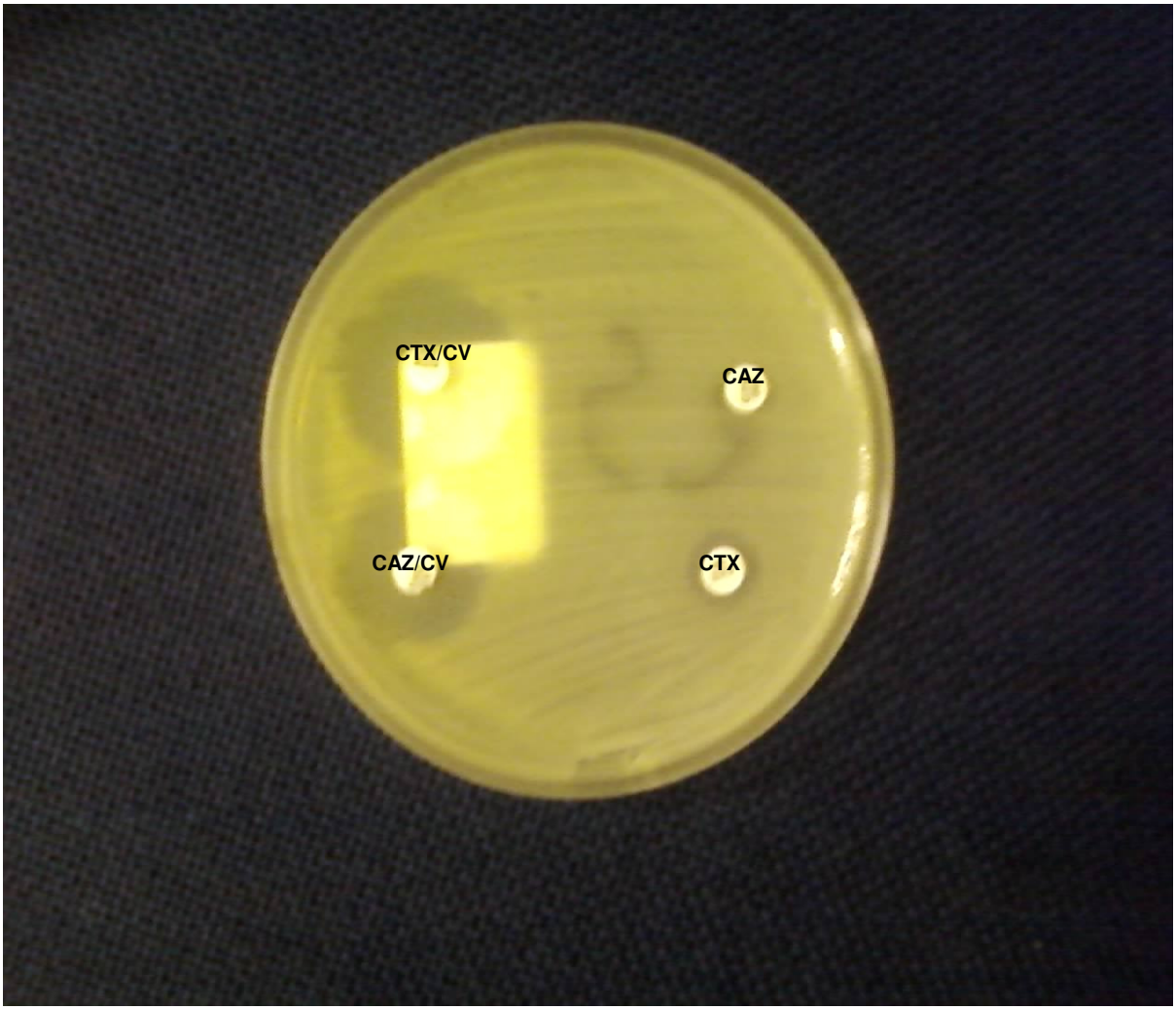

Figure 3.3 **The combination disc ESBL detection method showing an ESBL-positive isolate of *Klebsiella pneumoniae*. Note the expansion of the zone diameters around the cefotaxime (CTX) and ceftazidime (CAZ) discs when tested in the presence of clavulanic acid (CV) versus the zone diameters for the agents tested alone. An increase in ≥ 5 mm zone diameter for at least one combination disc indicates ESBL-production.**

Clonal relatedness of *Klebsiella pneumoniae* clinical isolates

In the following chapter the editorial style of the journal, FEMS Immunology and Medical Microbiology, was followed

CHAPTER 4

4.1 ABSTRACT

The ability of *Klebsiella pneumoniae* to spread rapidly amongst patients often leads to nosocomial outbreaks of infection, especially in neonatal units. Phenotypic methods developed for typing *K. pneumoniae* includes phage typing, bacteriocin typing and serotyping. However, due to the poor reproducibility of phenotypic methods newer molecular typing techniques, such as pulsed field gel electrophoresis, ribotyping and rep-PCR have emerged and have proven to be useful in typing bacterial isolates. A total of 150 consecutive *K. pneumoniae* clinical isolates were collected and investigated for clonal relatedness using the rep-PCR (ERIC, BOX and REP) fingerprinting method. In this study, the ERIC-PCR assay was found to have a higher resolution power than the BOX-PCR assay (22 groups versus 18 groups). The rep-PCR assay using ERIC-primers may be useful in confirming the presence of clonal populations among *K. pneumoniae* isolates. Furthermore, the rep-PCR assay can be an alternative method to PFGE in developing countries and resource-limited laboratories for rapidly determining the clonal relatedness of *K. pneumoniae* clinical isolates. The ESBL-producing and non ESBL-producing *K. pneumoniae* isolates were uniformly distributed in groups generated by ERIC and BOX PCR dendrogrammes.

Keywords: Clonal relatedness, dendrogramme, *K. pneumoniae*, rep-PCR, ERIC-PCR, BOX-PCR

4.2 INTRODUCTION

Klebsiella pneumoniae is an important hospital-acquired pathogen that causes severe morbidity and mortality among newborns and has the ability to spread rapidly, expecially in neonatal intensive care units (Macrae *et al*., 2001; Kuripati *et al*., 2004). Hospital outbreaks of *K. pneumoniae* are frequent and especially feared when caused by multidrug-resistant strains, such as extended-spectrum beta-lactamase (ESBL)-producers (Paterson and Bonomo, 2005). Outbreaks of *K. pneumoniae* infections often result from exposure to a common source of the aetiological agent (Olive and Bean, 1999). Infections with ESBL-producing *K. pneumoniae* strains are usually encountered in critical care units in the hospitals (Samaha-Kfoury and Araj, 2003).

Several phenotypic typing methods, such as biotyping, phage typing and antibiogram typing have been traditionally used to distinguish isolates of a given bacterial genus or species involved in outbreaks (Mohamudha *et al*., 2010). Unfortunately, because of inconsistently expressed phenotypic traits, these classical typing approaches are often not able to discriminate between related outbreak strains (Hamouda *et al*., 2003).

Researchers rely on a variety of DNA-based typing methods to determine the genetic relatedness of bacterial isolates (Silbert *et al*., 2004). These methods include plasmid profile analysis, pulsed field gel electrophoresis (PFGE), ribotyping and random amplified polymorphic DNA (RAPD) (Paterson, *et al*., 2004). The PFGE is the most commonly used DNA-based typing method because of its high discrimatory power (Hansen *et al*., 2002). However, PFGE is not easy to perform, the assay relies on specialized equipment, well-trained personnel and has a longer turnaround time (3 to 5 days) (Lin *et al*., 2008).

