Incidence of Bacterial Infections and Colonisation in Patients Admitted to a Tuberculosis Hospital

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Incidence of Bacterial Infections and Colonisation in Patients Admitted to a Tuberculosis Hospital

By

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DECLARATION:

In accordance with Rule G5.6.3, I hereby declare that the above-mentioned treatise/ dissertation/ thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

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TABLE OF CONTENTS

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ABSTRACT

Patients with drug resistant tuberculosis (TB) are treated with multiple antibiotics including moxifloxacin, linezolid, and meropenem, which puts them at greater risk for colonisation by multi-drug resistant (MDR) bacteria. The objectives of this study were to: (i) assess the antimicrobial prescribing patterns practiced within the hospital by retrospective patient file review; (ii) determine the spectrum of bacterial colonisation in TB patients upon admission and during hospitalisation; (iii) identify bacterial isolates and evaluate antimicrobial susceptibility profiles; (iv) detect antimicrobial resistance genes in the bacterial isolates by PCR and DNA sequencing; and (v) investigate genetic relatedness of *Klebsiella pneumoniae* isolates using Multi Locus Sequence Typing. Nasal, groin and rectal swabs [for the detection of extended spectrum beta lactamases (EBSLs), carbapenem-resistant Enterobacteriaceae (CRE), vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA)] were analysed from a cohort of patients (n=37) admitted either from the community (n = 28) or from other healthcare facilities (n=9) to a TB hospital. Swab samples were collected at admission and at four week intervals thereafter during hospitalization. Identification and antimicrobial susceptibility testing of bacterial isolates (n=62) were determined at the National Health Laboratory Services (NHLS) by the VITEK-MS and Vitek 2 systems respectively. Additional antimicrobial susceptibility testing was conducted by Sensititre Gram Negative Xtra (GNFX2) MIC plates. PCR and DNA sequencing were used for detection of resistance genes. Patients (n=13/37; 35%) were colonized by MDR bacteria (ESBLs [n=11], MRSA [n=2]) on admission. Colonization rates were lower in patients admitted from the community (9/28; 32%) compared to those transferred from other healthcare facilities (4/9; 44%). All admitted patients who did not exhibit colonization at baseline and who were resident within the hospital for longer than 4 weeks (17/37; 46% of total patients) became colonised by an ESBL-producing Enterobacteriaceae species. No patients acquired MRSA during hospitalisation. Among ESBL Enterobacteriaceae, *Escherichia coli (*41/62; 66%) and *K. pneumoniae* [14/62; 23%]) predominated. Nineteen percent (7/37) of patients demised during their hospitalization. Both the Vitek system and Sensititre Gram Negative Xtra (GNFX2) MIC plates susceptibilities were similar for most antimicrobials, however there were discrepancies for tigecycline susceptibility profiles. A high number of isolates exhibited resistance to aminoglycosides and fluoroquinolones. Genes

encoding for ESBLs (CTX-M-14, CTX-M-15, SHV-28, OXA-1, and OXY-2-9) were detected among ESBL Enterobacteriaceae. Two Enterobacteriaceae isolates with reduced carbapenem susceptibility did not contain carbapenemase-encoding genes. MLST revealed unique sequence types and genetic diversity among the *K. pneumoniae* isolates from hospitalised patients. However, the source and colonization routes of these isolates could not be determined, which requires further investigation. This study provides insight into the spectrum of bacterial pathogen colonisation in hospitalised TB patients and suggests a review of infection control programs and practices at the TB hospital.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

aac(6')-Ib-cr	Aminoglycoside acetyltransferase for ciprofloxacin resistance
ABC	ATP-binding cassette efflux pumps
AES	Advanced Expert System
AMX	Amoxicillin-clavulanic acid
AMP	Ampicillin
AmpC	Ampicillin cephalosporinase
AMI	Amikacin
AMS	Antimicrobial stewardship
AMX	Amoxicillin-clavulanic acid
armA	Gene encoding ArmA methylase
AST	Antimicrobial Susceptibility Testing
ATC	AmpC-type
ATP	Adenosine triphosphate
AZT	Aztreonam
β	Beta
$BaCl_2$	Barium chloride
bla	β-lactam antibiotic gene
***bla*CTX-M**	Gene encoding cefotaxime β-lactamase
***bla*CTX-M-9**	Gene encoding cefotaxime β-lactamase variant 9
***bla*CTX-M-14**	Gene encoding cefotaxime β-lactamase variant 14
***bla*CTX-M-15**	Gene encoding cefotaxime β-lactamase variant 15
***bla*CTX-M-22**	Gene encoding cefotaxime β-lactamase variant 22
***bla*GES**	Gene encoding Guiana extended-spectrum β-lactamase
***bla*IMI**	Gene encoding imipenem-hydrolysing β-lactamase
***bla*IMI-2**	Gene encoding imipenem-hydrolysing β-lactamase variant 2
***bla*IMP**	Gene encoding Imipenemase
***bla*KPC**	Gene encoding *Klebsiella pneumoniae* carbapenemase
***bla*NDM-1**	Gene encoding New Delhi metallo-β-lactamase variant 1
***bla*NMC**	Gene encoding non-metallo-enzyme carbapenemase
***bla*OXA-1**	Gene encoding oxacillinase variant 1
***bla*OXA-23-like**	Gene encoding oxacillinase variant 23-like
***bla*OXA-24**	Gene encoding oxacillinase variant 24
***bla*OXA-48**	Gene encoding oxacillinase variant 48
***bla*OXA-51-like**	Gene encoding oxacillinase variant 51-like
***bla*OXA-58**	Gene encoding oxacillinase variant 58
***bla*SHV**	Gene encoding Sulfhydryl variable β-lactamase
***bla*SME**	Gene encoding *Serratia marcescens* enzyme
BLAST	Basic Local Alignment Search Tool
***bla*TEM**	Gene encoding Temoneira β-lactamase
***bla*VIM**	Gene encoding Verona integron-mediated metallo-β-lactamase
***ble*MBL**	Gene encoding bleomycin
bp	Base pair(s)
BSI	Blood stream infections
°C	Degrees Celsius
C	Cytosine
CAF	Central Analytical Facility
CDC	Center for Disease Control
CFA	Cefuroxime axetil
CFX	Cefuroxime
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute
CMY	Cephamycin β-lactamase
COL	Colistin
CRE	Carbapenem-resistant Enterobacteriaceae
CTX	Cefotaxime
CTX-M	Cefotaxime β-lactamase / cefotaximase
CTX-M-1	CTX-M variant 1

CTX-M-2	CTX-M variant 2
CTX-M-3	CTX-M variant 3
CTX-M-8	CTX-M variant 8
CTX-M-9	CTX-M variant 9
CTX-M-14	CTX-M variant 14
CTX-M-15	CTX-M variant 15
CTX-M-22	CTX-M variant 22
CTX-M-25	CTX-M variant 25
CTX-M-27	CTX-M variant 27
CTXM1-F	Forward primer for *bla*CTX-M group I genes
CTXM1-2	Reverse primer for *bla*CTX-M group I genes
CTXM2-F	Forward primer for *bla*CTX-M group II genes
CTXM2-R	Reverse primer for *bla*CTX-M group II genes
CTXM3-F	Forward primer for *bla*CTX-M group III/V genes
CTXM3-R	Reverse primer for *bla*CTX-M group III/V genes
CTXM4-F	Forward primer for *bla*CTX-M group IV genes
CTXM4-R	Reverse primer for *bla*CTX-M group IV genes
CXA	Cefuroxime axetil
ddH2O	Double distilled water
DNA	Deoxyribonucleic acid
DOR	Doripenem
DOX	Doxycycline
EC-DOH	Eastern Cape – Department of Health
EDTA	Ethylene-diamine-tetra-acetate
ERT	Ertapenem
ESBL	Extended-spectrum β-lactamase
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
F	Female
FDA	Food and Drug Administration
FEP	Cefepime
FIDSSA	Federation of Infectious Diseases Societies of Southern Africa
FIM	Florence Imipenemase
FOT	Cefotaxime
FOX	Cefoxitin
xg	Gravitational force
g	Gram(s)
G	Guanine
***gap*A**	glyceraldehyde-3-phosphate dehydrogenase gene
***gap*A-F**	Forward primer for *gap*A
***gap*A-R**	Reverse primer for *gap*A
GEN	Gentamicin
GES	Guiana extended-spectrum β-lactamase
GES-F	Forward primer for *bla*GES gene
GES-R	Reverse primer for *bla*GES gene
GES-5	GES variant 5
GIM	German imipenemase
gyrA	Gene encoding gyrase subunit A
gyrB	Gene encoding gyrase subunit B
h	Hour(s)
HAI	Hospital-acquired infection
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid
HIV	Human immuno-deficiency virus
ICU	Intensive care unit
IMI	Imipenem-hydrolysing β-lactamase
IMI-2	IMI variant 2
IMI-F	Forward primer for *bla*IMI gene
IMI-R	Reverse primer for *bla*IMI gene

IMP	Imipenemase
IMP-F	Forward primer for *bla*IMP gene
IMP-R	Reverse primer for *bla*IMP gene
***inf*B**	Translation initiation factor IF-2 gene
***inf*B-F**	Forward primer for *inf*B
***inf*B-R**	Reverse primer for *inf*B
IPM	Imipenem
IS	Insertion sequence
K	Wobble base
kb	Kilobase(s)
kDa	Kilodalton(s)
KPC	*Klebsiella pneumoniae* carbapenemase
KPC-F	Forward primer for *bla*KPC gene
KPC-R	Reverse primer for *bla*KPC gene**L** Litre(s)
LEVO	Levofloxacin
MAC	MacConkey agar
MATE	Multidrug and toxic compound extrusion
MBL	Metallo-β-lactamase
mcr	Mobilized colistin resistance gene
***mcr*-1**	*mcr* variant 1
MDR	Multidrug-resistant / resistance
mdh	Malate dehydrogenase gene
***mdh*-F**	Forward primer for *mdh*
***mdh*-R**	Reverse primer for *mdh*
MER	Meropenem
MF	Major facilitator
MexAB-OprM	Multidrug efflux system AB- Outer membrane protein M found in *P. aeruginosa*
mexAB-oprM	Operon encoding MexAB-OprM
MexCD-OprJ	Multidrug efflux system CD- Outer membrane protein J found in *P. aeruginosa*
MexR	Repressor protein found in *P. aeruginosa*
mexR	Gene encoding repressor protein MexR
MexXY-OprM	Multidrug efflux system XY- Outer membrane protein M found in *P. aeruginosa*
mg	Milligram(s)
MH	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
min	Minute(s)
MIN	Minocycline
MLST	Multilocus sequence typing
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
n	Total number
NAL	Nalidixic acid
ND	Not determined
NDM	New Delhi metallo-β-lactamase
NDM-F	Forward primer for *bla*NDM gene
NDM-R	Reverse primer for *bla*NDM gene
NDM-1	NDM variant 1
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Diseases
NIT	Nitrofurantoin
nm	Nanometre(s)
NMC	Non-metallo-enzyme carbapenemase
NMC-F	Forward primer for *bla*NMC gene
NMC-R	Reverse primer for *bla*NMC gene
OMP	Outer membrane protein(s)
OXA	Oxacillinase
OXA-1	OXA variant 1
OXA-2	OXA variant 2
OXA-10	OXA variant 10
OXA-23	OXA variant 23

OXA-23-like	OXA variant 23-like
OXA-23-like-F	Forward primer for *bla*OXA-23-like gene
OXA-23-like-R	Reverse primer for *bla*OXA-23-like gene
OXA-24	OXA variant 24
OXA-24-like-F	Forward primer for *bla*OXA-24-like gene
OXA-24-like-R	Reverse primer for *bla*OXA-24-like gene
OXA-40	OXA variant 40
OXA-48	OXA variant 48
OXA48-F	Forward primer for *bla*OXA-48 gene
OXA48-R	Reverse primer for *bla*OXA-48 gene
OXA-51-like	OXA variant 51-like
OXA-51-like-F	Forward primer for *bla*OXA-51-like gene
OXA-51-like-R	Reverse primer for *bla*OXA-51-like gene
OXA-58	OXA variant 58
OXA-58-like-F	Forward primer for *bla*OXA-58-like gene
OXA-58-like-R	Reverse primer for *bla*OXA-58-like gene
OXA-F	Forward primer for *bla*OXA gene
OXA-R	Reverse primer for *bla*OXA gene
%	Percentage
P	Probability value (indicating statistical significance when smaller than alpha)
parC	Gene encoding topoisomerase IV subunit C
parE	Gene encoding topoisomerase IV subunit E
PBP	Penicillin-binding protein
PBP2α	PBP variant 2α
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR restriction fragment length polymorphism
PFGE	Pulsed-field gel electrophoresis
pgi	Glucose-6-phosphate isomerase gene
***pgi*-F**	Forward primer for *pgi*
***pgi*-R**	Reverse primer for *pgi*
pH	Indicates acidity (<7), neutrality (=7) or alkalinity (>7)
***pho*E**	Outer membrane pore protein E precursor *pho*E
***pho*E-F**	Forward primer for *pho*E
***pho*E-R**	Reverse primer for *pho*E
PJP	*Pneumocystis jiroveci* pneumonia
PMQR	Plasmid-mediated quinolone resistance
Pro	Provincial
PT4	Piperacillin-tazobactam
QepA	Quinolone efflux pump A
qepA	Gene encoding QepA efflux pump
Qnr	Quinolone resistance protein
qnr	Gene encoding Qnr protein
qnrA	Gene encoding QnrA protein
qnrB	Gene encoding QnrB protein
qnrC	Gene encoding QnrC protein
qnrD	Gene encoding QnrD protein
qnrS	Gene encoding QnrS protein
R	Wobble base
RAPD	Random amplified polymorphic DNA
rep-PCR	Repetitive extragenic palindromic element PCR
RND	Resistance-nodulation-cell division
rpm	Revolutions per minute
***rpo*B**	β subunit RNA polymerase gene
***rpo*B-F**	Forward primer for *rpo*B
***rpo*B-R**	Reverse primer for *rpo*B
RR-TB	Rifampicin-resistant TB
rRNA	Ribosomal ribonucleic acid
s	Second(s)
SAASP	South African Antibiotic Stewardship Programme

SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHV	Sulfhydryl variable β-lactamase
SHV-1	SHV variant 1
SHV-2	SHV variant 2
SHV-5	SHV variant 5
SHV-7	SHV variant 7
SHV-11	SHV variant 11
SHV-12	SHV variant 12
SHV-19	SHV variant 19
SHV-20	SHV variant 20
SHV-21	SHV variant 21
SHV-22	SHV variant 22
SHV-28	SHV variant 28
SHV-F	Forward primer for *bla*SHV gene
SHV-R	Reverse primer for *bla*SHV gene
SIM	Seoul imipenemase
SMART	Study for the Monitoring of Antimicrobial Resistance Trends
SME	*Serratia marcescens* enzyme
SMR	Staphylococcal multidrug resistance
SPM	Sao Paulo metallo-β-lactamase
spp.	Plural of species
SSI	Surgical site infection
ST	Sequence Type
SXT	Trimethoprim-sulfamethoxazole
T	Thymine
TB	Tuberculosis
TAZ	Ceftazidime
TE	Tris-EDTA buffer
TEM	Temoneira β-lactamase
TEM-1	TEM variant 1
TEM-F	Forward primer for *bla*TEM gene
TEM-R	Reverse primer for *bla*TEM gene**TGC**
TGC	Tigecycline
TIM2	Ticarcillin-clavulanic acid
tet	tetracycline efflux pump gene
TOB	**Tobramycin**
***ton*B**	Protien tonB gene
***ton*B-F**	Forward primer for *ton*B
***ton*B-R**	Reverse primer for *ton*B
µg	Microgram(s)
µL	Microlitre(s)
µm	Micrometre(s)
U	Units
UPGMA	Unweighted Pairs Geometric Matched Analysis
USA	United States of America
UTI	Urinary tract infection
UV	Ultraviolet light
V	Voltage
***van*HAX**	Vancomycin-resistance determinant gene cluster
VIM	Verona integron-mediated metallo-β-lactamase
VIM-F	Forward primer for *bla*VIM gene
VIM-R	Reverse primer for *bla*VIM gene
VIM-2	VIM variant 2
VRE	Vancomycin-resistant Enterococci
WGS	Whole genome sequencing
WHO	World Health Organization
w/v	Weight per volume
Zn2+	Zinc cation
XDR	Extensively drug-resistant

XDR-TB	Extensively drug-resistant Tuberculosis

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Nosocomial infections, also known as hospital-acquired infections (HAIs), are infections acquired by patients while seeking treatment for an unrelated illness within a healthcare facility or setting (McQuoid-Mason, 2012). HAIs are associated with high mortality rates (Brink *et al.* 2006) and most are caused by bacteria (McQuoid-Mason, 2012). The most common types of hospital acquired conditions are, surgical site infection (SSI), urinary tract infection (UTI), blood stream infection (BSI), and pneumonia (Weinstein *et al*. 2005). Major bacterial nosocomial pathogens include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), *Escherichia coli*, *Acinetobacter baumannii* and *Enterobacter* spp. (Duse, 2005; Nyasulu *et al.* 2012).

HAIs result in increased treatment costs, as nosocomial infection patients spend an average of 2.5-times longer in hospital placing a high financial burden on healthcare facilities (Zimlichman, *et al.* 2013; Spellberg *et al*. 2011), especially in developing countries such as South Africa (Brink *et al.* 2006). The consequences for both patients and the health care system are significant. Patients suffer, endure prolonged hospitalisation and in some cases, die. For hospitals, it is an additional burden on overcrowded and short-staffed wards. It also means that fewer beds are available for new patients, particularly in the intensive care unit (Dramowski, 2017).

Infection control is the primary option for preventing the increased costs associated with HAIs. It is our concern that patients in tuberculosis (TB) hospitals may be at elevated risk at developing HAIs. Infection control in a TB hospital focuses mainly on airborne transmission precautions. While there are infection control precautions and antibiotic stewardship guidelines approved by the South African Department of Health, it is unclear whether these programs are executed routinely and effectively. TB patients are at high risk of spreading and developing invasive infections of multi-drug

resistant bacteria if infection control and prevention are inadequate. Although the prevalence and spread of nosocomial tuberculosis infections is well documented, there is a severe lack of information available regarding other nosocomial bacterial infections within tuberculosis patients.

1.2 INFECTION CONTROL AND ANTIMICROBIAL STEWARDSHIP

Infection control is crucial within healthcare settings prevent the transmission of life threatening infections, (Khan *et al.* 2017). Optimum infection control of HAIs is dependent on three main factors, namely surveillance, prevention, and appropriate action when infection takes place (Ducel, *et al.* 2002). Surveillance and prevention are far more desirable and cheaper strategies for the prevention of development and spread of HAIs within a healthcare facility, however appropriate action such as quarantine and effective antimicrobial treatment programs are necessary to ensure patient safety. For the purpose of surveillance, the Center for Disease Control (CDC) have classified HAIs into 13 distinct types, with 50 different infection sites, based on clinical and biological criteria (Khan *et al.* 2015). HAIs are spread among patients through three major routes namely contact, droplet, and airborne spread (Brink *et al*. 2006). Contact spread occurs through person to person transmission, interaction with infected surfaces or objects and consumption of contaminated food or water. Preventing contact spread may pose a challenge within a specialised TB hospital which is concerned mainly with airborne transmission and respiratory protection (Khan *et al.* 2015; Punjabi, 2016). Therefore, patients being treated in such specialised settings may be at high risk of colonization by other nosocomial pathogens, and this further highlights the need for strictly executed infection control programs. Figure 1.1 illustrates the steps and factors required for effective infection prevention to take place within a healthcare facility environment.

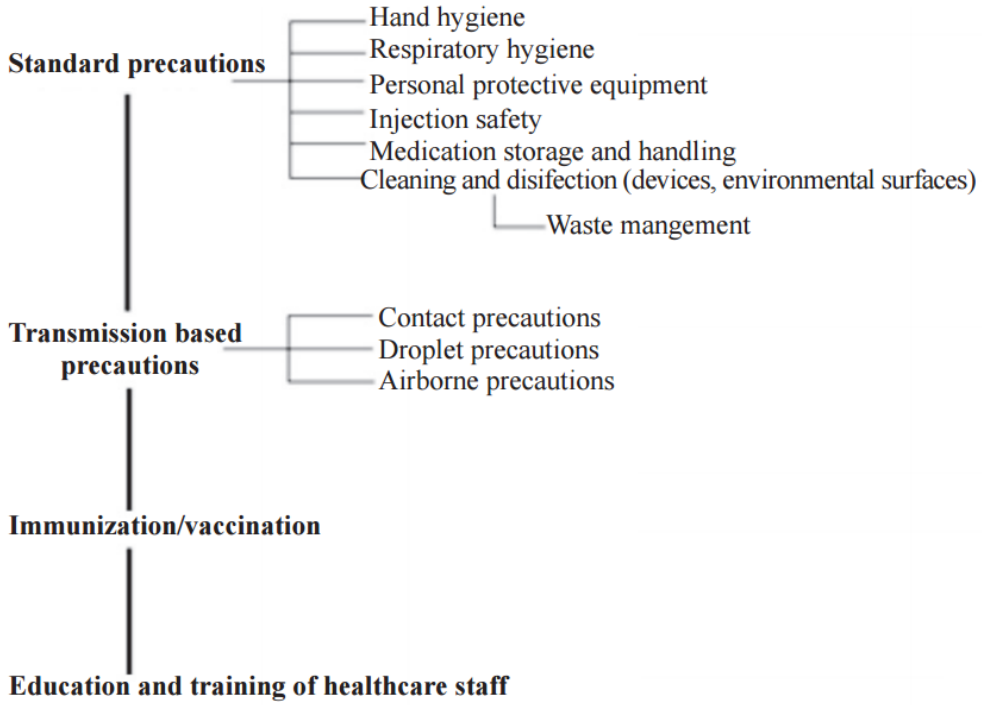

Figure 1.1: Infection control program guideline (Khan *et al.* 2017).

Society is currently in dire need of new antibiotics due to the rampant spread of antimicrobial resistance. However, the production of new drugs by the pharmaceutical industry is delayed by financial cost and lengthy clinical trials, which often take 10 years or more to complete (Fishman, 2006). Therefore, the prevention of antimicrobial resistance development has become just as important, if not more desired, than the development of new treatments in order to combat antimicrobial resistant infections. Limitation of antimicrobial resistance development can be achieved through antimicrobial stewardship. Antimicrobial stewardship is defined as optimal drug selection, dosage level, and duration of antimicrobial treatment which results in optimal patient outcomes with minimal toxicity to the patient and minimal impact on antimicrobial resistance (Fishman, 2006; Doron and Davidson, 2011). The implementation of antimicrobial stewardship programs hopes to achieve three distinct goals (Doron and Davidson, 2011). The first goal is to ensure that patients receive the most appropriate antibiotic to treat their infection along with the correct dose and duration. This is to achieve optimal patient care, and through scrutinized antibiotic use financial savings can be achieved within healthcare facilities (Doron and Davidson, 2011). The second and third goals of antimicrobial stewardship are to prevent antibiotic abuse and development of antimicrobial resistance respectively, due to antimicrobial misuse and overuse (Doron and Davidson, 2011). Antimicrobial abuse

occurs when antibiotics are prescribed when they are not needed; when an inappropriate antibiotic is used to treat a particular infection; or when a broad-spectrum antibiotic is prescribed when a more specific option is available (Doron and Davidson, 2011). Inappropriate and indiscriminate use of antibiotics is the major driving force behind the selection of antimicrobial resistant pathogens (Nagel *et al.* 2016). Recently applied antimicrobial stewardship programs have shown improvement in appropriate antibiotic use and cure rates, as well as improved patient outcome rates and lower healthcare costs (Nagel *et al.* 2016).

As indicated the goals of infection control and antimicrobial stewardship not only align but also overlap, and it has been stated that “antibiotic stewardship and infection control need to be seen as inseparable sides of the same coin” (Carling and Polk, 2011).

1.3 NOSOCOMIAL BACTERIAL PATHOGENS

“Nosocomial” is a term used to describe any disease acquired by a patient while receiving medical treatment for an unrelated condition within a healthcare setting (McQuoid-Mason, 2012). The most concerning and common occurring bacterial nosocomial pathogens, both worldwide and in South Africa, include *Pseudomonas aeruginosa*, *Enterococcus* spp., *Staphylococcus aureus*, and Enterobacteriaceae family members, such as *Escherichia coli* and *Klebsiella pneumoniae* (Nyasulu *et al.* 2012; Khan *et al.* 2015). These pathogens are described in detail according to their taxonomy, treatment, and epidemiology in the sections below.

1.3.1 *Enterobacteriaceae*

A number of bacterial species belonging to the family of *Enterobacteriaceae* are common members of the human intestinal microflora. However, many of these species are also opportunistic pathogens, which account for a large portion of reported HAIs (Nordmann *et al*. 2012). *Enterobacteriaceae* are found within phylum Proteobacteria, class Gammaproteobacteria, order *Enterobacteriales* and are a family of non-spore forming, oxidase negative, facultative anaerobic, Gram-negative bacilli (Willey *et al.* 2011). The family consists of 44 different genera; however, it can be difficult to differentiate the genera from each other due to their similarity in morphology, and so biochemical tests are utilized to achieve accurate differentiation. These tests include

sugar fermentation, lactose and citrate utilization, hydrogen sulphide and indole production, and urea hydrolysis (Willey *et al.* 2011). Many human pathogens are found within the Enterobacteriaceae which cause illnesses such as typhoid fever, pneumonia, and plague (Willey *et al.* 2011). A significant amount of HAIs are caused by the Enterobacteriaceae, including *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter* species (Weinstein *et al*. 2005; Peleg and Hooper, 2010; and Jacob *et al.* 2013).

Enterobacteriaceae infections are frequently treated with β-lactam antibiotics, however with the increasing trend of antimicrobial resistance among the genera of this family older generations of β-lactams are becoming obsolete and last line β-lactam drugs, such as carbapenems, are swiftly becoming the default choice to treat such infections (Delgado-Valverde *et al.* 2013). Other β-lactam drugs coupled with β-lactamase inhibitors may be used as the primary treatment of infections due to extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae. However, carbapenem treatment is associated with lower levels of mortality, especially in bacteraemic patients (Vardakas, *et al.* 2012). This is likely due to the fact that many of the ESBLs are not affected by β-lactamase inhibitors, such as clavulanic acid (Queenan and Bush, 2007).

Multiple drug resistance mechanisms expressed by the *Enterobacteriaceae*, have resulted in Carbapenem Resistant Enterobacteriaceae (CRE) infections (Queenan and Bush, 2007). These mechanisms will be discussed in greater detail in the next section. It would appear that in the case of extreme CRE infections that the antibiotics of choice are combinations of carbapenems, tigecycline, aminoglycosides, and colistin (Falagas *et al.* 2014). However even with the use of these very powerful last line antibiotics the mortality rates of patients are still relatively high, (Morrill *et al*. 2015) and last resort antibiotics, such as colistin, can often be as dangerous to the patients' health as the infection itself (Falagas *et al.* 2014; Morrill *et al.* 2015).

1.3.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a species of Gram-negative, non-spore forming, oxidative, motile bacilli which is found within the phylum Proteobacteria, class Gammaproteobacteria, order *Pseudomonadales*, family *Pseudomonadaceae*

(Palleroni, 1994; Willey *et al.* 2011). *Pseudomonas aeruginosa* has minimal nutrition requirements which has led to the microorganism's broad environmental distribution (Solh and Alhajhusain, 2009). The genus *Pseudomonas* is currently made up of approximately 60 different species and the genus can be further divided into seven subgroups based on rRNA-DNA hybridization. These subgroups can be further categorized through properties such as glucose utilization, presence of poly-β-hydroxybutyrate (PHB), fluorescent pigment production, and accumulation of arginine dihydrolase (Tayeb *et al*. 2005; Willey *et al.* 2011). *P. aeruginosa* belongs to rRNA group I, the "fluorescent group", which are known not to accumulate PHB and produce the water-soluble, fluorescent pigment pyoverdin (Cox and Adams, 1985; Willey *et al.* 2011). Interestingly, pyoverdin acts as a siderophore and is believed to be related to the pathogenicity of *P. aeruginosa* (Cox and Adams, 1985).

P. aeruginosa is a common nosocomial pathogen and the species may account for up to 17% of all HAIs, and up to 23% of all HAIs in ICU settings (Giamarellou, 2002; Driscoll *et al*. 2007). *P. aeruginosa* is associated with all major nosocomial infections such as BSIs, SSIs, and UTIs and it is one of the most prominent causative agents for healthcare-associated pneumonia and ventilator-associated pneumonia (Driscoll *et al*. 2007). It has also been reported to cause meningitis in patients after neurosurgery (Giamarellou, 2002). *P. aeruginosa* also causes conditions such as ulcerative keratitis, otitis externa, and chronic sinopulmonary colonisation in cystic fibrosis patients (Driscoll *et al*. 2007). Immunocompromised and neutropenic patients are at high risk of infection by *P. aeruginosa*, patients suffering from HIV, leukaemia, and diabetes are known to be readily colonized and at high risk of mortality (Kang *et al*. 2003; Driscoll *et al*. 2007).

Antibiotics often prescribed to treat *Pseudomonas* spp. infections include ticarcillin, ureidopenicillins, ciprofloxacin, aztreonam, aminoglycosides, ceftazidime, cefepime, and carbapenems (Giamarellou, 2002; Solh and Alhajhusain, 2009). With the development of multidrug resistant *P. aeruginosa,* treatment options are rapidly becoming scarce. Combination drug therapy has been advised to combat MDR infections, with drugs such as carbapenems, fluoroquinolones, cefepime, and colistin showing effectiveness against MDR cefepime-intermediate *P. aeruginosa* (Driscoll *et al*. 2007; Solh and Alhajhusain, 2009; Heil *et al.* 2015). Treatment of *P. aeruginosa*

can be tricky as the species is intrinsically resistant to many antibiotics, and can rapidly acquire additional resistance mechanisms (Henrichfreise *et al*. 2007). While carbapenemase production results in high levels of resistance, it has been observed that carbapenem resistance can result, and often does, from a combination of other mechanisms such as poor drug permeability and increased drug efflux (Rodríguez-Martínez *et al*. 2009; Meletis *et al.* 2014). With the increased incidence of carbapenemase over production in MDR *P. aeruginosa,* in many cases carbapenem treatment is no longer an option and treatment with polymyxins, such as colistin, is the only line of defence left (Nordmann *et al*. 2011). It has now been demonstrated that the MCR-1 gene that has been identified as the sole cause of colistin resistance in *E. coli* and *K. pneumoniae* is readily transferred and maintained within *P. aeruginosa* (Castanheir *et al.* 2016; Liu *et al.* 2016). This indicates a significant threat to one of the last line antibiotics.

1.3.3 *Staphylococcus aureus*

Staphylococcus aureus is one of the most currently discussed nosocomial pathogens. *S. aureus* is found within the phylum Firmicutes, class *Bacilli*, order *Bacillales*, family *Staphylococcaceae,* and are non-spore forming, catalase positive, halo-tolerant, facultative anaerobic, Gram-positive cocci (Foster, 1996; Wiley *et al*. 2011). DNA-rRNA hybridization and oligonucleotide analysis of the staphylococci 16S rRNA has demonstrated that the genus forms a coherent group, this group occurs within the *Bacillus-Lactobacillus-Streptococcus* cluster of the low G + C Gram-positive bacteria (Foster, 1996).

Approximately 30% of the human population is likely to be colonized with *S. aureus*, with the primary location of colonization being the anterior nares (Brown *et al.* 2013). *S. aureus* is associated mainly with osteoarticular infections, skin and soft tissue infections such as boils, furuncles, and styes (Foster, 1996). However, *S. aureus* is also associated with more severe infections such as, pleuropulmonary infections, pneumonia, meningitis, device-related infections such as joint prostheses and cardiovascular devices, and is also the leading cause of both bacteraemia and infective endocarditis (Foster, 1996; Tong *et al.* 2015). The clinical manifestations of *S. aureus* are highly pervasive; this highlights the species' threat as a nosocomial pathogen. The groups of people most at risk from *S. aureus* infections are those

naturally colonized with the bacteria, people younger than 13 or older than 70 years of age, patients undergoing haemodialysis, and patients suffering from an immunosuppressing disease, such as HIV (Naber, 2009; Tong *et al.* 2015). The vast amount of different infections caused by *S. aureus* highlights the clinical importance of this pathogen.

The majority of clinical *S. aureus* isolates are now resistant to early generation penicillin drugs, therefore β-lactamase resistant penicillins, such as flucloxacillin, dicloxacillin, are the antibiotics of choice when treating methicillin susceptible infections (Rayner and Munckhof, 2005). Cephalosporins, such as cefazolin, cephalothin, and cephalexin, and erythromycin are also options for treating skin and soft tissue infections, and for patients with penicillin hypersensitivity (Rayner and Munckhof, 2005). However, with the increasing incidence of methicillin resistant *S. aureus* (MRSA) infections, penicillin and cephalosporin-based treatments are becoming less and less viable (Moellerin, 2008). The major therapeutic agents currently used to treat MRSA infections include vancomycin, daptomycin, and linezolid (Purrelloa *et al*. 2016). It has also been suggested that rifampin may be successful through combination therapy in order to treat MRSA (Forrest and Tamura, 2010). However, since 2002 several cases of vancomycin-resistant *S. aureus* (VRSA) have been reported, therefore illustrating yet another threat to the dwindling options of therapeutic agents available to combat MRSA (Howden *et al.* 2010). Vancomycin-intermediate *S. aureus* (VISA) is also a concern, as these isolates exhibit a relatively poor response towards glycopeptides, and VISA has become considerably wide spread (Appelbaum, 2006; Zhu *et al.* 2015). However, there are a few newly developed antibiotics which have demonstrated activity against MRSA. These include lipoglycosides, ceftaroline, ceftobiprole, and torezolid (Purrelloa *et al*. 2016).

Hospitalised patients with TB have high rates of HIV co-infection in sub-Saharan Africa that may increase risk of MRSA colonisation and infection. A high prevalence of multidrug-resistant MRSA nasal carriage was found among TB patients with advanced HIV admitted to a rural hospital in South Africa (Heysell *et al.* 2011).

1.3.4 *Enterococcus species*

The *Enterococcus* genus is located within the phylum Firmicutes, class *Bacilli*, order *Lactobacillales*, family *Enterococcaceae,* and consists of catalase negative, facultative anaerobic, non-spore forming, Gram-positive cocci (Fisher and Phillips, 2009; Wiley *et al*. 2011). The *Enterococci* belong to a group known as the lactic acid bacteria (LAB), which are known for the production of bacteriocins, they are also categorized among the low G + C Gram-positive bacteria (Fisher and Phillips, 2009). Phenotypically, it is difficult to distinguish the Enterococci from other catalase negative, Gram-positive cocci. However, 16S rRNA sequencing, identified 28 *Enterococcus* species, of which *Enterococcus faecalis* and *Enterococcus faecium*, are the two most clinically relevant accounting for approximately 80 to 90% and 5 to 15% of Enterococci infections respectively (Cetinkaya *et al.* 2000; Fisher and Phillips, 2009; Kristich *et al*. 2014).

