β-lactam resistance mechanisms in *Enterobacter* spp. isolates from Tygerberg Hospital

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

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Declaration

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Summary

Background

Resistance to carbapenem antibiotics in Gram-negative bacteria is a major public health problem due to limited treatment options. In *Enterobacter* species, carbapenemase production and reduced membrane permeability, resulting from reduced expression of outer membrane proteins OmpF and OmpC, in combination with extended-spectrum β-lactamase (ESBL) production, hyper-production of chromosomal *bla*AmpC β-lactamases and / or over-expression of the AcrAB/TolC efflux pump have been speculated to promote carbapenem resistance. This study investigated the mechanisms that promote ertapenem resistance in clinical *Enterobacter cloacae* isolates from Tygerberg Hospital.

Materials and methods

Twenty ertapenem non-susceptible clinical *E. cloacae* isolates, four ertapenem susceptible controls and five wild-type controls were selected based on the VITEK2®AES. Ertapenem MICs were determined using broth microdilution (BMD) and gradient diffusion. Resistance mechanisms were characterized using the phenotypic assays VITEK2®AES, Mastdiscs D68C, D69C and D63C combination sets, Rapidec® Carba NP kit and a synergy assay using disc diffusion in the presence of an efflux pump inhibitor (EPI). Molecular assays included multiplex PCR to identify carbapenemases, multiplex PCR and sequencing to identify ESBLs, SDS-PAGE to characterize OmpC and OmpF abundance and RT-qPCR to quantify expression of *bla*AmpC, *ompC*, *ompF* and *acrB*.

Results and Discussion

Seventeen (85%) ertapenem non-susceptible isolates were confirmed non-susceptible by BMD and six (30%) by gradient diffusion, suggesting possible undercalling of ertapenem resistance by gradient diffusion and overcalling by VITEK2®AES.

Seven ertapenem non-susceptible isolates were predicted to be carbapenemase producers by the VITEK2®AES and one by the Rapidec® Carba NP kit, however no carbapenemases were detected by PCR. Nineteen ESBL producers were identified by the VITEK2®AES, eight by the D68C combination set and eleven by the D63C combination set. ESBLs were detected in 12 (60%) isolates by PCR and sequencing; of which eight were *bla*CTX-M, three were *bla*SHV-12 and one isolate contained both genes.

Twelve (60%) ertapenem non-susceptible isolates were predicted to be derepressed *bla*AmpC producers by the VITEK2®AES, thirteen (65%) by the D68C combination set and six (30%) by the *bla*AmpC RT-qPCR.

OmpC and OmpF abundance could not be quantified using SDS-PAGE, however, reduced expression of *ompC* was detected in eleven (55%) ertapenem non-susceptible isolates, and *ompF* in thirteen (65%). Expression of both genes was reduced in seven (35%) ertapenem non-susceptible isolates.

The synergy assay did not detect increased antibiotic susceptibility in the presence of EPI. Increased efflux activity was observed in two (10%) ertapenem non-susceptible isolates based on *acrB* expression.

Overall, 11 of the 17 ertapenem non-susceptible isolates confirmed by BMD showed ESBL production and reduced membrane permeability; three of these were derepressed chromosomal *bla*AmpC producers and two showed increased efflux activity. Carbapenemase production was detected in one isolate which also showed hyper-production of chromosomal *bla*AmpC and reduced membrane permeability. One additional isolate showed hyper-production of chromosomal *bla*AmpC and reduced membrane permeability, while two showed only reduced membrane permeability and one only hyper-production of chromosomal *bla*AmpC. No tested resistance mechanisms was detected in one ertapenem non-susceptible isolate, suggesting that alternative mechanisms may have contributed to resistance.

Conclusion

Ertapenem resistance in the tested clinical *E. cloacae* isolates was mediated through several mechanisms, predominantly reduced membrane permeability and ESBL production, and not carbapenemase production. Additional unknown mechanisms may be involved in ertapenem resistance in some isolates, hence further investigation is needed.

Opsomming

Agtergrond

Karbapenem-weerstandige Gram-negatiewe bakterieë is 'n alomvattende publieke gesondheidsprobleem weens ‘n beperking van toepaslike mediese behandelings. In *Enterobacter* spesies word ertapenemweerstandigheid toegeskryf aan karbapenemase-produksie en/of verminderde membraandeurlaatbaarheid, as gevolg van onderdrukking van die OmpF- en OmpC-buitemembraanproteïene, in kombinasie met uitgebreide spektrum β-laktamase (ESBL) produksie, oorproduksie van chromosomale *bla*AmpC β-laktamases en/of die ooruitdrukking van die AcrAB/TolC efflukspomp. Hierdie studie ondersoek die meganismes wat ertapenemweerstandigheid veroorsaak in kliniese *Enterobacter cloacae* isolate vanaf Tygerberg hospitaal.

Materiale en metodes

Twintig ertapenem-nie-vatbare kliniese *E. cloacae*-isolate, vier ertapenem-vatbare kontrole en vyf wilde-tipe kontrole is ingesluit op grond van VITEK2®AES resultate. Die vlak van ertapenem weerstandigheid is bepaal deur middel van die antibiotiese-mikroverdunning (BMD) metode, asook gradiëntdiffusie. Weerstandigheidsmeganismes is bepaal met verskeie fenotipiese toetse, soos VITEK2®AES, “Mastdiscs” D68C-, D69C- en D63C-kombinasiestelle, Rapidec®Carba NP-kit en 'n sinergie-toets met behulp van skyfdiffusie in die teenwoordigheid van 'n efflukspompinhibeerder (EPI). ‘n “Multiplex” polimerasiekettingreaksie (PKR) is toegepas om ESBLs en karbapenemases te identifiseer. Die hoeveelheid OmpC en OmpF proteïene is met SDS-PAGE bepaal en die vlak van uitdrukking van *bla*AmpC, *ompC*, *ompF* en *acrB* is met RT-qPKR bepaal.

Resultate en Bespreking

Sewentien (85%) ertapenem-nie-vatbare isolate is bevestig as ertapenem-nie-vatbaar deur BMD en ses (30%) deur gradientdiffusie. Dit dui aan dat ertapenemweerstandigheid moontlik deur gradientdiffusie onderskat word en oorskat word deur VITEK2® AES.

Sewe ertapenem-nie-vatbare isolate is voorspel om karbapenemase-produseerders te wees deur die VITEK2® AES en een volgens die Rapidec Carba NP-kit, maar geen karbapenemases is deur PKR waargeneem nie. Negentien ESBL-produseerders is deur die VITEK2® AES geïdentifiseer, agt deur die D68C-kombinasiestel en elf deur die D63C-kombinasiestel. ESBLs is in 12 (60%) isolate deur middel van PKR en DNA-volgordebepaling geïdentifiseer, waarvan agt *bla*CTX-M bevat, drie *bla*SHV-12 bevat en een isolaat beide gene bevat.

Twaalf (60%) ertapenem-nie-vatbare isolate is deur VITEK2® AES voorspel om on-onderdrukte *bla*AmpC-produseerders te wees, dertien (65%) deur die D68C-kombinasiestel en vyf (25%) deur *bla*AmpC RT-qPKR.

Die hoeveelheid OmpC en OmpF kon nie deur SDS-PAGE bepaal word nie, maar verlaagde *ompC*- en *ompF* uitdrukking is onderskeidelik in elf (55%) en twaalf (60%) van die ertapenem-nie-vatbare isolate waargeneem. Die uitdrukking van beide gene is verlaag in sewe (35%) van die ertapenem-nie-vatbare isolate.

Die sinergie-toets het nie verhoogde antibiotiese vatbaarheid in die teenwoordigheid van die EPI waargeneem nie. Verhoogde effluksaktiwiteit gebaseer op die uitdrukking van *acrB* is in twee (10%) van die ertapenem nie-vatbare isolate waargeneem.

Altesaam het 11 van die 17 BMD bevestigde ertapenem-nie-vatbare isolate ESBL-produksie en ‘n verminderde membraandeurlaatbaarheid getoon; twee hiervan is chromosomale *bla*AmpC-produseerders, en twee het verhoogde effluksaktiwiteit getoon. Karbapenemaseproduksie is waargeneem in een isolaat, wat ook die oorproduksie van AmpC en verminderde membraandeurlaatbaarheid getoon het. Een isolaat het oorproduksie van chromosomale *bla*AmpC en verminderde membraandeurlaatbaarheid getoon, terwyl twee slegs verminderde membraandeurlaatbaarheid en een slegs die oorproduksie van chromosomale *bla*AmpC getoon het. In een ertapenem-nie-vatbare isolaat is geen weerstandsmeganismes opgespoor nie (ERD 13), wat daarop dui dat alternatiewe meganismes bydra tot die weerstandigheid in hierdie isolaat.

Gevolgtrekking

Ertapenemweerstandigheid in hierdie kliniese *E. cloacae* isolate is deur middel van 'n kombinasie van meganismes bemiddel; oorwegend verminderde membraandeurlaatbaarheid en ESBL produksie, en nie karbapenemase produksie nie. Onbekende ertapenem-weerstandigheidsmeganismes kan by sommige isolate betrokke wees en daarom is verdere navorsing nodig.

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List of Abbreviations

AAC-1	Ambler class C-1
ABC superfamily	Adenosine triphosphate-binding cassette superfamily
ACT	AmpC type
ACN	Acetonitrile
AcrA	Acriflavine resistance protein A
AcrB	Acriflavine resistance protein B
AIM	Australian imipenemase Metallo β-lactamases
AmpC	Ampicillin Class C
AMR	Antimicrobial Resistance
AR1	*Acinetobacter* resistant to imipenem
AST	Antimicrobial Susceptibility Testing
BARDA	Biomedical Advanced Research and Development Agency
BLAST	Basic Local Alignment Search Tool
BHI	Brain heart infusion
βLIs	β-lactamase inhibitors
BMD	Broth microdilution
CAF	Central Analytical Facility
CAMHB	Cation-adjusted Mueller-Hinton broth
cDNA	Complementary deoxyribonucleic acid
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CMY	Cephamycins
CRPs	Carbapenem resistant pathogens
CRE	Carbapenem Resistant Enterobacteriaceae
C_t	Comparative threshold cycles
DHA	Dhahran Hospital in Saudi Arabia

DHP-1	Renal dehydropeptidase I
DNA	Deoxyribonucleic acid
2D-DIGE	2D-Difference Gel electrophoresis
EDTA	Ethylenediamine tetra-acetic acid
EPIs	Efflux pump inhibitors
ESBLs	Extended-spectrum β-lactamases
EST	Expressed sequence tag (EST) assay
FOX	Cefoxitin
GES	Guiana extended-spectrum enzymes
GIM	German imipenemase Metallo β-lactamases
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
HAI	Healthcare-associated infections
HEPES	4 - (2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid Chromatography
HREC	Health Research Ethics Committee
ICUs	Intensive care units
IMI	Innovative Medicines Initiatives
IMI carbapenemases	Imipenem hydrolyzing carbapenemases
IMP	Imipenem resistant enzymes
JPIAMR	Joint Programming Initiative on Antimicrobial Resistance
KHM enzymes	Kyorin Health Science Metallo β-lactamases
KPC	*Klebsiella pneumoniae* carbapenemase
LC-MS	Liquid Chromatography Tandem Mass Spectrometry
MATE superfamily	Multidrug and toxic compound extrusion superfamily
MBLs	Metallo β-lactamases
MBK	Microbank
MDR	Multi-drug resistant