In contrast to PFGE, the repetitive extragenic palindromic sequence (rep)-PCR genotyping techniques are inexpensive and are easy to perform (Versalovic and Lupski, 1998). The rep-PCR based typing technique uses primers complementary to naturally occurring, highly conserved, repetitive DNA sequences (Tacao *et al*., 2005). These non-coding sequences are present in multiple copies in the genomes of diverse bacterial species (Tacao *et al*., 2005). Examples of these repetitive elements are the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX sequences (Wieser and Busse, 2000). The rep-PCR method amplifies diverse regions of DNA

flanked by the repetitive sequences, leading to amplicon patterns specific for an individual bacterial strain (Rademaker and De Bruijn, 1997). The rep-PCR method can be used for genotyping of different bacterial strains (McLellan *et al*., 2003; Tacao *et al*., 2005). Studies which have compared REP-PCR to other typing methods, such as multilocus enzyme electrophoresis (Woods *et al.,* 1992) and ribotyping (Vila *et al.,* 1996) have shown REP-PCR to be superior to these methods. Studies have shown REP-PCR to have good correlation with PFGE results (Liu and Wu*,* 1997). Sampaio and colleagues (2006) concluded that the ERIC-PCR assay is a simple, reproducible and affordable assay in determining the genetic relatedness of rapidly growing mycobacteria of the *M. chelonae-abscessus* group. The DiversiLab (DL) system (Bacterial BarCodes, bioMérieux, Athens, USA), which is a semi-automated rep-PCR typing system has become commercially available (Shutt *et al*., 2005). The advantage of the DL system is that it provides internet-based data analysis, user specific data storage and a data retrieval system (Healy *et al*., 2005).

Typing of microbial pathogens is of importance for medical microbiologists and hospital epidemiologists. By adequate laboratory analysis of bacterial strains, valuable insight into the local, national or international spread of pathogens can be obtained. This study was designed to investigate the clonal relatedness of 150 *K. pneumoniae* clinical isolates obtained from a large tertiary hospital in Pretoria.

4.3 MATERIALS AND METHODS

4.3.1 Study setting

The study was conducted at the University of Pretoria, Department of Medical Microbiology/ NHLS, Pretoria, South Africa. This was a second part of a study, which was approved by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (protocol number S21/2010).

4.3.2 Sampling methods

Between September and December 2009, 150 consecutive clinical isolates of *K. pneumoniae* obtained from clinical specimens sent to the Medical Microbiology laboratory of NHLS for analysis from a large tertiary hospital in Pretoria were collected. The collected isolates were

subcultured in 3 ml of Brain heart infusion broth (Oxoid, England) and incubated at 37˚C (Shangai Lishen Scientific Equipment, China) for 18 to 24 hrs. Following incubation, 200 µl of the cultured broth was used for DNA extraction and the remaining broth culture was mixed with 50% glycerol and stored at -70˚C (New Brunswick Scientific, Germany) for future use.

4.3.3 DNA extraction

The DNA was manually extracted using a commercial DNA extraction kit (Zymo Research, USA). The DNA extraction was performed according to the manufacturer's protocol.

4.3.4 Typing techniques

Enterobacterial repetitive intergenic consensus (ERIC) PCR, repetitive extragenic palindromic sequence (REP) PCR and BOX-A1-based repetitive extragenic consensus (BOX) PCR assays were used to determine the clonal relatedness of the collected *K. pneumoniae* clinical isolates. The nucleotide sequences of the primers used for the ERIC, REP and BOX-PCR assays are shown in Table 4.1. All the primers were manufactured by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

4.3.4.1 Reaction mix and cycling conditions for the ERIC, REP and BOX PCR assays

The total reaction volume for the ERIC and BOX PCR assay was 50 µl and it was made up of the following PCR components: 25 µl of Dream*Taq* Green PCR Master Mix (Fermentas, Lithuania), 1 µl of 20 pmol/µl forward primer, 1 µl of 20 pmol/µl reverse primer, 18 µl of RNase-free water (Fermentas, Lithuania) and 5 µl of template DNA. The amplification reaction was performed using a Mastercycler (Eppendorf, Germany). The ERIC and REP PCR assays were performed using the following cycling conditions: initial activation step at 95˚C for 7 min, 35 cycles of denaturation at 94˚C for 1 min, annealing at 52˚C for 1 min and extension at 65˚C for 8 min, followed by a single final extension cycle at 65˚C for 16 min, then cooling at 4˚C (De Bruijn, 1992).

The total reaction volume for the BOX PCR assay was 50 µl and it consisted of of the following PCR components: 25 µl of Dream*Taq* Green PCR Master Mix (Fermentas, Lithuania), 1 µl of 20 pmol/µl forward primer, 19 µl of RNase-free water (Fermentas,

Lithuania) and 5 µl of template DNA. The amplification reaction was done using a Mastercycler (Eppendorf, Germany) with the following cycling conditions: initial activation step at 95˚C for 7 min, 35 cycles of denaturation at 94˚C for 1 min, annealing at 53˚C for 1 min and extension at 65˚C for 8 min, followed by a single final extension cycle at 65˚C for 15 min and cooling at 4˚C (Louws *et al*., 1994).

4.3.4.2 Procedure for detection and visualization of ERIC, REP and BOX-PCR amplification products

Fingerprinting analysis was done using a 1% (m/v) agarose gel (Seakem, USA) in a 1X TBE buffer [10 mM Tris-HCl (Calbiochem, Merck, Canada), 10 mM Boric acid (Analar, Merck, Canada) and 1mM EDTA (Promega, Madison, USA), pH 8.0], which was electrophoresed at 80 V for 120 min. The fingerprints were visualized using the Ultraviolet Light Box (UVP products, USA), which was made possible by the addition of 5 µl of ethidium bromide (10 mg/ml) (Promega, Madison, USA) in the gel before it solidified. On each gel, a 1 Kb DNA ladder (Fermentas, Lithuania) was included in three specified lanes as reference lanes. Each gel image was captured and stored for analysis.

4.3.5 Analysis of ERIC and BOX PCR fingerprints

Gel images were analyzed and compared using the GelCompar*II* software programme (Applied Math, Belgium). The positions of the bands on each gel were normalized using the 1 Kb DNA ladder (Fermentas, Lithuania) as a normalization reference. The distance matrix for ERIC-PCR was constructed using the Dice coefficient with band optimization of 0.61% and tolerance of 2.3%. The distance matrix for BOX-PCR was also constructed using the Dice coefficient but with band optimization and tolerance of 0.67% and 3.9% respectively. Dendrogrammes were constructed using the unweighted pair group method with arithmetic mean algorithm (UPGMA) on the basis of averaged levels of similarity of the matrix. Genetic relatedness between the isolates was determined on the basis of the percentage similarity, isolates were considered to be highly related if they showed ≥ 80% similarity on the dendrogramme. Groups were constructed with 60% similarity for ERIC-PCR and BOX-PCR dendrogramme and 50% similarity for the composite (ERIC and BOX PCR) dendrogramme.

4.4 RESULTS AND DISCUSSION

Two samples were excluded from the study due to contamination. As a result only 148 samples (85 ESBL-positive and 63 ESBL-negative isolates) were available for analysis. The rep-PCR DNA fingerprinting technique using the ERIC, REP and BOX primer sets was used to determine the clonal relatedness of the 148 *K. pneumoniae* clinical isolates. However, the REP-PCR primer set failed to yield any fingerprint and therefore was excluded from the analysis. The ERIC and BOX primer sets yielded complex genomic fingerprints.