Enterococci exhibit intrinsic resistance to many of the commonly used antibacterial therapeutic agents (Kristich *et al*. 2014). Enterococci are known to exhibit resistance against penicillin, ampicillin, most cephalosporins, all semi-synthetic penicillins, and clindamycin (Kristich *et al*. 2014). Trimethoprim-sulfamethoxazole has been shown to be effective against Enterococci *in vitro*, however has failed to show significant activity in animal models (Zervos and Schaberg, 1985). Combination treatment with quinupristin-dalfopristin is known to be effective against clinical *E. faecium* infections, however this is not a viable treatment for naturally resistant *E. faecalis* (Kristich *et al*. 2014). Enterococci also exhibit an intrinsic resistance to clinical concentrations of aminoglycosides, this prevents use of aminoglycosides as a single treatment agent, however aminoglycosides, such as gentamycin, is a common treatment option when used in combination therapy with penicillins and glycolpeptides (Sierra-Hoffman *et al.* 2012; Kristich *et al*. 2014). With the emergence of vancomycin-resistant enterococci (VRE) fewer antimicrobial drugs are available to treat such enterococcal infections. Drugs such as quinupristin-dalfopristin, linezolid, daptomycin, and tigecycline are viable treatment options for VRE treatment, however none of these drugs have been observed to be truly free from resistance amongst the Enterococci (Kristich *et al*. 2014).

1.4 ANTIMICROBIAL DRUG RESISTANCE MECHANISMS

The development of antibiotic resistance among bacterial pathogens is an emerging public health crisis worldwide, with many antibiotic resistant species, such as *S. aureus* and *M. tuberculosis* being described as pandemics (Spellberg *et al.* 2008). The major cause of antibiotic resistance is the misuse and overuse of antibiotics which has caused a selective pressure favouring bacteria harbouring antibiotic resistance genes.

Multiple antibiotic resistance mechanisms have been identified among bacteria, however the mechanisms depend on the bacterial species as well as the antibiotic being utilized. Though resistance mechanisms differ from species to species, they often involve drug efflux from the bacterial cell; decreased influx of the drug into the bacterial cell; degradation of the antimicrobial; or a structural change to the antimicrobials' target (Cartwright *et al.* 2013; Kristich *et al.* 2014; Meletis *et al*. 2014; Liu *et al.* 2016). The specific resistance mechanisms of each of the nosocomial bacterial pathogens are described in detail in the sections below.

1.4.1 Extended-Spectrum β-Lactamases & Carbapenemases

The *Enterobacteriaceae* species, (*E. coli* and *K. pneumoniae)*, and *P. aeruginosa* share very similar drug resistance mechanisms, and this is not surprising as they are often treated with similar antimicrobial agents as was outlined in sections 1.3.1 and 1.3.2. The major class of antibiotics utilized against these Gram-negative bacteria are the β-lactam antibiotics due to their broad spectrum of activity and high safety profile (Abdallah *et al.* 2015). β-lactam antibiotics function by binding to the peptidoglycan cross-linking enzymes, also known as penicillin binding proteins (PBPs), such as transpeptidases and carboxypeptidases, which prevents peptidoglycan crosslinking within the bacterial cell wall (Heesemann, 1993). The β-lactam-PBP complex also induces the release of autolytic enzymes which further damage the bacterial cell wall, and cell death is achieved through cell lysis (Heesemann, 1993). Bacteria can achieve resistance to β-lactams through the presence of β-lactam insensitive cell wall transpeptidases and the active efflux of the β-lactam molecules from the bacterial cell through efflux pumps (Abdallah *et al.* 2015). However, the most effective way for bacteria to counteract the effect of β-lactam antibiotics is by the acquisition and production of β-lactam hydrolysing enzymes known as β-lactamases, which include

extended-spectrum β-Lactamases (ESBLs) and carbapenemases (Kong *et al.* 2010). ESBLs have the ability to hydrolyse penicillins, cephalosporins, and aztreonam, but cannot hydrolyse carbapenems, however, carbapenemases do have the ability to degrade carbapenem antibiotics (Abdallah *et al.* 2015).

β-lactamases are traditionally classified by their functional characteristics or by their primary molecular structure, with the simplest and most commonly used classification being based on the enzymes' amino acid sequence by identifying conserved and characteristic amino acid motifs (Queenan and Bush, 2007; Bush and Jacoby, 2010). The classification scheme based on amino acid homology has resulted in four major molecular classes of β-lactamases, namely molecular classes A, B, C, and D (Bush and Jacoby, 2010). Classes A, C, and D β-lactamases are characterized by the serine residue which is contained and plays a vital role within the enzyme active-site, while class B, known as the metalloenzymes, utilize active-site divalent zinc ions in order to achieve β-lactam hydrolysis (Bush and Jacoby, 2010). The serine based β-lactamases function by forming a covalent acyl intermediate at the active site serine residue and the β-lactam antibiotic, the intermediate is then deacylated which causes the C-N bond of the β-lactam ring within the antibiotic to be hydrolysed, which in turn inactivates the antibiotic (Fig. 1.2) (Queenan and Bush, 2007).

The functional-based classification of β-lactamases is a system developed in 1989, (Queenan and Bush, 2007) which considers the spectrum and rates spectrum of β-lactam hydrolysis and the inhibitor profiles of the β-lactamases (Queenan and Bush. 2007; Bush and Jacoby, 2010). The functional analysis has resulted in four distinct groups, namely Group 1 Cephalosporinases, Group 2 Serine β-lactamases, Group 3 Metallo-β-lactamases, and Group 4 uncategorized β-lactamases (Bush and Jacoby, 2010). While the structural classification is simpler and less controversial than the functional classification, the latter classification provides a more relevant way of associating β-lactamases with their clinical significance, as in the past it has been the functionality of β-lactamases which determine their level of concern within a medical setting, rather than the molecular structure of the enzyme (Bush and Jacoby, 2010).

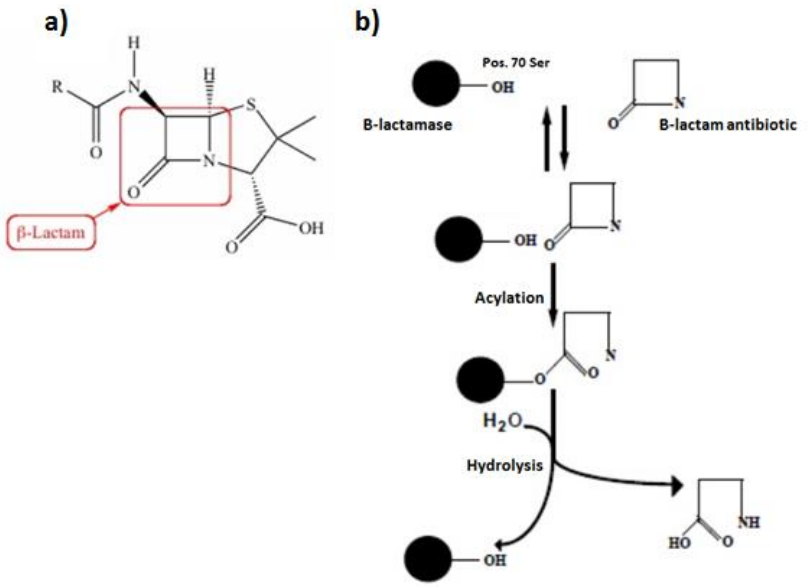

Figure 1.2: **a)** Molecular structure of penicillin as an example of the molecular structure of β-lactam antibiotics. **b)** Mechanism of action exhibited by molecular class A, C, and D β-lactamases (Rao, 2012).

Group 1 β-lactamases are cephalosporinases which belong to the molecular class C, they are encoded chromosomally and are found mainly within the *Enterobacteriaceae* and a few other similar species, such as *Pseudomonas aeruginosa* (Bush and Jacoby, 2010). Group 1 cephalosporinases are active against cephalosporins, aztreonam, cephamycins, cephalothin, cefazolin, most penicillins and generally are not inhibited by clavulanate, tazobactam, or other β-lactamase inhibitors (Jacoby, 2009; Bush and Jacoby, 2010). Interestingly, when produced in large amounts group 1 enzymes can confer resistance to carbapenem drugs, especially to ertapenem (Bush and Jacoby, 2010). The most well-known group 1 β-lactamase is AmpC and it was the first β-lactamase ever reported (Jacoby, 2009). AmpC is produced by a number of organisms including *E. coli, Enterobacter cloacae, P. aeruginosa, Acinetobacter baumannii,* and a number of other *Enterobacteriaceae* species (Bush and Jacoby, 2010). While the group 1 cephalosporinases are classically known as being chromosomally encoded some transmissible plasmids have acquired genes for AmpC-like enzymes, such as variants of the cephamycinase (CMY), AmpC-type (ACT), Dhahran (DHA), Cefoxitin (FOX), and Miriam Hospital (MIR) β-lactamases, however these plasmid-encoded group 1 β-lactamases are far less common than the plasmid-encoded group 2 β-lactamases (Jacoby, 2009; Bush and Jacoby, 2010).

Group 2 β-lactamases are known as the serine β-lactamases, are made up of enzymes belonging to the A and D molecular class, and represent the largest and fastest growing functional group of β-lactamases (Bush and Jacoby, 2010). As the name suggests, this group's mechanism of β-lactam hydrolysis relies on an active-site serine residue at position 70 (Queenan and Bush, 2007). There exists a significant number of subgroups within the serine β-lactamases. Subgroup 2a represents the β-lactamases that are predominantly observed in Gram-positive cocci, such as in *Staphylococcus* and *Enterococcus* species, and are chromosomally encoded (Bush and Jacoby, 2010). Subgroup 2a enzymes are known to show a limited β-lactam hydrolytic spectrum, and are able to hydrolyse penicillin and penicillin derivatives, however they exhibit extremely low hydrolysis rates for all other β-lactam drugs, and experience inhibition from clavulanate and tazobactam (Bush and Jacoby, 2010). Examples of a 2a β-lactamases would be the Exo and PC1 β-lactamases (Bush, 1989).

Subgroup 2b serine β-lactamases confer resistance to penicillins and early cephalosporin drugs, however are strongly inhibited by tazobactam and clavulanic acid (Bush and Jacoby, 2010). The 2b subgroup enzymes are the most common plasmid-encoded β-lactamases, and the most widespread of these enzymes are the TEM and SHV β-lactamases families (Bush and Jacoby, 2010; Zaniani *et al.* 2012). The production of serine β-lactamases such as TEM and SHV are observed mainly within the *Enterobacteriaceae* species, with *K. pneumoniae* and *E. coli* being the major producing species (Turner, 2005; Zaniani *et al.* 2012). The subgroup 2br share the same spectrum of activity as the 2b subgroup, however the 2br subgroup enzymes are characterised by their resistance to clavulanic acid inhibition (Bush and Jacoby, 2010). This subgroup includes 36 TEM enzymes and 5 SHV enzymes (Bush and Jacoby, 2010). The 2be subgroup share very similar characteristics and spectrum of action as seen in the 2b subgroup, however the 2be subgroup ESBLs are also active against one or more of the oxyimino-β-lactams, such as ceftazidime, cefotaxime, and aztreonam (Bush and Jacoby, 2010). The 2be subgroup ESBLs also include enzymes of the TEM and SHV families, however they contain amino acid substitutions which differentiate the 2be variants from the 2b subgroup enzymes, it is these amino acid substitutions which is believed to confer the hydrolytic ability against the oxyimino-β-lactams (Bush and Jacoby, 2010). Another type of highly prolific type of ESBL seen

within the 2be subgroup are the CTX-M β-lactamases. Along with penicillins and cephalosporins, the CTX-M enzymes are able to hydrolyse cefotaxime, ceftazidime, and cefepime, they also experience a higher level of inhibition from tazobactam over clavulanic acid (Bush and Jacoby, 2010). Other clinically relevant group 2be ESBLs include BEL-1, BES-1, SFO-1, TLA-1, TLA-2, PER, and VEB enzyme families, however the major enzymes of concern of the 2be group remain the TEM, SHV, and CTX-M families (Bush and Jacoby, 2010). Finally, there exists a small and rare group of ESBLs which belong to the subgroup 2ber. As the name suggests these enzymes combine the extended hydrolysis spectrum of the 2be subgroup with the clavulanate resistance of the 2br subgroup (Bush and Jacoby, 2010). These ESBLs are known as Complex Mutant TEM or CMT (Bush and Jacoby, 2010).

Subgroup 2c consists of penicillinases which have the ability to hydrolyse carbenicillin, ticarcillin, benzylpenicillin, cloxacillin, and oxacillin, and are observed to be readily inhibited by both tazobactam and clavulanic acid (Bush and Jacoby, 2010). Subgroup 2ce contains the fairly newly described ESBL, RTG-4, which in addition to the subgroup 2c spectrum of action, is also active against cefepime and cefpirome (Bush and Jacoby, 2010). As carbenicillin is an infrequently used antibiotic, this may suggest why few other subgroup 2c type β-lactamases have been recently described (Bush and Jacoby, 2010).

Subgroup 2d consists of the OXA-related β-lactamases and makes up the second largest group of the β-lactamases (Bush and Jacoby, 2010). The OXA-type enzymes are generally plasmid encoded, they have the ability to hydrolyse oxacillin, and this is where the OXA name is taken from, they also have the ability to hydrolyse cloxacillin, benzylpenicillin, and carbenicillin and they experience little inhibition form clavulanic acid but can be inhibited by NaCl (Queenan and Bush, 2007; Bush and Jacoby, 2010). The subgroup 2de consists of OXA-family enzymes which exhibit an extended spectrum of action. The spectrum of activity of the 2de subgroup is the same as the 2d subgroup, however they are also able to hydrolyse oxyimino-β-lactams (Bush and Jacoby, 2010). Many of the 2de β-lactamases are derived from OXA-10, showing between 1 and 9 amino acid substitutions, these include the enzymes OXA-11 and OXA-15 (Bush and Jacoby, 2010). The majority of these enzymes have been isolated from *P. aeruginosa* (Bush and Jacoby, 2010). The 2df subgroup includes the OXA-

type enzymes which include carbapenems within their spectrum of action and generally do not exhibit inhibition by clavulanic acid (Bush and Jacoby, 2010). The majority of the 2df subgroup β-lactamases are chromosomally encoded and have been observed within *A. baumannii*, however OXA-23 and OXA-48 are plasmid-encoded and have been observed within a number of other species, mainly the *Enterobacteriaceae* species (Bush and Jacoby, 2010). Enzymes such as OXA-23 and OXA-48 are of fairly high clinical concern due to their mobile nature and ability to hydrolyse a broad spectrum of β-lactams, including last line drugs.

Subgroup 2e includes extended spectrum cephalosporinases, they have the ability to hydrolyse extended spectrum cephalosporins and are known to be inhibited by clavulanic acid and tazobactam (Bush and Jacoby, 2010). These enzymes are often confused with the group 1 cephalosporinases; however, they can be distinguished by a low affinity for aztreonam (Bush and Jacoby, 2010). The most common subgroup 2e enzymes known are chromosomally encoded and produced by *Proteus* species (Bush and Jacoby, 2010).

Subgroup 2f are made up of the molecular class A carbapenemases. Apart from molecular structure these carbapenemases differ from the more aggressive carbapenemases seen in group 3 due to their susceptibility to inhibition by clavulanic acid and tazobactam (Bush and Jacoby, 2010). These enzymes are able to hydrolyse a wide variety of β-lactams, however show a low affinity for the extended spectrum cephalosporins, such as ceftazidime (Bush and Jacoby, 2010). The 2f subgroup include both chromosomally and plasmid-encoded β-lactamases. Chromosomally encoded 2f enzymes include SME, IMI-1, and NMC-1, while the more clinically concerning plasmid-encoded 2f β-lactamases include the KPC and some GES enzymes (Bush and Jacoby, 2010). The KPC enzymes are of particular concern as they are associated with many worldwide outbreaks of multidrug resistant *K. pneumoniae* and other Gram-negative pathogens (Queenan and Bush, 2007; Bush and Jacoby, 2010).

The functional group 3 β-lactamases are made up of the metallo-β-lactamases belonging to molecular class B with the molecular class being divided into class B1, B2, and B3, these enzymes are unique in both structure and functionality when

compared to the other groups of β-lactamases (Queenan and Bush, 2007; Bush and Jacoby, 2010). The metallo-β-lactamases make use of Zn^{2+} ions and hydrolysis of the β-lactam ring is achieved by cleavage of the β-lactam amide bond by attack of the hydroxide ion on the carbonyl carbon, a basic depiction of the mechanism can be observed in figure 1.3 (Palzkill, 2013).

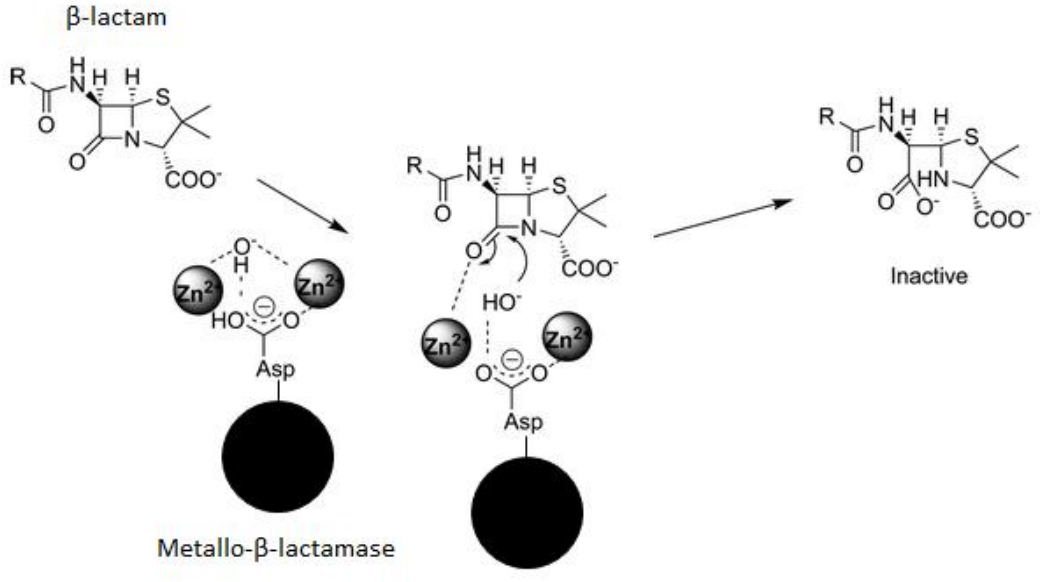

Figure 1.3: Mechanism of β-lactam hydrolysis by metallo-β-lactamases (Wright, 2011).

Group 3 metallo-β-lactamases have the ability to hydrolyse carbapenems, penicillins, and cephalosporins, however they do show low affinity for monobactams such as aztreonam (Queenan and Bush, 2007; Bush and Jacoby, 2010). Another defining feature of the metallo-β-lactamases is that to date there are no clinically viable inhibitors, with clavulanic acid and tazobactam being ineffective against the enzymes (Queenan and Bush, 2007). However, these enzymes are inhibited by metal chelators such as EDTA, dipicolinic acid, and phenanthroline due to their reliance on Zn^{2+} ions (Bush and Jacoby, 2010). It has been suggested that the functional group 3 should be further divided into two subgroups, namely subgroup 3a and 3b, with the molecular group B1 and B3 metallo-β-lactamases being found in functional subgroup 3a and molecular group B2 metallo-β-lactamases being found in functional subgroup 3b (Bush and Jacoby, 2010).

Subgroup 3a is the largest of the subgroups and includes the plasmid-encoded enzymes such as VIM, IMP, and GIM which are observed mostly in non-fermentative

Gram-negative bacteria but also in Enterobacteriaceae (Queenan and bush, 2007; Bush and Jacoby, 2010). These enzymes require two Zn^{2+} ions to be bound to ensure the broad-spectrum activity the metallo-β-lactamases are known for (Bush and Jacoby, 2010). Subgroup 3b is a relatively small subgroup. The main characteristic of this subgroup is that it requires only one Zn^{2+} ion to be bound in order to show maximal activity, and the presence of a second Zn^{2+} ion in fact acts as an enzymatic inhibitor (Bush and Jacoby, 2010).

Group 4 consists of β-lactamases which are poorly studied and as a result their characteristics are poorly understood (Bush and Jacoby, 2010). Thus, group 4 is essentially a placeholder group and its members are not clinically relevant.

1.4.2 Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

Staphylococcus aureus exhibits resistance to β-lactam drugs by two main mechanisms, through the expression of chromosomally encoded β-lactamases and the acquisition of genes encoding for β-lactam-resistant penicillin binding proteins (PBPs) (Llarrull *et al*. 2009). However, the β-lactamases produced by *S*. *aureus* are limited in their spectrum of activity and as a result it is the acquisition of the variant PBPs that confer the greatest resistance of β-lactam drugs in *S*. *aureus* (Bush and Jacoby, 2010; Elhassan *et al.* 2015). MRSA isolates are generally identified by the presence of the *mec* genes encoding for the variant PBP known as variant PBP2α, which has an extremely low affinity for β-lactam antibiotics which then allows for cell wall synthesis to continue even in the presence of high β-lactam antibiotic concentrations (Ba *et al.* 2014). The most common genes encoding for PBP2α variant are the *mec*A and *mec*C genes. These genes are located on the genetic mobile elements, known as staphylococcal cassette chromosome *mec* (SCC*mec*) (Cartwright *et al.* 2013).

1.4.3 Vancomycin Resistance (*van)* Gene Cluster

Glycopeptide resistance was first described in Enterococci in 1988 and glycopeptide-resistant *E. faecalis* and *E. faecium* have been a major cause of HAIs (Hollenbeck and Rice, 2012). Vancomycin functions by binding to the D-ala-D-ala terminal of the peptidoglycan pentapeptide precursor and prevents cross linking of the peptidoglycan subunits which in turn compromises the integrity of the bacterial cell wall (Cetinkaya

et al. 2000; Miller *et al*. 2014). Glycopeptide resistance is conferred and mediated by the vancomycin resistance gene clusters or *van* clusters. The vancomycin resistance genes are located on a transposon, which are often found within a plasmid. The vancomycin resistance gene clusters are made up of the genes *van*R, *van*S, *van*H, *van*X, *van*Z, and a gene encoding for a ligase, which is ultimately responsible for vancomycin resistance (Cetinkaya *et al.* 2000). There are five variants or phenotypes of the ligase gene namely, *van*A, *van*B, *van*C, *van*D, and *van*E, and these gene variants account for the five different phenotypes of VRE (Cetinkaya *et al.* 2000; Hollenbeck and Rice 2012).

1.4.4 Alternative Resistance Mechanisms

Drug efflux is a well understood mechanism of drug resistance, and many bacterial species are able to exhibit some degree of drug efflux. There are five distinct families of efflux pumps, namely resistance-nodulation-cell division (RND), staphylococcal multidrug resistance (SMR), ATP-binding cassette (ABC), major facilitator (MF), and multidrug and toxic compound extrusion (MATE) pumps (Alekshun and Levy, 2007). The efflux pump RND, SMR, MF, and MATE families function by proton motive force and achieve drug efflux through secondary transport, while ATP hydrolysis drives the ABC pumps and their drug efflux is classified as primary transport (Alekshun and Levy, 2007). Efflux pumps can be further divided into two different types. Firstly, there are single component efflux systems which have a narrow spectrum of action, they are generally active against a single antimicrobial or a single class of antimicrobials, such as the tetracycline (*tet*) pumps seen within the *Enterobacteriaceae* (Alekshun and Levy, 2007). Secondly, there are the multicomponent pumps which require additional proteins in order for the pumps to function, such as the RND family pumps (Alekshun and Levy, 2007). Multicomponent efflux pumps have the ability to bind and transport many structurally unrelated antimicrobials and as a result can confer broad spectrum drug resistance (Alekshun and Levy, 2007).

Cell permeability is another potential mechanism by which antibiotic resistance may be achieved. The bacterial cell membrane acts as a barrier to both hydrophobic and hydrophilic molecules and therefore is able to exclude many antibiotics from the cell. However, bacteria produce transmembrane porins, such as OmpF in *E. coli* and OprD in *P. aeruginosa,* which allow non-specific transport of small organic molecules into

the cell, which includes antibiotics (Alekshun and Levy, 2007). Mutation and decreased expression of these membrane porins result in antibiotic resistance (Alekshun and Levy, 2007). Another mechanism related to antibiotic resistance and reduced permeability is the tendency of many bacterial species to form biofilms. This is relevant in many nosocomial pathogens such as *P. aeruginosa* and *K. pneumoniae*. The biofilm polysaccharide matrix prevents antimicrobial agents from reaching the bacterial cells, the antimicrobials cannot perform their mechanisms of action and therefore the bacterial population remains largely unaffected (Mah, 2012).

1.5 ANTIMICROBIAL SUSCEPTIBILITY TESTING

Determination of antimicrobial susceptibility and resistance is a crucial part in determining appropriate therapy options and is therefore a critical step in antimicrobial stewardship (Abbot *et al*. 2013). Knowing both the species of the pathogen responsible for an infection and the antimicrobial susceptibility profile of that particular pathogen can aid treatment tremendously. It allows for the patient to be treated with specific antibiotics, rather than with broad spectrum antibiotics, and at appropriate doses. Not only does this vastly improve patient outcomes but also aids the prevention of antimicrobial resistance development (Doron and Davidson, 2011). Laboratory antimicrobial susceptibility techniques include broth microdilution, agar dilution, and disk diffusion (Jorgensen and Turnidge, 2015). Multiple rapid automated systems are available for antimicrobial susceptibility testing, these include the bioMerieux Vitek system, the Thermo Scientific Sensititre system, and the Becton Dickinson Phoenix system (Karlowsky and Richter, 2015). Antimicrobial susceptibility methods and breakpoints are determined and validated according to standardized guidelines set out by organisations such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) (Kahlmeter, 2014; Jorgensen and Turnidge, 2015). With technological developments, the rapid and accurate detection of antimicrobial resistant infections are becoming increasingly available, and this provides a significant contribution to evidence based treatment and infection control (Reller *et al.* 2009).

1.6 MOLECULAR TYPING

Molecular typing encompasses a number of techniques, which make use of microbes' genotypes or portions of their DNA to distinguish between different strains of the same organism (Olive and Bean, 1999). Bacteria of the same species share common characteristics such as virulence factors, biochemical characteristics, and genomic patterns, however there is enough genetic variance at species level that bacteria of the same species isolated at different times or from different locations can be distinguished from each other and be classified into different strains or types (Olive and Bean, 1999).

Molecular based typing methods have become indispensable in epidemiological studies of bacterial pathogens, as they can be used to assess an outbreak and determine if the causative agents are clonally related or the degree to which the organisms are genetically similar (Olive and Bean, 1999; Ranjbar *et al*. 2014). The major methods of molecular typing used are pulsed-field gel electrophoresis (PFGE) and PCR-based methods, the most commonly used PCR-based methods include multilocus sequence typing (MLST), repetitive extragenic palindromic element (REP)-PCR, PCR restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD) assays (Ranjbar *et al*. 2014), and more recently, whole genome sequencing (WGS).

1.7 SCOPE AND OBJECTIVES

Patients with TB are often transferred from acute care facilities following a period of hospitalisation during which they may have received broad spectrum antibiotics. In addition, they may receive broad spectrum antibiotics such as moxifloxacin, linezolid and meropenem as part of their TB treatment. They are therefore at higher risk of developing multi-drug resistant bacterial colonisation. In a TB hospital, infection control is focused on airborne transmission precautions. There are no screening procedures in place to evaluate the risk of multi-drug resistant bacterial colonisation together with risk factors. These patients are at high risk of spreading multi-drug resistant bacteria if Infection Prevention and Control is not well practiced. They are also at risk of developing invasive infections from multi-drug resistant bacteria.

It has been determined that a major factor in the development of antimicrobial resistance among bacteria is the selective pressure applied by overuse of the antimicrobials in question from the cell (Levy, 2002). Tuberculosis patients, especially patients with advanced and drug resistant infections, are often exposed to high concentrations of powerful antimicrobials, such as isoniazid and rifampicin, as treatment (Alsaad *et al.* 2014). Therefore, this study investigated antimicrobial susceptibility profiles and antimicrobial resistance genes present within HAI pathogens isolated from patients within a TB hospital.

The primary aim of the study is to determine the incidence of bacterial infections and colonisation in patients admitted to a Tuberculosis hospital.

1.7.1 Hypotheses to be tested

It was hypothesized that:

(i) TB patients under high antibiotic stress show high incidence of colonization by antimicrobial resistant bacteria,

(ii) Enterobacteriaceae are responsible for the majority of the HAIs within the TB hospital,

(iii) Carbapenem resistant isolates will exhibit carbapenemase production, such as VIM, and

(iv) Isolates of the same species obtained from patients in the hospital will be genetically similar

1.7.2 Objectives

The following objectives were established to test the above hypotheses:

(i) To assess the antimicrobial prescribing patterns practiced in the hospital by retrospective patient file review,

(ii) To determine the spectrum of bacterial colonisation in patients upon admission and during hospitalisation from nasal, groin and rectal swabs,

(iii) To identify bacterial isolates and evaluate antimicrobial susceptibility profiles by Vitek system and Sensititre assay,

(iv) To detect antimicrobial resistance genes in the bacterial isolates by PCR and DNA sequencing, and

(v) To investigate genetic relatedness of *K. pneumoniae* isolates using MLST.

CHAPTER TWO

ANTIMICROBIAL PRESCRIBING PATTERNS AMONG HOSPITALISED PATIENTS WITH TUBERCULOSIS

2.1 INTRODUCTION

The increase of antimicrobial resistance highlights the need for the development of novel antimicrobial drugs. However, many studies have illustrated that the development of new drugs has been in decline since the end of the of the 20th century (Spellberg *et al.* 2004; Conly and Johnston, 2005; Freire-Moran *et al.* 2011). Since the turn of the millennium, several new drugs have been developed and released for clinical use, such as gemifloxacin, daptomycin, linezolid, and bedaquiline, with the latter three drugs even exhibiting novel mechanisms of action (Conly and Johnston, 2005; Deoghare, 2013). However, these numbers pale in comparison to the explosion of antimicrobial resistance development that was seen in the latter half of the 20th century. The major reason behind the decline of antibiotic drug development is believed to be the cost and time required, as bringing a new drug to the market entails an average cost of US$800 million, and a minimum of 10 years in development and clinical trials (Fishman, 2006; Freire-Moran *et al.* 2011). Additionally, pharmaceutical companies currently display a focus for the development of chronic medication, which are utilized far longer per patient when compared to antibiotics (Freire-Moran *et al.* 2011).

Studies have shown that the incorrect antibiotic and treatment duration are prescribed in 30 to 50% of cases, and a U.S.A. based study showed that only in 7.6% of 17400 community-acquired infection cases, was the responsible pathogen identified (Centers for Disease Control and Prevention, 2013; Luyt *et al.* 2014; Ventola, 2015). Additionally, it is believed that 30 to 60% of the antibiotics prescribed to ICU patients are inappropriate, unnecessary, or suboptimal (Luyt *et al.* 2014; Ventola, 2015). This illustrates how antimicrobial drugs are frequently misused and highlights the need for careful and considerate antibiotic use.

Antimicrobial stewardship (AMS) and infection control are crucial in preventing antimicrobial resistance. Antimicrobial stewardship is defined as the optimal drug selection, dosage level, and duration of antimicrobial treatment, with the main goals of optimal patient treatment and outcomes, and the decrease of antibiotic abuse which leads to the development of antimicrobial resistance (Fishman, 2006; Doron and Davidson, 2011). Infection control goes hand in hand with AMS, reducing the cost associated with the treatment of antibiotic resistant infections, and the risk to patient health and greatly increases the quality of patient outcomes (Khan *et al.* 2017). Additionally, effective infection control programs reduce the need to utilize antibiotics and therefore reduce the antibiotic pressure which is selecting for drug resistant pathogens.

Africa is currently the continent with the least AMS activities by a considerable margin (Huttnera *et al.* 2014). Some AMS activity has been carried out within South Africa, however this is limited primarily to the private Sector, with Netcare Ltd implementing AMS programs within 55 of its hospitals since 2010 to address the threat posed by carbapenemase-producing enterobacteria (Mendelson *et al.* 2012). The implementation of the AMS program suggested a 12.1% reduction in the daily prescriptions per 100 patient-days by the beginning of 2014 (Huttnera *et al.* 2014). There are several successes due to the implementation of AMS programs in order to combat antimicrobial resistance. While most of these have occurred in developed countries, such as the USA, France, Scotland, Australia, and Sweden, AMS programs have shown to be effective in developing countries, such as Taiwan and Vietnam (Huttnera *et al.* 2014). In 2015 “A Pocket Guide to Antibiotic Prescribing for Adults in South Africa” outlined by Wasserman and colleagues was published by the South African Antibiotic Stewardship Programme (SAASP). This guide outlines the principles of rational antibiotic prescribing behaviours and outlines how to manage many different situations were antimicrobial treatment is required (Wasserman *et al.* 2015).

Several studies have been conducted in South Africa in order to demonstrate the antimicrobial prescribing patterns and behaviours present throughout the different medical environments within the country. Analysis of prescribing antimicrobials patterns among 83 655 patients from nine primary healthcare facilities indicated that antimicrobials were prescribed in 72% of the cases, with penicillin drugs accounting

for 38.17% of the prescriptions while sulphonamides, antiprotozoals, and tetracyclines were prescribed 22.49%, 9.88%, and 9.34% respectively (Katende-Kyenda *et al.* 2007). It was revealed that in a significant amount of cases that antimicrobials were prescribed in inappropriate situations where patients did not require antibiotics.

Tuberculosis remains a major public health problem worldwide. It is estimated that one third of the world population is colonized by *Mycobacterium tuberculosis (*Sandhu, 2011). South Africans make up less than one percent of the total world population, however we account for approximately 17% and 25% of the recorded worldwide cases of HIV and HIV-TB infection, respectively (Abdool Karim *et al.* 2009; Wood *et al.* 2010). In South Africa 50% of new TB cases have been determined to be associated with HIV co-infection (Abdool Karim *et al.* 2009). If TB patients are immunocompromised this would cause elevated risk towards the contraction of community and healthcare-acquired infections (HAIs). RR-TB is defined as TB that shows monoresistance resistance to rifampicin, MDR-TB is defined as TB resistant to at least two first-line antimycobacterial drugs such as isoniazid and rifampicin, and XDR-TB is defined as TB resistant to isoniazid, rifampicin, one fluoroquinolone, and one or more of the second-line drugs (amikacin, kanamycin, or capreomycin) (Centers for Disease Control and Prevention, 2016). Understanding the type of TB infection present within a patient is extremely important as it can greatly affect the antimicrobial treatment prescribed to that patient, as demonstrated within the following results section.