ME	Major Error
MFS superfamily	Major Facilitator superfamily
MH	Mueller Hinton
MHT	Modified Hodge test
MICs	Minimum inhibition concentrations
MiE	Minor Error
MicF RNA	mRNA interfering complementary RNA
MIR-1	Miriam Hospital in Providence, R.I.
MLST	Multi-locus sequence typing
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NDM	New Delhi Metallo β-lactamases
NHSN	National Healthcare Safety Network
NICD	National Institute for Communicable Diseases
NIH	National Institutes of Health
NMC-A	Non-metallo-carbapenemase-A
NoRT	No Reverse Transcriptase
OMPs	Outer membrane proteins
OXAs	Oxacillinases
PaβN	Phenylalanine-arginine β-naphthylamide
PBPs	Penicillin-Binding Proteins
PCR	Polymerase chain reaction
PER	*Pseudomonas* extended resistant enzymes
PFGE	Pulsed-field gel electrophoresis
rep-PCR	Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction
ROMP	Rapid outer membrane protein

RNA	Ribonucleic acid
RND superfamily	Resistance-nodulation-cell division superfamily
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SHV	Sulfhydryl derivatives
SIM	Seoul imipenemase Metallo β-lactamases
SMR superfamily	Small multidrug resistance superfamily
SME	*Serratia marcescens* enzyme
SPM	Sao-Paulo Metallo β-lactamases
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBA	Tryptone bile agar
TBH	Tygerberg Hospital
TEMED	N, N, N′, N′-Tetramethylethylenediamine
TMP-SMZ	Trimethoprim-sulfamethoxazole
TolC	Tolerance Colicin E1
TSA	Trypticase Soy Agar
T3SS	Type III secretion system
UDP	Uridine diphosphate
VIM	Verona integron-encoded Metallo β-lactamases
VME	Very Major Error
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild-type
ZOI	Zones of inhibition

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Chapter 1 : Literature Review

1.1: Introduction

Antibiotics play an important role in treating life-threatening bacterial infections. Since the introduction of the first penicillin antibiotics in the 1940s, bacteria have developed resistance to several antibiotics. This can be partly attributed to certain risk factors and practices. These include overuse of antibiotics as growth promoters in the agricultural sectors, over-prescription of broad-spectrum antibiotics by clinicians, pro-longed hospital stays in specialized wards including intensive care units (ICUs) and burns units, inter-country travels for tourism and healthcare purposes and poor sanitation practices and personal hygiene (Watkins and Bonomo, 2016). Antimicrobial resistance is a major problem globally due to limited treatment options to deal with multi-drug resistant (MDR) pathogens, and is particularly concerning in cases such as cancer chemotherapy and organ transplantation which rely heavily on antibiotics.

In 2013, the Centers for Disease Control and Prevention (CDC) reported that annually approximately 23,000 deaths could be attributed to antimicrobial resistance (AMR) in the United States of America (USA) (CDC, 2013). Recently, the death rate has risen to about 1 million per year, and is projected to increase to 10 million globally in 2050, if appropriate interventions are not in place (United Nations and Social Affairs, 2015). AMR also impacts the economy of most countries. For example, it costs the US healthcare system about 20 billion USD every year to control bacteria resistant to first-line antibiotic treatment (Smith and Coast, 2013). This global economic impact is expected to increase to 100 trillion USD by 2050, if there are no interventions from both the public and private sectors to deal with the current situation (United Nations and Social Affairs, 2015).

Despite the impact of AMR on mortality and the economy, there has been underinvestment from both the public and private sections in the development of new antibiotics. A recent review by Renwick *et al* (2016), confirmed that out of 38 billion USD invested in pharmaceutical research and development from 2003 to 2013, only 1.8 billion was invested in antimicrobial research. Similarly, from 2009 to 2014, the US National Institutes of Health (NIH) allocated only 1.2% of grant funding to AMR-related research, compared to 18.6% towards cancer research (O'Neill, 2015). Very few antibiotics have been produced or introduced since the 1980s, especially against Gram-negative bacteria (GNB) (Payne *et al* 2007).

Most pharmaceutical companies focus on research areas with higher commercial returns such as cancer, instead of antibiotic research. In 2014, about 800 first-in-line cancer drugs were produced, compared to less than 50 new antibiotics currently in the pipeline (Kinch and Patridge, 2014), suggesting that AMR will continue to be a problem.

After recognizing AMR as one of the leading causes of mortality, in 2015, the World Health Organization (WHO) initiated a global action plan on AMR to improve awareness and understanding, to strengthen knowledge through surveillance and research, to reduce incidence of infection, to optimize the use of antimicrobial agents and to develop the economic case for sustainable investment (WHO, 2015). In addition, funding into antimicrobial research to promote the production of new drugs, especially against MDR GNB has been provided by the European Joint Programming Initiative on AMR (JPIAMR), US Biomedical Advanced Research and Development Agency (BARDA) and Innovative Medicines Initiatives (IMI) (Eichberg, 2015). However, these initiatives are not adequate to drastically tackle AMR worldwide.

In South Africa, there is an enforcement of the Antimicrobial Stewardship Programme (Brink *et al* 2016), and screening for carbapenemases (carbapenem-hydrolyzing enzymes) in Enterobacteriaceae by the National Institute for Communicable Diseases (NICD), however AMR is still a major problem. Investigation of other possible mechanisms through which these MDR GNB become resistant to broad-spectrum β-lactams, is limited in the current surveillance system (Singh-Moodley and Perovic, 2016). Therefore, research involving all possible AMR mechanisms is needed to improve surveillance, and knowledge for appropriate treatment options in dealing with these pathogens.

1.2 β-lactam Antibiotics

β-lactams are one of the safest, broad-spectrum antibiotics, active against mostly GNB. All β-lactam antibiotics are bactericidal agents, meaning, they are able to kill their target micro-organisms with minimal effect on the host cells. β-lactams act by inhibiting the synthesis of the cell walls of their targets. This mechanism involves preventing the crosslinking of peptidoglycan by the transpeptidase enzymes, thereby affecting the growth rates and survival of targeted pathogens (Lambert, 2011). The β-lactam ring is a common structure found in all β-lactam antibiotics. β-lactam antibiotics are divided into four main classes namely: penicillins, monobactams, cephalosporins and carbapenems (Figure 1.1). This classification is based on alterations to the side chains adjacent to the β-lactam ring (Lambert, 2011).

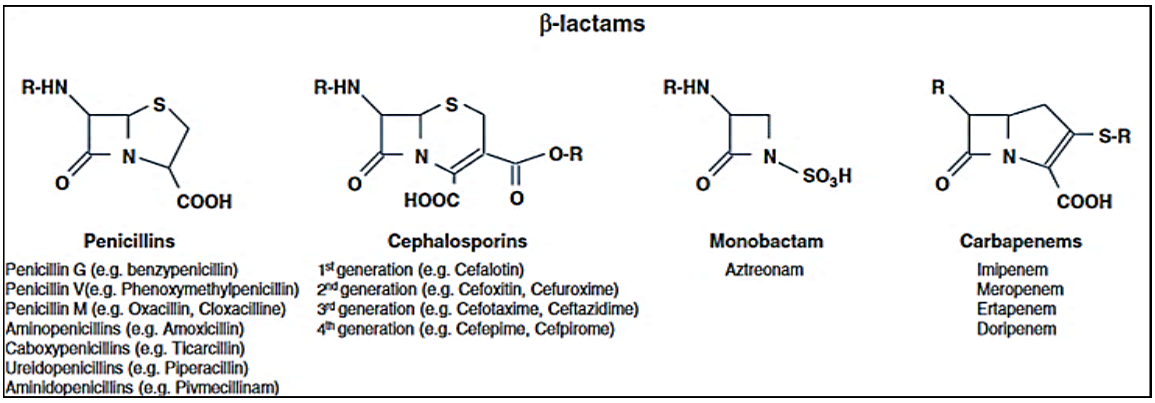

Figure 1.1: Chemical structures of β-lactam antibiotics (Nordmann *et al* 2012)

1.2.1 Penicillins

Penicillin, often referred to as the 'wonder drug', was produced by the fungus *Penicillium notatum*, which was identified by Dr. Alexander Fleming in 1929. Penicillins consist of a five-membered ring adjacent to the β-lactam ring (Figure 1.1).

Penicillins are considered effective drugs based on two factors:

(1) They bind to penicillin-binding proteins (PBPs) which act as transpeptidases and assist with peptidoglycan synthesis, thereby inhibiting transpeptidation.

(2) Penicillin-treated cells activate autolytic enzymes called peptidoglycan hydrolases, which are capable of degrading peptidoglycan, thereby leading to cell death (Levinson, 2014).

There are four major groups of penicillins, namely narrow penicillins, moderate penicillins, extended penicillins, and moderate penicillins with β-lactamase inhibitors (Miller, 2002).

Narrow penicillins are produced naturally from molds. They were the first penicillins to be produced, are easily degraded by β-lactamases, and have the narrowest spectrum of activity against some Gram-positive bacteria (GPB). An example is phenoxymethylpenicillin (Miller, 2002).

Moderate penicillins have activity against GNB, and are used as the backbone for developing extended penicillins. Examples include amoxicillin, ampicillin and bacampicillin (Miller, 2002).

Extended penicillins have broad-spectrum activity against both GNB and GPB. This is based on their high affinity for PBPs and ease of penetration. Examples include azlocillin, mezlocillin and piperacillin (Selwyn, 1982; Lambert, 2011).

Moderate penicillins are often combined with β-lactamase inhibitors (βLIs) to enhance their activity. βLIs are substances which inhibit the activity of β-lactamases, by binding irreversibly to their active site, preventing the degradation of the β-lactam antibiotics (Bebrone *et al* 2010). Early βLIs include clavulanic acid, sulbactam, and tazobactam (Brown *et al* 1976; English *et al* 1978; Aronoff *et al* 1984). Newly identified βLIs include relebactam (MK-7655) (Young *et al* 2009), avibactam (NXL104), a non-β-lactam βLI (Coleman, 2011), and vaborbactam (RPX7009), and a novel boronic acid β-lactamase inhibitor (Rempex Pharmaceuticals, 2012).

The four most common β-lactam/β-lactamase inhibitor combinations used for treating certain types of β-lactamase-producing organisms include amoxicillin/clavulanate, ticarcillin/clavulanate, ampicillin/sulbactam, and piperacillin/tazobactam (Bush, 1988), whilst some novel combinations include ceftazidime/avibactam (Shazad *et al* 2010), ceftaroline/avibactam (Mushtaq *et al* 2010), ceftolozane/tazobactam (Craig & Andes, 2013), and imipenem/relebactam (Lob *et al* 2017).

1.2.2 Monobactams

Monobactams lack a membered ring structure adjacent to the β-lactam ring, hence, referred to as 'monocyclic β-lactams' (Sykes *et al* 1981) (Figure 1.1). These agents were first isolated in the mid-1970s from soil dwelling bacteria including *Chromobacterium*, *Gluconobacter*, *Agrobacterium*, *Flexibacter* and *Acetobacter* species. The naturally occurring monobactams were found to be unstable (Sykes, 1985); however, variation of their side chains led to the discovery of more stable monobactams with a broader spectrum of activity, such as aztreonam. Monobactams are resistant to most β-lactamases, and do not induce the production of these enzymes. Monobactams are only active against aerobic Gram-negative bacilli, and are potent against *Neisseria gonorrhoeae* and non-enteric bacilli (Sykes *et al* 1982).

1.2.3 Cephalosporins

Cephalosporins are made by molds belonging to the genus *Cephalosporium*. However, a few are from the actinomycete *Streptomyces*. Cephalosporins have a six-membered ring adjacent to the β-lactam ring (Levinson, 2014) (Figure 1.1).