With the ERIC-PCR primer set, the profiles generated consisted of three to ten bands, the bands were evenly distributed over the entire fingerprint profile (Figure 4.1). The size of amplicons generated by the ERIC primers ranged from 250 to 10 000 base pairs (bp). The ERIC-PCR dendrogramme revealed 22 distinct groups of *K. pneumoniae* isolates, designated group A to V (Figure 4.3). The majority of these groups consisted of clusters and subclusters with high similarity scores (≥ 80% similarity values). Group A had five clusters (A1 to A5) with cluster A1 containing 14 isolates, 13 of the 14 isolates shared 100% similarity. From the 13 isolates sharing 100% similarity, 11 of the isolates were ESBL-producing isolates. Furthermore, cluster A5 was also found to have four isolates with a similarity of 100%, again all these 4 isolates were ESBL-producers. Group B consisted of 27 isolates and it had eight clusters (B1 to B8). In group B, 67% (18/27) of the isolates were ESBL-producing isolates. Cluster B1 was the largest cluster in group B, it consisted of four isolates, three of the four isolates were ESBL-producing isolates. Cluster B3 consisted of three isolates, two of the three isolates were non ESBL-producing isolates. Cluster B2, B4, B5, B6, B7 and B8 consisted of only two isolates per cluster. In addition, the dendrogramme generated by ERIC-PCR showed that 91% (77/85) of the ESBL-positive *K. pneumoniae* isolates shared similarity values of ≥ 80%. This high level of similarity among ESBL-producing isolates may be due to the nosocomial spread of a single clone. Five isolates (isolate, 35, 48, 57, 59 and 146) were found not to belong to any of the twenty-two major groups, thus making these isolates unique.

The BOX primer set generated DNA fragments of three to nine bands on each fingerprint (Figure 4.2). The size of the bands generated by the BOX-PCR ranged from 500 to 5 500 bp. The dendrogramme generated by the analysis of the BOX-PCR DNA fragments revealed 18 distinct major groups, designated group A to R (Figure 4.4). In the BOX-PCR dendrogramme, only three isolates (isolate 18, 101 and 104) were found to be unique, thus not

belonging to any of the nineteen groups. As in the case of ERIC-PCR, each major group consisted of several clusters and subclusters. Group A was the biggest group; consisting of 40 isolates distributed into seven clusters (A1 to A7). In this group, 58% (23/40) of the isolates were ESBL-producing *K. pneumoniae* isolates. Group B was the second largest group in BOX-PCR dendogramme and it consisted of 19 isolates. In group B, 32% (6/19) of the isolates were ESBL-producing isolates. The BOX-PCR dendrogramme revealed that 50% (9/18) of the groups had at least two isolates possessing a 100% similarity level and most of those isolates were ESBL-producers. The BOX-PCR assay was found to have a slightly lower discriminatory power when compared to the ERIC-PCR assay. In addition, the dendrogramme generated by the BOX-PCR data revealed comparable but not similar results to the ERIC-PCR dendrogramme.

The composite dendrogramme (generated using BOX-PCR and ERIC-PCR data) revealed 18 distinct major groups designated group A to R (Figure 4.5). Most of the isolates were clustered in group A, within group A, 18 clusters (A1 to A18) with a more than 75% similarity were obtained. Three isolates (18, 35 and 48) did not cluster into any group and were found to be unique. Interestingly, isolate 48 was also found to be unique in the dendrogramme generated by the ERIC PCR data, while isolate 48 clustered to group A in the dendrogramme generated by BOX PCR data. When compared to the individual dendrogrammes (ERIC or BOX PCR dendrogramme), the composite dendrogramme revealed the highest resolution power among the ESBL-positive isolates. In the composite dendrogramme, only two isolates (isolates 147 and 149) were shown to share 100% similarity. Furthermore, only one (isolate 149) of these two isolates was ESBL-positive. In addition, both these two isolates were found to be 100% similar in dendrogrammes generated by either of the two PCR assays (ERIC / BOX PCR). A composite dendrogramme (based on more than one fingerprinting method) may be useful in confirming the presence of clonal populations among *K. pneumoniae* isolates.

The REP primer set which was successfully used by Stumpf and colleagues (2005) to fingerprint bacterial isolates failed to yield any fingerprint profile in the present study. The inability of the REP primer set to yield any DNA fingerprint might be due to problems with the synthesis of these primers. The BOX-PCR and ERIC-PCR fingerprinting assays generated characteristic banding profiles, which were successfully used in clustering and grouping the *K. pneumoniae* clinical isolates. The results of this study corroborated the

assertion by Versalovic *et al.* (1991) that the direct amplification and agarose gel electrophoresis of PCR products can provide genomic fingerprints of sufficient complexity to distinguish species and strains. The study also demonstrated that BOX and ERIC like sequences are present in many Gram-negative bacteria as observed by Versalovic *et al.* (1994). The DNA fingerprinting assay employing REP, BOX and ERIC sequences as PCR primer binding sites can be used to study the distribution of repetitive sequences in different bacterial genomes (Ishii and Sadowsky, 2009).

The rep-PCR DNA fingerprinting assay is fast becoming the most widely used typing technique, in this study this assay was successfully used in determining the clonal relatedness of *K. pneumoniae* clinical isolates. This assay has also been successfully used to differentiate strains of *Enterobacter aerogenes* (Georghiou *et al.,* 1995), *Neisseria meningitidis* and faecal *Escherichia coli* (Mohapatra *et al*., 2007). In a study by Silbert *et al*. (2004) involving the typing of 20 *Stenotrophomonas maltophilia* isolates collected from six Latin American countries, the results of PFGE were found to be consistent with those obtained by ERIC-PCR. A study by Cartelle *et al*. (2004) which included the genotyping of 43 clinical strains of *K. pneumoniae* and seven ATCC strains of *K. pneumoniae* obtained 17 genotypes by ERIC-PCR that were identical to those obtained by PFGE and rapid amplified polymorphic DNA, thus showing the discriminatory power and typing capacity of the ERIC-PCR assay.

The distinguishing feature of the rep-PCR fingerprinting technique when compared to other molecular typing methods is that it is relatively cheap and easy to perform (Versalovic and Lupski, 1998; Ishii and Sadowsky, 2009). A semi-automated typing system called DiversiLab (Bacterial BarCodes, bioMérieux, Athens, USA) has been developed and it has the potential to track and monitor antibiotic resistant bacteria in real-time basis (Shutt *et al*., 2005; Pitout *et al*., 2009). In a study by Pitout and colleagues (2009), the DL system successfully identified the CTX-M-15-producing *Escherichia coli* clone ST131 and was able to distinguish it from other *E. coli* isolates.

4.5 CONCLUSION

This study demonstrated that the rep-PCR fingerprinting technique using BOX and ERIC primer sets is an effective, rapid and easy to perform method to determine the clonal relatedness of bacterial isolates. Furthermore, the rep-PCR technique evaluated in this study

can be an alternative to PFGE in developing countries and resource-limited laboratories for rapidly determining the clonal relatedness of *K. pneumoniae* clinical isolates. The ERIC-PCR assay has a better discriminatory power than the BOX-PCR assay.

The ESBL-producing and non ESBL-producing *K. pneumoniae* isolates were mixed in different clusters in both the ERIC and BOX PCR dendrogramme, thus ruling out the possibility of an ESBL-producing *K. pneumoniae* outbreak. It can be concluded that the high prevalence of ESBL-producing *K. pneumoniae* isolates in the Pretoria region might be due to the inappropriate use of extended spectrum cephalosporins rather than a spread of one clone in the hospital. Strict infection control measures, such as a strong and effective antibiotic policy is warranted and needed.

4.6 ACKNOWLEDGEMENTS

The authors would like to thank the Department of Medical Microbiology, UP/NHLS for the financial support provided for this research project. Mr GI Manenzhe would like to thank the South African Medical Research Council (MRC) and National Research Foundation (NRF) for the financial support received. The authors would also like to thank the Diagnostic Division of the National Health Laboratory Services (NHLS), Pretoria, South Africa.