The focus of this chapter was to assess the antimicrobial prescribing patterns within a specialized treatment centre for patients with drug resistant TB.

2.2 MATERIALS AND METHODS

A retrospective, quantitative study was conducted at Jose Pearson TB hospital (a treatment centre for patients with drug resistant TB) in the Eastern Cape, using patient medication records. The hospital had the capacity to accommodate 230 patients at any given time. A modified data collection tool (Appendix A) based on the design of a previous study by (Chunnilall *et al.* 2015), was used to review the antibiotic prescribing patterns.

Patient records (*n*=50) were randomly selected and evaluated using the data collection tool to record relevant data such as patient demographics (gender, age), diagnosis, and antimicrobials prescribed, treatment duration, type of TB infection (MDR, HIV status, and co-morbidities) was collected. All types of prescribed antimicrobials were recorded, this included antibacterial, antifungal, and antiviral. In addition, the list of prescriptions were also noted whether systemic, topical, prophylactic, or standard therapy. The inclusion criteria were patients (n=50) admitted to Jose Pearson TB hospital between August to December 2016. Patients had to be hospitalized for three months or longer and be older than 18 years of age to be included in the study.

Ethical approval was obtained from the Research Ethics Committee (Human) at Nelson Mandela University (NMU) (Reference number: H15-HEA-PHA-017) and the University of Cape Town (UCT) (HREC REF: 573/206), and permission from both the Eastern Cape Department of Health and CEO of Jose Pearson TB Hospital were obtained to access patient records (Appendix 1).

2.3 RESULTS

2.3.1 Patient Demographic and Clinical Information

Retrospective analysis of patient files indicated that the cohort comprised 50% (*n*=25) males and 50% (*n*=25) females respectively. The youngest patient was 16 and the oldest 59 years old [Fig. 2.1 (A)]. The majority of patients were between 20 and 49 years (88%; 44/50). Only two patients were <20 years of age, while four were >50 years of age. The median age was 35, while the average age was 36 years. Three different degrees of TB infections were identified [Fig. 2.1 (B)]: rifampicin-resistant TB (RR-TB), multidrug-resistant TB (MDR-TB), and extensively drug-resistant TB (XDR-TB). MDR-TB [54% (*n*=27)] was the highest in the cohort of patients, followed by XDR-TB [34% (*n*=17)] and RR-TB [12% (*n*=6)].

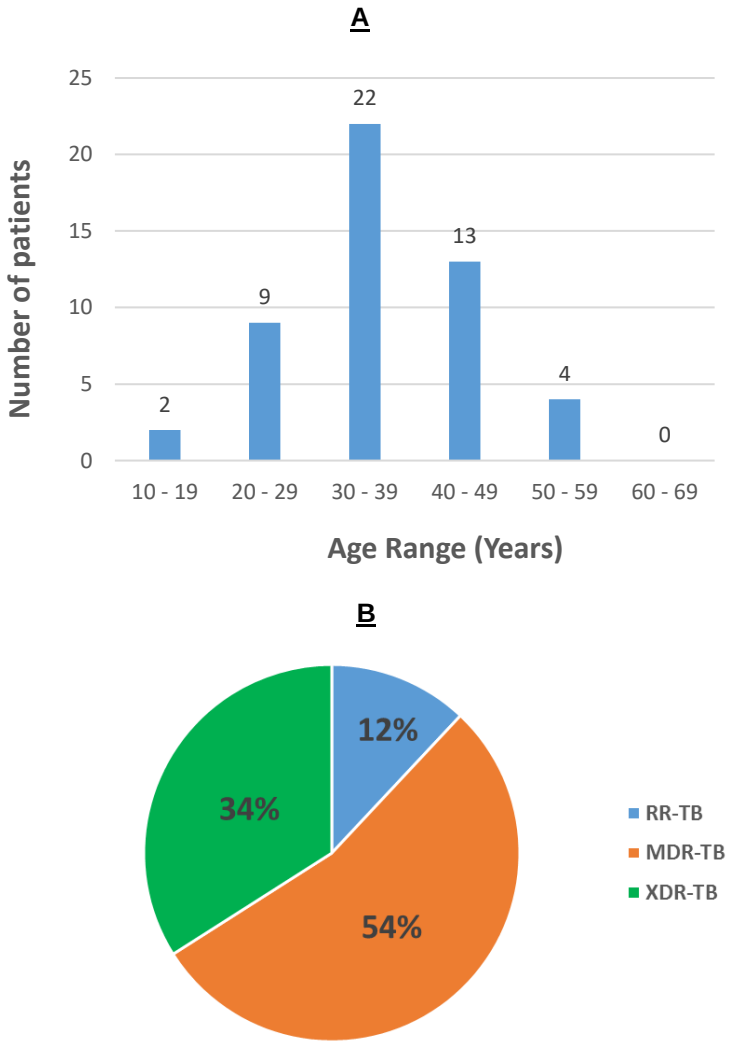

Figure 2.1: Distribution of patients (*n*=50) screened for antimicrobial prescribing patterns. (A): Age groups, (B): Types of TB infections.

RR-TB - rifampicin-resistant TB, MDR-TB - multidrug-resistant TB, and XDR-TB: extensively drug-resistant TB.

All patients admitted at Jose Pearson TB hospital undergo HIV testing, as HIV status can significantly affect the patient treatment. Majority of patients were HIV-positive [84% (*n*=42)], and 8 patients were HIV-negative (Fig. 2.2A). Other co-morbidities among patients included hypertension, diabetes, hospital-acquired infections with *K. pneumoniae*, and *Candida* spp. (oral and vaginal). Comparison of the HIV status of patients with the type of TB (Fig. 2.2B) showed that 16 patients with XDR-TB and all patients with RR-TB (*n*=6) were HIV-positive, with one HIV-negative in the XDR-TB group. The MDR-TB patients (*n*=27) showed diversity in HIV status, with 74% (20/27) of patients being HIV-positive and 26% (7/27) being HIV-negative.

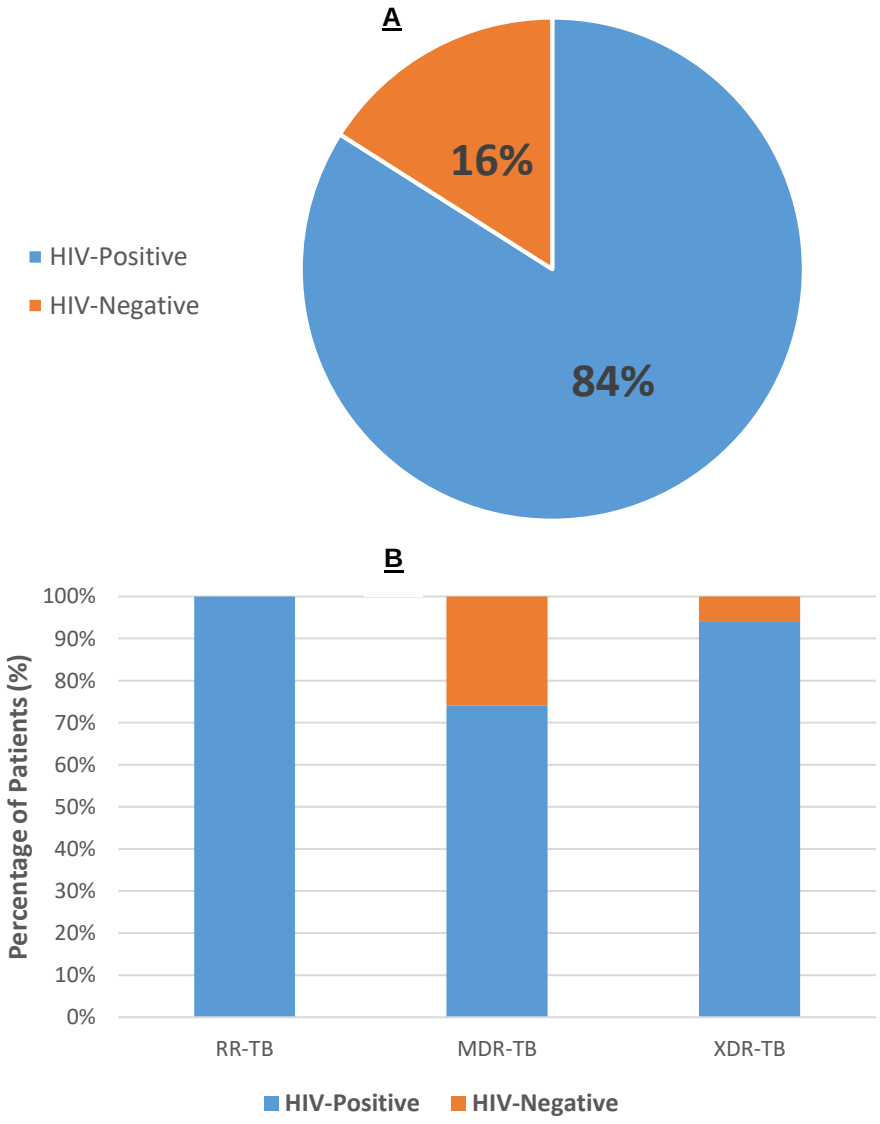

Figure 2.2: (A): HIV-status among the patients (*n*=50) screened for antimicrobial prescribing patterns. (B): HIV infection and type of drug resistant TB (p = 0.168).

RR-TB - rifampicin-resistant TB, MDR-TB - multidrug-resistant TB, and XDR-TB: extensively drug-resistant TB.

2.3.2 Antimicrobial Prescribing Patterns

From the patient records, the antimicrobial prescriptions and treatments of 50 patients during their residence at Jose Pearson TB hospital were collected and reported. The antimicrobial prescriptions reports considered the antimycobacterial drug regimen, as well as any other antimicrobial prescriptions, which included any antibacterial, antifungal, and antiviral drugs that were additionally prescribed by physicians. Prophylaxis was also considered. Both the median and mean number of antibacterial drug prescriptions among patients was 8, with patients individually being prescribed between 6 and 11 antimicrobials (excluding antivirals) at any one time during their hospitalization.

2.3.2.1 Antimycobacterial drugs

In total, there were 14 different antimycobacterial drugs prescribed to patients (Fig. 2.3). The specific drugs prescribed to patients differed greatly depending on the type of TB exhibited by that patient, as illustrated in Fig 2.3. The RR-TB patients (*n*=6) were mainly treated with moxifloxacin (66.7%; 4/6), linezolid (66.7%; 4/6), ethionamide (83.3%; 5/6), isoniazid (83.3%; 5/6), and terizidone (100%; 6/6). The only antimycobacterial that was not applied as treatment among the RR-TB patients was delamanid. The MDR-TB patients (*n*=27) received a broad range of drugs, as 13 antimycobacterial drugs were prescribed within this group. The major antimycobacterials used to treat the MDR-TB patients were pyrazinamide (100%; 27/27), terizidone (100%; 27/27), levofloxacin (88.9%; 24/27), bedaquiline (85.2%; 23/27), ethambutol (81.5%; 22/31), and linezolid (66.7%; 18/27). The XDR-TB patients had a smaller range of antimycobacterials available due to the highly antibiotic resistant nature of XDR-TB infections. Amikacin, ethionamide, and isoniazid were not prescribed in a single XDR-TB case. One XDR-TB patient (5.9%; 1/17) received a combination of moxifloxacin and delamanid, however no other XDR-TB patient received either of these drugs. Bedaquiline, clofazimine, linezolid, and aminosalicylic acid were prescribed among 94.1% (16/17) of XDR-patients.

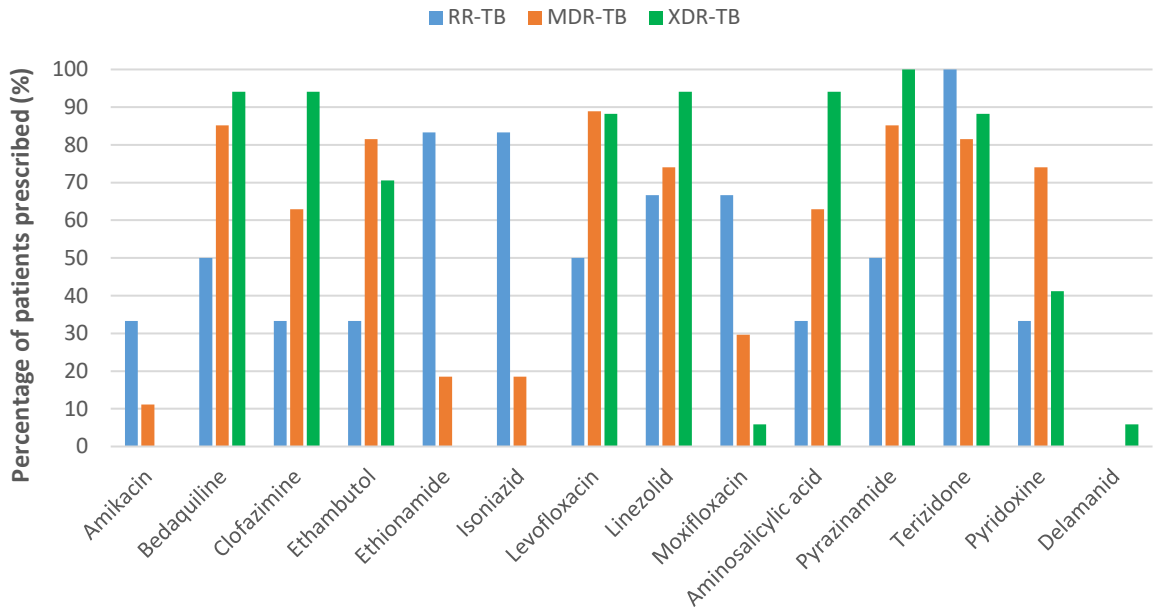

Figure 2.3: Prescription rates of antimycobacterials among patients (*n*=50) screened for antimicrobial prescribing patterns based on the types of TB ($p < 0.001$).

2.3.2.2 Other antibacterial drugs

Nine additional antibacterial drugs were used among the 50 screened patients. These drugs were prescribed for reasons other than the treatment of TB infection (Fig 2.4). The most prescribed antibacterial was cotrimoxazole (trimethoprim-sulfamethoxazole). This drug was prescribed among 36% (18/50) of the patients. The second most prescribed antibacterial was rifabutin, with 10% (5/50) of patients being prescribed this drug. While rifabutin is an antimycobacterial, it is often prescribed as prophylaxis to prevent the atypical mycobacterial infection *Mycobacterium avium* complex (MAC) in immunocompromised patients. The remaining five antibacterial drugs included carbapenem, penicillin, fluoroquinolone, macrolide, tetracycline, and cephalosporin class antibiotics. Ertapenem and amoxicillin were both prescribed twice. The remaining antibacterial drugs were only prescribed once respectively, each in separate patient cases.

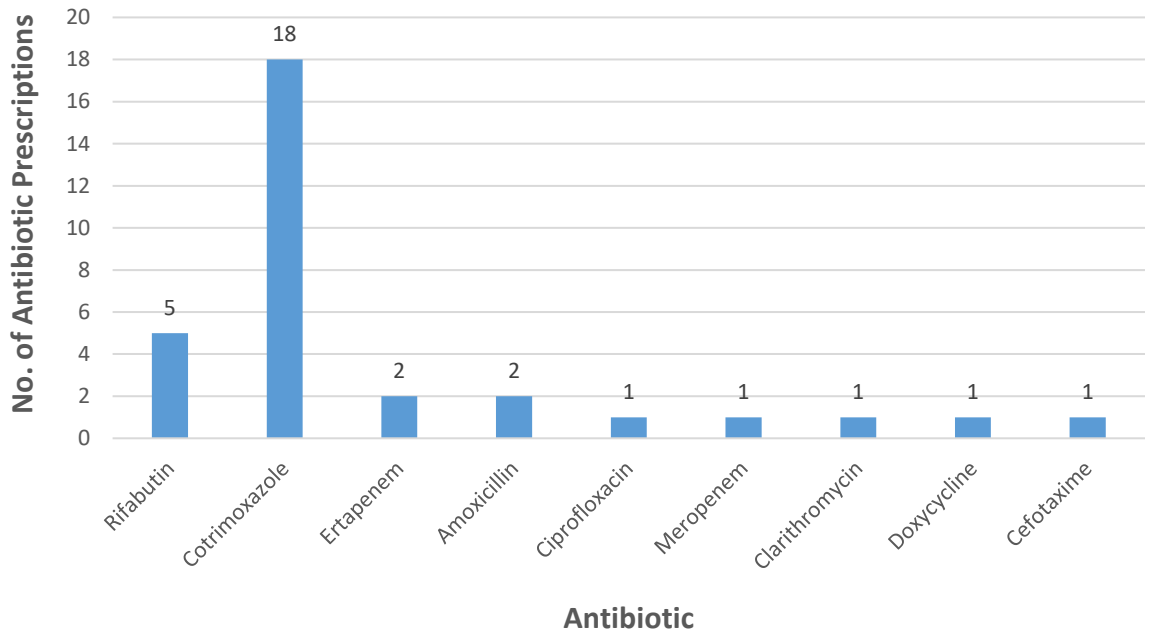

Figure 2.4: Summary of prescriptions of non-tuberculosis antibacterial drugs (*n*=32) among the patients (*n*=50) screened for antimicrobial prescribing patterns.

2.3.2.3 Antifungal drugs

Antifungal drugs were the least prescribed of the antimicrobials with only 13 prescriptions among 11 of the 50 patients (Fig. 2.5).

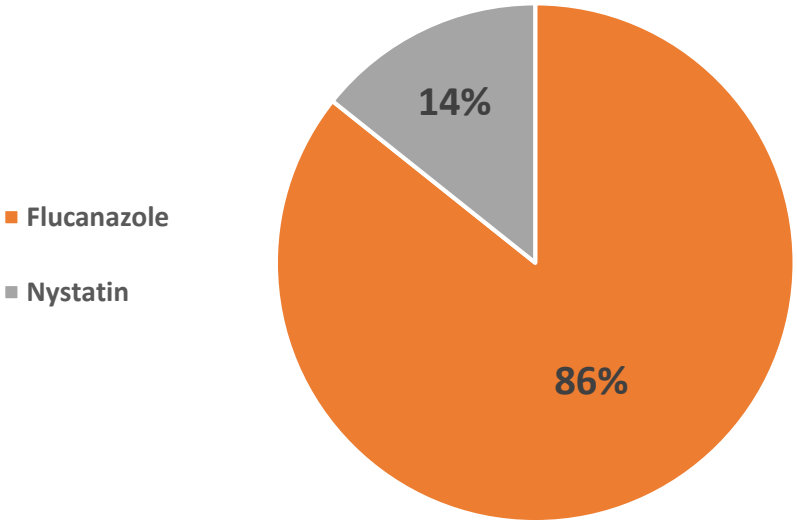

Figure 2.5: Antifungal drug prescriptions (*n*=7) among the patients (*n*=50) screened for antimicrobial prescribing patterns.

Among the antifungals fluconazole was the most used antifungal. Fluconazole accounted for 86% (6/7) of the antifungal prescriptions. Nystatin was prescribed once, accounting for 14% of the total antifungal prescriptions.

2.3.2.4 Antiviral drugs

Antivirals were only prescribed among the HIV patients (*n*=42). However, the antiviral treatments for three patients could not be recovered, therefore a total of 39 antiviral prescription records were analysed. All the drugs shown in Fig 2.6 are antiretroviral (ARV) drugs, primarily used to treat HIV, with the one exception being aciclovir which is primarily used to treat the varicella-zoster and herpes simplex viruses. In total, there were 122 antiviral drug prescriptions among 39 patients. Most patients (66.7%; 26/39) were treated with three different antivirals, patients who were treated with two and four antiviral drugs both made up 15.4% of patients (6/39) respectively. The antiviral that was prescribed the most was lamivudine which was prescribed to 87.2% (34/39) of patients. Second was nevirapine followed by tenofovir with 69.2% (27/39) and 56.4% (22/39) prescription rates respectively.

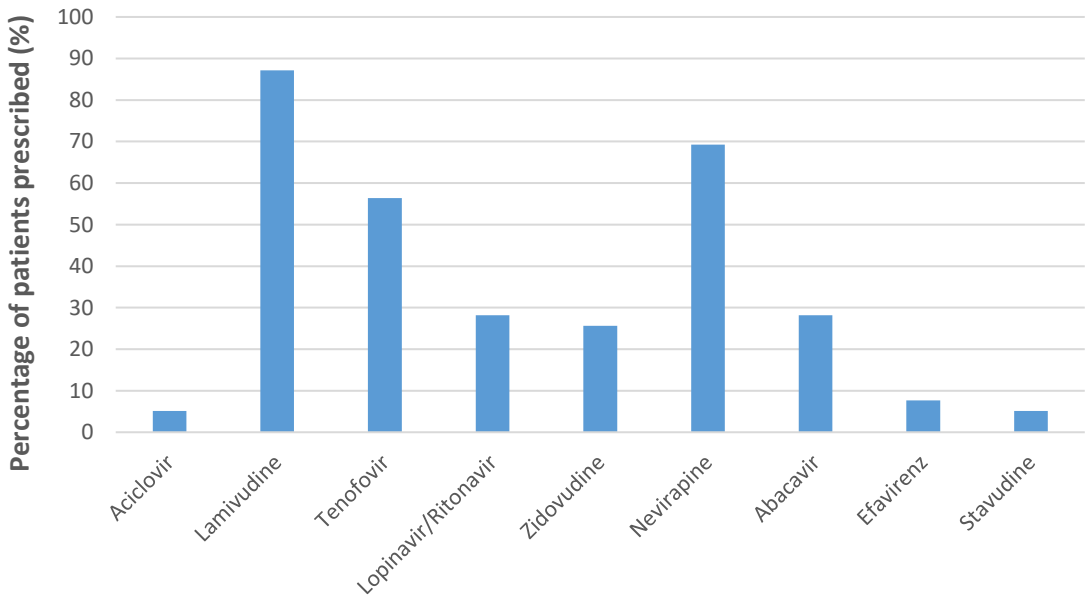

Figure 2.6: Antiviral drug prescriptions among HIV patients (*n*=39) screened for antimicrobial prescribing patterns.

2.4 DISCUSSION

Antimicrobial resistance development among pathogens associated with hospital-acquired infections (HAIs) is currently one of the major public health concerns worldwide. The misuse and irresponsible use of antibiotics are the major driving forces behind this phenomenon. It is our concern that patients receiving treatment at a tuberculosis hospital may be at high risk of acquiring HAIs. This is exacerbated by the treatment regimens applied to TB patients, which are known to consist of highly concentrated and broad-spectrum antibiotics. In addition, many TB patients undergo prophylactic treatment to prevent other infections. These antimicrobial prescribing practices may result in an environment of extremely high antibiotic selection pressure which may lead to the development of highly drug resistant nosocomial pathogens within the hospital. This study provides insight into the antimicrobial prescribing patterns within Jose Pearson TB hospital.

There was an equal distribution of female and male patients among the cohort of files analysed and it would appear that this observation is consistent with the numbers admitted to Jose Pearson. With regard to age, most patients were in the 30 - 49 years age category. Furthermore, a study that compared the TB incidence rates among patients from Europe, Asia, and Somalia, found that there was a higher incidence of TB in younger patients within Europe, while patients from Asia and Somalia both exhibited a much higher incidence of TB within patients 25 – 64 years of age (Zhang *et al.* 2011).

The majority of patients were HIV positive. This is consistent with what is known for TB patients in South Africa (Bekker and Wood, 2011). However, 84% of patients is higher than what is generally observed among TB patients. As of 2012 12.2% of South Africa's population and 11.6% of the Eastern Cape's population were HIV positive (Shisana *et al.* 2012). The HIV incidence rate among the analysed cohort of patients from Jose Pearson was 84%. This is not surprising as HIV or immune system suppression are known to be the primary risk factors in the development of TB infection (Naidoo *et al*. 2011; Pawlowski *et al.* 2012).

Jose Pearson is a hospital for the treatment of patients with drug resistant TB. The patients are separated into three categories, namely rifampicin-resistant TB (RR-TB),

multidrug-resistant TB (MDR-TB), and extensively drug-resistant TB (XDR-TB). RR-TB is the least drug resistant of the infections and XDR is the most resistant. In 2012 15 419 and 1 596 cases of MDR-TB and XDR-TB were diagnosed respectively in South Africa, a ratio of almost 10:1 (Churchyard *et al.* 2014). At Jose Pearson over half (54%; 27/50) of the patients were MDR-TB patients, however one third (34%; 17/50) of the admitted patients were infected with XDR-TB. This is highly concerning as this is a ratio of 1.5:1 and this may indicate a growing incidence of XDR-TB cases over the past five years. However, prospective studies would be required to determine whether the number of XDR-TB cases have been consistently on the rise. The high incidence of XDR-TB patients at this hospital would suggest higher prescription rates of powerful and last-line antibiotics. This would result in exposure of patients at Jose Pearson to unusually high antibiotic pressure.

A wide variety of antimycobacterial drugs were prescribed to patients depending on the type of TB with which they were infected. Of the 14 antimycobacterials, five drugs were prescribed consistently among all patients, these were terizidone, pyrazinamide, linezolid, levofloxacin, and bedaquiline. Interestingly, there was quite a diversity of prescribed antibiotics among the RR-TB and MDR-TB patients compared to the XDR-TB patients. Among the XDR-TB patients there was lower diversity of prescribed antibiotics classes.

While many of the antibiotics prescribed to the patients are specific for the treatment of TB, such as bedaquiline, clofazimine, ethambutol, ethionamide Isoniazid, aminosalicylic acid, pyrazinamide, terizidone, and delamanid, it is unclear if these antibiotics have any influence on other species of nosocomial pathogens. Very limited literature is available on the specific biochemical and genetic effects that antimycobacterial drugs have on other bacterial species, however the incorrect prescription of drugs is a known factor in the development of antimicrobial resistance (Ventola, 2015). It is known that subtherapeutic and subinhibitory concentrations of prescribed antibiotics can promote the development of antibiotic resistance by inducing increased gene expression and promoting mechanisms such as horizontal gene transfer and mutagenesis (Viswanathan, 2014). It is suggested that in the presence of non-specific and subtherapeutic levels of antibiotics that the bacterial general stress response, mediated by the alternate sigma factor RpoS, is triggered

and results in the stabilization of the error-prone DNA polymerase PolIV and a decrease in MutS-dependent DNA mismatch repair (Viswanathan, 2014). This mechanism may account for the rapid development of mutations which result in antibiotic resistance, such as decreased drug influx due to mutations in genes encoding for bacterial membrane porins.

Linezolid is an interesting antibiotic which exhibits a broad spectrum of activity among the Gram-positive bacteria, and is often the treatment of choice against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and can be included in the treatment regimen for MDR-TB (Livermore, 2003; Maartensa and Benson, 2015). While resistance towards linezolid is rare, it has been observed among clinical MRSA and VRE isolates (Quiles-Melero *et al.* 2013; de Almeida *et al.* 2014). This is concerning as linezolid is a last-line antibiotic used to treat multidrug-resistant infections. It was observed that the prescription rate of linezolid among Jose Pearson patients was extremely high, but it is unclear to what effect the high utilization of linezolid would have on other colonizing Gram-positive pathogens.

Amikacin was prescribed among 10% of the TB patients at Jose Pearson and in many settings where resources are limited, such as developing countries, aminoglycoside inclusive MDR-TB treatment regimens are the standard practice (Modongo *et al.* 2014). It has long been demonstrated that extended and unrestricted use of aminoglycosides, such as gentamycin and amikacin, leads to the development of aminoglycoside resistance, especially among Gram-negative species like *K. pneumoniae* (Young *et al.* 1985). While many of the antibiotics used as treatment for TB are antimycobacterial-specific, such as bedaquiline, aminoglycosides are concerning as they are often used to treat infections caused by other MDR pathogens. Therefore, if patients are routinely, treated with amikacin or other aminoglycosides, the selection pressure will potentially promote patient colonization by aminoglycoside-resistant bacteria.

The fluoroquinolones: levofloxacin and moxifloxacin were prescribed to the majority of the patients as part of their routine TB therapy. The fluoroquinolones are broad spectrum antibiotics and are primarily used in the treatment of Gram-negative infections, however later generations include activity against some Gram-positive

pathogens (Oliphant and Green, 2002). Levofloxacin and moxifloxacin have long been used in the treatment of TB and studies have shown the possibility of fluoroquinolone inclusive regimens to reduce the necessary treatment duration of drug-susceptible TB infections (Thee *et al.* 2015). It may be ill advised to use fluoroquinolones, especially older generations, as substitutes in place of other antimycobacterial drugs in MDR-TB patients (Moadebi *et al.* 2007; Ziganshina *et al*. 2013; Thee *et al.* 2015). However, treatment of MDR-TB can be a complicated affair and often drug toxicity can be an issue with standard MDR-TB treatment regimens (Gillespie, 2016). Therefore, the fluoroquinolones are very useful in the treatment of patients unable to tolerate the standard regimen (Gillespie, 2016). Among the patients at Jose Pearson moxifloxacin was mainly prescribed among RR-TB patients, and it was prescribed to several MDR-TB patients and one XDR-TB patient. On the other hand, levofloxacin was one of the most prescribed drugs among MDR and XDR-TB patients, and it was also prescribed to half of the RR-TB patients.

Resistance to fluoroquinolones is becoming a world-wide problem and prescribing guidelines generally recommend the reservation of fluoroquinolone use, however resistance rates to this class of antibiotics is still on the rise (Redgrave *et al.* 2014). The high rate of fluoroquinolone prescription among TB patients at Jose Pearson is concerning as there does appears to be little benefit to the use of fluoroquinolones over conventional antimycobacterial drugs (Moadebi *et al*. 2007; Thee *et al*. 2015). Additionally, the high utilization of fluoroquinolones is likely contributing to a high antibiotic pressure environment and potentially selecting for other antibiotic resistant strains of bacteria within the TB patients. However, as previously stated, fluoroquinolones are often required for MDR-TB cases due to toxicity issues and few other alternative options are available. The acquisition of fluoroquinolone resistance has long been associated with the use of fluoroquinolones, and resistance to fluoroquinolones is often accompanied with a multidrug-resistant profile among bacterial isolates (Pallecchi *et al.* 2012). Increased expression of efflux pumps is often observed in isolates exposed to fluoroquinolones and is known to decrease fluoroquinolone sensitivity (Redgrave *et al.* 2014). Increased drug efflux from exposure to fluoroquinolones may also account for the decreased sensitivity to other antibiotics. The most common mechanism of high level fluoroquinolone resistance is mutation within the quinolone resistance-determining region (QRDR) of the type II

topoisomerase genes (*gyr*A, *gyr*B, *par*C, and *par*E) (Redgrave *et al.* 2014). However, there are a various number of transferable or acquired mechanisms accounting for fluoroquinolone resistance, the most common being plasmid-mediated quinolone resistance (PMQR) genes, with the *qnr* genes being the archetypal examples (Deng *et al.* 2015). Furthermore, β-lactamase genes, such as KPC, TEM, and CTX-M, are highly associated with *qnr*-encoding plasmids and therefore with the dissemination of fluoroquinolone resistance, comes β-lactam resistance (Richter *et al.* 2010; Jacoby *et al.* 2014). Additionally, *qnr* genes are often incorporated into integrons which carry sulfonamide and aminoglycoside resistance genes (Jacoby *et al.* 2014; Deng *et al.* 2015). This again illustrates how overuse of one antibiotic class can result in resistance to many.

The study revealed that antibiotics were the most common accounting for 68.1% of the prescriptions, followed by antifungals at 27.7%, and antivirals at 4.3%. Cotrimoxazole, was the most prescribed non-antimycobacterial antibiotic among the Jose Pearson patients, fluconazole was the major antifungal drug prescribed, and the only non-antiretroviral treatment prescribed was aciclovir which was prescribed in two cases. Fluconazole and aciclovir are outlined as the primary antifungal and antiviral drug to be prescribed as treatment for HAIs within the “Standard treatment guidelines and essential medicines list for South Africa” (South African Department of Health, 2015). While the Standard treatment guidelines do promote antimicrobial stewardship for treating HAIs it has limited information treating MDR-HAIs.

Trimethoprim-sulfamethoxazole is used as a prophylaxis treatment to prevent the development of *Pneumocystis jiroveci* pneumonia (PJP) co-infection among TB patients, and as a result significantly reduces morbidity and mortality among TB patients (UNAIDS/WHO, 2000). The need for prophylaxis is further exacerbated with the high incidence of HIV. It was common practice for Jose Pearson patients treated with trimethoprim-sulfamethoxazole prophylaxis to receive this antibiotic daily for a month or longer. In some cases, patients were treated with trimethoprim-sulfamethoxazole daily for the length of their entire hospitalization. Due to the antibiotic resistance selection effect trimethoprim-sulfamethoxazole can have on other species of bacteria, such as *Escherichia coli*, it may be wise to consider other prophylactic treatments, such as pentamidine and other “second-line” alternatives, as the

preventative treatment for PJP among HIV and TB patients (Castro and Morrison-Bryant, 2010; Egwuatu *et al.* 2016). However, many of the alternative forms of treatment are not readily available nor practical. Therefore, it may be wise to routinely review the patient's need for prophylaxis treatment, especially if the patient is on antiretroviral treatment.

A 2016 Lagos-based study conducted by Egwuatu *et al.* assessed the effect of everyday trimethoprim-sulfamethoxazole prophylaxis on the antibiotic resistance and carriage rates of *Escherichia coli* among HIV patients over a twelve-month period. It was determined that 54% of patients were colonized with *E. coli* at the start of the prophylaxis treatment while 46% were not, however by the third month 84% of patients were colonized and by the ninth month, 99% of patients were colonized by *E. coli* (Egwuatu *et al.* 2016).

A 2017 study conducted by Ncube e*t al.* analysed 166 821 insurance reports from 2013 where patients were treated for acute bronchitis. The etiological agents included *Mycoplasma pneumoniae*, *Haemophilus influenza*, *Streptococcus* spp., and five different viruses, though in 86.6% of patients the causative agent was not identified (Ncube e*t al.* 2017). In 52.9 % of the cases antimicrobials were prescribed and prescription rates did not differ between bacterial, viral, or unspecified bronchitis patients (Ncube e*t al.* 2017). It was observed that β-lactam antibiotics accounted for 71% of the antimicrobial prescriptions, followed by macrolides and tetracyclines (11%), quinolones (4.8%), and sulphonamides (1.5%) (Ncube e*t al.* 2017). In both studies, published 10 years apart, we see the high utilization of β-lactam drugs as well as the disregard for the pathogen of consequence in the application and prescription of medication, in the South African Health Sector. Unfortunately, very little data is available regarding the antimicrobial prescribing patterns within public South African health sector. This indicates a need for a greater understanding of the antimicrobial prescription patterns throughout both the public and private healthcare sectors in South Africa.