There are five generations of cephalosporins (first to fifth), distinguished based on how effective they are against a wide range of organisms. First and second generation cephalosporins are early generation cephalosporins, whilst third to fifth generation cephalosporins are extended-spectrum cephalosporins.

First-generation cephalosporins are active against Gram-positive cocci such as *Staphylococci* and *Streptococci*, except Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Enterococci*. Examples of this group are cephalothin, cefazolin and cephalexin.

Second-generation cephalosporins are active against some Gram-negative rods such as *Haemophilus influenzae, Neisseria gonorrhoeae*, some strains of *Enterobacter* and *Serratia, Klebsiella pneumoniae, Escherichia coli* and anaerobes. Examples include cefuroxime, cefoxitin, and cefotetan.

Third-generation cephalosporins have a wide spectrum of activity. Examples are ceftazidime, moxalactam, cefotaxime and ceftriaxone. Cefotaxime is active against most members of Enterobacteriaceae family. Ceftazidime is active against *P. aeruginosa*. Ceftriaxone has similar activity against Enterobacteriaceae as cefotaxime. Moxalactam is mostly active against *Bacteroides fragilis*.

Fourth-generation cephalosporins are active against enteric Gram-negative rods, and but also have enhanced Gram-positive activity. Examples are: cefepime and cefpirome. Both agents also have enhanced activity against *Pseudomonas* spp.

Fifth-generation cephalosporins are active against MRSA isolates. Some examples include ceftaroline and ceftobiprole which are both active against GPB including *S. aureus* isolates resistant to methicillin antibiotics (Anderson and Gums, 2008; Biek *et al* 2010). Ceftolozane is a novel agent of this group which is highly active against enterobacterials and *P. aeruginosa* (Craig and Andes, 2013).

1.2.4 Carbapenems

Carbapenems differ from penicillins in that a carbon atom is replaced with a sulphur atom at position C1 of the 5-membered ring structure, attached to the β-lactam ring (Norrby, 1995). They have the broadest spectrum of activity of the β-lactams. Carbapenems are stable against most β-lactamases except carbapenemases, due to a trans-α-1-hydroxylethyl substituent at the 6th position, compared to a *cis* configuration found in cephalosporins and penicillins (Kahan *et al* 1983; Moellering *et al* 1989).

Carbapenems have enhanced activity against both GNB and GPB because, they have high affinity for several essential PBPs, including PBPs1a, 1b, 2, and 3, from a broad range of bacteria, compared to penicillins, cephalosporins and monobactams, whose primary target is PBP3 (Kahan *et al* 1983).

Thienacymin, produced by *Streptomyces cattleya* was the first carbapenem identified in the mid-1970s (Kahan *et al* 1979). The instability of thienacymin led to the production of newer carbapenems such as imipenem, meropenem, ertapenem, biapenem, doripenem and tebipenem.

Imipenem, which is an N-formimidoyl derivative, was the first new carbapenem to be produced. Imipenem possesses a methylene group instead of sulphur in the β-lactam ring (Leanza *et al* 1979). Imipenem is mostly active against Gram-positive cocci including *Streptococci* and *Staphylococci*, Gram-negative cocci such as *Neisseria* spp., and Gram-negative rods. Although imipenem is effective, it is rapidly degraded by dehydropeptidase-1 (DHP-1), an enzyme located in the proximal renal tubules of mammals. In view of this, cilastatin, a DHP-1 inhibitor, is administered with imipenem to inhibit DHP-1 activity and to prevent nephrotoxicity (Leanza *et al* 1979). Imipenem is an exceptionally rapid permeant due to its small size and zwitterionic property (Matsumura *et al* 1999).

Meropenem differs from imipenem by having a pyrrolidinyl substituent at position 2. Meropenem is more stable against DHP-1 activity than to imipenem (Tanio *et al* 1987). Meropenem is slightly more effective against Gram-negative aerobic bacteria compared to Gram-positive bacteria.

Ertapenem is structurally similar to meropenem and imipenem, but possesses a meta-substituted benzoic acid group at position 2 (Shah and Isaacs, 2003). Possession of this group accounts for its extensive protein binding and slow outer membrane permeation in GNB (Nikaido *et al* 1983). Ertapenem is used for treating complicated community-acquired urinary tract infections, skin and intra-abdominal infections caused by Enterobacteriaceae, some GPB and anaerobes; however, it lacks activity against non-fermenting GNB such as *P. aeruginosa* and *Acinetobacter* spp. (Livermore *et al* 2001).

Biapenem is one of the newest carbapenems, first approved for usage in Japan in the early 2000s (Rempex Pharmaceuticals, 2012). It has a similar spectrum of activity to meropenem and imipenem antibiotics.

Doripenem has a sulfamoylaminoethyl-pyrrolidinylthio group in its side chain at position 2, increasing its activity against non-fermentative GNB and GPB (Tsuji *et al* 1998). Doripenem has a strong affinity for PBP targets that are species-specific. For example, PBP3 in *P. aeruginosa*, PBPs 1, 2 and 4 in *S. aureus*; and PBP2 in *E. coli* (Hanaki *et al* 1996).

Tebipenem, the newest carbapenem, has a 1-(1, 3-thiazolin-2yl) azetidin-3-ylithio group at position 2. Tebipenem is highly stable against DHP-1. This agent has been showed to have activity against *Streptococcus pneumoniae* (Kobayashi *et al* 2005).

1.3 *Enterobacter* species

1.3.1 The organism

The genus *Enterobacter*, belonging to the family Enterobacteriaceae, was first described by Hormaeche and Edwards in 1960 (Hormaeche and Edwards, 1960). *Enterobacter* spp. are aerobic Gram-negative bacilli. They share some characteristics with *Klebsiella* spp.; however, they produce ornithine decarboxylase and are motile; although some rare non-motile *Enterobacter* spp. have been identified (Ehrhardt *et al* 1993). *Enterobacter* species act as co-host dwellers in the gastrointestinal tract of humans and animals, and are also distributed in the environment in soil, water, and sewage (Dudley *et al* 1980).

There are about 14 subgroups under the genus *Enterobacter*, but not all are known to cause infections in humans or animals (Farmer *et al* 1995). Modern techniques used to identify these subgroups include sequencing of the *rpoB* gene (for differentiating between closely related species) (Hoffmann and Roggenkamp, 2003), microarray-based comparative genomic hybridization analysis (Paauw *et al* 2008), matrix-assisted laser desorption ionization time-of-flight mass spectrometry and real time PCR (Pavlovic *et al* 2012).

Among these subgroups, *E. cloacae* (Figure 1.2) and *E. aerogenes* are of great clinical importance; however, most infections in humans are caused by *E. cloacae* species (Mezzatesta *et al* 2012). Other species such as *E. gergoviae*, *E. sakazakii*, *E. amnigenus*, *E. taylorae*, and *E. asburiae*, are also isolated from clinical samples (Brenner *et al* 1980; Farmer *et al* 1980; Izard *et al* 1981; Farmer *et al* 1985; Brenner *et al* 1986). Other strains such as *E. nimipressuralis* have been identified as plant pathogens and do not affect humans (Brenner *et al* 1986).

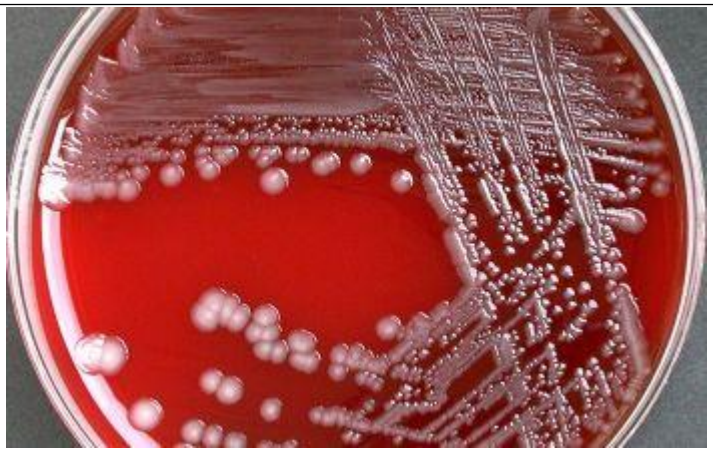

Figure 1.2: *Enterobacter cloacae* cultured on a tryptose blood agar plate (https://i.pinimg.com/originals/10/5d/fc/105dfcea8661c5ed9f6415acaf11dfc8.jpg).

Characterization of *Enterobacter* strains was performed in the past using traditional methods such as biotyping, serotyping and phage typing methods (Aber *et al* 1981). Recent molecular-based techniques used include pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984), rep-PCR (Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction) (Versalovic *et al* 1991), multi-locus sequence typing (MLST) (Maiden *et al* 1998), and whole genome sequencing (Van El *et al* 2013)

1.3.2 Epidemiology and clinical manifestations of *Enterobacter* infections

During the pre-antibiotic era, *Enterobacter* species were not considered to be nosocomial pathogens until 1976, during a nationwide outbreak of septicemia associated with contaminated intravenous solutions in some American hospitals (Maki *et al* 1976). Since their discovery, *Enterobacter* spp. have become important pathogens. The National Healthcare Safety Network (NHSN) report from 2006 to 2007 also confirmed *Enterobacter* spp. as the eighth most common cause of all healthcare-associated infections (HAI) (Hidron *et al* 2008). Several outbreaks of *E. cloacae* infections have been reported in the neonatal ICUs in the Gauteng province of South Africa (Nierop *et al* 1998) and in Brazil (Dalben *et al* 2008). Cases of community-acquired *Enterobacter* infections among female outpatients with confirmed urinary infections have been reported in India (Kothari & Sagar, 2008).

Enterobacter spp. are known to cause common clinical infections including: mediastinitis (Akl *et al* 1985), bacteremia (Weischer & Kolmos, 1992), sepsis amongst neonates and adults (Bhutta, 1996; Hervas *et al* 2001; Lai, 2001), meningitis (Huang *et al* 2001), central nervous system infections, meningitis (Huang *et al* 2001), urinary tract infections (Allan, 2003), and intra-abdominal infections (Paterson *et al* 2005).

1.3.3 Pathogenesis and Risk factors

Enterobacter spp. possess several virulence factors including endotoxins, enterotoxins, adhesins, α-hemolysin and thiol-activated pore-forming cytotoxins similar to Shiga-like toxin II (Barnes *et al* 2001). Additional virulence factors include the type III secretion system (T3SS) which plays an important role in host-pathogen-interactions (Stuber *et al* 2003), and ‘‘curli fimbriae’’ (proteinaceous extracellular fibers) which help to mediate host cell adhesion and invasion (Kim *et al* 2012).

Enterobacter spp. are opportunistic pathogens which cause infections when the host immune system is impaired. Often times, patients in special units (ICUs, burns and wounds, and surgery) are most prone to *Enterobacter* infections (Mayhall *et al* 1979; Burchard *et al* 1986). Infections caused by *Enterobacter* spp. are acquired from both endogenous and exogenous sources. Endogenous sources are the commonest, usually through the skin, gastrointestinal and urinary tracts (Flynn *et al* 1987),

whilst exogenous sources are from hospital devices such as central intravenous catheters, stethoscopes, fluids (such as contaminated intravenous fluid) (Matsaniotis *et al* 1984), and blood (Stenhouse, 1992).