4.7 REFERENCES

Cartelle M, Tomas MM, Pertega S, Beceiro A, Dominguez MA, Velasco D, Molina F, Villanueva R and Bou G (2004) Risk factors for colonization and infection in a hospital outbreak caused by a strain of *Klebsiella pneumoniae* with reduced susceptibility to expanded-spectrum cephalosporins. *Journal of Clinical Microbiology* **42**: 4242-4249.

De Bruijn FJ (1992) Use of repetitive (repetitive extragenic palondromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology* **58**: 2180-2187.

Georghiou PR, Hamill RJ, Wright CE, Versalovic J, Koeuth T, Watson DA and Lupski JR (1995) Molecular epidemiology of infections due to *Enterobacter aerogenes*: identification of hospital-associated strains by molecular techniques. *Clinical Infectious Diseases* **20**: 84-94.

Hamouda TB, Foulon T, Masmoudi ABC, Fendri C, Belhadj O and Mahrez KB (2003) Molecular epidemiology of an outbreak of multiresistant *Klebsiella pneumoniae* in a Tunisian neonatal ward. *Journal of Medical Microbiology* **52**: 427-433.

Hansen DS, Benedi SR, Sperling V and Kolmos HJ (2002) *Klebsiella* typing: pulsed field gel electrophoresis (PFGE) in comparison with O: K-serotyping. *Clinical Microbiology and Infections* **8**: 397-404.

Healy M, Huong J, Bittner T, Lising M, Frye S, Raza S, Schrock R, Manry J, Renwick A, Nieto R, Woods C, Versalovic J and Lupski JR (2005) Microbial DNA typing by automated repetitive-sequence-based PCR. *Journal of Clinical Microbiology* **43**: 199–207.

Ishii S and Sadowsky MJ (2009) Applications of the rep-PCR fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental Microbiology* **11**: 733-740.

Kurupati P, Chow C, Kumarasinghe G and Poh CL (2004) Rapid Detection of *Klebsiella pneumoniae* from Blood Culture Bottles by Real-Time PCR. *Journal of Clinical Microbiology* **42:** 1337-1340.

Lin CW, Chiou CS, Chang YC and Yang TC (2008) Comparison of pulsed field gel electrophoresis and three REP-PCR methods for evaluating the genetic relatedness of *Stenotrophomonas maltophilia* isolates. *Letters in Applied Microbiology* **47**: 393-398.

Liu PYF and Wu WL (1997) Use of different PCR-based DNA fingerprinting techniques and pulsed-field gel electrophoresis to investigate the epidemiology of *Acinetobacter calcoacetics-Acinetobacter baumannii* complex. *Diagnostic Microbiology and Infectious Diseases* **28**: 19–28.

Louws FJ, Fulbright DW, Stephens CT and De Bruijn FJ (1994) Specific genomic fingerprints of phytopathogenic *Xanthomas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* **60**: 2286-2295.

Macrae MB, Shannon KP, Rayner DM, Kaiser AM, Hoffman PN and French GL (2001) A simultaneous outbreak on a neonatal unit of two strains of multiple antibiotic resistant *Klebsiella pneumoniae* controllable only by ward closure. *Journal of Hospital Infections* **49**: 183-192.

McLellan SL, Daniels AD and Salmore AK (2003) Genetic characterization of *Escherichia coli* populations from host sources of fecal pollution by using DNA fingerprinting. *Applied and Environmental Microbiology* **69**: 2587-2594.

Mohamudha PV, Srinivas AN, Rahul D, Harish BN and Parija SC (2010) Molecular epidemiology of multidrug resistant extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* outbreak in a neonatal intensive care unit. *Journal of collaborative Research on Internal Medicine and Public Health* **2**: 226-237.

Mohapatra BR, Broersma K and Mazumder A (2007) Comparison of five rep-PCR genomic fingerprinting methods for differentiation of fecal *Escherichia coli* from humans, poultry and wild birds. *FEMS Microbiology Letters* **277**: 98-106

Olive MD and Bean P (1999) Principles and applications of methods for DNA-based typing of Microbial organism. *Journal of Clinical Microbiology* **37**: 1661-1669.

Paterson DL, Ko WC, Gottberg AV, Mohapatra S, Casellas J M, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG and Yu VL (2004) Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of beta-lactamases. *Clinical Infectious Diseases* **39**: 31-39.

Paterson DL and Bonomo RA (2005) Extended spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews* **18**: 657-686.

Pitout JDD, Campbell L, Church DL, Wang PW, Guttman DS and Gregson DB (2009) Using a commercial DiversiLab semiautomated repetitive sequence-based PCR typing technique for identification of *Escherichia coli* clole ST131 producing CTX-M15. *Journal of Clinical Microbiology* **47**: 1212-1215.

Rademaker JLW and De Bruijn FJ (1997) Charaterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. In: DNA markers, protocols, applications and overviews. New York, *John Wiley and Sons*, pp: 151-171.

Samaha-Kfoury JN and Araj GF (2003) Recent developments in beta-lactamases and extended spectrum beta-lactamases. *British Medical Journal* **327**: 1209-1213.

Sampaio JLM, Viana-Niero C, De Freitas D, Hofling-Lima AL and Leao SC (2006) Enterobacterial repetitive intergenic consensus PCR is a useful tool for typing *Mycobacterium chelonae* and *Mycobacterium abscessus* isolates. *Diagnostic Microbiology and Infectious Diseases* **55**: 107-118.

Shutt C K, Pounder JI, Page SR, Schaecher BJ and Woods GL (2005) Clinical evaluation of the DiversiLab microbial typing system using repetitive-sequence-based PCR for characterization of *Staphylococcus aureus* strains. *Journal of Clinical Microbiology* **43**: 1187–1192.

Silbert S, Pfaller MA, Hollis RJ, Barth AL and Sader HS (2004) Evaluation of three molecular typing techniques for nonfermentative gram-negative bacilli. *Infection Control and Hospital Epidemiology* **25**: 847-851.

Stumpf AN, Roggenkamp A and Hoffmann H (2005) Specificity of enterobacterial repetitive intergenic consensus and repetitive extragenic palindromic polymerase chain reaction for the detection of clonality within the *Enterobacter cloacae* complex. *Diagnostic Microbiology and Infectious Diseases* **53**: 9-16.

Tacao M, Alves A, Saavedra MJ and Correia A (2005) BOX-PCR is an adequate tool for typing *Aeromonas* spp. *Antonie van Leeuwenhoek* **88**: 173-179.

Versalovic J, Koeuth T, and Lupski JR (1991) Distribution of repetitive DNA sequences in Eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research* **19**: 6823-6831.

Versalovic J, Schneider M, Bruijn FJ and Lupski JR (1994) Genomic fingerprinting of bacteria using the repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cell Biology* **5**: 25-40.

Versalovic J and Lupski JR (1998) Interspersed repetitive sequences in bacterial genomes. In bacterial genomics: physical structure and analysis. New York, USA. *Chapman and Hall* pp: 38-48.

Vila, JM, Marcos A and Jimenez de Anta MT (1996) A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus A. baumannii* complex. *Journal of Clinical Microbiology* **44**: 482–489.

Wieser M and Busse HJ (2000) Rapid identification of *Staphylococcus epidermidis*. *International Journal of Sytematic and Evolutionary Microbiology* **50**: 1087-1093.

Woods CR, Versalovic J, Koeuth T and Lupski JR (1992) Analysis of relationships among isolates of *Citrobacter diversus* by using DNA fingerprints generated by repetitive sequence-based primers in polymerase chain reaction. *Journal of Clinical Microbiology* **30**: 2921–2929.