Among the other prescribed antibiotics, the most common type of antibiotic seen was the β-lactam class, with amoxicillin, cefotaxime, ertapenem, and meropenem, prescribed in 10 percent of the patients. It would appear that a careful attitude is taken

towards the prescribing of additional antimicrobial drugs by the physicians of Jose Pearson TB hospital as there was a fairly limited amount of additional antimicrobial prescriptions. Carbapenems prescribed in several cases, this suggests two possibilities. Firstly, that the physicians are aware that the infections they are treating are highly antibiotic resistant, and prescribe last-line carbapenem antibiotics. Secondly, that they are prescribing carbapenems before identification of the etiological agent of the infection. Either this implies that multidrug resistant bacteria (excluding *M. tuberculosis*) are already present and causing infection within Jose Pearson TB hospital, or physicians are misusing carbapenem antibiotics and contributing to a high antibiotic pressure environment. Both outcomes are worrying and undesirable. While meropenem is sometimes used as part of XDR-TB treatment, in this case it was not indicated that meropenem was prescribed for the treatment of the patients TB infection.

Consultation with the healthcare professionals at Jose Pearson TB hospital, revealed that there are guidelines set out for the usage of antimicrobials in public sector hospitals within South Africa, as outlined in “Standard treatment guidelines and essential medicines list for South Africa” (2015). However, there is no formal or standardized antimicrobial stewardship (AMS) or de-escalation program outlined for the use by hospital staff, and all antimicrobial prescriptions in the hospital are at the discretion of the physicians on duty. This is concerning as we have outlined that there is already a wide variety and number of antimicrobials being utilized as part of the standard TB treatment at Jose Pearson, and it would be imperative to have an AMS analysis and procedure to minimize unnecessary use of antimicrobials.

The long exposure time to broad spectrum antimicrobials no doubt has an adverse effect and greatly disrupts the healthy gut microbiota. This will not only increase the susceptibility of patients towards colonization and infection by opportunistic pathogens, but may also have an adverse effect on the immune systems of the TB patients. The next chapter explores the spectrum of bacterial colonisation in TB patients upon admission and during hospitalisation.

CHAPTER THREE

SPECTRUM OF BACTERIAL COLONISATION IN HOSPITALISED TUBERCULOSIS PATIENTS

3.1 INTRODUCTION

Hospital-acquired infections are responsible for approximately 4% of morbidity and mortality in all hospitalized patients (Sligl *et al*. 2015). Hospitals and healthcare facilities differ in their needs and challenges because of their specific purposes and the types of patients they treat. For example, a surgical practice is expected to experience increased pressure from different etiological agents compared to a tuberculosis clinic, and therefore the infection control policies should differ in ways which reflect this. However, there are general practices that must be maintained throughout all conditions. These general practices include adequate hand hygiene, the use of protective apparel (facial protection, gowns, and gloves), respiratory hygiene and cough etiquette, effective environmental cleaning and cleanliness, and appropriate waste disposal (World Health Organization. 2006). In larger general hospitals or healthcare-facilities different infection control protocols may differ throughout different wards or departments, however the general infection control policies must always be maintained.

In this study, the chosen sample site was a specialised tuberculosis (TB) hospital. The major concern was the treatment of MDR TB patients and prevention of further spread of nosocomial TB amongst patients and healthcare professionals at the hospital. In TB hospitals infection control is geared more towards airborne spread and other transmission routes, such as contact and vehicle transmission, may be neglected (Dusé. 2005). With this in mind, and coupled with the extremely high antibiotic pressure present amongst TB patients it may be that TB hospitals are potentially a critical location in the development and spread of multidrug resistant bacteria pathogens.

It is well known that other underlying conditions can greatly increase a patient's susceptibility to nosocomial infections, especially to that of multidrug resistant Gram-

negative bacilli, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Zhao *et al*. 2015). However, information regarding the colonization of TB patients by other nosocomial pathogens is extensively limited. This may be due to low incidence of colonization due to the potent antibiotics that are prescribed to these patients or the bias towards TB based studies amongst these patients. However, with explosion of antimicrobial resistance amongst bacteria, many TB patients may now be at increased risk from nosocomial etiological agents.

The Gram-negative bacilli are a large group of organisms which account for a large portion of HAIs. Gram-negative organisms are highly concerning as they are proficient at up-regulating, sharing, and acquiring antibiotic resistance genes, especially under antibiotic selection pressure (Peleg and Hooper. 2010). Major Gram-negative bacilli pathogens include: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Peleg and Hooper, 2010). The conditions most associated with the Gram-negative pathogens are pneumonia, gastrointestinal infectious, and bacteraemia (Peleg and Hooper, 2010; Sligl *et al*. 2015). Pneumonia caused by Gram-negative bacteria is a threat to TB patients as they are already immunocompromised by TB infection. In addition, many TB patients are HIV positive, and at risk for gastrointestinal and blood-stream infections (Bekker and Wood, 2011).

Surveillance of antimicrobial resistance patterns throughout the South African population has indicated an increasing trend of resistance. The growing concern of antimicrobial resistance was highlighted in 2011 when *K. pneumoniae* bacteria was isolated exhibiting resistance to all available clinical antibiotics (SA-DOH, 2015). Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococci* (VRE), and ESBL and carbapenemase-producing Gram-negative bacteria, which include the *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Actinobacteria baumannii* have been reported in South Africa and globally (Bamford *et al.* 2011; Naidoo *et al.* 2013).

Antimicrobial resistance surveillance data taken from 16 sentinel sites in SA revealed 880 clinical *Enterobacteriaceae* isolates, with 15 different species, identified as being carbapenemase producers (Perovic and Chetty, 2016). Antimicrobial resistant Gram-negative pathogens have been extensively reported amongst clinical isolates from

healthcare facilities in Port Elizabeth, South Africa (Brink *et al.* 2012; Gqunta, 2014; Masunda, 2014; Govender *et al.* 2015; Annear *et al.* 2017). These reports include ESBL and carbapenemase-producing *Enterobacteriaceae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. In many of these cases patient outcomes were not favourable (Annear, 2015).

Worldwide, *S. aureus* is considered the number one cause of hospital acquired infections, with a large proportion of these infections being MRSA (Stefani *et al.* 2012). It is estimated that the worldwide mortality rate of invasive MRSA infections is approximately 20%, however this number may fluctuate based on the specific environment and population (Stefani *et al.* 2012; Fortuin-de Smidt *et al.* 2015). In a South African antimicrobial susceptibility study performed in 2010, it was realised that between 30 - 60% of *S. aureus* isolates obtained from the blood cultures of patients from public hospitals were MRSA (Bamford *et al.* 2011). From September 2012 to September 2013, MRSA rates in four South African hospitals ranged between 24 and 58%, and accounted for 36% of the total cases of bacteraemia (Fortuin-de Smidt *et al.* 2015). Several clinical MRSA isolates have been reported within both public and private hospitals in Port Elizabeth (Marais *et al.* 2009; Moodley *et al.* 2010). MRSA isolates in Port Elizabeth have been observed carrying the Type X SSC*mec* cassette chromosome (Moodley *et al.* 2010).

The aim of this chapter was to determine the spectrum of bacterial colonisation of TB patients upon admission and while receiving treatment at Jose Pearson TB hospital.

3.2 MATERIALS AND METHODS

3.2.1 Study Site and Patient Population

This study was a prospective cohort study of patients admitted (during the period of August to December 2016) to the public-sector Jose Pearson TB hospital in the Eastern Cape. Ethical approval was obtained from the Research Ethics Committee (Human) at Nelson Mandela University (NMU) (Appendix 1 – Reference number: H15-HEA-PHA-017) and the Human Research Ethics Committee at the University of Cape Town (UCT) (Appendix 1 – Reference number: HREC REF: 573/2016), and permission from both the Eastern Cape Department of Health and CEO of Jose Pearson TB Hospital were obtained. Patients had to be 18 years or older and diagnosed with drug-resistant TB to be included in the study. Upon admission, patients were approached by Jose Pearson healthcare professionals about participation within the study. Signed informed consent was given by all participating patients. All patients admitted from acute care facilities were included together with selected patients admitted directly from the community. Patients admitted directly from the community to the Tuberculosis (TB) hospital were labelled as “Community patients” while patients admitted to the TB directly from another healthcare facility were labelled as “Healthcare patients”.

3.2.2 Base Data and Clinical Isolate Collection

Patient demographic information was recorded at baseline. Other information regarding medical care and antibiotic therapy received in the past month, and whether any invasive devices were used was also collected.

Nasal, groin and rectal swabs [for the detection of EBSLs, carbapenem-resistant Enterobacteriaceae (CRE), vancomycin-resistant enterococci (VRE) and MRSA)] were taken from each patient by dedicated study nurses, at admission and at four-week intervals thereafter until discharge or transfer to another facility. Swabs were then delivered to the National Health Laboratory Services (NHLS) where they were processed by culture on selective blood agar plates. Cultured isolates were then tested by the VITEK-MS system at NHLS to identify the isolates’ species and to determine their antimicrobial susceptibility profile. Gram-positive and Gram-negative organisms were identified and antibiotic sensitivity tests were performed. The cultured isolates

were analysed for detection of antibiotic resistance genes by PCR and DNA sequencing (Chapter 4).

3.2.3 Vitek-MS Antimicrobial Susceptibility Testing

Antimicrobial susceptibility profiles with MICs and resistance phenotype reports from the VITEK-MS system were used. Susceptibilities of the clinical isolates to 19 different antimicrobial agents were tested. The MICs of the following antimicrobial agents were determined: ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, cefuroxime axetil, cefoxitin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, tigecycline, nitrofurantoin, colistin and trimethoprim-sulfamethoxazole. The recommended quality control strains were used by NHLS in the VITEK-MS system tests, namely *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *E. coli* ATTC 35218.

3.2.4 Data Analysis

The data was captured onto Microsoft Excel®, and subjected to general descriptive statistics, measures of central tendency (mean, median and mode), frequency distribution and standard deviation.

3.3 RESULTS

3.3.1 Bacterial Isolates and Colonization Rates

A total of 37 patients were recruited to the study from August to December 2016. 28 of these patients were admitted directly from the community and 9 patients were transferred in from other healthcare facilities. A total of 62 MDR bacterial isolates were collected from patients being treated for MDR TB at Jose Pearson TB hospital in Port Elizabeth. A total of 15 MDR bacterial isolates were obtained from 13 patients upon admission. Patients (13/37; 35%) were colonized by an MDR pathogen (ESBL, MRSA) on admission. Two patients were colonized by two different species upon admission. All other patients (24; 65%) appeared to free of colonization by an MDR pathogen on admission (Fig 3.1-A). Colonization rates were lower in patients admitted from the community (9/28; 32%) compared to those transferred from other healthcare facilities

(4/9; 44%) (Fig. 3.1-B-C). A total of 48 MDR bacterial isolates were obtained from patients during hospitalization. All admitted patients who did not exhibit colonization at baseline and who were resident within the hospital for longer than 4 weeks (17/17; 100%) became colonised by an ESBL-producing Enterobacteriaceae species. Only two patients exhibited MRSA colonisation, both cases were at admission.

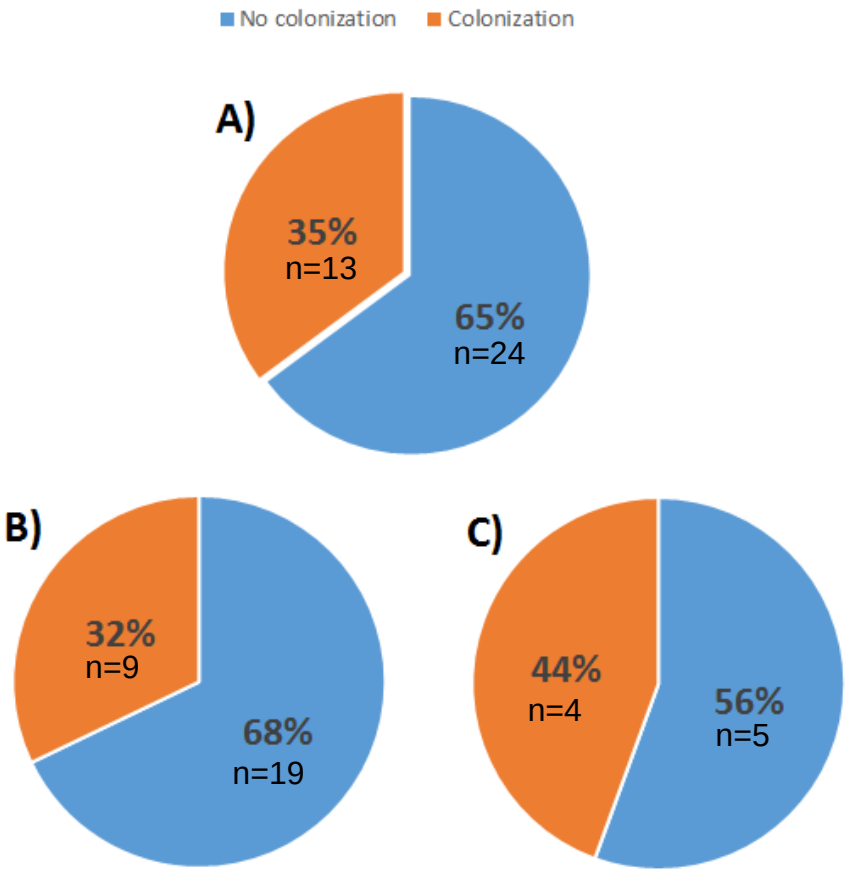

Figure 3.1: Distribution of patients that exhibited colonization of MDR pathogens upon hospital admittance. **A)** All patients included in the study (*n*=37); **B)** Patients admitted from the community (*n*=28); **C)** Patients admitted from previous healthcare facilities (*n*=9). (p = 0.28)

Swab screening of patients occurred at baseline (when patients were admitted to the hospital) and then after every four weeks that a patient remained within hospital. The most isolates were obtained after the first four weeks of patient hospitalisation (22/62; 35.8%). The bulk of isolates were obtained at baseline and during the first two months of patient hospitalisation (53/62; 85.5%). The number of isolates obtained from patients appeared to decrease after 8 weeks, however this is because there were far less patients that underwent screening at 12 and 16 weeks.

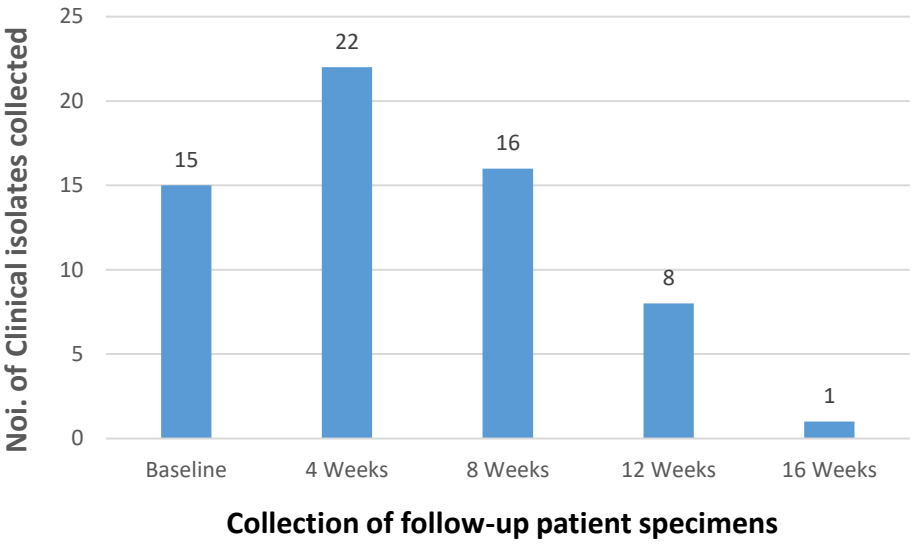

Figure 3.2: Collection of bacterial isolates (*n*=62) based on duration of patient hospitalization (August to December 2016).

3.3.2 Patient Information (Demographic and Clinical)

From the study population, 78% of patients (29/37) were female, while 22% of patients were male (8/37). In total, 75% (6/8) males and 62% (18/29) females were colonised by MDR bacteria (Fig. 3.3). MRSA colonisation was only observed in male patients. Due to the small size of Jose Pearson TB hospital all patients were taken from two wards. All female patients were located within New Block ward, while all male patients were located within the Jackie Richter ward.

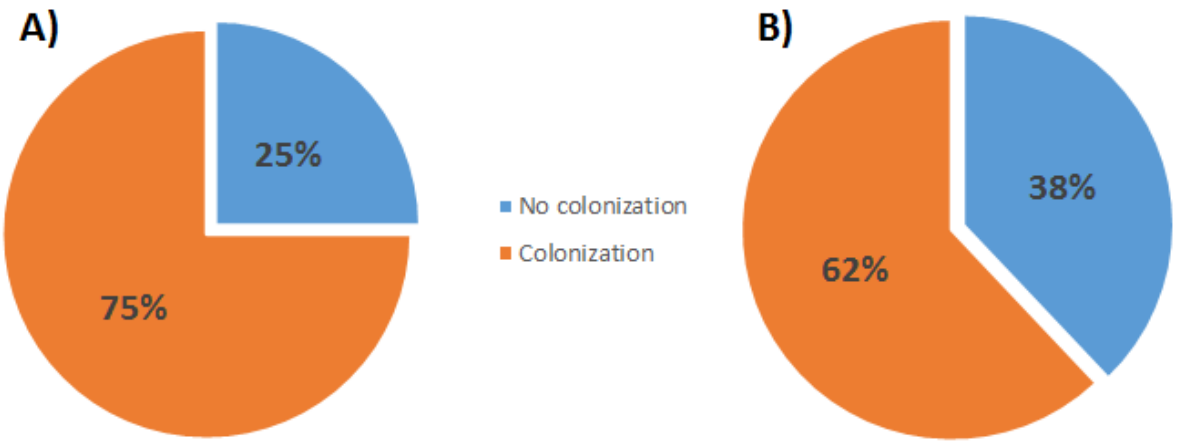

Figure 3.3: Distribution of patient colonization based on gender. **A)** Rates amongst males (*n*=8); **B)** Rates amongst females (*n*=29). (*p* = 0.09)

Amongst the patients screened in this study the average age was 35 years, the youngest patient was 18 years. The oldest patient was 58 years, and the largest group was 30 – 39 years (Fig. 3.4).

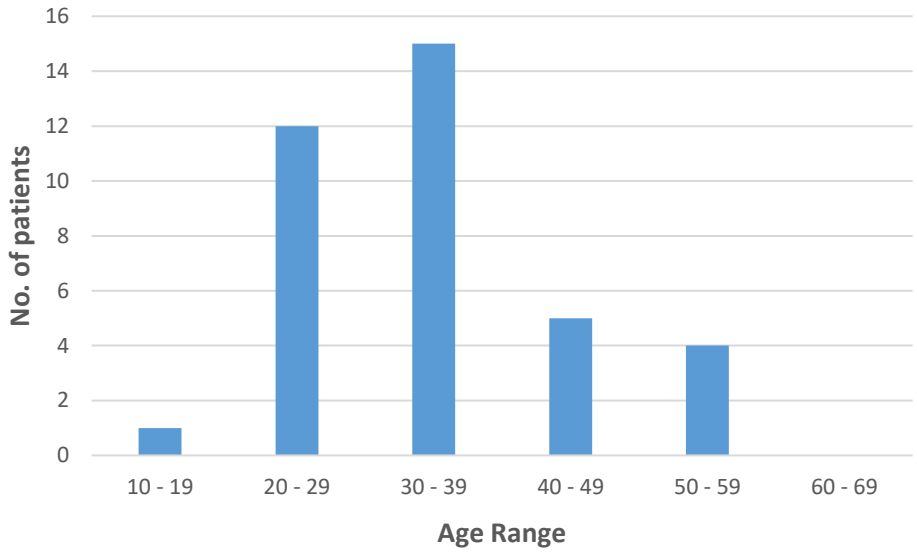

Figure 3.4: Distribution of patients (*n*=37) admitted to Jose Pearson TB hospital and screened for MDR pathogens based on age

The majority of patients included within this study were admitted directly from the community (Fig. 3.5). A ratio of approximately 1:3 was observed of patients admitted from other healthcare facilities compared to patients admitted from the community. The healthcare admitted patients were all transferred from one of four other healthcare facilities which included two major public hospitals within the Port Elizabeth area.

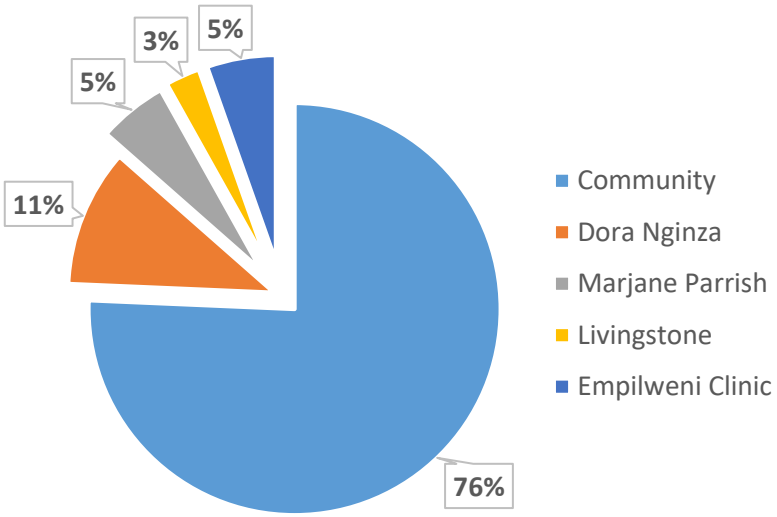

Figure 3.5: Distribution of the origin of patients (*n*=37) admitted to Jose Pearson TB hospital and screened for MDR pathogens.

Co-morbidities other than HIV (n=24; 64.9%) included oral thrush (n=3), epilepsy (n=2), vaginal thrush (n=1), syphilis (n=1), hypertension (n=1) and MRSA (n=1). There was one patient reported to have an intravenous line inserted, but there was no complication. Nineteen percent (7/37) of patients demised during their hospitalization.

All patients admitted to Jose Pearson were on a regimen of antibiotics for the treatment of MDR-TB. The median number of antibiotics prescribed was seven. The most prescribed antibiotics were pyrazinamide, terizidone, and bedaquiline, which were prescribed amongst 93%, 90%, and 76% of patients respectively (Fig. 3.6). The least prescribed antibiotics were penicillin and clofazimine, in 3% and 24% of patients respectively (Fig. 3.6). Penicillin was prescribed in only one case where the patient had syphilis. Throughout the duration of the study, patients were treated with between five and ten of the antibiotics listed in figure 3.6 at any one time.

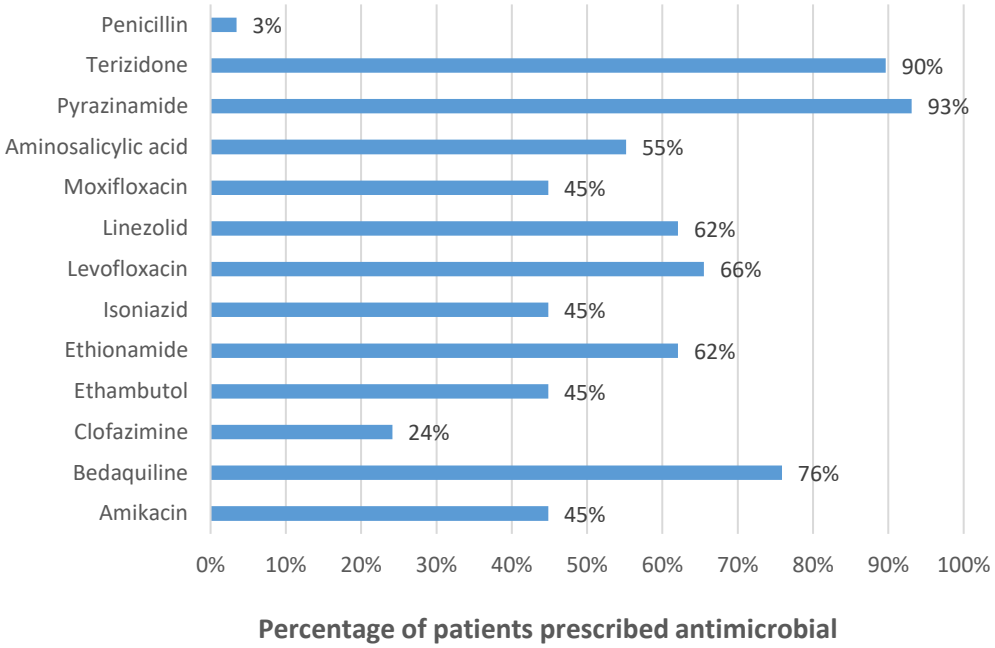

Figure 3.6: Antibiotics prescribed to patients (*n*=37) during their time of treatment within Jose Pearson hospital.

While the majority of patients (21/37; 57%) were discharged from the hospital, the other patients had less favourable outcomes (Fig. 3.7). Seven patients passed away during their hospitalization at Jose Pearson (7/37; 19%). In addition, five patients chose to withdraw from the study at some point during their hospitalisation (5/37; 14%), while two patients were transferred to other facilities (5%), and two patients were still present within the hospital (5%) at the conclusion of the study (Fig. 3.7). The average and median patient length of stay at the hospital were 10 weeks and 9.85 weeks respectively. The shortest patient length of stay was 1.5 weeks and the longest was 20 weeks.

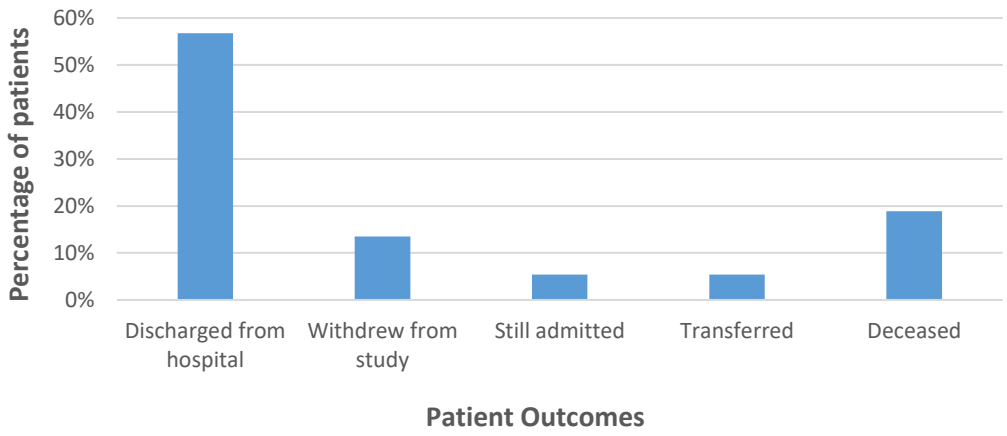

Figure 3.7: Clinical outcomes of patients (*n*=37).

3.3.3 Spectrum of Bacterial Colonisation

The majority of antibiotic resistant bacteria were isolated from rectal swab samples (60/62, 96.8%). Only two MRSA isolates were obtained from nasal swabs (2/62, 3.2%), while none of the remaining nasal swabs and groin swabs yielded any MDR pathogens. *E. coli* was the only species isolated every month, and *K. pneumoniae* was isolated for three months consecutively. All other species were isolated sporadically over the period of patient screening (Fig. 3.8).

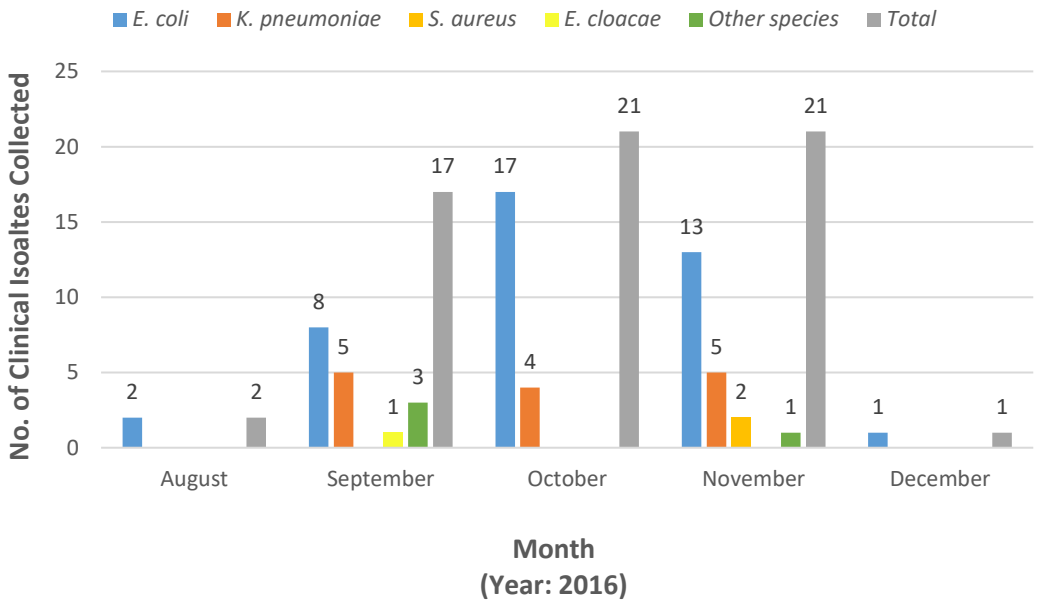

Figure 3.8: Collection of bacterial isolates (*n*=62) during the period of August to December 2016 by bacterial species

Among the isolates identified from the colonisation screening, Enterobacteriacae*, i.e. Escherichia coli* (42/62, 67.7%) and *Klebsiella pneumoniae* (14/62, 22.6%) predominated, followed by *Enterobacter cloacae* (1/62, 1.6%), *Citrobacter braakii* (1/62, 1.6%), *Citrobacter freundii* (1/62, 1.6%), *Klebsiella oxytoca* (1/62, 1.6%), and *Proteus mirabilis* (1/62, 1.6%),) (Fig. 3.9). *Staphylococcus aureus* isolates made up 3.2% (2/62). No vancomycin-resistant enterococci (VRE) were identified. While all isolates were identified as being multidrug-resistant, no carbapenem-resistant Enterobacteriaceae (CRE) were observed. In the following chapter, the antimicrobial

susceptibilities of the Enterobacteriaceae will be discussed in greater detail in the next chapter.

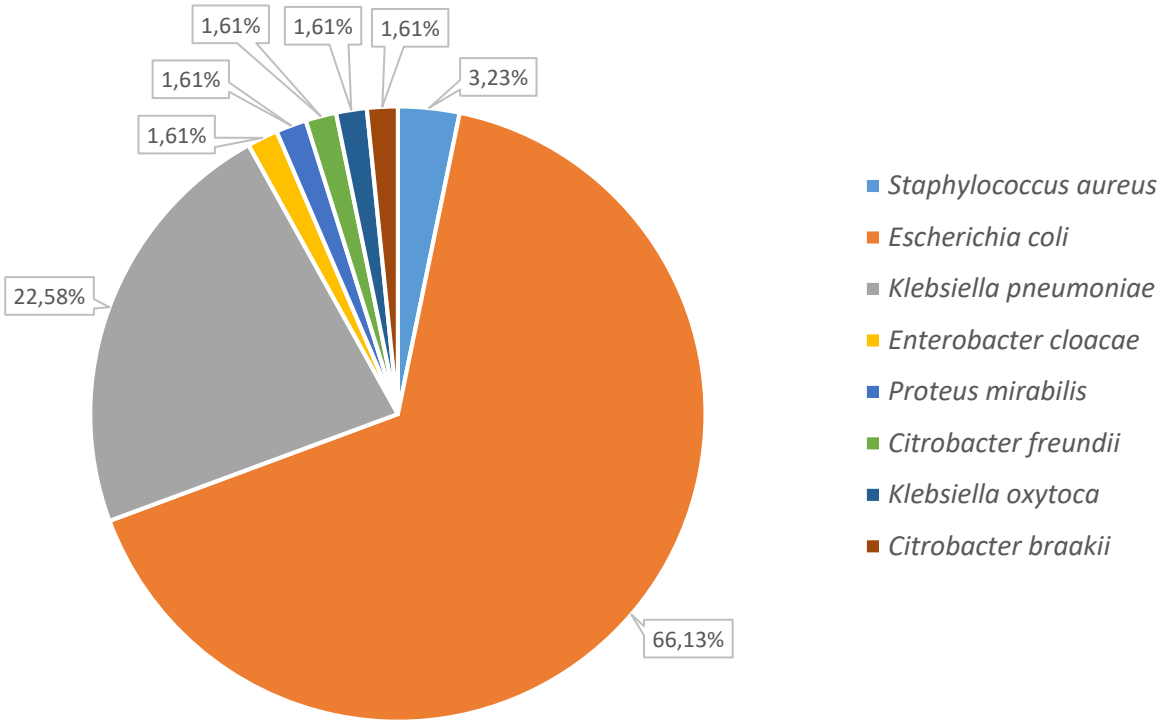

Figure 3.9: Species distribution of the MDR pathogens (*n*=62) isolated from the screened patients (*n*=37) admitted to Jose Pearson hospital

3.4 DISCUSSION

The development of multidrug-resistant (MDR) pathogens, nosocomial or hospital-acquired infections are swiftly becoming extremely costly to healthcare facilities and life threating to patients. The known risk factors associated with patient colonization of MDR nosocomial bacterial pathogens include advanced age, underlying disease, prolonged hospitalisation, surgery, invasive devices, and exposure to antimicrobial drugs (Kim *et al.* 2011). This chapter provides some insight into the types of nosocomial bacterial pathogens, which colonize MDR-TB patients while receiving treatment within a specialized TB hospital setting.

Incidence rates of nosocomial pathogen colonization appeared to be independent of gender, as outlined in the previous chapter that there were equivalent amounts of males and females admitted to Jose Pearson for treatment. However, in this patient cohort, there were a higher number of females. In both cases this is unusual as the literature outlines the disparity of TB infection rates between males and females. Studies report a far higher incidence of TB infection among males, with approximately double the number of males being infected by TB than females (Jiménez-Corona *et al.* 2006; Thorson *et al.* 2007; Olivier and Quintana-Murci, 2009). In this phase of the project on the colonisation, the higher number of female patients could be due to the patient recruitment by enthusiastic physicians assigned to the female ward (New Block). This highlights a challenge of clinical research where both patient and physician co-operation and assistance are essential.

As of 2015, the HIV-positive population of the Eastern Cape was 796 634, which was 11.51% of the province's total population (Crowther-Gibson *et al*. 2016). Of the 37 screened patients 65% were HIV positive. HIV infection can cause patients to be 20 times more likely to contract TB (Naidoo *et al*. 2011; Pawlowski *et al.* 2012). While South Africa's population makes up less than one percent of the world's total population it accounts for 17% percent of all HIV cases and approximately 25% of all HIV-TB co-infected patients worldwide (Naidoo *et al*. 2011). HIV rates between the both patient cohorts (i.e prescribing patterns and colonisation) varied, however, there was a similar age distribution among the patients which is likely due to the low patient sample sizes.