1.3.4. Antibiotic resistance in *Enterobacter* spp.

Enterobacter infections are treated with β-lactams (such as penicillins, βLIs, narrow and extended-spectrum cephalosporins), fluoroquinolones, aminoglycosides, colistin, tigecycline and TMP-SMZ (trimethoprim-sulfamethoxazole). Resistance to most of these antibiotics has been reported in *Enterobacter* spp. (Neonakis *et al* 2003; Wilke *et al* 2005; Daurel *et al* 2009).

In general, *Enterobacter* spp. are intrinsically resistant to aminopenicillins, and may become resistant to narrow-spectrum cephalosporins upon continuous exposure, which leads to constitutive production of chromosomal Ampicillin class C (*bla*AmpC) β-lactamases, and can result in resistance to other broad-spectrum antibiotics (Jacoby, 2009). Hyper-production of these enzymes may occur in either derepressed or inducible *Enterobacter* mutants.

In addition, *Enterobacter* spp. can acquire other hydrolyzing enzymes (β-lactamases) such as extended-spectrum β-lactamases (ESBLs) (Bradford, 2001), which have a broad-spectrum of activity against extended-spectrum cephalosporins, resulting in the use of carbapenems, which are effective against ESBL-producing *Enterobacter* spp. (Queenan and Bush, 2007).Carbapenem resistant pathogens (CRPs) have been reported for over a decade now, posing a major public health problem due to limited effective treatment options (Nordmann & Poirel, 2002). Recently, the World Health Organization has listed CRPs in the category of most prioritized of bacterial species (WHO, 2017). CRPs are therefore treated with the ‘very last resort drugs’ such as colistin and tigecycline although some toxicity effects have been associated with these agents, and resistance to colistin is more frequently seen (Falagas and Kasiakou, 2005; Falagas and Petros Kopterides, 2007; Falagas *et al* 2014).

In South Africa, the presence of β-lactamases in GNB and other bacterial species has been reported in several provinces (Osei Sekyere, 2016). ESBL production (due to resistance to extended-spectrum cephalosporins and β-lactamase inhibitors) (Bradford, 2001), plasmid-mediated *bla*AmpC enzyme (due to resistant to β-lactamase inhibitor combinations; except possibly when combined with tazobactam) (Philippon, Arlet & Jacoby, 2002) and carbapenemase production (due to resistant to all carbapenems) are the major β-lactamase resistance mechanisms reported in South Africa. There are a variety of these hydrolyzing enzymes occurring which is a reflection of overuse of available effective antimicrobial agents. For example, ESBL types such as: TEM-26, TEM-55, TEM-145, TEM-146, CTX-M-1, SHV-2, SHV-5, SHV-12 (Pitout *et al*, 1998; Mocktar, *et al* 2009; Rubin, *et al*

2014), plasmid-mediated *bla*AmpC enzymes (CMY-20) (Mocktar *et al* 2009) and carbapenemases (oxacillinases and New Delhi Metallo-β-lactamases identified as common types) (Osei Sekyere, 2016) have been reported.

1.4 Carbapenem Resistance Mechanisms

Carbapenem resistance is driven by a number of mechanisms (Figure 1.3) and the types of mechanisms depend on the bacterial species. In *Enterobacter* spp., carbapenem resistance may be mediated through the acquisition of carbapenemases and / or reduced outer membrane permeability, in combination with ESBLs, hyper-production of chromosomal *bla*AmpC β-lactamases and / or over-expression of efflux pumps (Szabó *et al* 2006; Yang *et al* 2012).

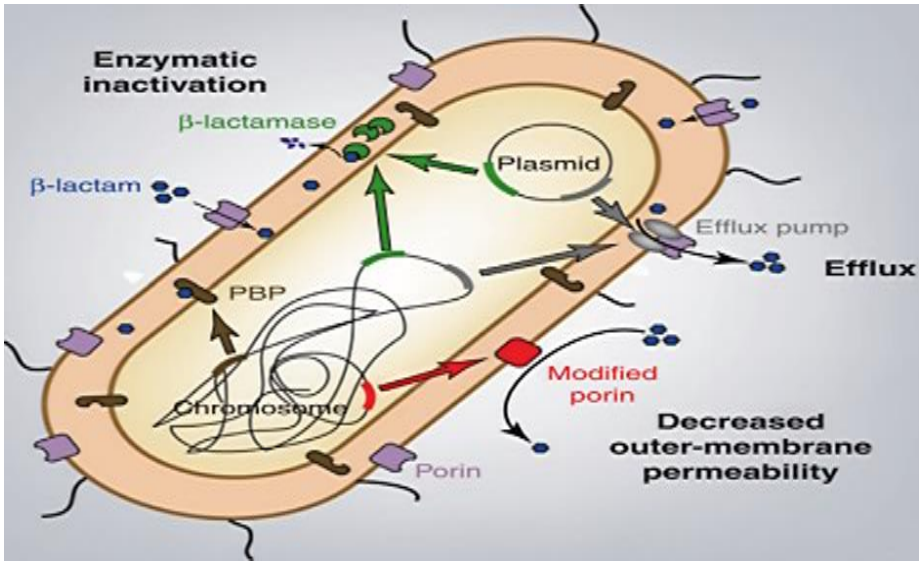

Figure 1.3: Bacterial resistance mechanisms (Nordmann *et al* 2012)

1.4.1. β-lactamases

β-lactamases are enzymes capable of hydrolyzing the β-lactam ring of β-lactam antibiotics, and can be either plasmid-mediated or encoded on chromosomes (Abraham & Chain, 1940). There are two major classifications of β-lactamases: functional (based on substrate and inhibitor profiles), and molecular (based on their amino acid sequences) (Bush *et al* 1995).

The molecular classification differentiates β-lactamases into four major classes (A, B, C and D). Classes A, C and D utilize a serine active site, whilst class B (Metallo-β-lactamases) uses divalent zinc atoms during β-lactam hydrolysis. Class C enzymes have about 360 amino acids in their coding sequences compared to less than 310 amino acids in both classes A and D (Jaurin and Grundstrom,

1981). Class A enzymes have a conserved glutamic acid at sequence position number 166 whereas class D enzymes have a carbamylated lysine residue in its active site (Herzberg *et al* 1987; Maveyraud *et al* 2000).

The functional classification distinguishes β-lactamases into Bush group 1, groups 2b, 2be, 2br, 2ber, 2c, 2d, 2f and groups 3 and 4. The specific substrates, inhibitor profiles and representative enzymes from these groups are described in Table 1.1.

Table 1.1: Molecular and functional classification of β-lactamases. Adapted from Bush *et al* 1995

Functional group	**Molecular class**	**Preferred substrate**	**Inhibition**		**Representative enzymes**
			Clavulanic acid	**EDTA**	
1	C	Cephalosporins	_	_	*bla*AmpC enzymes from Gram-negative bacteria; MIR-1
2a	A	Penicillins	+	_	Penicillinases from Gram-positive bacteria
2b	A	Penicillins, cephalosporins	+	_	TEM-1, TEM-2, SHV-1
2be	A	Penicillins, narrow-spectrum and extended-spectrum cephalosporins, monobactams	+	_	TEM-3 to TEM-26, SHV-2 to SHV-6, *Klebsiella oxytoca* K1
2br	A	Penicillins	±	_	TEM-30 to TEM-36, TRC-1
2c	A	Penicillins, carbenicillin	+	_	PSE-1, PSE-3, PSE-4
2c	A	Cephalosporins	+	_	Inducible cephalosporinases from *Proteus vulgaris*
2d	D	Penicillins, cloxacillin	±	_	OXA-1 to OXA-11, PSE-2, (OXA-10)
2f	A	Penicillins, cephalosporins, carbapenems	+	_	NMC-A from *E. cloacae*, SME-1 from *Serratia marcescens*
3	B	Most β-lactams including carbapenems	_	+	L1 from *Xanthomonas maltophilia*, CcrA from *Bacteroides fragilis*
4	Not determined	Penicillins	+	Unk.	Penicillinase from *Pseudomonas cepacia*

+ = positive, - = negative, ± = either positive or negative, Unk. = unknown

1.4.1.1 Extended-spectrum β-lactamases (ESBLs)

ESBLs are plasmid-mediated β-lactamases capable of hydrolyzing penicillins, narrow to extended-spectrum cephalosporins and monobactams, but not carbapenems (Bradford, 2001). These plasmids can contain other resistance genes, hence, promoting resistance to other classes of antibiotics.

ESBLs fall under the '2be' functional subgroup. The '2be' classification indicates that they were first derived from the '2b' subgroup which contains TEM-1, TEM-2 and SHV-1 β-lactamases, but have extended-spectrum of activity (Bush *et al* 1995). ESBLs are inhibited by βLIs such as clavulanic acid, sulbactam and tazobactam.

The three major ESBL family genes are: *bla*TEM, *bla*CTX-M and *bla*SHV (Bradford, 2001). Other ESBL types include *bla*PER (Nordmann and Naas, 1994), *bla*SFO-1 (Matsumoto and Inoue, 1999) *bla*VEB (Poirel *et al* 1999), *bla*BES-1 (Bonnet *et al* 2000), , *bla*TLA-1 (Silva *et al* 2000), and *bla*PER (Yong *et al* 2003).

CTX-M

CTX-M ESBLs originated from an environmental *Kluyvera* spp.; but are currently also found in enterobacterials and other bacterial species worldwide, due to their high dissemination rates (Poirel *et al* 2002). There are about 30 different variants classified into five main groups based on their amino acid sequences. These groups are: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Bonnet, 2004). In general, CTX-M enzymes have potent hydrolytic activity against cefotaxime, however, some common variants such as CTX-M-15 are capable of hydrolyzing ceftazidime (Poirel *et al* 2002).

TEM

TEM designation was due to the first isolation of a TEM-1 *E. coli* producing isolate from a patient called Temoneira, from Greece (Datta and Kontomichalou, 1965). The TEM-1 enzyme is the commonest TEM type. TEM-2, which is considered to be the first derivative of TEM-1, differs from the latter by a single amino acid substitution (Barthelemy *et al* 1985). TEM-1 and TEM-2 β-lactamases serve as progenitors for other TEM ESBL types. Currently, there are over 200 new derivatives of TEM-type β-lactamases; most of which are ESBLs (http://www.lahey.org/studies/temtable.asp). These derivatives are commonly found in *E. coli* and *K. pneumoniae*; but can be found in other enterobacterials and some non-Enterobacteriaceae GNB (Palzkill *et al* 1995; Mugnier *et al* 1996).

Some of these derivatives are commonly isolated in some countries. For example, *bla*TEM-3 is common in France (Soilleux *et al* 1996) and *bla*TEM-47 in Poland (Gniadkowski *et al* 1998).

SHV

SHV enzymes are of sulfhydryl variation due to the varied response to inhibition by *p*-chloromercuribenzoate substrate used for detection (Sykes and Bush, 1982). Substitutions on the *bla*SHV-1 gene sequence give rise to SHV ESBL types. There are over 100 SHV derivatives presently described (http://www.lahey.org/studies/). The variants SHV-5 and SHV-12 are among the most common SHV ESBL types (Paterson *et al* 2003). Most SHV ESBL types are identified based on the replacement of glycine with serine at the 238th position, or glutamate with lysine at the 240th position on the *bla*SHV-1 gene (Ambler *et al* 1991). Replacement of glycine with serine, or glutamate with lysine, enables the variant to be effective against cefotaxime and ceftazidime antibiotics respectively (Huletsky *et al* 1993).

1.4.1.2 Ampicillin Class C (*bla*AmpC) β-lactamases

*bla*AmpC β-lactamases are also known as 'cephalosporinases'. These enzymes are mainly found on the chromosomes of many members of the Enterobacteriaceae family such as *Serratia marcescens, Morganella morganii, Providencia* spp., *Enterobacter* spp., and *Citrobacter freundii* (Jacoby, 2009).