LIST OF TABLES

Table 4.1: Nucleotide sequences of primers used for REP-PCR, ERIC-PCR and BOX-A PCR assays (Louws *et al*., 1994)

Primer	Primer Sequence	Length
REPIR-PLS	5'- III ICG ICG ICA ICI GGC- 3'	18 mer
REP2-PLS	5'- ICG ICT TAT CIG GCC TAC- 3'	18 mer
ERIC1R-PLS	5'-ATG TAA GCT CCT GGG GAT TCA C- 3'	22 mer
ERIC2-PLS	5'- AAG TAA GTG ACT GGG GTG AGC G- 3'	22 mer
BOXAIR-PLS	5'- CTA CGG CAA GGC GAC GCT GAC G- 3'	22 mer

All the primers were manufactured by Inqaba Biotech, Pretoria

LIST OF FIGURES

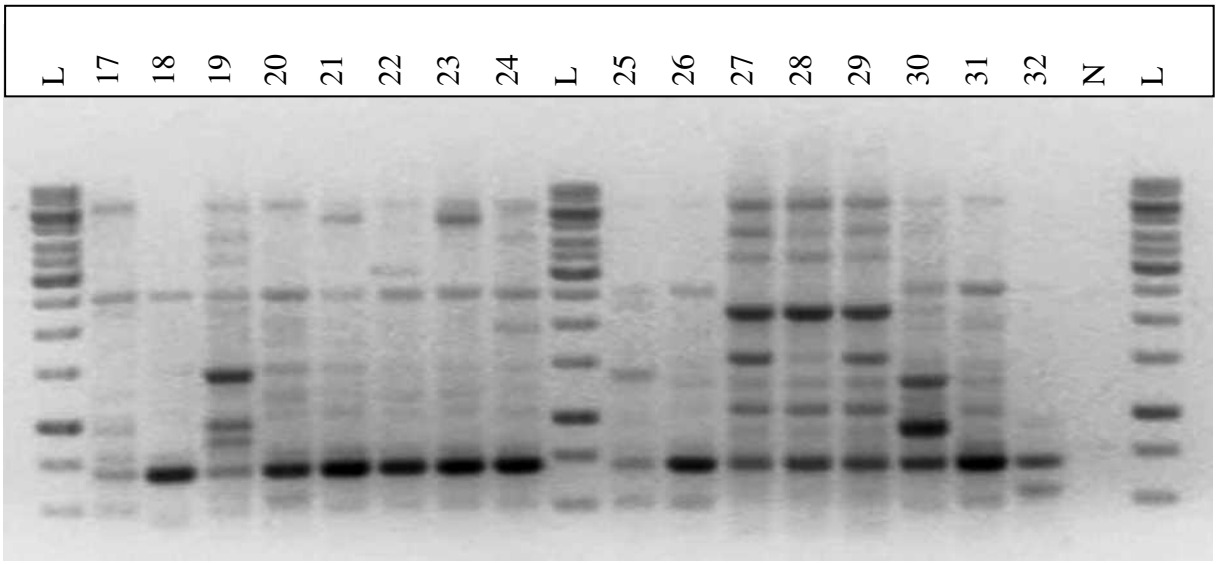

Figure 4.1: **The agarose gel electrophoresis results visualized under UV light showing the DNA fragments generated by ERIC-PCR. Lanes 17 to 32 correspond to *K. pneumoniae* isolates number 17-32, L = 1 Kb DNA ladder and N is a negative control.**

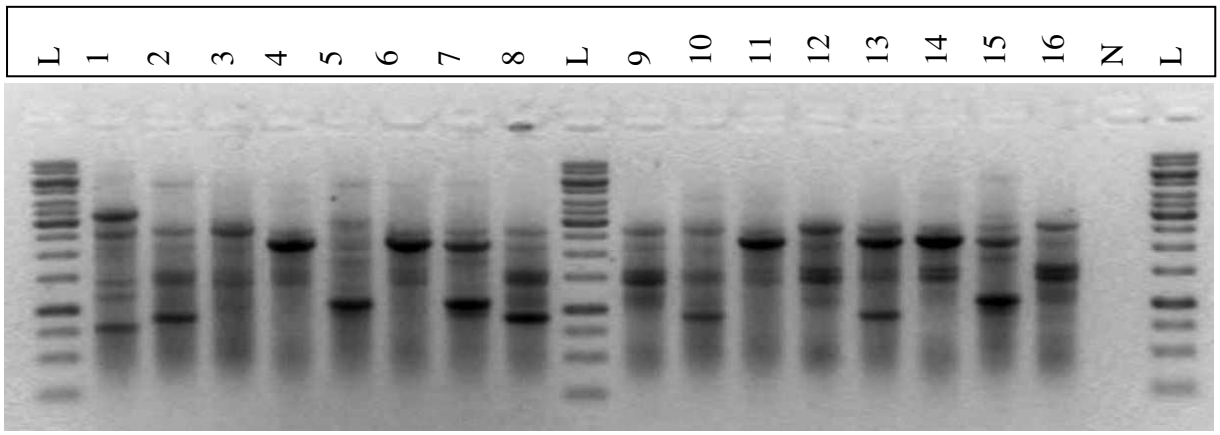

Figure 4.2: **The agarose gel electrophoresis results visualized under UV light showing the DNA fragments generated by BOX-PCR. Lanes 1 to 16 corresponds to *K. pneumoniae* isolate number 1-16, L = 1 Kb DNA ladder and N is a negative control.**