Several patients withdrew from the study, possibly due to the nature of the swab screening procedure, while seven demised during the study. In most cases, the specific cause of death could not be determined, though in two patients death was due to XDR-TB complications and to epilepsy, respectively. In most cases of death, the concerned patients were admitted to hospital in a poor state of health. In addition, many patients experienced a diarrhoeal episode before they demised. Diarrhoea particularly in HIV-positive patients may be due to various causes, including antibiotic-associated diarrhoea (AAD), *Clostridium difficile* infection, and diarrhoea due to nosocomial pathogens such as *K. pneumoniae* (Francino, 2016). While these patients were known to be colonized with MDR Enterobacteriaceae, *K. pneumoniae*, it is

unclear whether this was the cause of death. In most cases, no follow up investigation was done at the time of patient death which resulted in little available information and evidence. This posed a great challenge in determining the exact cause of death.

Patients were less likely to be admitted to Jose Pearson TB hospital already colonized with a MDR pathogen. Among all the patients, 35% were colonized by a MDR or ESBL-producing pathogen upon admittance. As expected, a slightly higher incidence of MDR pathogen colonization on admittance was observed among the patients that were transferred from other healthcare facilities compared to patients who were admitted directly from the community.

The study revealed that the *Enterobacteriaceae* were by far the major cause of multidrug-resistant (MDR) nosocomial colonisation within hospitalized TB patients. *Escherichia coli* and *Klebsiella pneumoniae* were the significant etiological agents accounting for 66.1% and 22.6% of colonisation respectively. With the exception of two isolates (which were community-acquired), all pathogens were members of the *Enterobacteriaceae* family. This is not surprising as treatment regimens of the Jose Pearson patients rarely included surgical intervention and the use of invasive devices, which greatly decreases the colonization risk of pathogens associated with surgical site infections (SSIs), blood stream infections (BSIs), and urinary tract infections (UTIs). A truly concerning occurrence was that 100% of patients that resided within the hospital for 4 weeks or more were colonized with a MDR *Enterobacteriaceae* pathogen.

Staphylococcus aureus, *Enterobacter cloacae*, *Citrobacter braakii*, *Citrobacter freundii*, and *Proteus mirabilis* were only isolated and identified from patients at baseline which implies these isolates were either community-acquired infections (CAIs) or HAIs obtained from the previous healthcare facilities. All these isolates were identified as ESBL producers by the Vitek system. This is an interesting observation as these isolates may have been hyper-producing *Amp*C, and as a result may have been native intestinal flora. Further information regarding *S. aureus*, *E. cloacae*, *C. braakii*, *C. freundi* and *P. mirabilis* can be seen in the accompanying data (Appendix 6: Table A6 and Table A7). *E. coli*, *K. pneumoniae*, and *Klebsiella oxytoca* were acquired by patients during their hospitalization at Jose Pearson. A few cases (n=9)

of *E. coli* and *K. pneumoniae* were observed among patients at baseline, however the majority of *E. coli* and *K. pneumoniae* isolates were hospital-acquired.

Within hospitals, there are multiple infection routes by which patients can become colonized by a nosocomial pathogen. There are four main routes of transmission, these include contact; common vehicle, respiratory droplet, and airborne transmission (Collins, 2008). In total 62 pathogen isolates were obtained, 48 isolates were hospital-acquired, while the remaining 14 isolates were either community-acquired or obtained by the patient at their previous healthcare facility. All 48 hospital-acquired pathogens were *Enterobacteriaceae* species. At a specialized TB hospital, the focus is on the treatment of MDR and XDR-TB patients, and nosocomial spread of tuberculosis bacilli is a constant threat. Therefore, the most likely transmission routes by which the monitored patients obtained *Enterobacteriaceae* pathogens were either contact or vehicle transmission suggesting spread through patient-patient and patient-staff interactions. There is also the possibility of an environmental reservoir within the hospital. Education of patients and healthcare staff and further studies with a larger sample size are recommended for possible infection points and routes.

Only two MRSA isolates were obtained during the study from patients who were colonized before admittance to the hospital. This was uncommon, as *S. aureus* is known to be a major nosocomial pathogen, especially in immunocompromised hosts with a high rate of HIV infection (Khan *et al.* 2015). Further investigation would be required to identify of the antimicrobial resistance genes present.

A similar South African study was conducted where the incidence of nosocomial MRSA within the nasal canal was surveyed amongst TB patients in a rural hospital in Tugela Ferry, Kwazulu-Natal Province (Heysell *et al.* 2011). Amongst the 52 patients surveyed within their study, 11 of the patients were admitted to the hospital exhibiting colonization with *S. aureus*, 9 of these isolates were determined to be MRSA (Heysell *et al.* 2011). Upon patient follow up, 14 days after admission, it was observed that an additional 4 patients had been colonized with MRSA, and it was determined that the HIV-positive patients were far more likely to be colonized (Heysell *et al.* 2011). The findings of this study are similar to that of Heysell *et al* where TB patients were admitted to TB hospitals already colonized with MRSA, rather than be colonized during

their hospital stay. However, in the case of our study both patients found to be colonized with MRSA were HIV-negative. The extremely low incidence of MRSA amongst patients may be because of decolonization of MRSA amongst TB patients due to the high prescription of antimycobacterials, such as moxifloxacin, linezolid, and rifampicin which exhibit effective activity against MRSA (Rayner and Munckhof, 2005).

A healthy gut microbiome is crucial for optimal functioning of the human immune system, producing several essential vitamins and compounds required for healthy bodily functions (Francino, 2016). While TB treatment regimens are required to cure patients of their TB infection, the broad-spectrum antimicrobials used may also be promoting the colonisation and transmission of multi drug resistant bacteria, especially ESBL producing *Enterobacteriaceae* species. This highlights the need for antimicrobial stewardship programs and the elimination of the unnecessary use of broad spectrum antibiotics.

A South Korean case-control study published in 2011 attempted to elucidate the MDR bacterial infections faced by TB patients (Kim *et al.* 2011). In total 123 patient records at Seoul National University Hospital were screened and 321 non-mycobacterial nosocomial infections were identified, of these 59 TB patients were colonised by MDR pathogens and a total of 120 MDR clinical isolates were identified (Kim *et al.* 2011). 45.4% of MDR isolates were Gram-positive which included 32 Coagulase-negative staphylococci, 26 *S. aureus,* and 6 enterococci isolates (Kim *et al.* 2011). 31.1% of MDR isolates were Gram-negative and included 18 *P. aeruginosa*, 11 *A. baumannii*, 9 *E. coli*, 9 *Stenotrophomonas maltophilia*, 6 *Klebsiella* spp., and 3 *E. cloacae* isolates (Kim *et al.* 2011). In the Kim *et al* (2011) study the major sites of nosocomial infection were urinary tract (34.6%), respiratory tract (29.2%), skin and soft tissue (10.9%), and gastrointestinal tract (9.7%). In contrast, our study showed that colonization sites were either the respiratory tract (3.2%) or the gastrointestinal tract (96.8%).

Amongst the TB patients that acquired MDR nosocomial infections that were assessed by Kim *et al* (2011) only 2 patients (3.39%) were HIV-positive and only 8 (13.56%) were MDR-TB patients (Kim *et al.* 2011). In contrast, 63.89% of TB patients in this study were HIV positive and all patients were MDR-TB patients if not XDR-TB patients. In addtion, Kim *et al* observed a median prescription of 4 antimycobacterial drugs

amongst their patients with a range of 3 to 9 antimycobacterials prescribed (Kim *et al.* 2011). However, in this study we saw a median prescription of 7 antimycobacterials drugs amongst patients, with a prescription range of 5 to 10 antimycobacterials. This considerable difference in the antimicrobial treatment of patients may be responsible for discrepancy seen between our two studies in regards to the colonization rates of Gram-Positive and Gram-Negative infection.

There is no standardized protocol set in place to determine whether patients at Jose Pearson have been colonised by other drug resistant HAIs. Majority of patients treated at Jose Pearson are unemployed and most likely return to one of the multiple informal settlements or townships in Port Elizabeth. This is a significant concern to public health as it has been long understood that the conditions of informal settlements, such as lack of adequate sanitation systems, can facilitate the spread of disease, and in this case the spread of genes encoding for ESBLs, carbapenemases, and other antibiotic resistance mechanisms.

In conclusion, patients admitted to the Jose Pearson TB hospital experienced a “high force of colonisation” as all patients that were present in the hospital for one month or longer, became colonized by a MDR or ESBL-producing pathogen. The susceptibility profiles of these isolates and their resistance genes will be discussed in the next chapter.

CHAPTER FOUR

Antimicrobial Susceptibility and Resistance Genes of isolates colonising hospitalised TB patients

4.1 INTRODUCTION

The genetic events responsible for antibiotic resistance can be grouped very broadly into two categories. Firstly, there is the acquisition of new genes. Bacteria are capable of taking up mobile genetic elements, and incorporating them into their genome. This leads to a decrease in their susceptibility to antibiotics. Bacteria also undergo horizontal gene transfer where genetic information, often containing resistance genes, can be shared among bacteria within the same population or even between different species (Thomas and Nielsen, 2005). A good example of this is when bacteria acquire plasmids carrying a gene encoding for a carbapenemase, such as the New Delhi metallo-β-lactamase, which gives the bacteria the ability to hydrolyse many types of β-lactam antibiotics, including carbapenems, through the production of this enzyme. Secondly, antibiotic resistance can develop through a change in expression or mutation of an innate chromosomal gene. This is well illustrated by the upregulation of chromosomally encoded *amp*C β-lactamase gene or the mutation of the *rpo*B gene that encodes for the β-subunit of RNA polymerase, which prevents drugs, such as rifampicin, from binding and allows RNA biosynthesis to continue (Yue *et al.* 2003).

The identification of antimicrobial determinants carried by clinical bacterial isolates is important as it can assist the appropriate antimicrobial treatment. This is because resistance determinant genes usually provide resistance to a specific spectrum of drugs. For example, while metallo-β-lactamases are able to hydrolyse a very broad spectrum of monobactam drugs, they lack activity towards the monobactams (Zmarlicka *et al.* 2015). Due to the mobile nature of many of these resistance determinants it may indicate which infection control policies the concerned healthcare facility should put into focus to maximize patient safety. Measurement of the specific phenotypic antimicrobial susceptibilities of an isolate is also important, this is because it can indicate which drugs are appropriate to prescribe to a patient. While the genotypic contribution of antimicrobial resistance is relatively well understood, just

because a specific resistance gene is present or absent does not imply that the isolate will be susceptible or resistant to the associated antimicrobials of that genetic determinant. This type of information is critical for clinicians as they can then prescribe drugs based on evidence, and last-line antibiotics may be reserved for cases where both the genotypic and phenotypic data indicate it necessary.

Conducting antimicrobial susceptibility testing of clinical isolates is necessary, as it is crucial to confirm susceptibility or resistance to the chosen antimicrobial therapy (Reller *et al.* 2009). Antimicrobial susceptibility testing can be achieved through several different methods. Broth dilution tests are commonly used antimicrobial susceptibility testing methods which comprise mainly of the macrobroth or tube-dilution method, and microdilution tests (Reller *et al.* 2009). Broth dilution tests involve preparing a series of antimicrobial dilutions which are then inoculated with a standardized bacterial suspension. A quantitative result, i.e. the minimum inhibitory concentration (MIC), can then be determined by observing which dilutions, or concentrations, of antimicrobial inhibit bacterial growth (Reller *et al.* 2009). However, broth dilution tests can be time consuming and tedious as they require the manual preparation of antimicrobial dilutions, and this can also lead to errors in the concentration of the antimicrobial solutions (Reller *et al.* 2009). As a result, many laboratories make use of 96-well microdilution plates that contain pre-weighted amounts of antibiotics, which can be purchased from commercial suppliers (Reller *et al.* 2009) such as the Sensititre™ MIC plates.

The Sensititre System is a collection of products produced by Thermo Scientific which enables rapid and accurate species identification and antimicrobial susceptibility testing (AST) for a wide range of bacterial and yeast species. Sensititre™ MIC plates are included within the Sensititre System. Sensititre™ MIC plates consist of 96-well plates where each well has been pre-coated with a specific antimicrobial at a certain concentration. A defined microbial inoculation can then be added to each well of the MIC plate and left to incubate. Based on the presence or absence of microbial growth at a range of different antimicrobial concentrations, and coupled with MIC interpretive criteria, such as the CLSI or EUCAST antimicrobial breakpoints, the antimicrobial susceptibility profile of a microbial isolate can be determined (Reller *et al.* 2009).

Automated antimicrobial susceptibility systems are swiftly becoming widespread due to their ability to generate antimicrobial susceptibility profiles in a shorter period compared to manual readings, as they possess sensitive optical detection systems which allows for the detection of subtle changes in bacterial growth (Reller *et al.* 2009). There are several automated systems available, however in South Africa the Vitek System is widely used. The Vitek 2 System is highly automated and uses compact 64-well plastic reagent cards which contain microliter quantities of a range of antimicrobials, the system repetitively monitors bacterial growth within the 64 wells by optically measuring turbidity (Reller *et al.* 2009). The Vitek 2 system also works in conjunction with the Advanced Expert System (AES). The AES is software designed to analyse the raw data generated by the Vitek 2 system and validate the results (Sanders *et al*. 2001). Two major purposes of the AES are to identify discrepancies between the species identification of the isolate and the determined antimicrobial susceptibility results, and establish the antimicrobial phenotype of the isolate based on the MIC data generated by the Vitek system (Sanders *et al*. 2001). Recently, Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid, accurate, and cost-effective method for the identification of clinical microorganism isolates (Lévesque *et al*. 2015). This technology is used in the Vitek MS System and provides accurate species identification from clinical samples (BioMerieux, 2017).

The acquisition and production of enzymes that degrade antimicrobials is one of the major mechanisms of antibiotic resistance observed among the Gram-negative pathogens. The most notable of these enzymes are the extended spectrum β-lactamases (ESBLs) and the carbapenemases. ESBLs confer the ability to hydrolyse penicillin, cephalosporin, and monobactam class antibiotics, and carbapenemases confer the ability to hydrolyse penicillin, cephalosporin, and carbapenem class antibiotics, however they lack activity towards the monobactams (Bush and Jacoby, 2010). The most common ESBLs include the CTX-M (Cefotaximase), TEM (Temoneira), and SHV (Sulfhydryl variable) β-lactamases (Dhillon and Clark. 2011). CTX-M variants are currently the most dominantly produced ESBLs, and this is concerning due to the MDR phenotype exhibited by CTX-M producing isolates (Canton *et al.* 2012). In addition, CTX-M carrying plasmids often carry other genes that confer resistance to several antimicrobial classes, such as aminoglycosides and

fluoroquinolones (Vaidya. 2011; Canton *et al.* 2012). The most commonly observed carbapenemases include the group 2 enzymes, KPC (*Klebsiella pneumoniae* carbapenemase) and OXA-48 (Oxacillin-Hydrolysing Variant 48), and the group 3 metallo-β-lactamases, NDM (New Delhi Metallo-β-Lactamase), IMP (Imipenemase), and VIM (Verona Integron–Encoded Metallo-β-Lactamase) (Bush and Jacoby, 2010; Lutgring and Limbago, 2016).

The ESBL types most identified in South Africa included TEM, SHV, CTX-M, CMY, and OXA-1, with *Enterobacteriaceae* species being the major producers (Storberg, 2014). The ESBLs previously listed have become extremely common and it is expected to find one or more of their respective encoding genes within drug resistant isolates. Several carbapenemases have been identified within South Africa, these include OXA-48, GES, KPC, NDM, and VIM, which are produced by several Gram-negative pathogens (Brink *et al.* 2012). The carbapenemase species commonly recorded in South Africa include OXA-48-like, VIM, NDM, GES, KPC, and IMP, with NDM and OXA-48-like being the produced carbapenemases in most cases, 57.8% and 30.9% respectively (Perovic and Chetty, 2016).

Antimicrobial resistance has been extensively reported amongst clinical isolates from healthcare facilities within Port Elizabeth, South Africa (Brink *et al.* 2012; Gqunta, 2014; Masunda, 2014; Govender *et al.* 2015; Annear *et al.* 2017). These reports include ESBL-producing and highly drug-resistant *Enterobacteriaceae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. In many of these cases patient outcomes were not favourable (Annear *et al*. 2017). Within the Port Elizabeth locale, bacteria carrying the following ESBL genes have been reported; OXA-1, SHV-1, SHV-11, CTX-M-3, CTX-M-14, and CTX-M-15 (Gqunta, 2014). There have been reports on several carbapenemase producers in Port Elizabeth. These included species such as *E. cloacae, P. aeruginosa, K. pneumoniae*, and *A. baumannii* and the produced carbapenemases were as follows, OXA-23, OXA-48, OXA-51, OXA-58, IMI-2, and GES (Brink *et al.* 2012; Masunda, 2014; Gqunta, 2014). The *bla*VIM-2 gene has also been detected in carbapenem-resistant clinical *Pseudomonas aeruginosa* isolates in Port Elizabeth (Govender *et al.* 2015; Annear *et al*. 2017). The metallo-β-lactamase IMP has been detected in clinical isolates obtained from Eastern Cape hospitals,

however IMP has been reported in the Port Elizabeth area (Singh-Moodley *et al.* 2015).

This chapter analysed the antimicrobial susceptibility profiles, and resistance genes of isolates colonising hospitalised TB patients.

4.2 MATERIALS AND METHODS

4.2.1 Antimicrobial Susceptibility Testing

All isolates were tested through two different methods of antimicrobial susceptibility testing (AST). These included the Vitek® Automated System and Sensititre™ Gram Negative Xtra (GNFX2) MIC plates. In the interim pure cultures of each isolates were stored at -85°C in MicroBank™ beads (Davies Diagnostics). In total, 26 different antimicrobial drugs were tested. Both methods tested 13 of the same antibiotics (amikacin, piperacillin-tazobactam, cefepime, cefotaxime, ceftazidime, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole, tigecycline, colistin, meropenem, ertapenem, and imipenem), while the Vitek system tested six other antibiotics (ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefuroxime-axetil, cefoxitin, and nitrofurantoin) and the Sensititre plates testing an additional seven (tobramycin, ticarcillin-clavulanic acid, aztreonam, levofloxacin, doxycycline, minocycline, and polymyxin B). The selected ranges of the antimicrobials tested were selected so that resistance breakpoints could be interpreted through the Clinical and Laboratory Standards Institute (CLSI) criteria.

4.2.1.1 Vitek-MS antimicrobial susceptibility testing

Antimicrobial susceptibility profiles with MICs and resistance phenotype reports from the VITEK® MS system were used. Susceptibilities of the clinical isolates [Enterobacteriaceae (n=60)] to 19 different antimicrobial agents were tested using the Vitek AST-N255 card type. The MICs of the following antimicrobial agents were determined: ampicillin (16 – 32 µg/ml), amoxicillin-clavulanic acid (16 - 32 µg/ml), piperacillin-tazobactam (8-4 – 128-4 µg/ml), cefuroxime (64 µg/ml), cefuroxime axetil (64 µg/ml), cefoxitin(16 – 32 µg/ml), cefotaxime (2 – 64 µg/ml), ceftazidime (16 – 32 µg/ml), cefepime (16 – 32 µg/ml), ertapenem (1 – 32 µg/ml) , imipenem (8 – 32 µg/ml),

meropenem (16 – 32 µg/ml), amikacin (32 – 64 µg/ml), gentamicin (8 – 16 µg/ml), ciprofloxacin (2 – 4 µg/ml), tigecycline (8 µg/ml), nitrofurantoin (512 µg/ml), colistin (16 µg/ml) and trimethoprim-sulfamethoxazole (320 µg/ml). The recommended quality control strains were used by NHLS in the VITEK-MS system tests: *E. coli* ATCC 25922, and *E. coli* ATTC 35218.

4.2.1.2 Antimicrobial Susceptibility Testing by Sensititre™ Plates

Sensititre™ Gram Negative Xtra (GNFX2) MIC plates (Thermo Scientific, SepSci)) were also used to determine the MIC values of the Enterobacteriaceae isolates against 21 antimicrobial compounds according to the manufacturer's instructions. Twenty-one antimicrobials were tested (figure 4.1). These included: amikacin (4 – 32 µg/ml), ticarcillin-clavulanic acid (16-2 – 128-2 µg/ml), aztreonam (2 – 16 µg/ml), piperacillin-tazobactam (8-4 – 64-4 µg/ml), trimethoprim-sulfamethoxazole (0.5-9.5 – 4-76 µg/ml), gentamicin (1 – 8 µg/ml), cefepime (2 – 16 µg/ml), tobramycin (1 – 8 µg/ml), levofloxacin (1 – 8 µg/ml), doxycycline (1 – 16 µg/ml), ciprofloxacin (0.25 - 2 µg/ml), minocycline (2 – 16 µg/ml), meropenem (1 – 8 µg/ml), cefotaxime (1 – 32 µg/ml), tigecycline (0.25 – 8 µg/ml), ertapenem (0.25 – 4 µg/ml), imipenem (1 – 8 µg/ml), doripenem (0.12 – 2 µg/ml), colistin (0.25 – 4 µg/ml), polymyxin B (0.25 – 4 µg/ml), ceftazidime (2 – 16 µg/ml), cefotaxime (2 – 32 µg/ml), and tigecycline (1 – 8 µg/ml). Each plate contained three positive controls (POS).

	1	2	3	4	5	6	7	8	9	10	11	12
A	AMI 4	AZT 2	SXT 0.5/9.5	FEP 2	LEVO 1	CIP 0.25	MERO 1	DOR 0.12	DOR 0.25	DOR 0.5	DOR 1	DOR 2
B	AMI 8	AZT 4	SXT 1/19	FEP 4	LEVO 2	CIP 0.5	MERO 2	COL 0.25	COL 0.5	COL 1	COL 2	COL 4
C	AMI 16	AZT 8	SXT 2/38	FEP 8	LEVO 4	CIP 1	MERO 4	POL 0.25	POL 0.5	POL 1	POL 2	POL 4
D	AMI 32	AZT 16	SXT 4/76	FEP 16	LEVO 8	CIP 2	MERO 8	TAZ 1	TAZ 2	TAZ 4	TAZ 8	TAZ 16
E	TIM2 16/2	P/T4 8/4	GEN 1	TOB 1	DOX 2	MIN 2	FOT 1	FOT 2	FOT 4	FOT 8	FOT 16	FOT 32
F	TIM2 32/2	P/T4 16/4	GEN 2	TOB 2	DOX 4	MIN 4	TGC 0.25	TGC 0.5	TGC 1	TGC 2	TGC 4	TGC 8
G	TIM2 64/2	P/T4 32/4	GEN 4	TOB 4	DOX 8	MIN 8	ETP 0.25	ETP 0.5	ETP 1	ETP 2	ETP 4	POS
H	TIM2 128/2	P/T4 64/4	GEN 8	TOB 8	DOX 16	MIN 16	IMI 1	IMI 2	IMI 4	IMI 8	POS	POS

Figure 4.1: Plate format of the Sensititre™ Gram Negative (GNX2F) MIC plate.

Overnight cultures of the *Enterobacteriaceae* isolates were prepared on Mueller-Hinton (MH) plates at 37°C. A phosphate buffer saline (PBS) suspension (inoculum) was prepared from overnight cultures and the turbidity was adjusted to a 0.5 McFarland standard (1.175% $BaCl_2$ + 1% H_2SO_4). Ten microliters of the adjusted inoculum was then transferred into a 11ml tube of cation adjusted MH broth with TES buffer to give a suspension of $1x10^5$ cfu/ml. This MH broth suspension (50µl) was then added to each well of the Sensititre™ Gram Negative (GNFX2) MIC plate using a multi-channel pipette. The plates were sealed with tape, incubated overnight at 37^{o}C. After incubation, the Sensititre™ plates were visually assessed for growth. Minimum inhibitory concentrations (MIC) were interpreted using the Clinical and Laboratory Standards Institute (CLSI) 2017 MIC breakpoint criteria (Clinical and Laboratory Standards Institute, 2017).

4.2.2 DNA Extraction and Primers

Isolates that exhibited drug resistance were grown in appropriate culture media for DNA extraction. Plasmid DNA was extracted from cultures (200µl) using the QIAprep® Spin Miniprep kit (Qiagen). Extracted DNA was quantified using the NanoDropTM Spectrophotometer (Thermo Scientific) at 260 nm. Primers were synthesised by Integrated DNA Technologies (WhiteSci) and re-constituted in TE buffer (Lonza) according to the manufacturer's instructions.

4.2.3 Detection of Antibiotic Resistance Determinants

PCR screening for carbapenemase encoding genes and extended spectrum beta lactamases were performed using plasmid DNA as a template with primers and PCR conditions shown in Tables 4.1 and 4.2 respectively.

Table 4.1: PCR primers for the detection of carbapenemase encoding genes.

Gene	Primer	Sequence (5' to 3')	Size (bp)	GenBank Reference	Reference
Metallo-β-lactamses					
VIM	VIM-F	GATGGTGTTTGGTCGCATA	390	AF191564	Nordmann *et al.* (2011)
	VIM-R	CGAATGCGCAGCACCAG			
IMP	IMP-F	GGAATAGAGTGGCTTAATCTC	188	AB010417	Ellington *et al.* (2007)
	IMP-R	CCAAACACTAAGTTATCT			
NDM	NDM-F	GGTTTGGCGATCTGGTTTTC	621	FN396876	Ellington *et al.* (2007)
	NDM-R	CGGAATGGCTCATCACGATC			
Serine Carbapenemases					
KPC	KPC-F	ATGTCACTGTATCGCCGTCT	882	AF297554	Pillai *et al.* (2009)
	KPC-R	TTACTGCCCGTTGACGCCC			
SME	SME-F	AGATAGTAAATTTTATAG	1138	Z28968	Queenan *et al.* (2000)
	SME-R	CTCTAACGCTAATAG			
GES	GES-F	GTTTTGCAATGTGCTCAACG	371	AF326355	Weldhagen and Prinsloo (2004)
	GES-R	TGCCATAGCAATAGGCGTAG			
IMI	IMI-F	ATAGCCATCCTTGTTTAGCTC	818	DQ173429.1	Aubron *et al.* (2005)
	IMI-R	TCTGCGATTACTTTATCCTC			
Oxacillinase-Type Carbapenemases					
OXA-23-like	23L-F	GATCGGATTGGAGAACCAGA	474	AJ132105	Woodford *et al.* (2006)
	23L-R	ATTTCTGACCGCATTTCCAT			
OXA-24-like	24L-F	GGTTAGTTGGCCCCCTTAAA	246	JQ409996.1	Woodford *et al.* (2006)
	24L-R	AGTTGAGCGAAAAGGGGATT			
OXA-48	O48-F	GCGTGGTTAAGGATGAACAC	438	AY236073	Poirel *et al.* (2012)
	O48-R	CATCAAGTTCAACCCAACCG			
OXA-51-like	51L-F	TAATGCTTTGATCGGCCTTG	353	AJ309734	Woodford *et al.* (2006)
	51L-R	TGGATTGCACTTCATCTTGG			
OXA-58-like	58L-F	AAGTATTGGGGCTTGTGCTG	575	JQ409994.1	Woodford *et al.* (2006)
	58L-R	CCCCTCTGCGCTCTACATAC			

Table 4.2: PCR primers for the detection of ESBL encoding genes.

Gene	Primer	Sequence (5' to 3')	Size (bp)	GenBank Reference	Reference
Extended-Spectrum β-lactamase					
OXA-1	OXA-F	CCAAAGACGTGGATG	420	AF255921.1	Siu *et al.* (2000)
	OXA-R	GTTAAATTCGACCCCAAGTT			
TEM	TEM-F	ATGAGTATTCAACATTTCCGTG	861	JX129212.1	Essack *et al.* (2001)
	TEM-R	TTACCAATGCTTAATCAGTGAG			
SHV	SHV-F	ATGCGTTATATTCGCCTGTG	865	AF117743.1	Essack *et al.* (2001)
	SHV-R	TTAGCGTTGCCAGTGCTCG			
CTX-M-1	CTX-1-F	GACGATGTCACTGGCTGAGC	499	X92506	Priyadharsini *et al.* (2011)
	CTX-1-R	AGCCGCCGACGCTAATACA			
CTX-M-2	CTX-2-F	GCGACCTGGTTAACTACAATCC	351	X92507	Priyadharsini *et al.* (2011)
	CTX-2-R	CGGTAGTATTGCCCTTAAGCC			
CTX-M-3	CTX-3-F	CGCTTTGCCATGTGCAGCACC	307	AF189721	Priyadharsini *et al.* (2011)
	CTX-3-R	GCTCAGTACGATCGAGCC			
CTX-M-4	CTX-4-F	GCTGGAGAAAAGCAGCGGAG	474	AF252622	Priyadharsini *et al.* (2011)
	CTX-4-R	GCTGGAGAAAAGCAGCGGAG			

4.2.4 Agarose Gel Electrophoresis

A 2% (w/v) agarose gel containing 0.3 µg/mL ethidium bromide was used for the confirmation and analysis of PCR products. Tris-acetate EDTA buffer was used as running buffer. Each well of the gel was loaded with 10 µL of respective PCR reaction mixture and the electrophoresis was conducted at a voltage and current of 100 V and 400 mA respectively for 45 minutes. Ethidium bromide stained DNA products were visualised by UV transillumination and images were produced using the Alpha Imager™ 3400 gel system. A 100 base pair molecular weight marker (Bioline HyperLadder™ II), ranging from 100 bp to 1 kb, was included in one lane of each gel to determine the size of the PCR products.

4.2.5 Sequencing

The PCR reaction products were prepared for sequencing using the Wizard SV gel PCR clean-up kit, in accordance to the manufacturer's instructions. Purified DNA

samples were sequenced at the Central Analytical Facility, University of Stellenbosch. Sequence analysis was performed using MEGA 6 software and NCBI BLAST.

4.3 RESULTS

4.3.1 Antimicrobial Susceptibility Profiles

Enterobacteriaceae (n=60) and MRSA isolates (n=2) (as described in the previous chapter) underwent antimicrobial susceptibility testing. The 60 Enterobacteriaceae isolates included 41 *E. coli*, 14 *K. pneumoniae*, one *E. cloacae*, one *K. oxytoca*, one *P. mirabilis*, one *C. braakii*, and one *C. freundi*. Twelve of the isolates were obtained from patients at admission, to the hospital, while the other 48 isolates were acquired during hospitalisation. Susceptibility testing was achieved through two different methods, namely the Vitek® MS system and Sensititre™ Gram Negative Xtra (GNFX2) 96-well MIC plates.

The antimicrobial resistance rates among the different isolates are listed in Tables 4.3 (Vitek) and 4.4 (Sensititre plates). Detailed, antimicrobial susceptibility profiles per isolate are in the Appendix (Table A6). A higher number of β-lactam antibiotics, both penicillins and cephalosporins, were tested by the Vitek system. The Sensititre plates tested a variety of antimicrobial classes such as third and fourth generation cephalosporins and fourth generation penicillins, while the Vitek system tested several older generation β-lactam drugs.

According to the Vitek® MS system (Table 4.3) complete resistance to ampicillin (100%; *n*=60), cefuroxime (100%; *n*=60), cefuroxime-axetil (100%; *n*=60), cefepime (98%; *n*=59), cefotaxime (100%; *n*=60), ceftazidime (100%; *n*=60), ciprofloxacin (100%; *n*=60), and trimethoprim-sulfamethoxazole (98%; *n*=59) was observed. Almost all isolates retained sensitivity towards carbapenems and colistin, however one *Klebsiella pneumoniae* isolate exhibited intermediate resistance towards ertapenem and a *Proteus mirabilis* isolate towards meropenem. The *Proteus mirabilis* isolate was also intrinsically resistant to colistin.

Table 4.3: Antimicrobial resistance rates of the clinical Enterobacteriaceae isolates as determined through the Vitek® MS system. ($p < 0.01$)

Antimicrobial	Percentage Resistance (%)				
	Admission (*n*=12)	**Hospital-acquired (*n*=48)**	***E. coli* (*n*=41)**	***K. pneumoniae* (*n=14*)**	**Total *(n=60)***
AMP	100	100	100	100	100
AMX	75	77	76	79	77
PT4	25	54	34	100	48
CFA	100	100	100	100	100
CFX	100	100	100	100	100
FEP	92	100	100	100	98
FOT	100	100	100	100	100
FOX	75	77	73	79	77
TAZ	100	100	100	100	100
ERT	8	0	0	7	2
IPM	0	0	0	0	0
MER	8	0	0	0	2
COL	8	0	0	0	2
CIP	100	100	100	100	100
AMI	50	79	73	71	73
GEN	50	69	61	79	65
TGC	17	23	0	71	22
NIT	23	28	5	93	28
SXT	92	100	98	100	98

AMP – Ampicillin; AMX – Amoxicillin-clavulanic acid; PT4 – Piperacillin-tazobactam; CFA – Cefuroxime Axetil; CFX – Cefuroxime; FEP – Cefepime; FOT – Cefotaxime; FOX – Cefoxitin; TAZ – Ceftazidime; ERT – Ertapenem; IPM – Imipenem; MER – Meropenem; COL – Colistin; CIP – Ciprofloxacin; AMI – Amikacin; GEN – Gentamycin; TGC – Tigecycline; NIT – Nitrofurantoin; SXT – Trimethoprim-sulfamethoxazole

Among the isolates obtained from patients at admission only 25% were resistant to piperacillin/tazobactam, 17% to tigecycline, and 50% to gentamycin. The only antimicrobials that seemed to be considerably effective against a large amount of the hospital-acquired isolates, other than the carbapenems and colistin, were tigecycline and nitrofurantoin. It appeared that the hospital-acquired isolates exhibited resistance to a higher number of different antimicrobials than the isolates obtained at admission.

E. coli and *K. pneumoniae* were the two-dominant species isolated from patients. A total of 42 *E coli* isolates were collected. One (2.4%) isolate retained sensitivity towards trimethoprim/sulfamethoxazole, 10 (23.8%) isolates to amoxicillin/clavulanic acid, 11 (26.2%) to amikacin, 11 (26.2%) to cefoxitin, 16 (38.1%) to gentamycin, and 27 (29.3%) to piperacillin/tazobactam. All *E. coli* isolates were susceptible to carbapenems, tigecycline, and colistin. 98% of the *E. coli* isolates (n=39) were susceptible to nitrofurantoin, however two isolates showed intermediate resistance. The *K. pneumoniae* isolates were the group with the highest rates of resistance. Apart from the carbapenems and colistin, the *K. pneumoniae* isolates exhibited resistance rates of greater than 70% towards all other drugs, and 100% resistance to 9 of the 19 drugs tested by the Vitek® MS system. Among the 14 *K pneumoniae* isolates, nitrofurantoin was active against one (7%) *K. pneumoniae* isolate, amoxacillin-clavulanic acid against three (21%) isolates, cefoxitin against three (21%), and gentamycin against four (28%). Four isolates (28%) retained sensitivity toward amikacin, with the remaining 71% exhibiting intermediate resistance. None of the *K pneumoniae* isolates retained sensitivity toward cephalosporins. Of all the antimicrobials tested only the carbapenems and colistin were consistently effective against all *K. pneumoniae* isolates, however one isolate did exhibit intermediate resistance to ertapenem.