Plasmid-mediated *bla*AmpC types such as CMY (cephamycins), AAC-1 (Ambler class C-1), ACT (AmpC type), DHA (Dhahran Hospital in Saudi Arabia), FOX (cefoxitin), MIR-1 (Miriam Hospital in Providence, R.I.), and other families have been identified since 1989, but are less common than ESBLs (Philippon *et al* 2002). Some enterobacterials such as *K. pneumoniae* lack chromosomal *bla*AmpC, but can acquire these enzymes through transmissible elements and hence, can undergo *bla*AmpC-mediated resistance (Bauernfeind *et al* 1999).

*bla*AmpC β-lactamases are active against narrow-spectrum cephalosporins especially cephalothin, cefazolin and cefoxitin, and most penicillins (Jacoby, 2009). These enzymes are less inhibited by clavulanic acid, sulbactam and tazobactam; hence, cloxacillin, oxacillin and aztreonam are considered good inhibitors of *bla*AmpC β-lactamases (Bush *et al* 1995).

Regulation of *bla*AmpC expression

Chromosomal *bla*AmpC is normally expressed at low levels. Over-expression of *bla*AmpC can result from either induced expression or constitutive derepression of this gene. Inducible expression is due to prolonged exposure to specific β-lactams (imipenem, meropenem or cefoxitin), and βLIs (clavulanic acid); whilst derepression is due to mutations in the *bla*AmpC promoter regions or modifications in proteins involved in regulating *bla*AmpC expression (Livermore and Yang, 1987; Jacobs *et al* 1997).

During normal bacterial growth, a number of cell wall precursors (1, 6-anhydromuropeptides) are released, and transported to the cytoplasm through AmpG (an inner membrane permease). The muropentapeptide cell wall precursors are recycled into free peptides and anhydromuramic acid by AmpD (a cytosolic N-acetyl muramyl-L-alanine amidase), and transported back into the periplasmic space (Jacobs *et al* 1995). During cell wall synthesis, uridine diphosphate (UDP) is added to the pentapeptides to form the peptidoglycan precursors, UDP-MurNac-pentapeptides. Any excess UDP-MurNac-pentapeptides peptidoglycan precursors bind to AmpR (a transcriptional activator in the presence of certain β-lactams and repressor in their absence) which causes an inactive state (Lindquist *et al* 1989). The inactive state of AmpR then decreases the rate of *bla*AmpC transcription leading to low levels of *bla*AmpC production.

The induction of *bla*AmpC expression (Figure 1.4) occurs when inducing agents bind to specific PBPs generating excess ‘inducing’ muropentapeptides, instead of ‘repressed’ UDP-MurNac-pentapeptides. These ‘inducing’ muropentapeptides then bind to AmpR changing its inactive state into an active form. AmpR then binds to the promoter region of the *bla*AmpC gene. Transcription of the *bla*AmpC gene is then increased, leading to over-expression. The absence of these inducing agents is hypothesized to reverse the induction of *bla*AmpC expression (Lister *et al* 2009).

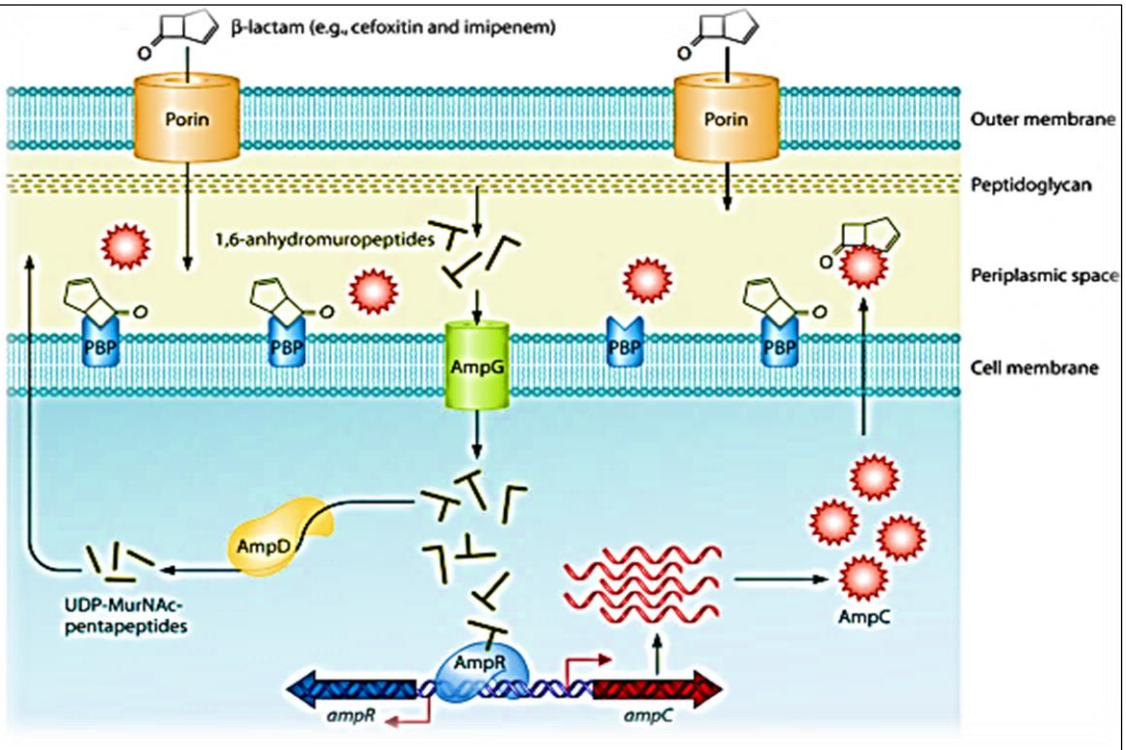

Figure 1.4: Induction of *bla*AmpC expression, as described in the text (Lister *et al* 2009).

The derepression of *bla*AmpC expression is driven by mutations in *ampD* and / or *ampR* (Figure 1.5). Mutations in the *ampR* gene results in the activation of AmpR, whilst mutations in *ampD* inactive AmpD. This process then leads to excess accumulation of muropentapeptides in the cytoplasm, which then bind to AmpR, converting it into a *bla*AmpC transcriptional activator, resulting in constitutively high production of *bla*AmpC transcripts, and *bla*AmpC over-expression (Jones *et al* 1997; Lister *et al* 2009). The absence of AmpG is hypothesized to prevent both induction and derepression of *bla*AmpC expression (Korfmann *et al* 1989).

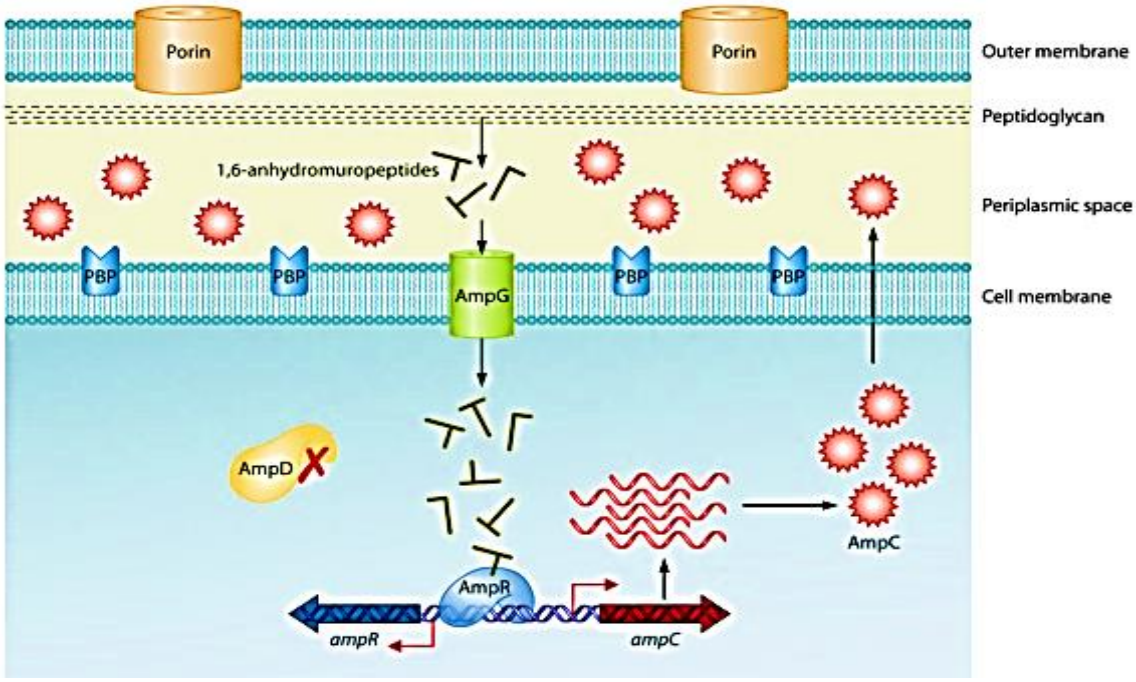

Figure 1.5: Derepression of *bla*AmpC expression, as described in the text (Lister *et al* 2009)

1.4.1.3 Carbapenemases

Carbapenemases are β-lactamases capable of hydrolyzing carbapenem antibiotics (Queenan and Bush, 2007). There are three Ambler classes under this group namely: A, B and D carbapenemases (Ambler, 1980) (Table 1.2).

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Table 1.2: Classification of carbapenemases and their respective substrates in Enterobacteriaceae. Nordmann *et al* 2012

Ambler class	Name of enzyme	Plasmid / chromosome	Hydrolysis spectrum						Inhibitor
			Penicillins	1st generation cephalosporin	2nd generation cephalosporin	3rd generation cephalosporin	Aztreonam	Carbapenems	
A	SME-1 to 3	Chromosome	++	++	_	+	+	+	Clavulanate
	NMC-A	Chromosome	++	++	_	+	_	++	Tazobactam, Sulbactam, NXL-104
	IMI-2	Plasmid	++	++	_	+	_	++	
	GES-4 to 6	Plasmid	++	++	+	+	_	+	
	KPC-2 to 12	Plasmid	++	++	_	++	+	++	Clavulanate, Tazobactam, boronic acid, Sulbactam.
B	IMP- 1 to -33	Plasmid	++	++	++	++	-	++	EDTA
	VIM- 1 to -33	Plasmid	++	++	++	++	-	++	
	NDM-1 to -6	Plasmid	++	++	++	++	-	+	
	KHM-1	Plasmid	++	++	++	++	-	++	
D	OXA-48	Plasmid	++	++	+/-	+/-	-	+	Sodium chloride
	OXA-181	Plasmid	++	++	+/-	+/-	-	+	

++ = complete hydrolysis, + = partial hydrolysis, - = no hydrolysis, + / - = partial or no hydrolysis

Ambler Class A carbapenemases

Ambler Class A carbapenemases consist of SME (*Serratia marcescens* enzyme), KPC (*Klebsiella pneumoniae* carbapenemase), IMI (imipenem hydrolyzing β-lactamases), NMC-A (Non-Metallo-carbapenemase-A) and GES (Guiana extended-spectrum) β-lactamases. SME, IMI and NMC-A are found on chromosomes, whilst KPC and GES are acquired on plasmids. Most members of this class hydrolyze all the four classes of β-lactams by a hydrolytic mechanism involving a serine active site (Ambler *et al* 1991).