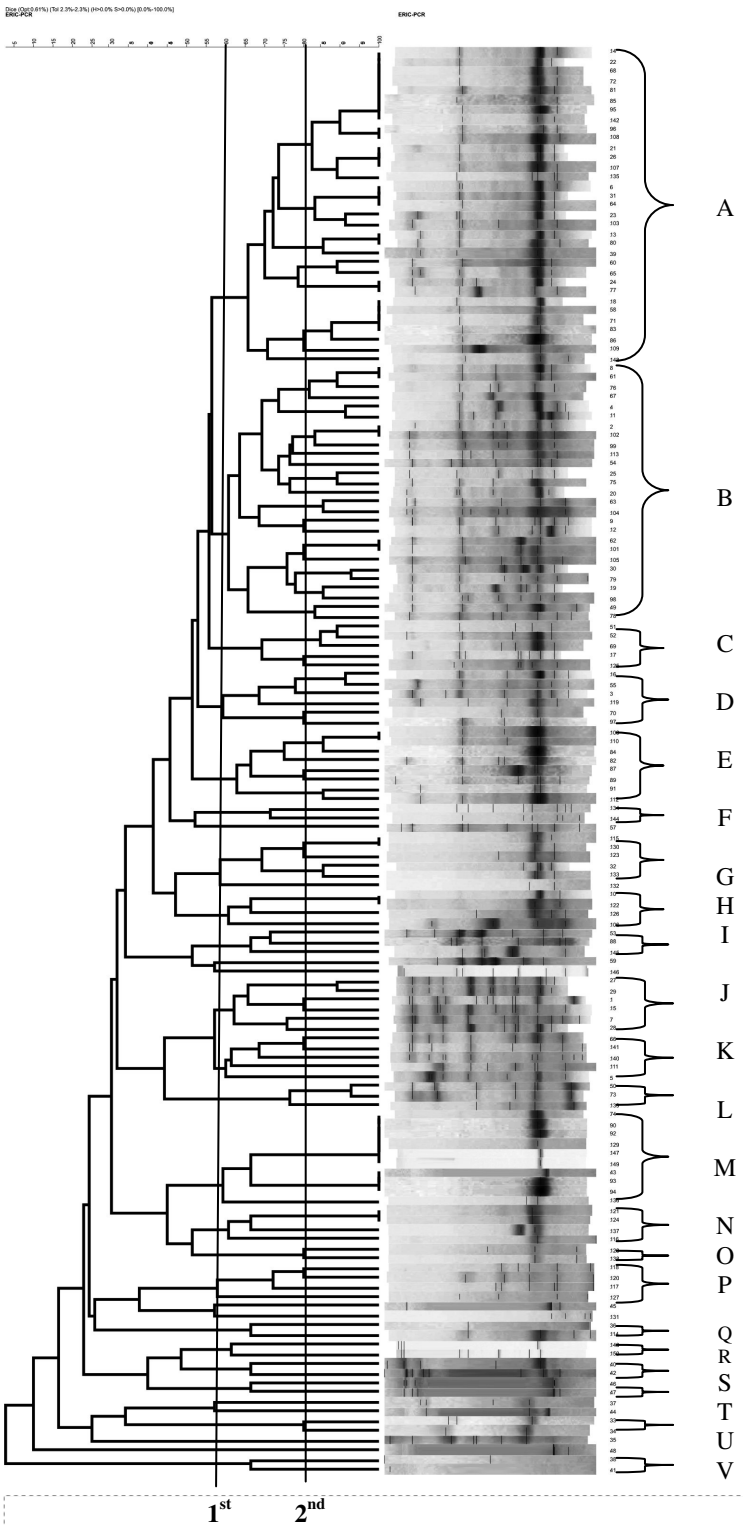

Figure 4.3: **Dendrogramme obtained for ERIC-PCR typing, depicting the clonal relationship of 148 *K. pneumoniae* clinical isolates. The 1st line represents 60% similarity for the groups and the 2nd line represents 80% similarity for the clusters. Twenty-two groups (A to V) were obtained.**

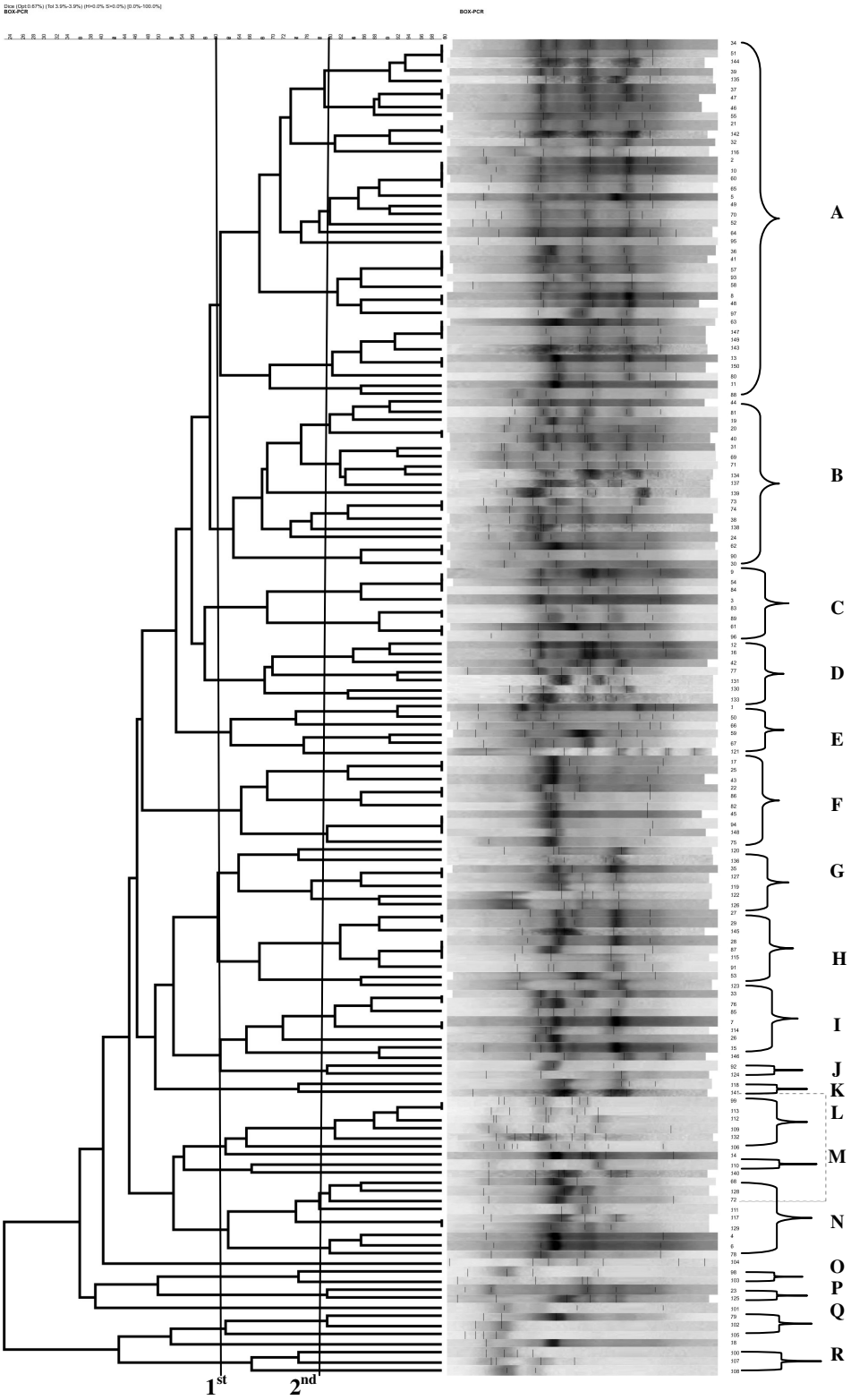

Figure 4.4: **Dendrogramme obtained for BOX-PCR typing, depicting the clonal relationship of 148 *K. pneumoniae* clinical isolates. The 1st line represents 60% similarity for the groups and the 2nd line represents 80% similarity for the clusters. Eighteen groups (A to R) were obtained.**