Table 4.4: Antibiotic resistance rates of the clinical Enterobacteriaceae isolates determined through the Sensititre™ Gram Negative Xtra (GNFX2) MIC plates. (p = 0.67)

Antimicrobial	Percentage Resistance (%)				
	Admission (*n*=12)	**Hospital-acquired (*n*=48)**	***E. coli* (*n*=41)**	***K. pneumoniae* (*n=14*)**	**Total *(n=60)***
PT4	50	46	34	79	47
TM2	100	100	100	100	100
FEP	100	77	80	86	82
FOT	100	98	83	100	98
TAZ	100	83	85	93	87
AZT	100	81	80	100	85
DOR	0	0	0	0	0
ERT	0	0	0	0	0
IPM	0	0	0	0	0
MER	0	0	0	0	0
COL	8	0	0	0	2
POL	17	2	0	14	5
CIP	100	100	100	100	100
LEVO	100	100	100	100	100
AMI	33	75	76	50	67
GEN	67	71	66	79	70
TOB	67	90	83	93	85
DOX	75	98	98	86	93
MIN	67	98	93	93	92
TGC	8	4	5	0	5
SXT	100	100	100	100	100

PT4 - Piperacillin-tazobactam; TIM2 - Ticarcillin-clavulanic Acid; FEP – Cefepime; FOT – Cefotaxime; TAZ- Ceftazidime; AZT – Aztreonam; DOR – Doripenem; ETP – Ertapenem; IPM - Imipenem; MER – Meropenem; COL – Colistin; POL - Polymyxin B; CIP – Ciprofloxacin; LEVO – Levofloxacin; AMI – Amikacin; GEN – Gentamycin; TOB – Tobramycin; DOX – Doxycycline; MIN – Minocycline; TGC – Tigecycline; SXT - Trimethoprim-sulfamethoxazole

The antimicrobial susceptibility profiles obtained by the two methods i.e., Vitek system and Sensititre plates had similar rates of resistance for, cefotaxime, fluoroquinolones, trimethoprim-sulfamethoxazole and piperacillin-tazobactam (Tables 4.3 and 4.4). However, there were several antimicrobials where the total rates of resistance differed noticeably, namely ertapenem (2% vs 0%), meropenem (2% vs 0%), ceftazidime (100% vs 87%), and tigecycline (22% vs 5%). The susceptibility results for tigecycline were the most discordant between the Vitek system and the Sensititre plates.

According to the Sensititre plates only 5% of the total isolates exhibited resistance to tigecycline, while the Vitek system determined that 22% of the total isolates exhibited resistance. This trend of discordance was greater in the *K. pneumoniae* isolates, as the Sensititre results reported that 100% were susceptible to tigecycline while in comparison, the Vitek system determined that 71% of the *K. pneumoniae* exhibited some degree of tigecycline resistance. However, doxycycline and minocycline were generally ineffective, as it was observed that respectively 93% and 92% of the total isolates were resistant to these drugs.

The Sensititre plates also tested the fourth-generation penicillin, ticarcillin-clavulanic acid and the monobactam, aztreonam, which exhibited total resistance rates of 100% and 85%, respectively.

For the aminoglycosides, the Sensititre plates indicated that of the total isolates 67% and 70% exhibited resistance to amikacin and gentamycin, respectively. While the Vitek system indicated that 73% and 65% of the total isolates were resistant to amikacin and gentamycin, respectively. While the results were very similar the Vitek system indicated higher resistance to amikacin, while the Sensititre plates indicated higher resistance to gentamycin. Sensititre plates indicated that tobramycin had a resistance rate of 85% among the Enterobacteriaceae isolates. This indicates high resistance levels to the aminoglycosides in the clinical isolates.

All isolates were susceptible to the carbapenems and colistin. The Sensititre™ plates also included doripenem, and all isolates (100%), were susceptible. The two isolates that exhibited resistance towards meropenem and ertapenem (*K. pneumoniae* and *P. mirabilis*) according to the Vitek results, were susceptible according Sensititre results. However, while these two isolates were susceptible they exhibited reduced susceptibility compared to the other isolates.

4.3.2 Antimicrobial Resistance Genes

Five different types of ESBL-encoding genes were detected in 93.3% isolates (n=56), namely, *bla*OXA-1, *bla*SHV-28, *bla*CTXM-14, 15 and *bla*OXY2 (Fig 4.2). The two isolates

with reduced carbapenem susceptibility on Vitek testing did not contain carbapenemase encoding genes.

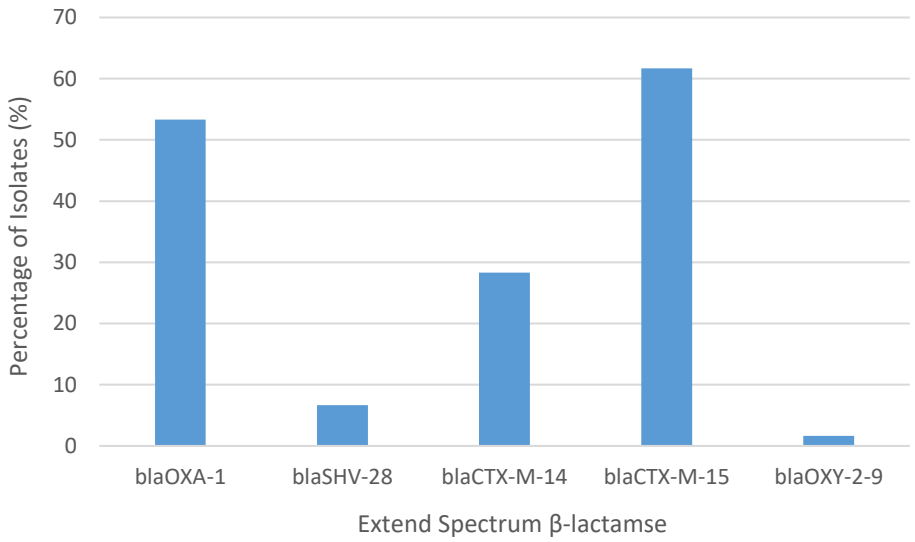

Figure 4.2: Distribution of antibiotic resistance genes among the Enterobacteriaceae isolates

The most prevalent ESBL-encoding gene was *bla*CTX-M-15 (Fig 4.3), detected in 61.7% (n=37) of the Enterobacteriaceae isolates followed by *bla*OXA-1 (Fig 4.4). The *bla*CTX-M-14 (Fig 4.5) and *bla*SHV-26 (Fig 4.6) genes were detected in *E. coli* (n=17) and *K. pneumoniae* isolates (n=4) respectively. The *bla*OXY-2 gene was found in a *K. oxytoca* isolate (Fig 4.7). Appendix 6 provides a list of the specific genes detected in each isolate.

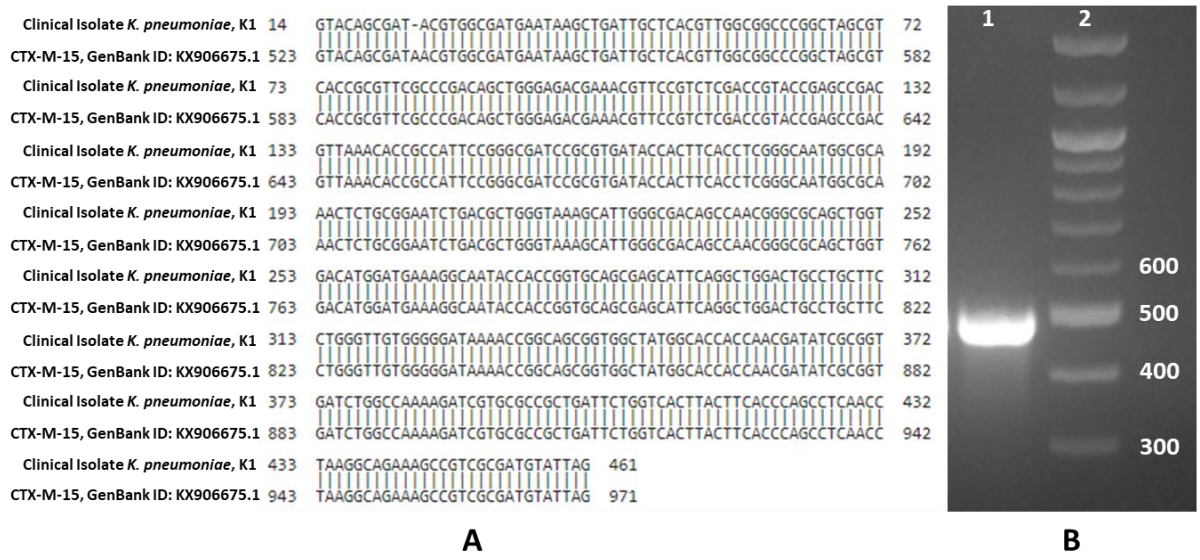

Figure 4.3: (A) DNA sequence of *bla*CTX-M-15 gene from *K. pneumoniae*, K1 aligned with a GenBank *Klebsiella pneumoniae* strain ESBL *bla*CTX-M-15 gene corresponding to position 523-971 with accession number: KX906675.1. The sequence identity = 99%. (B) Representative of PCR screening for *bla*$_{\text{CTX-M-15}}$ gene in clinical *Enterobacteriaceae* isolates; Lane 1: (K1) *bla*$_{\text{CTX-M-15}}$ gene (499bp); Lane 2: Molecular weight marker (100bp Bioline DNA ladder).

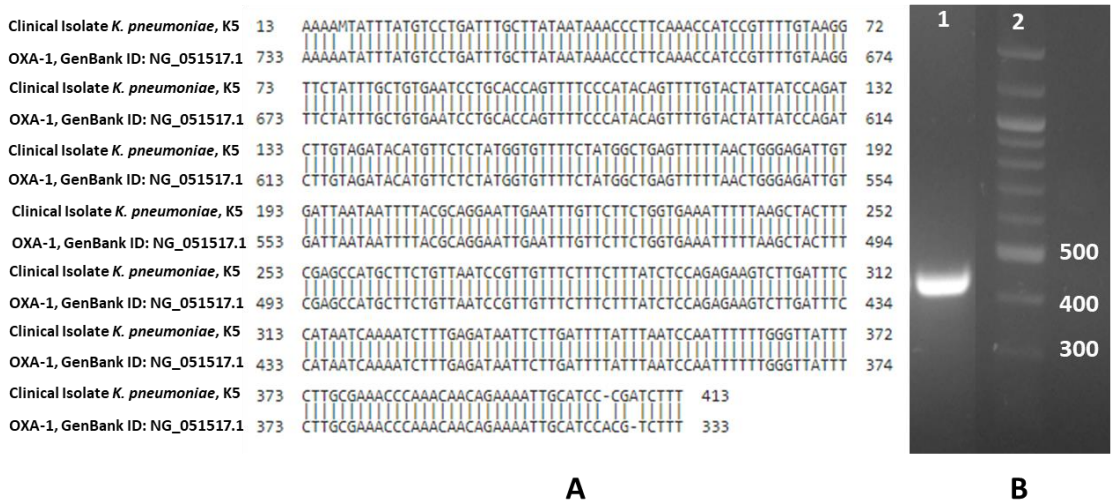

Figure 4.4: (A) DNA sequence of *bla*OXA-1 gene from *K. pneumoniae*, K5 aligned with a GenBank *Klebsiella pneumoniae* strain ESBL OXA-1 (*bla*OXA-1) gene corresponding to position 333-733 with accession number: NG_051517.1. The sequence identity = 99%. (B) Representative of PCR screening for *bla*$_{\text{OXA-1}}$ gene in clinical *Enterobacteriaceae* isolates; Lane 1: (K5) *bla*OXA-1 gene (420bp); Lane 2: Molecular weight marker (100bp Bioline DNA ladder).

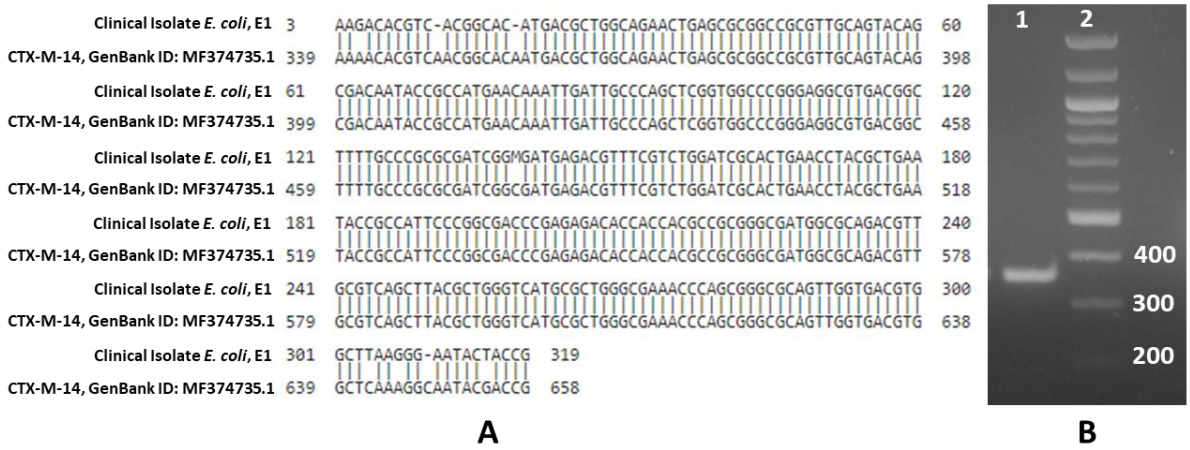

Figure 4.5: (A) DNA sequence of *bla*CTX-M-14 gene from *E. coli,* E1 aligned with a GenBank *Escherichia coli* strain ESBL CTX-M-14 (*bla*CTX-M-14) gene corresponding to position 339-658 with accession number: KX906675.1. The sequence identity = 98%. (B) Representative of PCR screening for *bla*CTX-M-15 gene in clinical *E. coli* isolates; Lane 1: (E1) *bla*CTX-M-14 gene (351bp); Lane 2: Molecular weight marker (100bp Bioline DNA ladder).

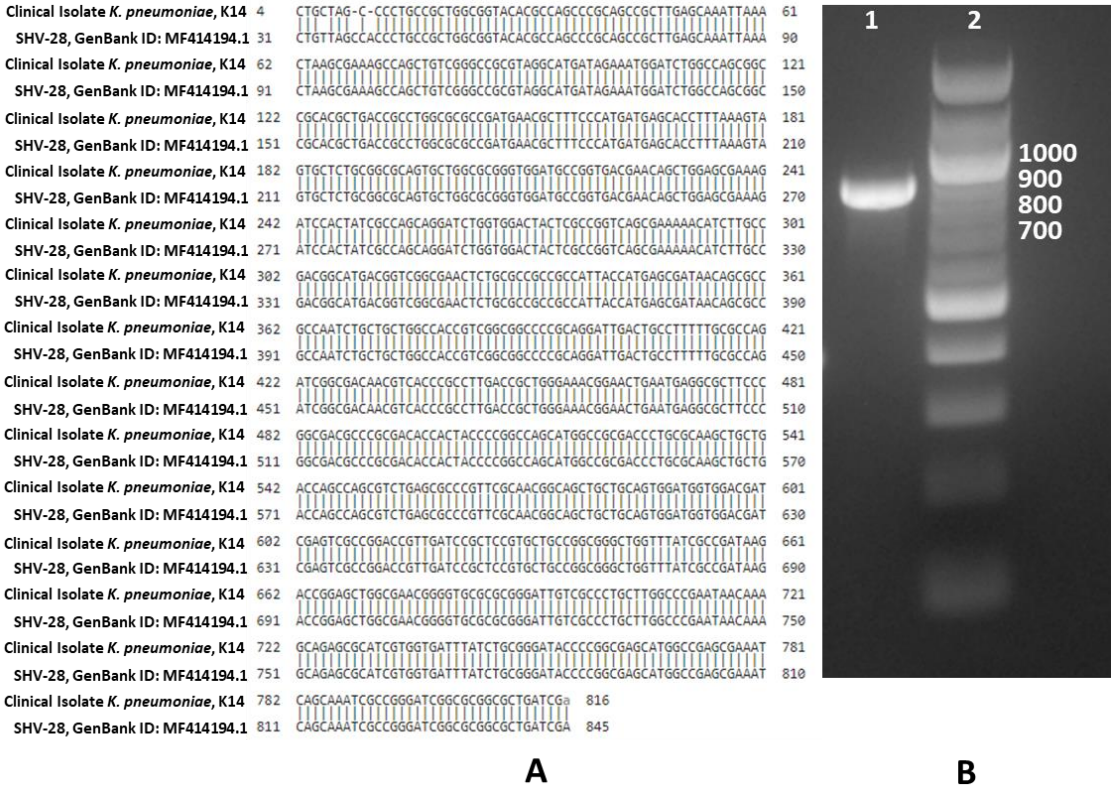

Figure 4.6: (A) DNA sequence of *bla*SHV-28 gene from *K. pneumoniae,* K14 aligned with a GenBank *Klebsiella pneumoniae* strain ESBL SHV-28 (*bla*SHV-28) gene corresponding to position 31-845 with accession number: MF414194.1. The sequence identity = 99%. (B) Representative of PCR screening for *bla*SHV-28 gene in clinical *K. pneumoniae* isolates; Lane 1: (K14) *bla*SHV-28 gene (865bp); Lane 2: Molecular weight marker (100bp Bioline DNA ladder).

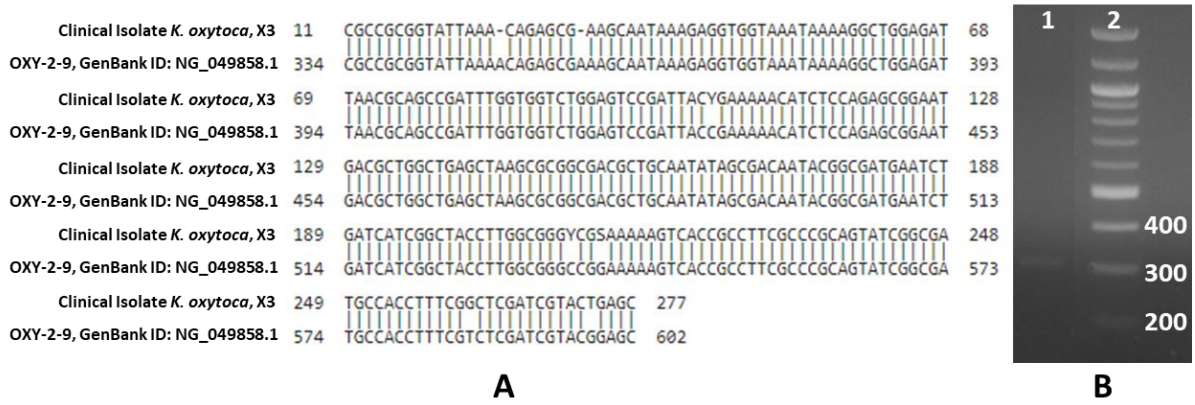

Figure 4.7: (A) DNA sequence of *bla*OXY-2-9 gene from *K. oxytoca,* X3 aligned with a GenBank *Klebsiella oxycota* strain ESBL OXY-2-9 (*bla*OXY-2-9) gene corresponding to position 334-602 with accession number: NG_049858.1. The sequence identity = 97%. (B) Representative of PCR screening for *bla*OXY-2-9 gene in the clinical *K. oxytoca* isolate; Lane 1: (X3) *bla*OXY-2-9 gene (351bp); Lane 2: Molecular weight marker (100bp Bioline DNA ladder).

4.4 DISCUSSION

At present antibiotic resistance is considered to be one of the greatest healthcare threats, with Gram-negative pathogens being of particular concern, as they possess the ability to become resistant to nearly all currently available antibiotics (Ventola, 2015). The Gram-negative bacilli have affected the practice of medicine in all fields, with extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae species, such as *Escherichia coli* and *Klebsiella pneumonia*, being the most common cause of antibiotic resistant hospital-acquired infections (Golkar *et al.* 2014; Ventola, 2015). ESBLs confer resistance towards the majority of β-lactams, including penicillins, cephalosporins, and monobactams (Bush and Jacoby, 2010). Due to the incorporation of ESBL-encoding genes within transferable genetic elements, this has allowed for the horizontal gene transfer of ESBLs, and as a result they have spread worldwide (Shaikh *et al.* 2015). Additionally, the genetic elements which carry ESBL-encoding genes are associated with other antibiotic determinant genes (Vaidya, 2011). Therefore, the spread of ESBL-encoding genes is often accompanied by the spread of other genes associated with antibiotic resistance towards classes of antibiotics other than the β-lactams. Many of the clinical isolates in this study, predominantly *E. coli* and *K. pneumoniae,* were resistant to multiple antibiotics including penicillins, cephalosporins, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole. The National Institute for Communicable Disease antimicrobial

surveillance reported in 2016 that the *E. coli* isolates were resistant to ampicillin/amoxycillin (86%) and trimethoprim-sulfamethoxazole (67%), while the *K. pneumoniae* isolates showed resistance to gentamycin (60%), piperacillin-tazobactam (49%), cefotaxime (69%), ceftazidime (68%), cefepime (68%), and levofloxacin (73%) (Perovic and Chetty, 2016). However, *Enterobacteriaceae* antimicrobial resistance rates from the MDR-TB patients in this study, were higher compared to data from the NICD report. The reason for this may be due to low sample number analysis of only suspected ESBL producers in this study. While the NICD report considered a higher sample numbers, and was not restricted to ESBL producers.

The *E. coli* isolates were susceptible to piperacillin-tazobactam, while all *K. pneumoniae* isolates were resistant according to the Vitek system. In contrast, two *K. pneumoniae* isolates were susceptible to piperacillin-tazobactam using the Sensititre plates. Regarding the other *Enterobacteriaceae* species, *E. cloacae*, *P. mirabilis*, *C. braakii*, and *C. freundi* all retained susceptibility against piperacillin-tazobactam. As indicated by both the Vitek and Sensititre results. It has been suggested that the increased resistance towards piperacillin-tazobactam among *K. pneumoniae* isolates may be due to the hyperproduction of plasmid-mediated *Amp*C and TEM variant ESBLs (Lee *et al.* 2013; Liu and Lui, 2016).

Cefoxitin was the only cephalosporin that retained antimicrobial activity against a considerable number of *Enterobacteriaceae* isolates (n=14), however the majority of isolates did exhibit resistance to this drug. This is not surprising as many ESBL-producers retain susceptibility to cefoxitin upon *in vitro* testing as cefoxitin remains stable against most ESBLs (Guet-Revillet *et al.* 2014). All isolates exhibited resistance to other cephalosporins, with the single exception being the *P. mirabilis* isolate which was susceptible against cefepime. The reasons behind the extremely high cephalosporin resistance rates among the *Enterobacteriaceae* isolates may be likely due to reduced outer membrane permeability though a decrease in the quantity of membrane porins, coupled with the acquisition and production of TEM, SHV, and CTX-M variant ESBLs (Livermore, 1987; Moosdeen, 1997; Gharout-Saita *et al.* 2015). We confirmed that the TEM, CTX-M, and SHV encoding genes were detected throughout the Enterobacteriaceae population analysed within this study. The only cephalosporin

to retain some activity was the second-generation drug, cefoxitin, while third and fourth-generation cephalosporins did not. While cefepime is known to be stable against functional group 1 β-lactamases, they are known to be hydrolysed by group 2 β-lactamases such as CTX-M, SHV, and TEM variants (Angelescu and Apostol, 2001; Bush and Jacoby, 2010). Cefoxitin is known to have several chemical modifications, which include a methoxy group and a carbamoyloxy group, which confers an increased stability towards β-lactamases (Nair and Cherubin, 1978). It may be due to cefoxitin being the only cephalosporin to retain activity, and it has also been suggested that cefoxitin may be used as an alternative treatment option, other than carbapenems, to treat ESBL producing *Enterobacteriaceae* infections (Guet-Revillet *et al.* 2014).

One monobactam class antibiotic, aztreonam, was tested by the Sensititre™ plates. Monobactams are interesting antibiotics as they are the only class of β-lactam antibiotics that are not hydrolysed by metallo-β-lactamases, however monobactam resistance can be conferred by the production of ESBLs, such as TEM and SHV variants (Bush and Jacoby, 2010; Li *et al*. 2017). The majority of Enterobacteriaceae isolates in this study were resistant to aztreonam.

P. mirabilis exhibited intermediate resistance towards meropenem, and one isolate (*K. pneumoniae)* exhibited intermediate resistance towards ertapenem, according to the Vitek results. These isolates were susceptible to these drugs using the Sensititre assay. However, Sensititre results did indicate that these isolates had elevated MICs compared to the other tested isolates, but these MIC values were within the susceptible range. The difference in susceptibility results may indicate variability between the two methods, especially when testing isolates which exhibit an intermediate resistance phenotype. The absence of CRE was unusual, because the high antimicrobial pressure exerted among MDR-TB patients through their treatment regimen would be expected to select for MDR bacteria. Furthermore, carbapenem-resistant and carbapenemase-producing bacteria have been extensively reported within the Port Elizabeth locale (Brink *et al*. 2012; Gqunta, 2014; Masunda, 2014; Govender *et al.* 2015; Annear *et al.* 2017). A low number of carbapenems were prescribed among patients at Jose Pearson (Chapters 2 and 3). This may be the reason for the low incidence of carbapenem resistance.

The fluoroquinolone drugs were ineffective against the *Enterobacteriaceae* isolates. It was observed that both ciprofloxacin and levofloxacin did not exhibit activity against any of the isolates, as outlined in tables 4.2 and 4.3 (Appendix 6). Fluoroquinolone use was documented as a major part of MDR-TB patient treatment therapy in both Chapters 2 and 3, and this is likely to have contributed to the selection of fluoroquinolone resistant isolates among the Jose Pearson patients. There are several transferrable plasmid-encoded determinants which encode for quinolone resistance, such as *Qnr*, *Oqx*AB, *Qep*A, and aminoglycoside acetyltransferase AAC(6′)-Ib-cr, and they are associated with ESBLs (Dalhoff, 2012). The *Qnr*B, *Qnr*S, and AAC(6′)-Ib-cr genes have been previously reported in Port Elizabeth (Gqunta, 2014). As the fluoroquinolone-resistance genes are associated with ESBL-encoding genes through the plasmids on which they are carried, the high use of fluoroquinolones may also be a factor in the spread of ESBLs among the isolates colonising patients at Jose Pearson. Therefore, resistance to ciprofloxacin and levofloxacin among ESBL producing *Enterobacteriaceae* was expected. This is in agreement with other studies where there was correlation between fluoroquinolone resistance and ESBL production (Paterson *et al.* 2000; Bidella *et al.* 2016).

With the exception of one *E. coli* isolate, all *Enterobacteriaceae* isolates were resistant to trimethoprim-sulfamethoxazole. Trimethoprim-sulfamethoxazole is widely used in South Africa to prevent *Pneumocystis* pneumonia in acquired immune deficiency syndrome (AIDS) patients. There was a high rate of HIV infection among the patients of Jose Pearson in this study. Furthermore, it was outlined in chapter 3 that prescription of trimethoprim-sulfamethoxazole among the patients at Jose Pearson was common practice. Resistance to trimethoprim-sulfamethoxazole can be conferred by multiple mechanisms which include cell permeability barriers, gene regulation changes, and mutational or acquired changes of target enzymes (Eliopoulos and Huovinen, 2001). In *E. coli*, resistance may be conferred by a single amino acid substitution in the chromosomally encoded *dhps* gene, while in *K. pneumoniae* two amino acid duplications in the *fol*P gene causes sulfamethoxazole resistance (Eliopoulos and Huovinen, 2001). The high resistance to trimethoprim-sulfamethoxazole may be that liberal prescription of trimethoprim-sulfamethoxazole among Jose Pearson patients is causing a selection pressure for bacterial strains carrying these mutations.

Three aminoglycosides antibiotics were tested against the *Enterobacteriaceae* isolates, which included amikacin, gentamycin, and tobramycin. Resistance rates of aminoglycosides were high. Aminoglycoside resistance is complex and involves multiple mechanisms, many of which can occur simultaneously (Ramirez and Tolmasky, 2010). However, the major resistance mechanism to aminoglycosides, which occurs in clinical settings, is known to be the acquisition of aminoglycoside modifying enzymes which catalyse the inactivation of the antibiotic molecule (Ramirez and Tolmasky, 2010). The AAC(6')-I enzymes have long been known to be present within *Enterobacteriaceae* species in South Africa, and the presence of these enzymes are strongly correlated to amikacin resistance (Miller *et al.* 1997). It is generally observed that Gram-negative pathogens show a greater susceptibility towards amikacin when compared to gentamycin (Gad *et al.* 2011). However, in this study the resistance rates for amikacin were similar to that of gentamycin. This may be attributed to the use of amikacin in the MDR-TB treatment regimen among patients at Jose Pearson.

Multiple tetracycline class antibiotics were tested against the Enterobacteriaceae isolates. This included tigecycline, doxycycline and minocycline, however only tigecycline was tested by the Vitek® MS system. High rates of resistance were observed when assessing both doxycycline and minocycline. However, increased susceptibilities to both these drugs were observed among the isolates obtained at admission. Tigecycline on the other hand, largely retained its activity towards the majority of isolates. Tetracycline resistance is attributed to drug efflux and ribosomal protection (Greer, 2006). The high level of tigecycline resistance among the *K. pneumoniae* isolates may suggest that most of these isolates may belong to single strain-type, or high expression of efflux pumps or has acquired encoding genes for effective ribosomal protection proteins.

Nitrofurantoin was an antimicrobial that was only tested by the Vitek® system. Nitrofurantoin has a unique mechanism of action that targets three different locations within the Kreb's cycle, thus disrupting bacterial metabolism and growth (Cunha *et al*. 2011). Nitrofurantoin is useful in the treatment of UTIs, especially when caused by *E. coli* and Enterococci species. However, species such as, *Proteus mirabilis* and

Pseudomonas aeruginosa, are known to be naturally resistant to the drug (Cunha *et al*. 2011). Nitrofurantoin has also been demonstrated as being effective against multidrug resistant pathogens, such as ESBL producing Gram-negative bacteria and vancomycin-resistant enterococci (VRE) (Grayson and Whitby, 2010). We observed that nitrofurantoin exhibited activity against 95% of *E. coli* isolates. While nitrofurantoin was extremely effective against the *E. coli* isolates, it was not active against the *K. pneumoniae* isolates. While nitrofurantoin has been recorded as being less effective among *K. pneumoniae* compared to *E. coli* isolates, the susceptibility rates of even ESBL-producing *K. pneumoniae* towards nitrofurantoin are known to be quite high (De Miranda *et al*. 2014; El Bouamri *et al.* 2015; Salvatore and Resman-Targoff, 2015). However, in this study almost all *K. pneumoniae* isolates exhibited resistance to nitrofurantoin. Resistance to nitrofurantoin among *E. coli* and other known susceptible species is uncommon, and the phrase “Low resistance potential” has been used to describe Nitrofurantoin (Cunha *et al*. 2011). Resistance to nitrofurantoin has been documented and is likely due to step-wise mutations within the type I nitroreductase genes, which reduces ability of the bacterium to reduce the drug to its active form (Sandegren *et al.* 2008).

Colistin and Polymyxin B were the polymyxin class antimicrobials that was tested. All isolates showed susceptibility towards colistin, except for *P. mirabilis* which is known to exhibit innate resistance. According to the Sensititre tests two *K. pneumoniae* isolates exhibited intermediate resistance to polymyxin B. Colistin is used as the final last line antibiotic and is generally active against even CRE isolates. Recently a mobile polymyxin resistance determinant has been identified, the *mcr*-1 gene. The *mcr*-1 gene has been identified within multiple *Enterobacteriaceae* and *P. aeruginosa* and has been demonstrated to be readily transmissible between these species (Gao *et al.* 2016). There have been multiple reports of *mcr*-1 harbouring *E. coli* isolates in South Africa in both the clinical and agricultural sector (Coetzee, 2008; Perretena *et al.* 2016; Poirel *et al.* 2017).

It was hypothesized that drug resistant isolates would be isolated from the TB patients, and while all isolates exhibited MDR phenotypes, there was a low incidence of resistance to last line antibiotics, such as the carbapenems and colistin. The lack of carbapenem-resistant (CRE) was surprising as it was hypothesized that CRE’s would

have been observed as carbapenem resistance among clinical Gram-negative isolates has extensively been reported within the Port Elizabeth locale (Brink *et al.* 2012; Gqunta, 2014; Masunda, 2014; Govender *et al.* 2015; Annear *et al.* 2017). However, in the previous two chapters it was demonstrated that carbapenems where prescribed to patients within Jose Pearson hospital. Therefore, the lack of a selection pressure from carbapenems is likely the reason for an observed lack of CRE.

In this study two different methods of antimicrobial susceptibility testing (AST) were conducted. The results generated by both methods were interpreted through the Clinical and Laboratory Standards Institute (CLSI) 2017 clinical MIC breakpoint criteria. The isolate antimicrobial susceptibility rates towards the 13 antibiotics that were tested by both methods were similar. However, one major difference was the resistance rates towards tigecycline. The resistance rates of *K. pneumoniae* to tigecycline, as observed through the Sensititre™ assay (Table 4.3), were lower (0%), compared to the Vitek® results, where 71% of the *K. pneumoniae* exhibited resistance to tigecycline (Table 4.2). This is not the first time this trend has been noted. It has been observed and reported by Marchaim *et al*. (2015), that MICs and resistance rates of Enterobacteriaceae isolates towards tigecycline were significantly higher when measured by the Vitek-2 system and compared to broth microdilution techniques (Marchaim *et al.* 2015). However, the explanation for this could not be determined, and it would appear that this is an issue unique to tigecycline (Marchaim *et al.* 2015). The Vitek system, like other automated AST systems, is an extremely useful tool due to its rapid turnover time in determining antimicrobial susceptibility profiles, and thus physicians can prescribe appropriate antimicrobial drugs based on the specific resistance phenotypes of the isolated pathogens. Due to the wide spread use of automated AST systems they have be thoroughly tested and validated. The Vitek system is no exception, and it has been determined that the system performs reliably in determining correct antimicrobial susceptibilities, especially regarding the Enterobacteriaceae (Otto-Karg *et al.* 2009; Bobenchik *et al.* 2015). This further suggests that the discrepancy in tigecycline susceptibilities seen in this study is likely due to characteristics of the antibiotic, rather than the AST methods.

In total six different antimicrobial resistance genes were detected, all of which were β-lactamase encoding genes. The most prevent was the *bla*CTX-M-15 gene. The CTX-

M type β-lactamases are the common and wide spread of the extended-spectrum β-lactamase (ESBL) enzymes so it was not surprising that CTX-M encoding genes were observed harboured within the isolates of this study (Bonnet, 2004). The two observed CTX-M encoding genes, *bla*CTX-M-14 and *bla*CTX-M-15, belonged to different CTX-M groups, groups 9 and 1 respectively (Bonnet, 2004). In this study *bla*CTX-M-15 was observed in different Enterobacteriaceae species, while *bla*CTX-M-14 was observed exclusively within the *E. coli* isolates. Both CTX-M-14 and CTX-M-15 have been previously reported within clinical Enterobacteriaceae isolates within Port Elizabeth, and extensively reported throughout South Africa (Gqunta, 2014).