SME-1 was originally identified in a *Serratia marcescens* isolate in London in 1982 (Yang *et al* 1990). Other variants (SME-2 and SME-3) have also been identified (Naas *et al* 1994). The IMI-1 enzyme was initially recovered from the chromosome of an *Enterobacter cloacae* isolate in southwestern USA (Rasmussen *et al* 1996). A variant of the IMI-1 enzyme, IMI-2 has been identified on plasmids from an environmental *Enterobacter asburiae* strain in some rivers in USA (Aubron *et al* 2005). The NMC-A enzymes were first identified among *E. cloacae* isolates from France (Nordmann *et al* 1993), but are currently rare (Pottumarthy *et al* 2003).

The KPC-2 variant was first identified in 1996, in the Eastern part of USA from a *K. pneumoniae* isolate (Yigit *et al* 2001). About 12 KPC subtypes (KPC-2 to KPC-13) have been identified, with KPC-2 and KPC-3 as the most common types (Navon-Venezia *et al* 2009). The GES-1 enzymes were first identified in a *K. pneumoniae* isolate from Guiana in 2000 (Poirel *et al* 2000). These enzymes have also been reported among members of the Enterobacteriaceae family. In South Africa, the GES-2 variant has been reported in *Pseudomonas aeruginosa* isolates from the Gauteng province (Poirel *et al* 2002).

Ambler Class B carbapenemases

Ambler Class B carbapenemases or Metallo β-lactamases (MBLs) have a broad-spectrum of activity to include all penicillins, cephalosporins and carbapenems, but not monobactams. Their hydrolytic mechanism requires a zinc ion in their active sites, and hence, they are inhibited by ethylenediamine tetra-acetic acid (EDTA). MBLs are classified into chromosomal and plasmid-mediated types. The chromosomal types (an example is L1) were the first to be identified and accounted for carbapenem resistance in *Bacillus cereus* and *Stenotrophomonas maltophilia* (Walsh *et al* 2005). The transferable types include VIM (Verona Integron-encoded Metallo β-lactamases), IMP (imipenem-resistant) and NDM (New Delhi Metallo β-lactamases).

Some rare MBLs are SPM (Sao Paulo Metallo β-lactamases) which were associated with hospital outbreaks in Brazil (Toleman *et al* 2002). GIM (German imipenemase) isolated in carbapenem resistant *P. aeruginosa* isolates from Germany (Castanheira *et al* 2004), SIM (Seoul imipenemase)

from *Acinetobacter baumannii* isolates from Korea (Lee *et al* 2005), AIM (Australian imipenemase) from *P. aeruginosa* isolates from Australia (Walsh *et al* 2007) and KHM-1 (Kyorin Health Science MBL) from a *Citrobacter freundii* isolate in Japan (Sekiguchi *et al* 2008).

IMP

IMP types were the first acquired MBLs to be identified. The IMP-1 variant was the first to be identified in *P. aeruginosa* isolates in 1988, and later in *S. marcescens* isolates in 1991, both from Japan (Watanabe *et al* 1991; Osano *et al* 1994). They have also been found in *Acinetobacter* spp. and Enterobacteriaceae. The *bla*IMP genes are associated with class 1 integrons, and co-exist with aminoglycoside resistance genes as *aacA4*, *aadA1* and *aadB*, and the chloramphericol resistance gene, *catBs* (Zhao & Hu, 2011).

VIM

The VIM-1 variant was first identified in Verona, Italy in 1997, in a *P. aeruginosa* isolate (Lauretti *et al* 1999). Another variant, VIM-2, was identified in a *P. aeruginosa* isolate from France, and is considered to be the most common type (Poirel *et al* 2000). Although VIM enzymes are mostly found in non-fermenting GNB, they have been increasingly reported among the family Enterobacteriaceae (Giakkoupi *et al* 2003).

NDM

The first report of the *bla*NDM-1variant was in Sweden, in *K. pneumoniae* isolates from an Indian patient previously hospitalized in New Delhi, India (Yong *et al* 2009). The *bla*NDM-1 variant is not associated with a single clone, species, or specific plasmid backbone, but has been identified in several unrelated GNB, and harbored on different plasmid types, accounting for its widespread distribution (Nordmann *et al* 2011).

Ambler Class D carbapenemases

The Ambler Class D carbapenemases or oxacillinases (‘OXAs’) can hydrolyze first generation cephalosporins, penicillins and βLIs, but have weak activity against carbapenems (Nordmann *et al* 2012). The variant OXA-23 or AR1-1 *(Acinetobacter* resistant to imipenem) was the first acquired oxacillinase to be identified, in an *Acinetobacter baumannii* isolate from Scotland in 1993 (Paton *et al* 1993).

More than 250 OXA types have been reported. The variant *bla*OXA-48, first isolated from *Pseudomonas aeruginosa,* is known to be involved in most nosocomial infections in Turkey (Carrer *et al* 2008). In general, the OXAs exhibit weak carbapenemase activity, which makes their detection

very difficult; however, other resistance mechanisms such as ESBLs, other carbapenemase types and or reduced membrane permeability enhance their detection (Walther-Rasmussen & Høiby, 2006).

The first report of carbapenem resistance in South Africa was in 2000, from a GES-2 producing *P. aeruginosa* isolate in the Gauteng province (Poirel *et al* 2002). The most common carbapenemases reported in the country are the New Delhi Metallo β-lactamases (NDMs), and oxacillinases (OXAs) carbapenemases (Osei Sekyere, 2016). Carbapenemase production has been reported to be rare in South Africa, based on monitoring by the NICD, and in Europe, among some *Klebsiella pneumoniae* and *E. coli* isolates (Perovic *et al* 2014; European Centre for Disease Prevention and Control, 2015).

1.4.2 Reduced permeability of the outer membrane

Reduced permeability of the outer membranes of GNB such as *Enterobacter* spp., in combination with other mechanisms such as ESBLs, hyper-production of chromosomal *bla*AmpC β-lactamases and over-expression of efflux pump systems have been shown by a number of studies to promote carbapenem resistance (Szabó *et al* 2006; Yang *et al* 2012). Reduced permeability can be attributed to the loss of some major outer membrane proteins (porins) and/or decreased expression due to mutations in the promoter regions of these porin genes (Koebnik *et al* 2000).

Porins form aqueous channels in the outer membranes of GNB or in the waxy outer layer of mycobacteria. This enables passive diffusion of hydrophilic substances including antimicrobials and nutrients, through the hydrophobic barrier. However, the rate of diffusion is affected by the osmolarity of the medium, temperature, and diameter of the channels depending on the type of porin present (Koebnik *et al* 2000).

The first two major osmoporins, OmpC and OmpF, were characterized in 1976 in an *E. coli* isolate (Nakae, 1976). Two porins, with molecular weights of 37 kDa, and 39 – 40 kDa, capable of permeating glucose and β-lactam antibiotics have been identified in *E. cloacae* (Sawai *et al* 1982; Kaneko *et al* 1984). The 37 kDa porin type with a radius of 0.6 nm, is assumed to be similar to OmpF in *E. coli*, which has a radius of 0.58 nm, and increased selectivity for positively charged particles.

The 39 – 40 kDa porin type, with a radius of 0.8 nm, is different in size compared to OmpC in *E. coli*, which has a radius of 0.54 nm, and has a weaker selectivity for charged molecules (Kaneko *et al* 1984; Renkin, 1954). These porins have been named OmpF and OmpC respectively. Several studies have described the involvement of these two major porins in ertapenem resistance in *E. cloacae* (Doumith *et al* 2009; Yang *et al* 2012).

Regulation of *ompF* and *ompC* genes

The *omp*R locus regulates the expression of the two major porin genes, *ompC* and *ompF*, during transcription (Hall & Silhavy, 1981). This locus encodes a two-component regulatory system, OmpR-EnvZ. EnvZ is a histidine kinase sensor, whilst OmpR is a cytosolic response regulator. EnvZ functions as an indicator in monitoring changes in the osmolarity of the external environment. This message is then conveyed to OmpR through phosphorylation and dephosphorylation, which then regulates the transcription of both *ompF* and *ompC*.

During low osmolarity due to reduced amounts of nutrients, *ompF* is upregulated, forming large pores which facilitate the influx of nutrients. Meanwhile, during high osmolarity and increased growth temperature, expression of *ompF* is repressed by the antisense MicF RNA (mRNA interfering complementary RNA), and *ompC* expression is upregulated (Hall and Silhavy, 1981; Forst and Inouye, 1988).

During osmolarity changes in the external environment, the relative production of these porins is modulated so that the total amount of porin in the outer membrane remains fairly constant (Kawaji *et al* 1979).

1.4.3 Over-expression of efflux pumps

Bacteria have evolved energy-dependent efflux systems which also play an important role promoting resistance in most GNBs. These efflux pump systems are usually encoded on the chromosomes, but some are found on plasmids (Nikaido and Pagès, 2012). Antimicrobial substrates of these pumps include antibiotics such as β-lactams, fluoroquinolones, and aminoglycosides (Webber and Piddock, 2003); and non-antibiotic substrates such as dyes, bile salt, detergents and biocides (Nikaido and Pagès, 2012).

Efflux pump systems are divided into five main superfamilies: Adenosine triphosphate (ATP)-binding cassette (ABC) superfamily (Lubelski *et al* 2007), Major facilitator superfamily (MFS) (Pao *et al* 1998), Small multidrug resistance (SMR) (Paulsen *et al* 1996), Multidrug and toxic compound extrusion (MATE) superfamily (Kuroda and Tsuchiya, 2009) and Resistance-nodulation-cell division (RND) superfamily (Tseng *et al* 1999). Among these superfamilies, RND efflux pump systems are known to cause resistance to different classes of antibiotics including β-lactams.

The AcrAB/TolC efflux pump system

The AcrAB/TolC efflux pump system, belonging to the RND superfamily, promotes antimicrobial resistance mainly in GNBs and has been shown by previous studies to play a role in antimicrobial resistance in *E. cloacae* isolates from Spain (Pérez *et al* 2007).

The AcrAB/TolC efflux pump system is a tripartite system. It consists of an inner membrane protein designated “Acriflavine resistance protein B” (AcrB), an outer membrane channel protein known as “Tolerance Colicin E1” (TolC), as well as a membrane fusion protein “Acriflavine resistance protein A” (AcrA) which connects AcrB and TolC (Figure 1.6) (Pos, 2009).