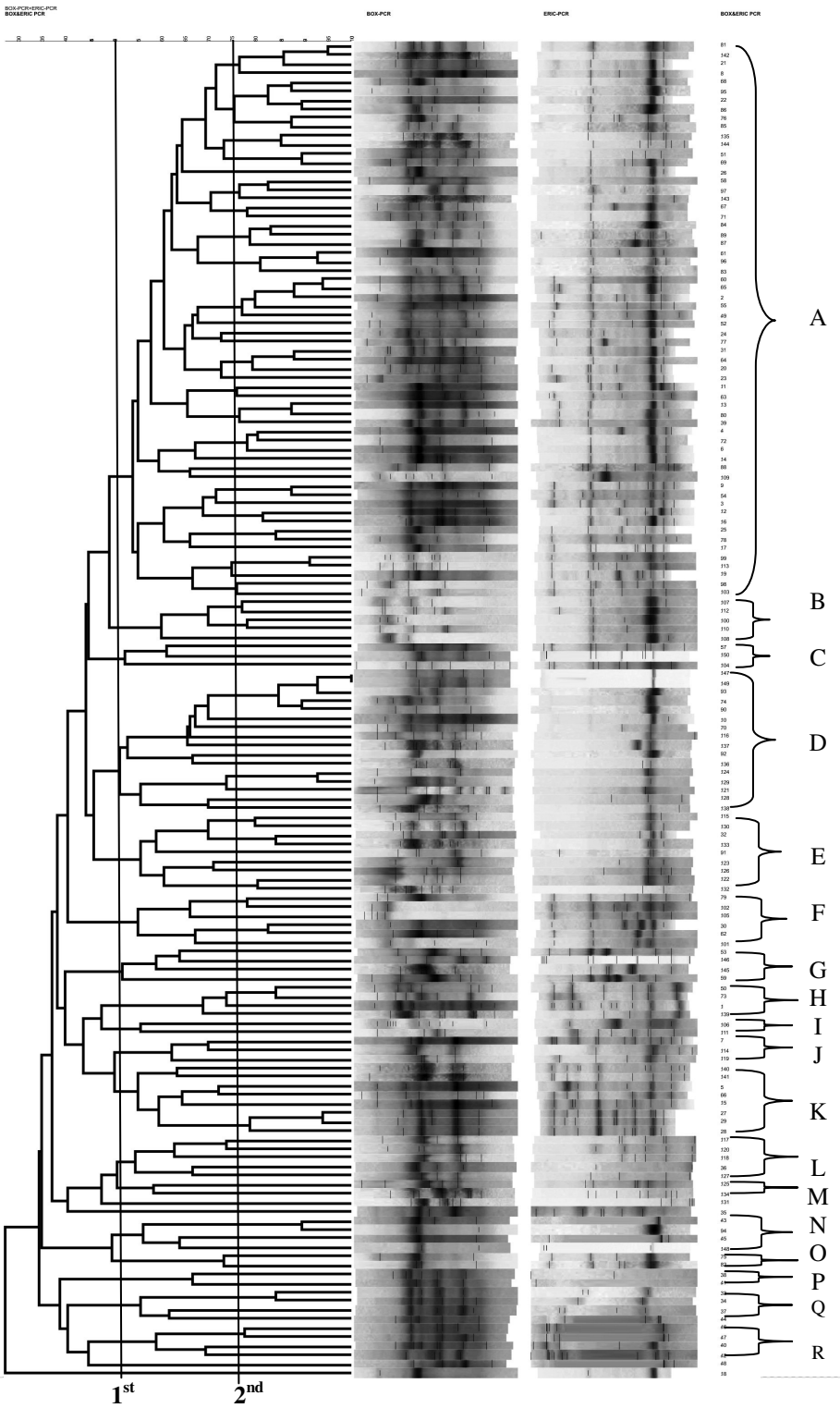

Figure 4.5: **Dendrogramme obtained for ERIC and BOX-PCR typing, depicting the clonal relationship of 148 *K. pneumoniae* clinical isolates. The 1st line represents 50% similarity for the groups and the 2nd line represents 75% similarity for the clusters. Eighteen groups (A to R) were obtained.**

CHAPTER 5

Concluding remarks

Klebsiella pneumoniae is among the most common Gram-negative bacteria encountered by physicians worldwide. It is the most common nosocomial bacterial pathogen, causing urinary tract infections, hospital-acquired pneumonia and intra-abdominal infections (Ko *et al*., 2002). The overuse of extended-spectrum cephalosporins and monobactams has allowed the emergence of ESBL-producing *K. pneumoniae* strains (Pfaller and Segreti, 2006). The ESBL-producing strains are highly efficient at inactivating the newer third-generation cephalosporins, such as cefotaxime and ceftriaxone (Dubois *et al*., 2002). In addition, ESBL-producing strains are frequently resistant to many classes of non-beta-lactam antibiotics, thus resulting in treatment failure (Stürenburg and Mack, 2003).

There are various methods for detecting ESBL-production by a bacterial isolate and the evaluations of these methods have been well published (Komatsu *et al*., 2003; Linscott and Brown, 2005). However, the combination disc test is currently regarded as a confirmatory test for detecting ESBL-production by *K. pneumoniae* isolates (CLSI, 2009). However, in a routine diagnostic laboratory, it is important to develop and evaluate a test that can be rapidly used to detect ESBL-producing bacteria (Gheldre *et al*., 2003).

The Vitek2 advanced expert system (AES), the combination disc method and a multiplex PCR assay were used to detect and characterize ESBL-producing *K. pneumoniae* clinical isolates. The prevalence of ESBL-producing *K. pneumoniae* isolates in the Tshwane region (South Africa) was determined. The study further investigated the clonal relationship of the 150 *K. pneumoniae* clinical isolates.

The combination disc method detected the production of ESBL in 57% (85/148) of *K. pneumoniae* isolates. The high prevalence of ESBL-producing *K. pneumoniae* in Tshwane region is likely to have been caused by the over-use of third generation cephalosporins. The observed high prevalence of ESBL-producing *K. pneumoniae* isolates warrants constant surveillance to monitor the spread and prevalence of ESBL-producing isolates. Knowing the overall prevalence of ESBL-producing isolates in the Tshwane region will help in monitoring the efficacy of infection control measures that are currently in place.

The combination disc method, which is recommended by the CLSI (2009) and CLSI (2011) guidelines as the confirmatory test in detecting ESBL-production by the bacterial isolates has the disadvantage of having a longer incubation period (18 to 24 hours versus 8 to 13 hours needed in the Vitek2 AES). Furthermore, the combination disc method can only be performed once the bacterial isolate has been identified.

Detection of ESBL-producing *K. pneumoniae* clinical isolates using the Vitek2 AES was completed within 8 to 13 hours. The Vitek2 AES has the advantage of identifying the bacteria while simultaneously detecting the presence of ESBL-production. In large diagnostic laboratories where there are a large number of requested tests, the Vitek2 AES can be used to detect ESBL-producing *K. pneumoniae* isolates without compromising the turnaround time.

The detection of ESBL-producing *K. pneumoniae* isolates using a multiplex PCR assay offers the advantage of detecting more than one ESBL gene in a single PCR reaction. The multiplex PCR assay is labour intensive and time consuming, since it requires extraction of bacterial DNA, amplification and lastly, the detection of amplified products using agarose gel electrophoresis. Furthermore, the assay can only be performed after the identification of the bacterial isolate. However, this assay with additional genes added need to be implemented in diagnostic laboratories in order to track and monitor the circulating ESBL-genes. This is vital for the detection of *Klebsiella pneumoniae* carbapenemases (KPC) and New Delhi Metallo-beta-lactamase-1 (NDM-1) producing *K. pneumoniae* isolates, which are usually missed by phenotypic methods.

The results of the Vitek2 AES and multiplex PCR assay were comparable with the results of the combination disc method; both assays yielded a high sensitivity and specificity. Furthermore, when taking the sequencing results into consideration the multiplex PCR assay was superior to the combination disc method, since it was able to detect ESBL-production in an inhibitor resistant isolates that co-produced an ESBL enzyme; this isolate was misidentified as ESBL-negative by the Vitek2 AES and the combination disc method.

There are various variants of sulfyhydry variable (SHV) (eg SHV-1) and temoneira (TEM) (eg TEM-1) genes that do no code for ESBL-production (Chaves, 2001). Due to the occurrence of SHV and TEM genes not associated with ESBL-production it is important to

sequence these genes in order to confirm if it is an ESBL genotype or not. Nevertheless, in contrast to the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes, the results of this study showed that the detection of the *bla*$_{CTX-M}$ gene in *K. pneumoniae* bacterial genome is usually associated with ESBL-production. This is in agreement with the review by Pitout and Laupland (2008) which outlined that the PCR amplification of the *bla*$_{CTX-M}$ gene without sequencing usually provides sufficient evidence that the bla$_{CTX-M}$ gene is responsible for ESBL-production.

In this study, the *bla*$_{SHV}$ was the most prevalent gene and it was detected in 79% (117/148) of the bacterial isolates. In a study conducted in Iran by Feizabadi and colleagues (2010) a *bla*$_{SHV}$ prevalence of 67.4% (60/89) among *K. pneumoniae* isolates was reported. The *bla*$_{CTX-M}$ gene was the second most detected gene with a prevalence of 57% (84/148) while in a study by Feizabadi *et al.* (2010) the second most detected gene was the *bla*$_{TEM}$ with a prevalence of 54% (48/89). Tollentino *et al*. (2011) in a study conducted in Brazil reported the *bla*$_{CTX-M}$ gene as the most prevalent gene in 70% (46/65) of *K. pneumoniae* isolates.