SHV-28 was observed exclusively within the *K. pneumoniae* isolates. SHV is known to be one of the more common ESBLs observed among *K. pneumoniae* (Babini and Livermore, 2000; Haanperä *et al.* 2008). This was not the first report of SHV within Port Elizabeth (Gqunta, 2014), however it was the first report of the SHV-28 variant. *K. pneumoniae* isolates simultaneously harbouring *bla*CTX-M-15, *bla*SHV-28, and *bla*TEM-1, as seen in this study, have been previously reported in Copenhagen, Denmark (Nielsen *et al.* 2011). While the SHV-28 variant has been reported in other African countries, such as Gabon and Tanzania, to the best of our knowledge, this appears to be the first report of the SHV-28 variant in South Africa (Ndugulile *et al.* 2005; Moussounda *et al.* 2017). Multiple studies have reported the *bla*SHV gene among *K. pneumoniae* isolates throughout South Africa, however the dominant variants appear to be SHV-2, SHV-5, and SHV-23 (Pitout *et al.* 1998; Paterson *et al*. 2003; Essack *et al.* 2004; Peirano *et al.* 2011; Kaba *et al.* 2016; De Francesco *et al.* 2017). As a group, the *K. pneumoniae* isolates exhibited the most drug resistant phenotypes out of all the Enterobacteriaceae isolates, however only four of the *K. pneumoniae* isolates harboured the *bla*SHV-28 gene. While these four isolates displayed resistant profiles, there could be other mechanisms in the *K. pneumoniae* isolates accounting for the broad antibiotic resistant phenotype.

The *bla*OXY-2 gene was detected in the one *Klebsiella oxytoca* isolate. The OXY-1 and OXY-2 group are chromosomally-encoded Ambler class A β-lactamases found within the *K. oxytoca* species (Sirot *et al.* 1998). Hyperproduction of *K. oxytoca* β-lactamases (OXY) is responsible for resistance to several β-lactam antibiotics, such as penicillins, cephalosporins, and aztreonam, additionally it has been observed that

a single amino acid substitution (Ser→Gly at position 130) in the enzyme results in reduced susceptibility to β-lactamase inhibitors (Sirot *et al.* 1998; González-López *et al.* 2009). *K. oxytoca* OXY-6 gene was previously reported in clinical isolates from South Africa (Fevre *et al.* 2005). However, this is the first report on the *bla*OXY-2 gene in South Africa. Interestingly, in this study the Vitek system flagged the *K. oxytoca* isolate harbouring the OXY-2 gene as an ESBL-producer. This may highlight an inaccuracy and limitation of the Vitek system as OXY-2 is not a true ESBL.

TEM-1 was observed in almost half of the Enterobacteriaceae isolates. TEM-1 was one of the first reported β-lactamase enzymes, and has been reported worldwide. The presence of this gene within the isolates of this study was not surprising. While TEM-1 (and TEM-2) is able to hydrolyse penicillins, and first and second generation cephalosporins, it lacks activity towards the extended-spectrum cephalosporins, and is therefore not regarded as a ESBL like the other TEM-variant enzymes (Ghafourian *et al.* 2015).

Isolates exhibited resistance to more antimicrobials if they contained a CTX-M encoding gene. No ESBL-encoding genes were detected among four isolates. Three of these isolates, two *E. coli* (E31 and E40) and the one *E. cloacae* (B1), which may have been conferred by the overproduction of chromosomally encoded *Amp*C β-lactamase (Jacoby, 2009). No ESBL encoding genes were detected in a *Proteus mirabilis* isolate which may have harboured a plasmid-mediated *Amp*C gene, or possibly even a serine-type ESBL-encoding gene which was not screened for in this study.

Analysis of the antimicrobial susceptibility profiles indicated that patients became colonised by MDR bacteria within one month of admission mostly with ESBL producing Enterobacteriaceae. ESBL encoding genes detected in resistant isolates included *bla*SHV, *bla*OXA-1, *bla*CTX-M-14, *bla*CTX-M-15, and *bla*OXY-9-2: while no carbapenem resistance was observed. It was thus important to investigate the genetic similarity and molecular epidemiology of the resistant isolates.

CHAPTER FIVE

Molecular Typing of Multidrug-Resistant *Klebsiella pneumoniae* Isolates Colonizing MDR-TB Patients

5.1 INTRODUCTION

Molecular typing is essentially a group of techniques for “fingerprinting” bacterial isolates based on an analysis of their genetic or nucleic acid characteristics (Foxman and Riley, 2001). Molecular typing techniques have become increasingly integrated with epidemiology, and as a result given rise to the concept of molecular epidemiology (Foxman and Riley, 2001). Molecular epidemiology has become an umbrella term, as it has been developed and applied in several different epidemiological fields, such as cancer epidemiology, environmental epidemiology, and infectious disease epidemiology (Foxman and Riley, 2001; Ioannidis, 2007). The definition of molecular epidemiology that was first published by Tompkins in 1994, and defined molecular epidemiology as “the application of molecular biology to the study of infectious disease epidemiology” (Foxman and Riley, 2001; Tompkins, 1994).

Molecular techniques can assist in infectious disease epidemiology by rapid pathogen identification, detection of virulence factors, and molecular typing (Eybpoosh *et al*. 2017).. The benefit of molecular typing can be that it allows for specific sequence types or strain identities to be assigned to the isolated pathogens, based on the sequence or structure of part or the whole genome of that isolated pathogen (Lin *et al.* 2014). This then allows for the comparison of the similarity and interrelatedness of pathogens isolated from the concerned patients, or even from samples taken from the environment surrounding those patients (Lin *et al.* 2014). If the information generated by molecular typing is applied effectively with patient data, many things may be elucidated concerning an infectious disease outbreak. This includes the identification or strain-type of the microorganism/s responsible for the outbreak, the source or environmental reservoir of the infectious disease, how the microbial strains circulate within the environment and transmit between the patients of the study, and the probability of spread of that specific microbial strain (Eybpoosh *et al*. 2017).

There are different types of molecular typing techniques of which the most commonly used include Pulsed-Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Automatic Ribotyping, rep-PCR, and Multi-Locus Sequence Typing (MLST) (Lin *et al.* 2014). Each technique exploits a different aspect or genetic characteristic in order to type the microbial isolate concerned and each has its own advantages and weaknesses. For example, while the RAPD and rep-PCR methods have very fast turn over times, they exhibit a low reproducibility, and while PFGE has high discriminatory power, it is also time-intensive and requires expensive, specialized equipment (Lin *et al.* 2014; Tabit, 2016). In this study we used the MLST technique.

Multilocus sequence typing (MLST) was first developed in 1998, and was used to type *Neisseria meningitidis* isolates (Belén *et al*., 2009). The basis of MLST is to use PCR to amplify several specific, well conserved genes. The selected genes customarily include housekeeping genes. The genes targeted in the MLST protocol depends on the species which is being analysed by MLST. The gene amplicons are then purified and sequenced. Based on the DNA sequences a specific allele number identity can be assigned to each gene. A sequence type can be assigned to each isolate based on the collection of allele numbers of that isolate. The major advantages of MLST are that it detects changes or differences within the isolates' genome at a nucleotide level, and that it makes use of readily available and accessible techniques and equipment, principally PCR, gel electrophoresis, and DNA sequencing. This makes MLST a very versatile and readily available method. Additionally, as DNA sequences are generated through the MLST method, this has allowed the development of online databases where researchers all around the world can access and share MLST data. This has made MLST a powerful epidemiological tool.

While MLST has become the "gold standard" of molecular typing for many species, it is traditionally conducted in a way that is time-consuming and expensive (Larsen *et al.* 2012). As the costs of whole-genome sequencing (WGS) continue to decline, this increases the availability of WGS to a greater number of scientists (Larsen *et al.* 2012; Salipantea *et al.* 2017). WGS can theoretically distinguish between strains which differ by a single nucleotide, and it has been demonstrated how the MLST protocol can be

applied to sequence data generated by WGS (Larsen *et al.* 2012; Salipantea *et al.* 2015). In addition to extremely high resolution molecular typing, WGS also provides a large amount of genetic data which would be extremely useful to infectious disease epidemiology. Theoretically, with the data generated by WGS one could identify the full spectrum of virulence and antimicrobial resistance genes present within the analysed microbial isolate. However, bacterial strains may undergo diversification in the duration of infection within an individual patient, and presently it is still unclear to what is the accepted number of genome-wide polymorphisms which identify isolates as belonging to the same outbreak (Salipantea *et al.* 2015). This is where the extremely high resolution of WGS may become somewhat of an issue, as isolates which are genetically distinct but very closely related may share a common source within an outbreak (Salipantea *et al.* 2015). Therefore, coupling MLST, or a similar method, with WGS may be quite beneficial, as MLST could assess the data generated by WGS in a resolution more applicable within a clinical setting, as currently the polymorphism rates within the genes assessed by MLST are understood in far greater detail than those of the entire genome of a species.

This chapter assessed the genetic relatedness of *K. pneumoniae* isolates and sought to establish the relatedness of the isolates, and whether isolates were being carried into the hospital by patients who were admitted to the hospital already colonized, or if the isolates were already present and circulating within the hospital environment.

5.2 MATERIALS AND METHODS

5.2.1 Chromosomal DNA Extraction

All *K. pneumoniae* isolates were grown on MacConkey culture media for DNA extraction. Chromosomal DNA was extracted from cultures (200µl) using the QIAprep® Blood and Tissue DNeasy kit (Qiagen). Extracted DNA was quantified using the NanoDropTM Spectrophotometer (Thermo Scientific) at 260 nm.

5.2.2 Molecular Typing of Isolates by Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) was performed according to the modified protocol as outlined by Diancourt *et al.* (2005) on the 14 multidrug-resistant *K. pneumoniae* isolates. Chromosomal DNA was used as the template for PCR amplification of the seven housekeeping genes (*rpo*B, *gap*A, *mdh*, *pgi*, *pho*E, *inf*B, and *ton*B). Primers were synthesised by Integrated DNA Technologies (WhiteSci) and re-constituted in TE buffer (Lonza) according to the manufacturer's instructions. PCR products were analysed by agarose gel electrophoresis (as described in Chapter 4, section 4.2.4) to confirm gene amplification.

Table 5.1: PCR primers for the amplification and sequencing of the *K. pneumoniae* MLST genes.

Gene	Sequence (5' to 3')		Size (bp)
	Forward	Reverse	
*rpo*B	GTTTTCCAGTCACGACGTTGTGG CGAAATGGCWGAGAACCA	TTGTGAGCGGATAACAATTTCGA GTCTTCGAAGTTGTAACC	501
*gap*A	GTTTTCCCAGTCACGACGTTGTAT GAAATATGACTCCACTCACG	TTGTGAGCGGATAACAATTTCCT TCAGAAGCGGCTTTGATGGCTT	450
mdh	GTTTTCCCAGTCACGACGTTGTAC CCAACTCGCTTCAGGTTCAG	TTGTGAGCGGATAACAATTTC CCGTTTTTCCCCAGCAGCAG	477
pgi	GTTTTCCCAGTCACGACGTTGTAG AGAAAAACCTGCCTGTACTGCTG GC	TTGTGAGCGGATAACAATTTCCG CGCCACGCTTTATAGCGGTTAA	432
*pho*E	GTTTTCCCAGTCACGACGTTGTA ACCTACCGCAACACCCAGTTCTTC GG	TTGTGAGCGGATAACAATTTCTG ATCAGAACTGGTAGGTGAT	420
*inf*B	GTTTTCCCAGTCACGACGTTGTAC TCGCTGCTGGACTATATTCG	TTGTGAGCGGATAACAATTTCCG CTTTCAGCTCAAGAACTTC	318
*ton*B	GTTTTCCCAGTCACGACGTTGTAC TTTATACCTCGGTACATCAGGTT	TTGTGAGCGGATAACAATTTCAT TCGCCGGCTGRGCRGAGAG	414
Universal sequencing primers	GTTTTCCCAGTCACGACGTTGTA	TTGTGAGCGGATAACAATTTC	

PCR Conditions: (2 mins at 94°C; 35 cycles of 30 secs at 94°C, 1 min at 50°C, and 30 secs at 72°C; 5 mins at 72°C for the final extension)

Table 5.2: Identities of the genes selected for the *Klebsiella pneumoniae* multilocus sequence typing protocol (Diancourt *et al.* 2005).

Locus	Gene Identity
*rpo*B	β subunit RNA polymerase
*gap*A	Glyceraldehyde-3-phosphate dehydrogenase
mdh	Malate dehydrogenase
pgi	Glucose-6-phosphate isomerase
*pho*E	Outer membrane pore protein E precursor
*inf*B	Translation initiation factor IF-2
*ton*B	Protein tonB

5.2.3 Sequencing and Phylogenetic Analysis

PCR products were purified using the SV Wizard PCR clean-up kit (Promega) in accordance with the manufacturer's instructions (as described in Chapter 4, section 4.2.4). Purified DNA samples were sequenced at the Central Analytical Facility (CAF), University of Stellenbosch. A sequence analysis was performed using Chromas 2.4.3 and MEGA 6 software. Based on the allele sequences each isolate was assigned a sequence type number using the allelic profiles available on the online *K. pneumoniae* MLST database (PasteurMLST, 2017). Dendrograms were constructed using the neighbour joining (NJ) method with the Kimura 2 parameter model with gamma correction and 1,000 bootstrap replicates for all sequences using the MEGA 6 software. Analyses were performed using the concatemer sequence of the seven sequenced genes used for the MLST assessment.

5.3 RESULTS

Multilocus sequence typing (MLST) was used to assess the interrelationship between the 14 *Klebsiella pneumoniae* isolates obtained through screening samples (n=14) isolated from MDR-TB patients at Jose Pearson hospital. The seven housekeeping genes (*rpo*B, *gap*A, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) were successfully amplified and sequenced, and allele numbers were assigned to each gene for each isolate so that sequence types (STs) could be assigned. Unfortunately, allele numbers could not be assigned for the *gap*A and *ton*B genes for isolates K8 and K9 as the sequencing results for these two genes were inconclusive. The isolates displayed eight different sequence types (Table 5.3). It was observed that the *K. pneumoniae* PasteurMLST

database did not contain STs that matched the allele profiles of the *K. pneumoniae* isolates observed with this study. Therefore, letter codes (A, B, C, etc.) were used to differentiate between the different STs while we await the profile and isolate information generated by this study to be incorporated into the PasteurMLST database. The observed sequence types were diverse, however several of the profiles were repeated namely, ST A, B, D, E, and F. Isolates K2 and K3, which were isolated from the same patient exhibited the same sequence type, ST B (Table 5.3). Isolates K5 and K14 were isolated from the same patient, however they exhibited different sequence types of ST C and ST H respectively (Table 5.3).

Table 5.3: Allelic profiles and sequence types (ST) assigned to the *Klebsiella pneumoniae* isolates according to the PasteurMLST multilocus sequence typing protocol and database. (n=14).

Isolate	Patient No.	Pathogen Source	Multilocus Sequence Type Profile							
			***rpo*B**	***gap*A**	***mdh***	***pgi***	***pho*E**	***inf*B**	***ton*B**	**ST**
K1	32	Hospital	46	3	1	1	177	3	329	**A**
K2	30	Hospital	109	4	2	52	177	1	7	**B**
K3	30	Hospital	109	4	2	52	177	1	7	**B**
K4	36	Baseline	46	3	1	1	177	3	329	**A**
K5	4	Hospital	109	2	1	3	177	6	77	**C**
K6	7	Hospital	114	3	6	1	177	4	40	**D**
K7	1	Hospital	137	1	1	1	177	1	1	**E**
K8	8	Hospital	146	-	154	166	177	115	-	**F**
K9	29	Hospital	146	-	154	166	177	115	-	**F**
K10	2	Hospital	114	3	6	1	177	4	40	**D**
K11	10	Baseline	137	1	1	1	177	1	1	**E**
K12	34	Hospital	67	3	1	1	177	3	382	**G**
K13	13	Baseline	137	1	1	1	177	1	1	**E**
K14	4	Hospital	131	2	2	1	177	3	54	**H**

Table 5.4: *Klebsiella pneumoniae* (n=14) MLST genotypes and ESBL genes with respective patient information.

Isolate	Sequence Type	Pathogen Source	ESBL/s	Patient No.	Patient Type	Patient Admission Date	Isolate Acquisition Date	Ward
K1	A	Hospital	CTX-M-15	32	Healthcare	05 August	02 September	1
K2	B	Hospital	CTX-M-15; SHV-28	30	Healthcare	15 August	13 September	1
K3	B	Hospital	CTX-M-15	30	Healthcare	15 August	13 September	1
K4	A	Baseline	CTX-M-15	36	Healthcare	23 September	23 September	1
K5	C	Hospital	OXA-1	4	Community	26 August	23 September	1
K6	D	Hospital	OXA-1; CTX-M-15	7	Community	01 September	01 October	2
K7	E	Hospital	CTX-M-15	1	Community	11 August	05 October	1
K8	F	Hospital	OXA-1; CTX-M-15; SHV-28	8	Community	23 September	21 October	1
K9	F	Hospital	CTX-M-15	29	Community	05 August	28 October	1
K10	D	Hospital	OXA-1; CTX-M-15	2	Community	05 August	28 October	1
K11	E	Baseline	OXA-1; CTX-M-15; SHV-28	10	Community	08 November	08 November	1
K12	G	Hospital	OXA-1; CTX-M-15	34	Community	07 September	08 November	1
K13	E	Baseline	OXA-1; CTX-M-15; SHV-28	13	Community	23 November	23 November	1
K14	H	Hospital	OXA-1; CTX-M-15	4	Community	26 August	23 November	1

Baseline: Isolates obtained upon patient admission

Hospital: Isolates obtained during patient hospitalisation

The majority of patients who became colonized with *K. pneumoniae* were present in Ward 1, as only one patient from Ward 2 was colonized by hospital-acquired *K. pneumoniae* (Table 5.4). Three of the patients who became colonized with *K. pneumoniae* were admitted to Jose Pearson from other healthcare facilities, one of these three patients exhibited colonization by *K. pneumoniae* at baseline (Table 5.4). All other patients were admitted from the community.

The DNA sequences used to determine the allelic profiles of the isolates were used to construct the phylogenetic tree or dendrogram (Figure 5.1). A number of clusters were detected among isolates (Figure 5.1). Although several different sequence types were observed, many of these isolates were genetically similar (K1, K2, K3, K4, K5, K6, K10, and K12) (Figure 5.1).

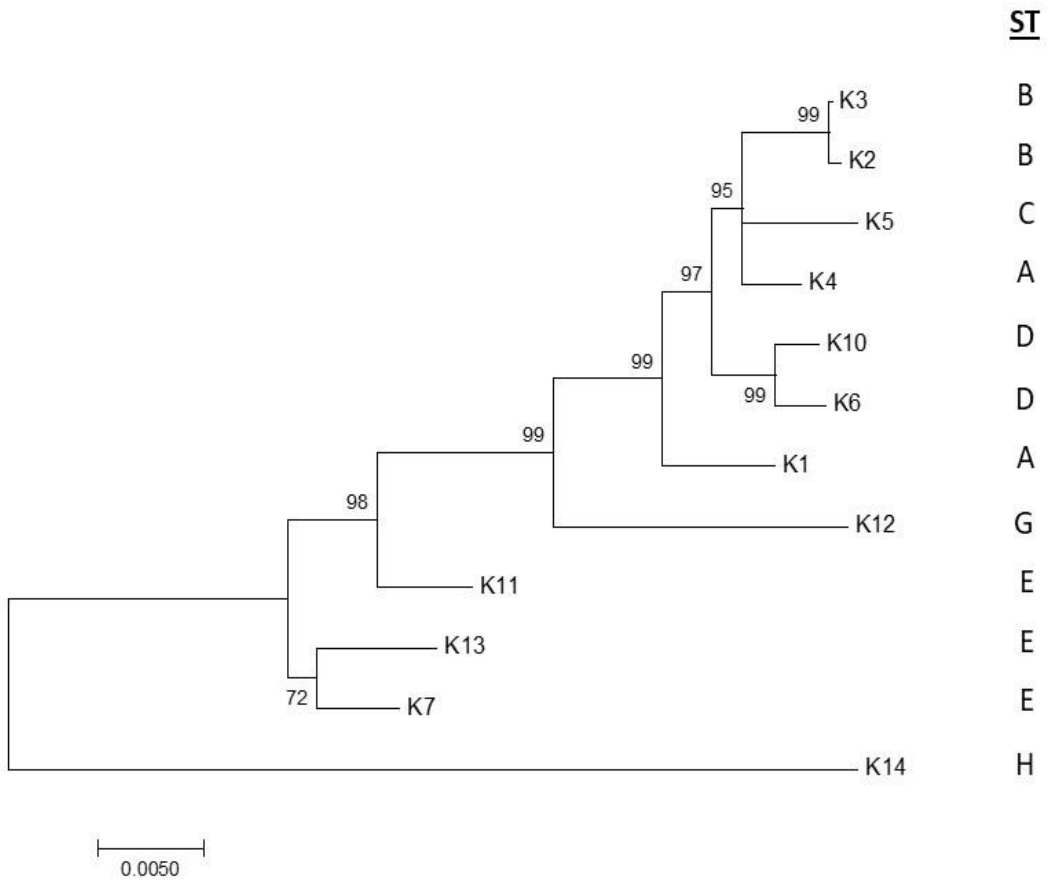

Figure 5.1: Neighbour joining dendrogram generated from the concatemer of the seven sequenced MLST genes of twelve of the *K. pneumoniae* isolates (n=12). The scale bar (0.0050) indicates sequence divergence by the number of nucleotide substitutions per site. Bootstrap percentages retrieved in 1000 replications are displayed at the branch nodes.

The optimal tree with the sum of branch length = 0.12915868 is shown (figure 5.1). The confidence probability multiplied by 100, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences, the concatemer of the seven sequenced MLST genes per isolate. All positions containing gaps and missing data were eliminated. There was a total of 2971 positions in the final dataset. Isolates K8 and K9 were excluded from the dendrogram, as the sequence data for two of the MLST genes were inconclusive.

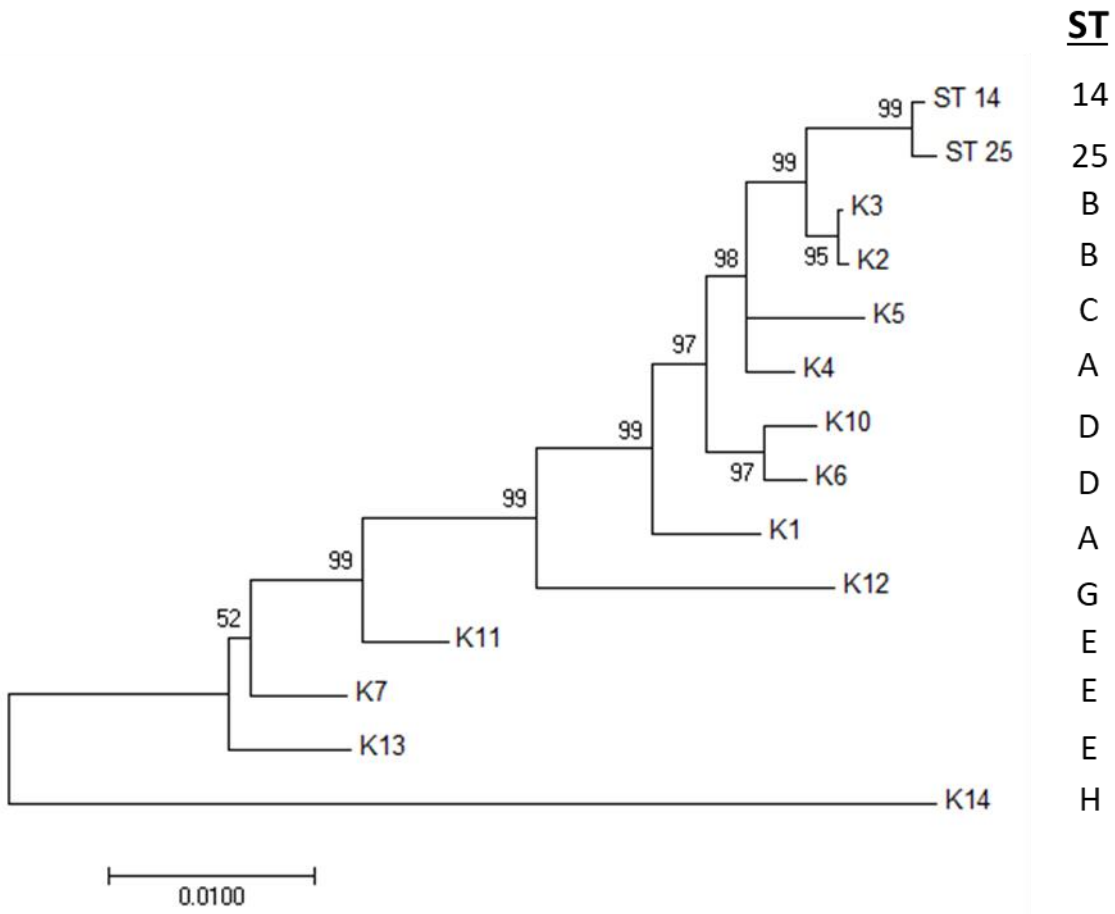

Figure 5.2: Neighbour joining dendrogram comparing the concatemer of the seven sequenced MLST genes of the twelve *K. pneumoniae* isolates of this study and of the concatemers of the two MLST sequence types observed among other South African *K.* pneumoniae isolates (ST 14 and ST 25) (Jacobson *et al.* 2015; Struve *et al.* 2015). The scale bar (0.0100) indicates sequence divergence by the number of nucleotide substitutions per site. Bootstrap percentages retrieved in 1000 replications are displayed at the branch nodes.

The optimal tree with the sum of branch length = 0.14312640 is shown (Figure 5.2). The confidence probability multiplied by 100, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences, 12 were the MLST concatemer of the isolates within this study and two were the MLST concatemer of the other *K. pneumoniae* sequence types observed in South Africa. All positions containing gaps and missing data were eliminated. There was a total of 2831 positions in the final dataset. All evolutionary analyses were conducted in MEGA7.

The same DNA sequences used to construct the phylogenetic tree (Figure 5.1) were used to construct an additional phylogenetic tree (Figure 5.2). However, figure 5.2 included two additional sequences in its construction. These sequences were the concatemers of the MLST gene sequences for the other two *K. pneumoniae* sequence types that have been observed within South Africa, namely ST 14 and ST 25 (Jacobson *et al*. 2015; Struve *et al*. 2015). This allowed for the genetic comparison of the *K. pneumoniae* isolates described in this study and the isolates described in Jacobson *et al.* (2015) and Struve *et al.* (2015). It was observed that ST 14 and ST 25 exhibited great genetic similarity, and that these sequence types also showed considerable genetic similarity to several the sequence types elucidated in this study (Figure 5.2).

5.4 DISCUSSION

Multilocus sequence typing (MLST) is a high-resolution genetic typing method, which is able to distinguish between different strain of pathogens which effect human health (Pérez-Losada *et al.* 2013). MLST is very useful in a developing country, such as South Africa. This is due to the versatility of the method, and the wide-spread availability of PCR and DNA sequencing. This chapter provides insight into the different strain types of *K. pneumoniae* present in Jose Pearson TB hospital.

The sequence types exhibited by the *K. pneumoniae* isolated in this study were unique, in the sense that isolates with matching allele profiles had not been previously reported. This is not surprising, as this appears to be the first study which has typed *K. pneumoniae* by MLST, in the Eastern Cape. There is one other published South African study which analysed *K. pneumoniae* through MLST, this study was based in Cape Town (Jacobson *et al.* 2015). Therefore, no other MLST data is available for *K. pneumoniae* within Port Elizabeth and the surrounding area. A similar situation was observed in a previous study where an outbreak of *P. aeruginosa* was typed through MLST where unique sequence types were observed (Annear *et al.* 2017).

In total, 14 K. *pneumoniae isolates* were obtained from 12 patients in the cohort of 37 patients, as described in chapter 3. The majority of *K. pneumoniae* isolates were hospital-acquired (*n*=11). Among the hospital-acquired isolates four sequence types were repeated, namely ST A, B, D and F while three sequence types were seen in one case each, namely ST C, G and H. In addition, two sequence types were exhibited by both baseline and hospital-acquired isolates, namely ST A and E.

Isolates K2 and K3 were isolated from the same patient and exhibited an identical sequence type of ST B. These isolates were obtained from the same swab sample, so it is highly likely that they were clonal repeats of the same organism. Isolates K6 and K10 were the only case of isolates that were obtained from different patients and that shared the same sequence type (ST D) that was only observed among the hospital-acquired isolates. Isolates K8 and K9 were isolated from two different patients at a similar time. Isolates K8 and K9 appeared to have the same sequence type as five of the seven allele numbers match, and the observed allele numbers of these isolates were different from the other isolates. However, sequence data obtained for two of the genes (*gap*A and *ton*B) for both K8 and K9 were inconclusive, and

therefore it cannot be determined whether these isolates truly shared the same sequence type. Isolates K5, K12, and K14 all exhibited unique sequence types, namely ST C, G, and H respectively. Isolates K5 and K14 were both isolated from the same patient, however they were isolated approximately two months apart from each other. This was the only observed case of a patient being colonized by two different genetically distant isolates.

Three of the *K. pneumoniae* isolates were isolated from patients at baseline, upon admission to the hospital. This suggests that these patients were colonized by ESBL-producing *K. pneumoniae* within the community or from a previous healthcare facility. Two of these baseline isolates, namely K11 and K13, exhibited identical sequence types. This is illustrated by both table 5.3 and figure 5.1. As these patients came into the hospital already colonized by these isolates, one would expect that they would be genetically distinct from isolates that appeared to be circulating within the hospital, however there appeared to be a hospital-acquired isolate, K7, that shared a sequence type with these two isolates. All patients colonized by isolates of ST E (K7, K11, and K13) were admitted to Jose Pearson from the community, this would suggest that these isolates were community acquired infections and present within the surrounding community. However, isolate K7 was only isolated from the patient after their second month of hospitalization, and this was approximately a month before the patients carrying isolates K11 and K13 were admitted to Jose Pearson. Interestingly, it appeared that the isolates exhibiting ST E were somewhat separate from the other strains. While the other sequence types appeared closely related the ST E seemed to differ genetically from the other isolates. Isolate K4 was interesting as it was isolated from a patient at baseline, however it shared a sequence type with K1, which was isolated from a patient during their hospitalization. Interestingly, the both patients carrying these isolates were transferred to Jose Pearson from the same previous healthcare facility. The patient carrying isolate K4 was admitted to Jose Pearson on 23/09/2016, however isolate K1 was collected on 02/09/2016. Therefore, K4 was not the first isolate of ST A to be present within Jose Pearson. As both these patients were transferred from the same hospital, it may be possible that isolate K1 colonized the patient during their previous hospitalization and was not detected by the baseline swabbing upon admission to Jose Pearson. Then after a month being exposed to the antibiotic selection pressure by the TB drug regimen, K1 may have become more prevalent within the patient, and was more easily detected.

During hospitalization, patients were committed to either Ward 1 or Ward 2. While the majority of patients in this study were confined to Ward 1, *K. pneumoniae* isolates were collected from patients of both wards. Isolate K6, which exhibited a sequence type of ST D, was isolated from a patient in Ward 2, one month after hospitalization. All other cases of *K. pneumoniae* were reported in patients either admitted to or present in Ward 1. There are several other wards in Jose Pearson, however this study did not contain patient representatives of these wards. This is due to the lack of involvement with this study of healthcare professionals assigned to these wards. Therefore, regular and consistent screening of these patients could not be achieved.

MLST can be used to group bacterial strains that are closely related into clonal complexes (Scally *et al.* 2005). A clonal complex can be defined as a group of isolates having the same sequence type, determined by the seven alleles analysed by the MLST protocol (Enright and Spratt, 1998). However, a clonal complex may include isolates which share at least four or five alleles out of seven of that observed in the an original “consensus” or “ancestral” clone (Feil *et al.* 2000; Feil *et al.* 2001). While eight different sequence types were observed within this study, many of the sequence types shared four of the seven allele identities. Furthermore, many of the isolates can be observed clustering together in the generated phylogenetic tree (K1, K2, K3, K4, K5, K6, K10, and K12) (Figure 5.1), suggesting a degree of genetic similarity between these isolates.

Unfortunately, due to the nature of the MLST results we can only state that the *K. pneumoniae* isolates seen in the hospital were genetically diverse. Due to the small sample size and lack of environmental samples we cannot comment whether there is a large number of different strains endemic to the hospital, or whether these isolates are being carried in from the community and spread by patients. A possible reason why several different sequence types were observed among the Jose Pearson patients could be that patients are often transferred to Jose Pearson from other healthcare facilities to receive specialized treatment for MDR-TB. Therefore, it may be possible that various strains of hospital-acquired pathogens are being carried into Jose Pearson by transferred patients. The small patient sample size was a great limitation within this study, as we only had access to 14 isolates to represent and compare the different strains of *K. pneumoniae* present within the hospital. The major limiting factor was the amount of time and funding available for the study. Molecular typing the *E. coli* isolates, as described in the previous chapters, may be an alternative option for

demonstrating the spread and dissemination of MDR Enterobacteriaceae isolates within Jose Pearson hospital, as there would have been a greater number of isolates to compare. However, this option was unavailable to us due the cost and time associated with typing 41 isolates using the MLST method.

Another limitation to this study was the lack of previous studies done on the genetic diversity of bacterial pathogens in the Eastern Cape, South Africa. This study lacks reference literature regarding the spread and genomic information of *K. pneumoniae*, and other Enterobacteriaceae, in Port Elizabeth and the surrounding Eastern Cape. At present, it is unclear as to what the common strain types of *K. pneumoniae* prevalent in hospitals and the community of the Port Elizabeth locale.