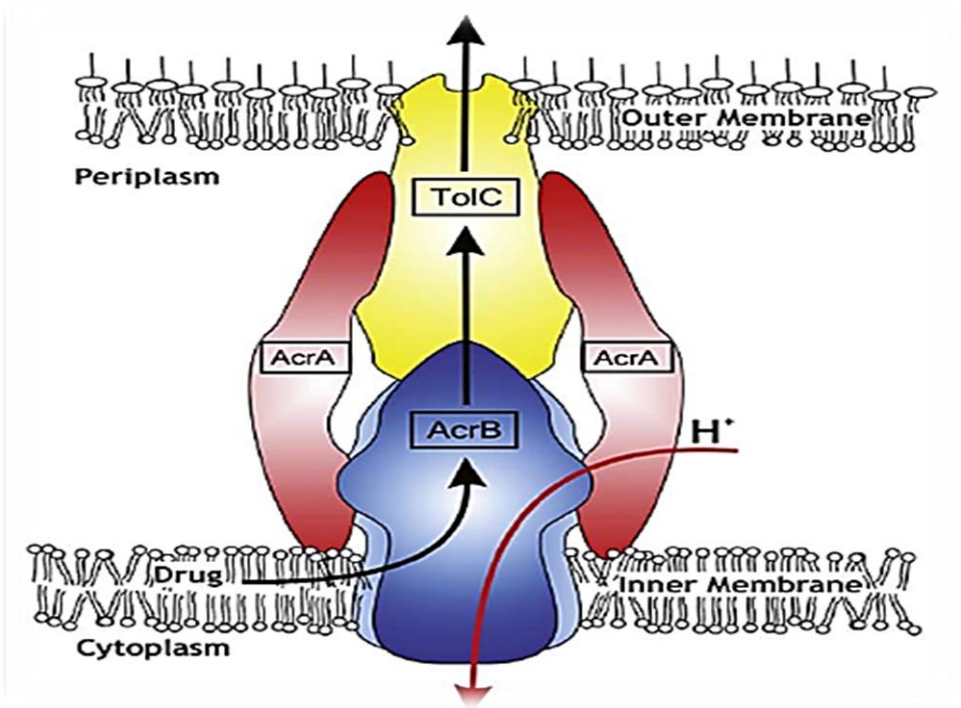

Figure 1.6: The structure of the AcrAB/TolC efflux pump system. (Pos, 2009)

AcrA functions as an adaptor protein in the AcrAB/TolC efflux system. It is an elongated molecule with three linearly arranged domains: a β-barrel, a central lipoyl and an α-helical coiled hairpin, and the 130 residues (the hitherto missing domain containing both the N- and C-termini) which is essential in the assembly and functions of the tripartite complex (Mikolosko *et al* 2006). Chimeric studies have confirmed that the C-terminal domain of AcrA interacts with the AcrB pump (Elkins and Nikaido, 2003; Ge *et al* 2009), serving as a direct activator of AcrB. Furthermore, strong AcrA stimulation of the AcrB pump has been found to extrude substrates (Zgurskaya and Nikaido, 1999). Finally, AcrA plays a key role in the assembly of the pump complex by driving TolC to fit the transporter complex (Bavro *et al* 2008).

The RND transporter (AcrB) is a homotrimer, with each monomer having a transmembrane domain to form a pore or porter domain (Murakami *et al* 2002). The proton translocation site of this transporter is composed of charged amino acid residues Asp 407, Asp 408 and Lys 940. These residues function as the proton relay network assisting in drug extrusion (Takatsuka and Nikaido, 2006). The AcrB transporter undergoes a three-step functional rotating mechanism: access (loose), binding (tight) and extrusion (open).

TolC is also a trimetric protein, each monomer having a 40 Å long β-barrel domain anchored to the outer membrane, and a 100 Å long α-helical domain that projects into the periplasm. In the assembled state, the monomers form an outer membrane pore, and a periplasmic tunnel (Koronakis *et al* 2004). Symmons *et al* (2009) proposed that when AcrA is bound to AcrB, TolC is positioned in the complex in such a way that, it opens and remains constitutively in the open state leading to increased activity of the efflux pump system.

The involvement of various RND systems in antibiotic resistance in mostly GNB has led to the development of efflux pump inhibitors (EPIs). These are molecules that significantly and rapidly increase the intracellular accumulation of different efflux-sensitive drugs, such as macrolides, quinolones, and chloramphenicol, in clinical isolates expressing different levels of efflux pumps, without affecting the integrity of the membrane (Pagès *et al* 2005). The first EPI, called Phenylalanine-arginine β-naphthylamide (PaβN), was described in 1999 (Renau *et al* 1999). This EPI was further characterized in 2001 due to its capability in reducing levofloxacin minimum inhibition concentrations (MICs) in *P. aeruginosa* isolates from the United Kingdom (Lomovskaya *et al* 2001). Synergy studies using PaβN at concentrations of 25 µg/ml and 40 µg/ml have shown a reduction in MICs of both fluoroquinolones and ertapenem antibiotics in *E. cloacae* isolates from USA and Taiwan respectively (Szabó *et al* 2006; Yang *et al* 2012).

The different components of the AcrAB/TolC system have also been shown by previous studies to promote resistance. For example, over-expression of both *acrA and acrB* have been determined to promote antibiotic resistance in *E. cloacae* (Pérez *et al* 2007), and ertapenem resistance in *E. cloacae* isolates (Szabó *et al* 2006).

1.5 Problem Statement

In South Africa, identification of Carbapenem Resistant Enterobacteriaceae (CRE) in various institutions is based on carbapenem MICs generated by automated systems (such as VITEK2® Advanced Expert System), gradient diffusion assays, or disc diffusion, with confirmation of these suspected carbapenem non-susceptible organisms by the NICD. However, only the presence of certain carbapenemases genes is investigated in these isolates.

Surveillance involving all possible carbapenem resistance mechanisms in *Enterobacter* spp. including carbapenemase production, reduced membrane permeability, ESBL production, chromosomal *bla*AmpC β-lactamase hyper-production and over-expression of efflux pumps, is yet to be established (Singh-Moodley & Perovic, 2016).

1.6 Aim and Objectives

1.6.1 Aim

The aim of this study is to investigate the carbapenem resistance mechanisms in clinical *Enterobacter cloacae* isolates from Tygerberg Hospital.

1.6.2 Objectives

1. To confirm ertapenem non-susceptibility
2. To screen for carbapenemases
3. To screen for ESBLs
4. To investigate *bla*AmpC hyper-production
5. To investigate outer membrane porin expression
6. To investigate over-expression of the AcrAB/TolC efflux pump system

Chapter 2: Identification and Antimicrobial Susceptibility Testing of Study Isolates

2.1 Introduction

The availability of limited treatment options for infections caused by Gram-negative bacilli is a major problem. Accurate identification of these pathogens and knowledge of their antimicrobial susceptibility profiles is critical in treating these infections. Several assays are used in determining the susceptibility profiles of bacteria.

The VITEK2® Advanced Expert System (VITEK2®AES, bioMérieux, France) is an automated system, which utilizes growth-based technology and biochemical assays to assign species identification and determine susceptibility profiles of common, rapidly growing Gram-positive and Gram-negative aerobic bacteria*;* as well as to detect antimicrobial resistance mechanisms. This system is one of the fastest ways of generating susceptibility profiles for bacterial isolates (Pincus, 2010).

Broth microdilution (BMD) is a semi-quantitative method for measuring the *in vitro* activity of an antimicrobial agent against a bacterial isolate (NCCLS, 2003), and is regarded as the gold standard method. Up to 12 different antibiotics can be tested in a range of 8 two-fold dilutions in a single tray (96-well plate). Some advantages of using this assay are that it is reproducible, and the end-point of this assay can be generated in the form of computerized reports when an automated panel reader is used. However, the BMD method is labor intensive and is only standardized for testing commonly isolated bacteria that grow well after an overnight incubation in cation-adjusted Mueller-Hinton broth (CAMHB).

The gradient diffusion assay uses the principle of establishing an antimicrobial concentration gradient in an agar medium, using commercialized plastic strips (gradient diffusion strips) as a means of determining susceptibility (NCCLS, 2003). One disadvantage of this assay is that the strips are costly, hence making it an expensive assay to perform if more than a few drugs are to be tested.

The Kirby Bauer disc diffusion assay is a simple and well-standardized method. It is performed by applying filter paper discs impregnated with antimicrobial agents onto Mueller Hinton agar plates swabbed with a standardized inoculum of bacteria, to generate zones of inhibition which can be interpreted to determine susceptibility (Bauer *et al* 1966).

The disc diffusion assay is the least costly of all the susceptibility assays and is standardised for testing various common organisms including Enterobacteriaceae, *P. aeruginosa*, *Acinetobacter* spp., *Staphylococcus* spp., and *Enterococcus* spp. (CLSI, 2016).

The aim of this chapter was to collect clinical *Enterobacter cloacae* isolates that were non-susceptible to carbapenems based on the VITEK2®AES, and to determine their antimicrobial susceptibility profiles using BMD and gradient diffusion assays. These isolates would then be further studied to characterize their underlying carbapenem resistance mechanisms, as described in the following chapters.

2.2 Materials and methods

2.2.1 Isolate Collection

Isolates were collected from specimens submitted to the National Health Laboratory Service (NHLS) diagnostic laboratory at Tygerberg Hospital (TBH) for routine diagnostic testing following clinical sampling from adult and paediatric patients. The NHLS laboratory receives specimens from the TBH as well as several regional and district hospitals and primary healthcare clinics in the Western Cape province. The specimens were processed according to standard protocols. Identification and susceptibility testing of the isolates was performed using the VITEK2®AES.

Non-duplicate clinical *E. cloacae* isolates that were non-susceptible to ertapenem antibiotics based on the VITEK2®AES results, with minimum inhibition concentrations (MICs) > 0.5 µg/ml were obtained from January 2015 to December 2016. In addition, nine ertapenem susceptible isolates (with MICs of ≤ 0.5 µg/ml) were collected. Of these nine, five were fully susceptible to all tested carbapenems (imipenem, ertapenem and meropenem) and extended-spectrum cephalosporins (cefotaxime, ceftazidime and cefepime), and were resistant to ampicillin, amoxicillin/clavulanate and second generation cephalosporins, and likely represent wild-type isolates (with inducible *bla*AmpC). The other four isolates were susceptible to tested carbapenems on the VITEK2®AES, but resistant to extended-spectrum cephalosporins. This phenotype suggests the presence of either an ESBL or derepressed *bla*AmpC, or both. The five wild-type isolates were chosen as controls with no acquired β-lactam resistance mechanisms; and the four ertapenem susceptible isolates were chosen as representatives of isolates with no acquired β-lactam resistance mechanisms, which do not affect susceptibility to carbapenems. In future chapters, these isolates will be referred to as wild-type (WT) controls; and ertapenem susceptible controls, respectively.

All study isolates were stored on Microbank (MBK) beads (Pro Lab Diagnostics, Canada) at -80°C for further analyses. Information on patient demographics such as gender, age and specimen type was retrieved from the Disa*Lab and TrakCare laboratory information systems of the NHLS.

This project was approved by the Health Research Ethics Committee (HREC), Faculty of Medicine and Health Sciences, Stellenbosch University with the ethics reference number, S16/04/068.

2.2.2 Antimicrobial Susceptibility Testing (AST)

MICs of clinically relevant antibiotics including extended-spectrum cephalosporins (cefepime, ceftazidime and cefotaxime) and carbapenems (imipenem, ertapenem and meropenem) were determined as part of routine diagnostic procedures using the VITEK 2 AST-N255 card on the VITEK2®AES platform. In addition, BMD had been performed by a previous student in our division, Sharona de Rijk, on all the 20 ertapenem non-susceptible isolates and susceptible controls identified by the VITEK2®AES to determine the ertapenem MICs.

Ertapenem MICs were also determined for all 20 ertapenem non-susceptible isolates and susceptible controls using gradient diffusion strips (Liofilchem s.r.l., Italy). Bacterial isolates were retrieved from MBK beads and inoculated onto fresh tryptose blood agar (TBA) plates (NHLS Media Laboratory, Greenpoint, South Africa). A single colony was restreaked twice onto TBA plates and incubated overnight at 37°C (ambient air) to obtain pure cultures. Two to three bacterial colonies of the same morphology were inoculated in 3 ml of saline solution in cylindrical cuvettes using sterile swabs and vortexed briefly. The turbidity of the solution was adjusted to 0.5 McFarland (equivalent to 1 - 2 x 10^8 CFU/mL) using the DensiCHEK (bioMérieux, France). The inoculum suspension was inoculated onto labelled Mueller Hinton agar plates (NHLS Media Laboratory, Greenpoint, South Africa) using fresh swabs, to cover the entire surface of the plates. The plates were allowed to dry on the bench for about 10 minutes, after which a single ertapenem gradient diffusion strip, able to determine MICs within the range of 0.002 – 32 mg/L, was placed on each of the inoculated plates with the aid of a sterile pair of forceps, and left on the bench to dry for 2 minutes. The plates were inverted and incubated for 16 - 20 hours at 37°C (ambient air). All activities were carried out around a Bunsen burner to reduce the risk of contamination.