The ERIC-PCR and BOX-PCR assays determined the clonal relatedness of 148 *Klebsiella pneumoniae* clinical isolates. The assays were found to be simple, rapid and reliable as reported by Versalovic and Lupski (1998), Rademaker *et al*. (2008) as well as Ishii and Sadowsky (2009). The use of a Dream*Taq*™ green PCR master mix further simplified the ERIC-PCR and BOX-PCR assays. The Dream*Taq*™ green master mix is supplemented with two tracking dyes and a density reagent that allows for direct loading of the PCR product on a gel. The dye did not interfere with the performance of the *Taq* polymerase.

The dendrogramme generated from the ERIC-PCR assay data revealed twenty-two distinct major groups of *K. pneumoniae* isolates. The majority of these groups consisted of clusters and subclusters with high similarity values (≥ 80% similarity). The dendrogramme generated by the BOX-PCR assay revealed 18 distinct major groups; interestingly, the composite dendrogramme (combined ERIC and BOX PCR data) revealed the same number of groups (18 distinct major groups). As in the case of the ERIC-PCR dendrogramme, each major group in the BOX-PCR dendrogramme consisted of several clusters and subclusters. Group A was the largest group in the BOX PCR dendrogramme; consisting of 40 isolates distributed into seven clusters. Furthermore, in this group (Group A) 58% (23/40) of the isolates were ESBL-producing isolates. In this study, there was a uniform distribution of ESBL and non ESBL-producing *K. pneumoniae* isolates in both the ERIC-PCR and BOX-PCR dendrogramme.

This might suggest that there was not an outbreak of ESBL-producing *K. pneumoniae* isolates during the period of this study.

The BOX-PCR assay was observed to have a low-resolution power compared to the ERIC-PCR assay. The reproducibility of the ERIC and BOX-PCR assays was not evaluated in this study. It would have been interesting to see if these assays are reproducible or not. The reproducibility of the assay can be determined by analyzing a certain number of isolates in duplicate in order to see if the duplicate isolates yield the same results.

Future research

Future studies should look at evaluating the Vitek2 AES with an additional panel of bacteria (eg *Escherichia coli*, *Proteus mirabilis* and other bacteria known to produce ESBLs) in order to assess the overall usefulness of the Vitek2 AES in detecting ESBL-producing bacterial isolates. The development of novel antibiotics that are resistant to hydrolysis by extended spectrum beta-lactamases enzymes are desired. The development and validation of a multiplex real-time PCR assay for the detection of ESBL genes is needed to reduce turnaround times.

The emergence and spread of ESBL and NDM-1-producing *K. pneumoniae* and *Escherichia coli* isolates is of great concern because such isolates are multidrug resistant (Mulvey *et al*., 2011). In developing country, the spread of NDM-1-producing isolates is facilitated by the unsanitary disposal of human excretion, faecal contamination of drinking water and inappropriate use of antibiotics (Promed post, 17 October 2011, http://www.deccanherald.com /content/198645/s-african-hospital-reports-superbug.html). In New Delhi, the NDM-1-producing isolates have been isolated from sewage and drinking water (Walsh *et al*., 2011). Proper infection control strategies such as hand washing and isolation of patients infected with multidrug resistant strains are warranted. These strategies may help in limiting the spread of ESBL-producing bacteria. The proper use of antimicrobial agents accompanied by the close monitoring of multidrug resistant isolates may play a crucial role in limiting the spread of these pathogens.

References

Chaves J, Ladona MG, Segura C, Coira A, Reig R and Ampurdanes C (2001) SHV-1 beta-lactamase is mainly a chromosomal encoded species-specific enzyme in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy* **45**: 2856-2861.

Clinical and Laboratory Standards Institute (2009) Performance standards for antimicrobial susceptibility testing: *Nineteenth Informational Supplement CLSI*, Wayne, PA, USA.

Clinical and Laboratory Standard Institute (2011) Performance standards for antimicrobial susceptibility testing; *Twenty-First Information Supplement CLSI* Wayne. PA, USA.

Coetzee J, Clay CG, Brink A (2011) Clinical microbiology considerations related to the emergence of New Delhi metallo beta-lactamases (NDM-1) and *Klebsiella pneumoniae* carbapenemases (KPC) amongst hospitalized patients in South Africa. *Federation of Infectious Diseases Societies of Southern Africa* (Special report).

Dubois V, Poirel L, Marie C, Arpin C, Nordmann P and Quentin C (2002) Molecular characterization of a novel class 1 integron containing bla_{Ges-1} and a fused product of acc (3)-Ib/aac (6')-Ib' gene cassettes in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **46**: 638-645.

Feizabadi MM, Delfani S, Raji N, Majnooni A, Aligholi M, Shahcheraghi F, Parvin M and Yadegarinia D (2010) Distribution of *bla*(TEM), *bla*(SHV) genes among clinical isolates of *Klebsiella pneumoniae* at Labbafinejad Hospital, Tehran, Iran. *Microbial Drug Resistance* **1**: 49-53.

Gheldre YD, Avesani V, Berhin C, Delmee M and Glupczynski Y (2003) Evaluation of Oxoid combination discs for detection of extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy* **52**: 591-597.

Ishii S and Sadowsky MJ (2009) Applications of the rep-PCR fingerprinting technique to study microbial diversity, ecology and evolution. *Environmental Microbiology* **11**: 733-740.

Ko WC, Paterson DL, Sagnimeni AJ, Hansen DS and Gottberg AV (2002) Community-Acquired *Klebsiella pneumoniae* bacteremia: Global differences in clinical patterns. *Emerging Infectious Diseases* **8**: 160-166.

Komatsu M, Aihara M, Shimakawa K, Iwasaki M, Nangasaka Y, Fukuda S, Matsuo S and Iwatani Y (2003) Evaluation of MicroScan ESBL confirmation panel for *Enterobacteriaceae*-producing, extended-spectrum beta-lactanases isolated in Japan. *Diagnostic Microbiology and Infectious Diseases* **46**: 125-130.

Linscott AJ and Brown WJ (2005) Evaluation of four commercially available extended-spectrum beta-lactamase phenotypic confirmation tests. *Journal of Clinical Microbiology* **43**: 1081-1085.

Mulvey MR, Grant JM, Plewes K, Roscoe D and Boyd DA (2011) New Delhi metallo beta-lactamase in *Klebsiella pneumoniae* and *Escherichia coli*, Canada. *Emerging Infectious Diseases* **17**: 103-106.

Pitout JDD and Laupland KB (2008) Extended-spectrum beta-lactamase producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infectious Diseases* **8**: 159-166.

Pfaller MA and Segreti J (2006) Overview of the Epidemiological Profile and Laboratory Detection of Extended spectrum beta-lactamase. *Clinical Infectious Diseases* **42**: 153-163.

Rademaker JLW, Louws FJ, Versalovic JV and De Bruijn FJ (2008) Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. Netherlands, *Springer* pp: 611-644

Stürenburg and Mack (2003) Extended-spectrum beta-lactamases: implications for the Clinical Microbiology laboratory, therapy and infection control. *Journal of Infection* **47**: 273-295.

Tollentio FM, Polotto M, Nogueira ML, Lincopan N, Neves P, Mamizuka EM, Remeli GA, De Almeida MT, Rubio FG and Nogueira MC (2011) High prevalence of *bla* (CTX-M) extended spectrum beta-lactamase genes in *Klebsiella pneumoniae* isolates from a tertiary care hospital: first report of *bla* (SHV-12), *bla* (SHV 31), *bla* (SHV-38) and *bla* (CTX-M-15) Iin Brazil. *Microbial Drug Resistance* **1**: 7-16.

Versalovic J and Lupski JR (1998) Interspersed repetitive sequences in bacterial genomes. In bacterial genomics: physical structure and analysis. New York, USA. *Chapman and Hall* pp: 38-48.

Walsh TR, Weeks J, Livermore DM and Toleman MA (2011) Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infectious Disease* **11**: 355-362.