This is the first Eastern Cape based study, which utilized MLST to type *K. pneumoniae* isolates. There are few other studies that have utilized the molecular typing method to analyse South African *K. pneumoniae* isolates. In a Cape Town based study, two clinical *K. pneumoniae* isolates were identified as belonging to ST 14 through MLST (Jacobson *et al.* 2015). However, in the Cape Town study only two isolates were analysed through MLST. While this demonstrates the presence of ST 14 *K. pneumoniae* isolates within South Africa, it provides very little insight into the diversity of *K. pneumoniae* isolates within South Africa. An international study, based in Denmark, analysed four hypervirulent *K. pneumoniae* strains isolated from pneumonia patients (Struve *et al*. 2015). Through MLST, it was observed that these patients exhibited a sequence type of ST 25 (Struve *et al*. 2015). Both ST 14 and ST 25 exhibit allele numbers of *inf*B: 1, *mdh*: 1, and *pgi*: 1 (PasteurMLST. 2017). These three allele numbers were observed in serval of the *K. pneumoniae* isolates within this study. However, other than the *inf*B, *mdh*, and *pgi* genes there was little similarity between the isolates observed in this study and ST 14 and ST 25 *K. pneumoniae*. However, the isolates that exhibited ST E (K7, K11, and K13) shared four of the seven allele numbers with that of the ST 14 isolates observed in the Cape Town study (Jacobson *et al.* 2015; PasteurMLST, 2017). This may indicate the possibility of a common ancestral genotype of hospital-acquired *K. pneumoniae* isolates existing within South Africa, however further studies would be required to explore this possibility. In figure 5.2 we compared the genetic relatedness between the isolates of this study and with that of ST 14 and ST 25. Firstly, it was observed that ST 14 and ST 25 are closely genetically similar to each other. Secondly, it was observed that ST 14 and ST 25 show considerable genetic

similarity to a number of the isolates observed within this study, especially the isolates that exhibited ST A, B, C and D (K1, K2, K3, K4, K5, K6, and K10). This may suggest that a predominant clonal complex of *K. pneumoniae* exists in South Africa, especially in the Western and Eastern Cape.

The *K. pneumoniae* isolates observed among patients at Jose Pearson TB hospital were genetically diverse, and there was no dominant sequence type identified among the group. It appears that there are multiple sources of *K. pneumoniae* colonization within Jose Pearson hospital, however the sources and routes of colonization by *K. pneumoniae* are unclear.

CHAPTER SIX

CONCLUSIONS

6.1 THE RESEARCH IN PERSPECTIVE

This study highlighted the spectrum of multidrug-resistant (MDR) Enterobacteriaceae colonization among hospitalized MDR and XDR-TB-patients with long-term prescription of broad-spectrum antibiotics such as fluoroquinolones and aminoglycosides. There was a low incidence of MRSA colonization among hospitalized MDR and XDR-TB patients, with a predominance of MDR ESBL-producing *E. coli* and K*. pneumoniae.* High rates of fluoroquinolone and aminoglycoside resistance were observed among the Enterobacteriaceae isolates with susceptibility to carbapenems. Detection of resistance genes encoding ESBLs included CTX-M-14, CTX-M-15, SHV-28 and OXA-1. The beta lactamase, OXY-2-9 was also identified in a *K. oxytoca* isolate, a first report of this in South Africa. Molecular typing using MLST revealed a high genetic diversity of drug resistant *K. pneumoniae* isolates colonizing patients at Jose Pearson TB hospital. This study appeared to be one of the first, in South Africa that examined the spectrum of hospital-acquired pathogens colonizing MDR-TB patients, and effects of the broad spectrum of antibiotics prescribed. In addition, the occurrence of genes encoding for the SHV-28 and OXY-2 ESBLs were not previously described in South Africa.

The high rates of colonization of MDR ESBL-producing Enterobacteriaceae colonizing multidrug-resistant (MDR) and extensively drug-resistant (XDR)-TB patients within Jose Pearson TB hospital is most concerning. All patients who were present in the hospital for 4 weeks or longer became colonized with an MDR ESBL-producing Enterobacteriaceae. In addition, majority of these patients were not colonised by ESBL-producers at admission. Colonization surveillance and infection control procedures need to be re-evaluated at the hospital to reduce emergence and transmission of ESBL-producing Enterobacteriaceae.

No carbapenem resistance was observed among the isolates of this study. However, due to the well documented incidence of carbapenem-resistant Enterobacteriaceae (CRE) within the Port Elizabeth locale, and the fact that carbapenems are one of the last treatment options for these infections, it seems unlikely that Jose Pearson TB hospital will remain free of

detectable carbapenem-resistance for long. Inadequate infection control may have contributed to the colonisation, spread and dissemination of ESBL-producing Enterobacteriaceae. Based on the results of the multilocus sequence typing (MLST) typing it would appear that the isolates present within the hospital are dominated by single genetic strain. This suggests that there may be multiple routes of infection and possibly multiple different sources infection within the hospital. There were several *K. pneumoniae* isolates in this study which showed genetic similarity to the other South African *K. pneumoniae* isolates that had been previously described (Jacobson *et al*. 2015; Struve *et al*. 2015).

The application of antimicrobial stewardship (AMS) at Jose Pearson poses a serious challenge as clinicians are faced with a “Catch-22” situation. All patients at hospital are being treated for a MDR or XDR-TB infection which includes the simultaneous prescription of several broad spectrum antibiotics. While the clinicians acknowledge the need to prescribe fewer antibiotics with a narrow spectrum of activity, they have limited options due to the severity of these TB infections. However, there are antimicrobial stewardship practices that could be implemented within the hospital. These include: (1) tracking and monitoring antibiotic prescribing patterns, (2) routine screening of patients for the colonization of hospital-acquired pathogens, (3) rapid response and antimicrobial susceptibility testing of hospital-acquired infections, (4) treatment with narrow spectrum antibiotics based on antimicrobial susceptibility profiles, and (5) the reservation of last line antibiotics, such as carbapenems, for the most dire of cases. The implementation of a carbapenem stewardship program at Jose Pearson is highly recommended, as they remain the single beta-lactam antibiotic class, and one of the few classes of antibiotics overall, still viable as treatment options against the present nosocomial infections.

While the burden of MDR and XDR-TB poses a challenge to antimicrobial stewardship, educating both hospital care staff and patients on proper infection control precautions, could decrease the spread of MDR Enterobacteriaceae isolates. Appendix 5 includes a list of the standard infection control practices that could be used to reduce the spread of MDR pathogens at Jose Pearson TB hospital.

The MIC data categorisation as suggested by the Vitek’s expert system, which is standard practice in diagnostic labs, was used. This could account for some of the discrepancies in MIC obtained using the Sensititre MIC plates since the Vitek system may alter the

categorisation irrespective of actual MIC value if it determines that there is a resistance mechanism present, e.g. amikacin often called intermediate or resistant even if MIC was less than breakpoint.

There were several limitations that were encountered within the duration of this study. Firstly, the small sample size of patients was a considerable limitation, as this small population size does not accurately represent the entire patient population of Jose Pearson. Furthermore, the study was only conducted at a single hospital and the results observed within this study many not be representative of the conditions present in other hospitals. It would have been desirable to include more patients within the study, however due to limited funding this could not be done. In addition, several patients withdrew from the study, and physicians were informed that patients found the swabbing procedure unpleasant. Finally, it was difficult to obtain assistance from some clinicians and nurses regarding patient recruitment and patient screening.

This study emphasises the need for longitudinal surveillance and antimicrobial susceptibility patterns of the hospital-acquired pathogens colonizing patients at Jose Pearson TB hospital. Implementation of more stringent infection control protocols is highly recommended.

6.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE WORK

The screening of a larger sample size/ cohort of patients could provide a better insight into the challenges and possible interventions that could assist in reducing mortality at the TB hospitals. It is suggested that similar studies be conducted at other TB hospitals in the country.

The molecular mechanisms of antibiotic resistance within the hospital-acquired pathogens obtained from Jose Pearson TB hospital could be analysed further to elucidate the specific drug resistance mechanisms in these isolates. The mechanisms of interest include: (1) analysis of plasmids carrying the ESBL-encoding genes detected in this study (*bla*CTX-M-14, *bla*CTX-M-15, *bla*OXA-1, and *bla*SHV-28) for detection of other associated antibiotic resistance determinants; (2) screening for *Amp*C β-lactamases (3) investigation of fluoroquinolone resistance-determinant genes such as the *Qnr* genes and aminoglycoside acetyltransferase AAC(6′)-Ib-cr; and mutations in the DNA gyrase and topoisomerase IV enzymes; (4) investigation of aminoglycoside resistance encoding genes such as the 16S rRNA

methylases and plasmid-encoded *arm*A methylase; and (5) observation of the expression levels of membrane porins and drug efflux pumps within the isolated hospital-acquired pathogens.

As the patients at Jose Pearson experienced high rates of colonization by MDR bacteria, further investigation into the molecular epidemiology of the pathogens present in the hospital is recommended. This study was limited by the available sample size of both isolates and patients. If a more in depth and comprehensive molecular study could be conducted into the colonizing pathogens, and environmental samples also be taken, then information regarding the routes transmission and environmental reservoirs within Jose Pearson could be determined.

Another potential area for future research, as identified in chapter 5, is that currently, broader information on the molecular epidemiology of both hospital-acquired and community-acquired pathogens throughout South Africa is lacking. This is not only true for Enterobacteriaceae pathogens, such as *K. pneumoniae* and *E. coli*, but for other major nosocomial pathogens such a *P. aeruginosa*, *A. baumannii*, and *S. aureus*. Broader molecular epidemiological studies of nosocomial pathogens present within the Port Elizabeth area would be beneficial as this would allow for the identification of the strains circulating the Port Elizabeth area, and help identify the infection routes and potential reservoirs of such strains. Furthermore, whole genome sequencing could identify strain, sequence types, and antimicrobial resistance/ virulence genes within the isolates.

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APPENDIX

1: NMU AND UCT ETHICS APPROVAL LETTERS & EC-DOH APPROVAL

Nelson Mandela Metropolitan University
for tomorrow

• PO Box 77000 • Nelson Mandela Metropolitan University
• Port Elizabeth • 6031 • South Africa • www.nmmu.ac.za

Chairperson: Research Ethics Committee (Human)
Tel: +27 (0)41 504-2235

Ref: [H15-HEA-PHA-017/Approval]

Contact person: Mrs U Spies

15 March 2016

Prof I Truter
Faculty of Health Sciences Department: Pharmacy South Campus

Dear Prof Truter

INCIDENCE OF NOSOCOMIAL INFECTIONS IN PATIENTS ADMITTED TO A DRUG-RESISTANT TB HOSPITAL

Your above-entitled application served at Research Ethics Committee (Human) for approval.

The ethics clearance reference number is **H15-HEA-PHA-017** and is valid for three years. Please inform the REC-H, via your faculty representative, if any changes (particularly in the methodology) occur during this time. An annual affirmation to the effect that the protocols in use are still those for which approval was granted, will be required from you. You will be reminded timeously of this responsibility, and will receive the necessary documentation well in advance of any deadline.

We wish you well with the project. Please inform your co-investigators of the outcome; and convey our best wishes.

Yours sincerely

CB Cilliers

Prof C Cilliers
Chairperson: Research Ethics Committee (Human)

cc: Department of Research Capacity Development

Faculty Officer: Health Sciences

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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Website: www.health.uct.ac.za/fhs/research/humanethics/forms

12 October 2016

HREC REF: 573/2016

Dr C Bamford
Division of Medical Microbiology
5th Floor
Falmouth Building-FHS

Dear Dr Bamford

PROJECT TITLE: INCIDENCE OF NOSOCOMIAL INFECTION IN PATIENTS ADMITTED TO A DRUG-RESISTANT TUBERCULOSIS HOSPITAL (masters-candidate-D Annear)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 October 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

We acknowledge that the student; D Annear will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

Eastern Cape Department of Health

Enquiries:	Madoda Xokwe	Tel No:	040 608 0830
Date:	04 April 2016	Fax No:	043 642 1409
e-mail address:	zonwabele.merile@echealth.gov.za		

Dear Mrs. R. Gaida

Re: Incidence of nosocomial infection in patients being admitted to a drug-resistant TB hospital (EC_2016RP1_50)

The Department of Health would like to inform you that your application for conducting a research on the abovementioned topic has been approved based on the following conditions:

1. During your study, you will follow the submitted protocol with ethical approval and can only deviate from it after having a written approval from the Department of Health in writing.
2. You are advised to ensure, observe and respect the rights and culture of your research participants and maintain confidentiality of their identities and shall remove or not collect any information which can be used to link the participants.
3. The Department of Health expects you to provide a progress on your study every 3 months (from date you received this letter) in writing.
4. At the end of your study, you will be expected to send a full written report with your findings and implementable recommendations to the Epidemiological Research & Surveillance Management. You may be invited to the department to come and present your research findings with your implementable recommendations.
5. Your results on the Eastern Cape will not be presented anywhere unless you have shared them with the Department of Health as indicated above.

Your compliance in this regard will be highly appreciated.

SECRETARIAT: EASTERN CAPE HEALTH RESEARCH COMMITTEE

PGDP EASTERN CAPE 2004 - 2014

Ikamva eliqaqambileyo!

2: ANTIMICROBIAL PRESCRIBING PATTERNS DATA COLLECTION TOOL

DATA COLLECTION TOOL

Patient number			**Type of TB**		
			TB lab culture		
Gender	F	M	**TB regimen**		
Age (in years)			**Substance use**	Y/N	Substance(s)
Weight on Administration (in kg)			**Patient smoking**	Y/N	
Weight on Discharge (in kg)			**Comorbidities**		
Height (in cm)			**Length of stay**(in months, using date of admission and date discharged)		

Date commenced	Date stopped	Antimicrobial prescribed	Antimicrobial indication	Other therapy	Therapy indication	Dose	Frequency	Route	Time administered	Prescribing Doctor

3: BASELINE DATA COLLECTION TOOL

BASELINE

DEMOGRAPHICS

1. Gender

☐ Female

☐ Male

2. Age: _____

RECENT MEDICAL CARE (PAST ONE MONTH)

3. HIV Positive

☐ **Yes, CD4 count: _____** ☐ **No**

4. Level of care received:_____

5. Duration of treatment/admission:

_____

6. Antibiotic therapy:

_____

_____

_____

7. Exposure to invasive devices:

_____

_____

_____

8. Co-morbidities:

_____

_____

_____

HOSPITAL CARE:

Ward:_____

BASELINE SWAB:

Swab performed	Location swab was taken	Date performed	Date cultured	Results

9. Infection present at baseline:

☐ **Yes** ☐ **No**

10. Site:_____

11. Pathogen:_____

12. Consensus on final diagnosis:_____

13. Antimicrobial treatment provided:

_____

_____

4: FOLLOW UP DATA COLLECTION TOOL

FOLLOW-UP

Patient number:_____

Ward:_____

DEMOGRAPHICS

1. Gender

☐ **Female**

☐ **Male**

2. Age:_____

SWABS:

Swab performed	Location swab was taken	Date performed	Date cultured	Results

INFECTION: ☐ **Yes** ☐ **No**

3. Site:_____

4. Pathogen:_____

Risk Factors:

Risk	Yes; which?	No
Invasive devices		
Antibiotic treatment (current/previous)		
Co-morbidities		
Length of hospital stay		
Ward		
Previous healthcare exposure		

Any previous colonisation results known	

5. Consensus on final diagnosis:_____
6. Antimicrobials treatment provided:

_____

_____

7. Infection resolved:

_____

_____

5: STANDARD INFECTION CONTROLS

Standard controls	
Hand washing	• Wash hands after touching blood, body fluid, secretions, excretions and contained items whether or not gloves are worn.
Gloves	• Wear gloves when touching blood, body fluid, secretions; excretions and contained items • Put on clean gloves before touching mucous membranes and non-intact skin • Change gloves between tasks and procedures on: – the same patient – after contact with material that may contain high concentration of microorganisms • Remove gloves promptly after use: – before touching non-contaminated items and environmental surfaces – before going to another patient • Wash hands immediately to avoid transfer of microorganisms to other patients and environments
Mask; eye protection; face shield	• Protects mucous membranes of the eyes, nose and mouth
Gown (plastic apron)	• Protects skin and prevents soiling of clothing during procedures and activities that are likely to generate splashes or sprays of blood, body fluid, secretion and excretions • Remove a soiled gown or apron as promptly as possible • Wash hands to avoid transfer of microorganisms to other patients and environments
Patient-care equipment	• Handle patient-care equipment soiled with blood, body fluids, secretions and excretions in a manner that: – prevents skin and mucous membrane exposures – contamination of clothing – transfer of microorganisms to other patients or environments • Ensure that reusable equipment is not used for the care of another patients until it has been cleaned and reprocessed appropriately • Ensure that single-use items are discarded properly

Environmental control	• Ensure that adequate procedures are in place for routine care, cleaning disinfection of: – environmental surfaces – beds and bedrails – bedside equipment – other frequently touched surfaces • Disinfection of environmental surfaces are not routinely required • Simple cleaning is adequate unless there has been significant soiling by potentially infectious body fluid
Linen	• Handle, transport and process used linen soiled with blood and body fluids, secretions and excretions in a manner that prevents: – skin and mucous membrane exposure – contamination of clothing – transfer of microorganisms to other patients and environments

6: ANTIMICROBIAL SENSITIVITY PATTERNS OF NOSOCOMIAL ISOLATES

Table A6: Antibiotic susceptibility profiles of all clinical Enterobacteriaceae isolates (n=62) determined by the both the VITEK system and Sensititre plates. Detected antimicrobial resistance determinant genes are also listed.

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 1	*E. coli*	S™	AMI; TOB; MER; DOR; ETP; IPM; COL; POL; TGC	TIM2; GEN; FEP; TAZ	AZT; PT4; SXT; LEVO; DOX; CIP; MIN; FOT	CTX-M-14;
		V®	AMX; PT4; FOX; ETP; IPM; MER; AMK; GEM; TGC; NIT; COL	TAZ; FEP	AMP; CFX; CFA; FOT; CIP; SXT	
E 2	*E. coli*	S™	AMI; GEN; TOB; DOX; MIN; MER; DOR; ETP; IPM; COL; POL; TGC	FEP	TIM2; AZT. PT4; SXT; LEVO; CIP; TAZ; FOT	CTX-M-15
		V®	AMX; PT4; FOX; ETP; IPM; MER; GEN; TGC; NIT; COL; SXT	AMK	AMP; CFX; CFA; FOT; TAZ; FEP; CIP	
E 3	*E. coli*	S™	AMI; GEN; FEP; TOB; MER; DOR; ETP; IPM; COL; POL	TIM2; TAZ	AZT; PT4; SXT; LEVO; DOX; CIP; MIN; FOT	CTX-M-14
		V®	AMX; PT4; FOX; ETP; IPM; MER; GEN; TGC; NIT; COL	TAZ; AMK	AMP; CFX; CFA; FOT; FEP; CIP; SXT	
E 4	*E. coli*	S™	AZT; FEP; TAZ; MER; DOR; ETP; IPM; COL; POL; TGC	TIM2	AMI; PT4; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; FOT	CTX-M-14; TEM-1
		V®	AMX; PT4; FOX; ETP; IPM; MER; TGC; NIT; COL	TAZ; FEP; AMK	AMP; CFX; CFA; FOT; GEN; CIP; SXT	
E 5	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC	PT4	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; CXT	
E 6	*E. coli*	S™	AZT; PT4; MER; DOR; ETP; IPM; COL; POL; TGC	TIM2; TAZ; FOT	AMI; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN	OXA-1
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; FEP; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; GEN; CIP; SXT	
E 7	*E. coli*	S™	AMI; PT4; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; AMK; GEN; TGC; NIT; COL	PT4	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 8	*E. coli*	S™	AMI; PT4; GEN; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15
		V®	PT4; ETP; IPM; MER; AMK; GEN; TGC; NIT; COL		AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 9	*E. coli*	S™	AZT; PT4; FEP; MER; DOR; ETP; IPM; COL; POL; TGC	TIM2	AMI; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	FOT; TAZ; FEP; AMK	AMP; AMX; CFX; CFA; FOX; GEN; CIP; SXT	
E 10	*E. coli*	S™	AZT; PT4; FEP; MER; DOR; ETP; IPM; COL; POL; TAZ; TGC	TIM2	AMI; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; FOT	OXA-1; CTX-M-14; CTX-M-15
		V®	AMX; PT4; FOX; ETP; IPM; MER; TGC; NIT; COL	TAZ; FEP; AMK	AMP; CFX; CFA; FOT; GEN; CIP; SXT	
E 11	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
E 12	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC	PT4	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
E 13	*E. coli*	S™	GEN; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	ETP; IPM; MER; TGC; GRN; NIT; COL	AMX; PT4; AMK	AMP; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 14	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; PT4	TGC	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	AMX; FOX; AMK	AMP; CFX; CFA; FOT; TAZ; FEP; GEN; CIP; SXT	
E 15	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC; PT4		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-14; CTX-M-15; TEM-1
		V®	AMX; PT4; FOX; ETP; IPM; MER; TGC; NIT; COL	TAZ; FEP; AMK	AMP; CFX; CFA; FOT; GEN; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 16	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC; PT4		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; TEM-1
		V®	PT4; FOX; ETP; IPM; MER; TIG; NIT; COL	AMX; AMK	AMP; CFX; CFA; FOT; TAZ; GEN; CIP; SXT	
E 17	*E. coli*	S™	PT4; AZT; FEP; MER; DOR; ETP; IPM; COL; POL; TAZ; TGC		AMI; TIM2; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; FOT	CTX-M-14; TEM-1
		V®	AMX; PT4; FOX; ETP; IPM; MER; TGC; NIT; COL	TAZ; FEP	AMP; CFX; CFA; FOT; GEN; CIP; SXT	
E 18	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC	PT4	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
E 19	*E. coli*	S™	AZT; FEP; MER; DOR; ETP; IPM; COL; POL; TAZ; TGC		AMI; TIM2; PT4; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; FOT	CTX-M-14; TEM-1
		V®	AMX; PT4; FOX; ETP; IPM; MER; AMK; TGC; NIT; COL	TAZ; FEP	AMP; CFX; CFA; FOT; GEN; CIP; SXT	
E 20	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL; TGC	PT4	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
E 21	*E. coli*	S™	GEN; AMI; PT4; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; AMK; GEN; NIT; COL	AMX	AMP; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 22	*E. coli*	S™	GEN; AMI; PT4; TOB; MER; DOR; ETP; IPM; COL; POL; TAZ; TGC	FEP	TIM2; AZT; SXT; LEVO; DOX; CIP; MIN; FOT	TEM-1
		V®	AMX; PT4; FOX; ETP; IPM; MER; AMK; GEN; TGC; NIT; COL	TAZ; FEP	AMP; CFX; CFA; CIP; SXT	
E 24	*E. coli*	S™	MER; DOR; ETP; IPM; COL; POL	TGC	AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-14; CTX-M-15
		V®	PT4; ETP; IPM; MER; GEN; TGC; NIT; COL	AMX; TAZ; FEP	AMP; CFX; CFA; FOX; FOT; AMK; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 25	*E. coli*	S™	AZT; PT4; MER; DOR; ETP; IPM; COL; POL; TAZ; TGC	TIM2; FEP	AMI; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; FOT	TEM-1
		V®	AMX; PT4; ETP; IPM; MER; TGC; NIT; COL	TAZ; FEP; AMK	AMP; CFX; CFA; FOT; GEN; CIP; SXT	
E 26	*E. coli*	S™	PT4; GEN; MIN; MER; DOR; ETP; IPM; COL; POL; TGC	AMI	TIM2; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-14
		V®	ETP; IPM; MER; GEN; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 27	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC	GEN	AMI; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-14; TEM-1
		V®	PT4; ETP; IPM; MER; TIG; NIT; COL	AMX; FOX; GEN	AMO; CFX; CFA; FOT; TAZ; FEP; AMK; CIP; SXT	
E 28	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	AMX; FOX; AMK	AMP; CFX; CFA; FOT; TAZ; FEP; GEN; CIP; SXT	
E 29	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-14; CTX-M-15
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; FEP; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; GEN; CIP; COL	
E 30	*E. coli*	S™	GEN; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15
		V®	ETP; IPM; MER; GEN; TGC; NIT; COL	AMX; PT4; AMK	AMP; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 31	*E. coli*	S™	PT4; FEP; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	FOT; TAZ; FEP; AMK	AMP; AMX; CFX; CFA; FOX; GEN; CIP; SXT	
E 32	*E. coli*	S™	PT4; FEP; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-14
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	AMX; FEP; AMK	AMP; CFX; CFA; FOX; FOT; TAZ; GEN; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 33	*E. coli*	S™	AMI; PT4; GEN; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-14; CTX-M-15
		V®	PT4; ETP; IPM; MER; AMK; GEN; TGC; NIT; COL	AMX; FOX	AMP; CFX; CFA; FOT; TAZ; FEP; CIP; SXT	
E 34	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC	FOT	AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ	OXA-1; CTX-M-14; TEM-1
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	AMX; TAZ; AMK	AMP; CFX; CFA; FOX; FOT; FEP; GEN; CIP; SXT	
E 35	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-14; TEM-1
		V®	PT4; ETP; IPM; MER; TGC; COL	FOX; AMK; NIT	AMP; AMX; CFX; CFA; FOT; TAZ; FEP; GEN;	
E 36	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	PT4; ETP; IPM; MER; TGC; COL	FOX; AMK; NIT	AMP; AMX; CFX; CFA; FOT; TAZ; FEP; GEN;	
E 37	*E. coli*	S™	AMI; PT4; GEN; TOB; MIN; MER; DOR; ETP; IPM; COL; POL; TGC	FEP	TIM2; AZT; SXT; LEVO; DOX; CIP; TAZ; FOT	CTX-M-14; TEM-1
		V®	PT4; ETP; IPM; MER; AMK; GEN; TGC; NIT; COL	AMX; FOX; TAZ; FEP	AMP; CFX; CFA; FOT; CIP; SXT	
E 38	*E. coli*	S™	GEN; MER; DOR; ETP; IPM; COL; POL; TGC	PT4	AMI; TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15; TEM-1
		V®	ETP; IPM; MER; GEN; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	
E 39	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC	GEN	AMI; TIM2; AZT; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15; TEM-1
		V®	PT4; ETP; IPM; MER; TGC; NIT; COL	AMX; FOX; AMK; GEN	AMP; CFX; CFA; FOT; TAZ; FEP; CIP; SXT	
E 40	*E. coli*	S™	PT4; GEN; FEP; TOB; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; LEVO; DOX; CIP; MIN; TAZ; FOT	
		V®	PT4; ETP; IPM; MER; AMK; GEN; TGC; NIT; COL	FOT; TAZ; FEP	AMP; AMX; CFX; CFA; FOX; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
E 41	*E. coli*	S™	PT4; MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	ETP; IPM; MER; TGC; NIT; COL	PT4; AMK	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
E 42	*E. coli*	S™	AMI; AZT; PT4; GEN; TOB; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; SXT; FEP; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-14; TEM-1
		V®	ETP; IPM; MER; AMK; GEN; TGC; NIT COL	PT4; FEP	AMP; AMX; CFX; CFA; FOX; FOT; TAZ; CIP; SXT	
K 1	*K. pneumoniae*	S™	AMI; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15
		V®	ETP; IPM; MER; AMK; COL		AMP; AMX; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; TGC; NIT; SXT	
K 2	*K. pneumoniae*	S™	AMI; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15; SHV-28; TEM-1
		V®	FOX; ETP; IPM; MER; TGC; COL	PT4; AMK; NIT	AMP; AMX; CFX; CFA; FOT; TAZ; FEP; GEN; CIP; SXT	
K 3	*K. pneumoniae*	S™	AMI; PT4; GEN; MER; DOR; ETP; IPM; COL; POL; TGC	FEP	TIM2; AZT; SXT; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15; TEM-1
		V®	FOX; ETP; IPM; MER; GEN; TGC; COL	PT4; AMK	AMP; AMX; CFX; CFX; FOT; TAZ; FEP; CIP; NIT; SXT	
K 4	*K. pneumoniae*	S™	AMI; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15; TEM-1
		V®	ETP; IPM; MER; AMK; TGC; COL		AMP; AMX; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; NIT; SXT	
K 5	*K. pneumoniae*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1
		V®	AMX; ETP; IPM; MER; COL	PT4; AMK	AMP; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; TGC; NIT; SXT	
K 6	*K. pneumoniae*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	ETP; IPM; MER; COL	AMX; FEP; AMK	AMP; PT4; CFX; CFA; FOX; FOT; TAZ; GEN; CIP; TGC; NIT; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
K 7	*K. pneumoniae*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15;
		V®	ETP; IPM; MER; COL	AMX; AMK	AMP; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; TGC; NIT; SXT	
K 8	*K. pneumoniae*	S™	AMI; PT4; GEN; FEP; TAZ; MER; DOR; ETP; IPM; COL; TGC		TIM2; AZT; SXT; TOB; LEVO; DOX; CIP; MIN; POL; FOT	OXA-1; CTX-M-15; SHV-28; TEM-1
		V®	ETP; IPM; MER; AMK; GEN; COL	AMX	AMP; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; TGC; NIT; SXT	
K 9	*K. pneumoniae*	S™	PT4; FEP; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; GEN; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	CTX-M-15
		V®	ETP; IPM; MER; AMK; GEN; COL	AMX	AMP; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; TGC; NIT; SXT	
K 10	*K. pneumoniae*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	ETP; IPM; MER; COL	AMX; FEP; AMK	AMP; PT4; CFX; CFA; FOX; FOT; TAZ; GEN; CIP; TGC; NIT; SXT	
K 11	*K. pneumoniae*	S™	MER; DOR; ETP; IPM; COL; POL; TGC		AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; SHV-28
		V®	ETP; IPM; MER; COL	AMK	AMP; AMX; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; TGC; NIT; SXT	
K 12	*K. pneumoniae*	S™	AMI; GEN; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	IPM; MER; AMK; GEN; COL	ETP	AMP; AMX; PT4; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; TGC; NIT; SXT	
K 13	*K. pneumoniae*	S™	AMI; DOX; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; CIP; MIN; TAZ; FOT	OXA-1; CTX-M-15; SHV-28
		V®	AMX; ETP; IPM; MER; AMK; GEN; NIT; COL	PT4	AMP; CFX; CFA; FOX; FOT; TAZ; FEP; CIP; TGC; SXT	
K 14	*K. pneumoniae*	S™	AMI; DOX; MIN; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; CIP; TAZ; FOT	OXA-1; CTX-M-15; TEM-1
		V®	FOX; ETP; IPM; MER; TGC; COL	AMK; NIT	AMP; AMX; PT4; CFX; CFA; FOT; TAZ; FEP; GEN; CIP; SXT	

Table A6 continued…

Isolate Code	Species	AST Method	Antimicrobial Resistance Profile			Resistance Genes
			Susceptible	Intermediate	Resistant	
B 1	*E. Cloacae*	S™	AMI; AZT; GEN; FEP; TOB; MER; DOR; ETP; IPM; COL; POL; TAZ; FOT; TGC	TIM2	PT4; SXT; LEVO; DOX; CIP; MIN	
		V®	PT4 ETP; IPM; MER; GEN; COL	FOT; TAZ; FEP; AMK	AMX; CFX; CFA; FOX; CIP; TGC; NIT; SXT	
X 1	*P. mirabilis*	S™	PT4; MER; DOR; ETP; IPM;	TIM2	AMI; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; COL; POL; TAZ; FOT; TGC	
		V®	AMX; PT4; FEP; IPM; MER	FOX; AMK; GEN	SMP; CFX; CFA; FOT; TAZ; CIP; TGC; NIT; COL; SXT	
X 2	*C. freundii*	S™	AMI; PT4; MER; DOR; ETP; IPM; COL; POL; TGC		TIM2; AZT; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN; TAZ; FOT;	CTX-M-15; TEM-1
		V®	PT4; ETP; IPM; MER; AMK; TGC; NIT; COL		AMP; CFX; CFA; FOX; FOT; TAZ; FEP; GEN; CIP; SXT	
X 3	*K. oxytoca*	S™	MER; DOR; ETP; IPM; COL; POL; TGC	TAZ; FOT	AMI; TIM2; AZT; PT4; SXT; GEN; FEP; TOB; LEVO; DOX; CIP; MIN	OXY-2-9; CTX-M-15
		V®	ETP; IPM; MER; NIT; COL	FOX; TAZ; FEP; AMK; TGC	AMP; AMX; PT4; CFX; CFA; FOT; GEN; CIP; SXT	
X 4	*C. braakii*	S™	DOX; MER; DOR; ETP; IPM; COL; POL; TGC	AMI; GEN	TIM2; AZT; PT4; SXT; FEP; TOB; LEVO; CIP; TAZ; FOT	OXA-1; CTX-M-15
		V®	PT4; ETP; IPM; MER; GEN; TGC; NIT; COL	AMK	CFX; CFA; FOX; FOT; TAZ; FEP; CIP; SXT	

AMP – Ampicillin; AMX – Amoxicillin-clavulanic acid; PT4 – Piperacillin-tazobactam; TIM2 - Ticarcillin-clavulanic acid; CFA – Cefuroxime axetil; CFX – Cefuroxime; FEP – Cefepime; FOT – Cefotaxime; FOX – Cefoxitin; TAZ – Ceftazidime; AZT – Aztreonam; DOR – Doripenem; ERT – Ertapenem; IPM – Imipenem; MER – Meropenem; COL – Colistin; POL - Polymyxin B; CIP – Ciprofloxacin; LEVO – Levofloxacin; AMI – Amikacin; GEN – Gentamycin; TOB – Tobramycin; DOX – Doxycycline; MIN – Minocycline; TGC – Tigecycline; NIT – Nitrofurantoin; SXT – Trimethoprim-sulfamethoxazole

S™ - Sensititre Antimicrobial Susceptibility Profiles; V® - Vitek Antimicrobial Susceptibility Profiles

Table A7: Antibiotic susceptibility profiles of the clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (n=2) as determined by the VITEK system.

Isolate Code	Species	Antimicrobial Resistance Profile		
		Susceptible	Intermediate	Resistant
S1	***Staphylococcus aureus***	LZD; TEC; VAN; FCA		BZP; OXC; GEN; CIP; MOX; ERM; CLD; TGC; RIF; SXT
S2	***Staphylococcus aureus***	MOX; ERM; CLD; LZD; TEC; VAN; FCA		BZP; OXC; GEN; CIP; TGC; RIF; SXT

BZP – Benzylpenicillin; OXC – Oxacillin; GEN – Gentamycin; CIP – Ciprofloxacin; MOX – Moxifloxacin; ERM – Erthromycin; CLD – Clindamycin; LZD – Linezolid; TEC – Tecoplanin; VAN – Vancomycin; TGC – Tigecycline; FCA - Fusidic Acid; RIF – Rifampicin; SXT – Trimethoprim-sulfamethoxazole

LIST OF CONFERENCE PRESENTATIONS

Poster Presentation

D. Annear, R. Gaida; J. Black, I. Truter, C. Bamford, and S. Govender. 2017. Incidence and antimicrobial susceptibilities of bacterial nosocomial pathogens among patients admitted to a Tuberculosis hospital. 7th FIDSSA Congress, Cape Town, South Africa. 9 – 11 November 2017.
Southern African Journal of Infectious Diseases **S59** 2017 Volume 32 Supplement

Satellite Session

Ordinary people doing extraordinary work to improve the clinical and programmatic management of DR-TB in South Africa

D. Annear, R. Gaida, J. Black, I. Truter, C. Bamford, and S. Govender. Incidence of nosocomial infections in patients admitted to a drug-resistant TB hospital / Incidencia de las infecciones nosocomiales en pacientes ingresados en un hospital para el tratamiento de la tuberculosis multirresistentel. 48th Union World Conference on Lung Health. Guadalajara, Mexico. 11 – 14 October 2017.
- Presented on my behalf by Dr Norbert Ndjeka