An ellipse shape was observed around the strip after incubation, and the MIC was determined by reading the concentration where the ellipse of bacterial growth intersects with the strip (Figure 2.1). If the ellipse intersected above the highest concentration on the strip (32 mg/L), the MIC value was recorded as > 32 mg/L.

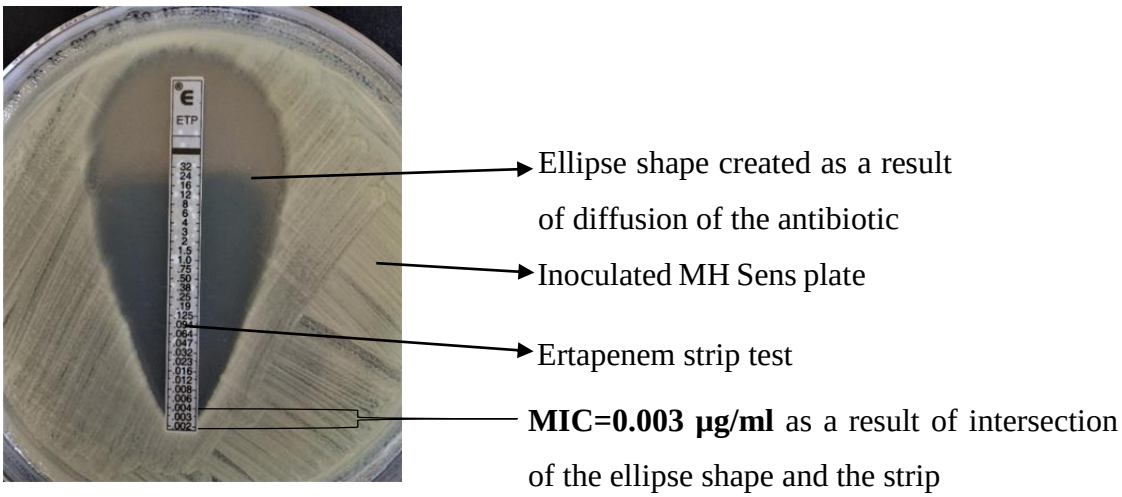

Figure 2.1: MIC determination using the gradient diffusion assay.

MIC breakpoints for ertapenem antibiotics were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) 2016 guidelines. An MIC value of ≤ 0.5 µg/ml indicates sensitive (S); 1 µg/ml, intermediate (I), and ≥ 2 µg/ml, resistant (R). Each assay was performed once.

The incidence of error for gradient diffusion assay and the VITEK2®AES for ertapenem susceptibilities compared to BMD was determined. The number of isolates (expressed as a percentage) under each category were determined. These categories include: very major error (when an isolate is resistant by BMD but susceptible by either VITEK2®AES or gradient diffusion assay), major error (when an isolate is susceptible by BMD but resistant by either VITEK2®AES or gradient diffusion assay) and minor error (when an isolate is intermediate by BMD but either resistant or susceptible by either VITEK2®AES or gradient diffusion assay).

2.3 Results

2.3.1 Sample collection and demographics of study isolates

A total of 20 ertapenem non-susceptible *E. cloacae* study isolates (based on VITEK2®AES), five wild-type controls and four ertapenem susceptible controls were included in this study. The majority of the study isolates, 16 (80%), were obtained from urine samples, and 1 (5%) each from sputum, pus, swab and pleural fluid samples. Eleven (55%) of the study isolates were obtained from males. Only 4 (20%) of the study isolates were from TBH, the rest (16, 80%) were from peripheral hospitals (Table 2.1).

Table 2.1: The demographics of study isolates

Sample IDs	**Sex**	**Age (years)**	**Specimen type**	**Date of specimen collection**	**Hospital**
ERD 1	Male	72	urine	25/02/2015	Helderberg
ERD 2	Female	33	urine	20/02/2015	Karl Bremer
ERD 4	Male	9	pleural fluid	10/02/2015	Khayelitsha District Hosp.
ERD 5	Male	14	urine	13/02/2015	Michael Mpongwana Day Hosp.
ERD 6	Female	52	urine	16/03/2015	Bishop Lavis Day
ERD 7	Female	86	sputum	10/03/2015	Eerste River
ERD 8	Female	35	urine	26/03/2015	Bloekombos PHC Clinic
ERD 9	Male	81	urine	15/03/2015	Karl Bremer
ERD 10	Female	70	urine	01/04/2015	Westfleur
ERD 11	Male	35	urine	05/04/2015	Tygerberg
ERD 12	Male	54	urine	18/03/2015	Khayelitsha District Hosp.
ERD 13	Female	48	urine	19/03/2015	Karl Bremer
ERD 14	Male	67	urine	01/06/2015	Karl Bremer
ERD 16	Male	34	pus	11/05/2015	Tygerberg
ERD 17	Female	8 days	urine	02/07/2015	Khayelitsha District Hosp.
ERD 18	Male	7 days	urine	01/07/2015	Khayelitsha District Hosp.
ERD 20	Female	36	urine	20/05/2015	Karl Bremer
ERD 29	Female	76	swab	14/02/2016	Citrusdal
ERD 34	Male	75	urine	15/03/2016	Tygerberg
ERD 42	Male	51	urine	20/04/2016	Tygerberg
Susceptible controls					
ERD 23	Male	26	urine	13/07/2015	Khayelitsha District Hosp.
ERD 25	Male	59	urine	12/07/2015	Worcester
ERD 35	Male	36	urine	15/03/2016	Ceres Hosp.
ERD 39	Female	NS	blood cultures	05/04/2016	Tygerberg

Wild-type controls					
WT 1	Male	71	bile	10/08/2016	Tygerberg
WT 2	Male	24	swab	10/08/2016	Helderberg Hosp.
WT 3	Female	44	sputum	10/08/2016	Worcester Hosp.
WT 4	Male	15	sputum	14/08/2016	Khayelitsha District Hosp.
WT 5	Female	48	sputum	15/08/2016	Tygerberg

NS – Not stated, Hosp. = Hospital

2.3.2 AST

Seventeen (85%) of the 20 study isolates were found to be ertapenem non-susceptible by BMD, and 6 (30%) by the gradient diffusion assay, in contrast to the VITEK2®AES results which had reported all 20 isolates as ertapenem non-susceptible (Figure 2.2). Ertapenem MICs range from 0.012 – 0.75 for the susceptible controls using the gradient diffusion (Refer to Appendix 1). Only three ertapenem non-susceptible *Enterobacter cloacae* isolates (ERD 6, ERD 7 and ERD 14) showed discrepant results for both BMD and gradient diffusion assays compared to the VITEK2®AES.

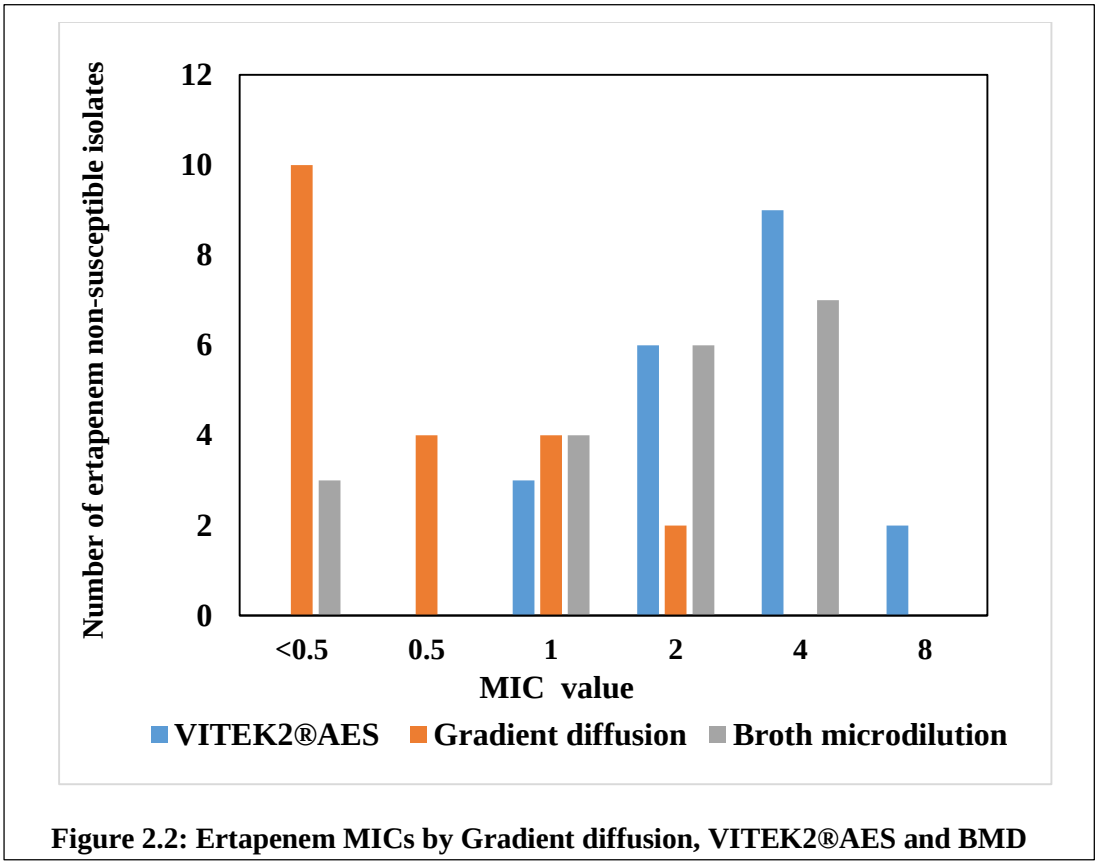

Figure 2.2: Ertapenem MICs by Gradient diffusion, VITEK2®AES and BMD

The incidence of error for the gradient diffusion assay and the VITEK2®AES susceptibilities for ertapenem, compared to the gold standard (BMD) were determined. When compared to BMD (the

gold standard), the ertapenem gradient diffusion assay showed a very major error rate of 35% and a minor error rate of 40% (Table 2.2). In comparison, the VITEK2®AES had no very major errors, but had major and minor error rates of 15% each.

Table 2.2: Incidence of error for methods used to determine ertapenem MICs

Error classification	VITEK2®AES	Gradient diffusion
Very Major Error (VME)	0	7 (35%)
Major Error (ME)	3 (15%)	0
Minor Error (MiE)	3 (15%)	8 (40%)

VME = broth microdilution (Resistant, R) and gradient diffusion / VITEK2®AES (Susceptible, S).
ME= broth microdilution (S) and gradient diffusion / VITEK2®AES (R).
MiE= broth microdilution (intermediate, I) and gradient diffusion / VITEK2®AES (R/S).

Based on the antimicrobial susceptibility profiles from the VITEK2®AES, all 20 ertapenem non-susceptible isolates were non-susceptible to ampicillin, co-amoxiclav and second generation cephalosporins antibiotics. Eighteen (90%) of the 20 ertapenem non-susceptible isolates were non-susceptible to ceftazidime and cefotaxime, 12 (60%) non-susceptible to cefepime, and 1 (5%) non-susceptible to each of imipenem, meropenem and colistin. For the other antibiotics, 55% were resistant to each of ciprofloxacin and gentamicin, 40% to tigecycline and 10% to amikacin (Figure 2.3).

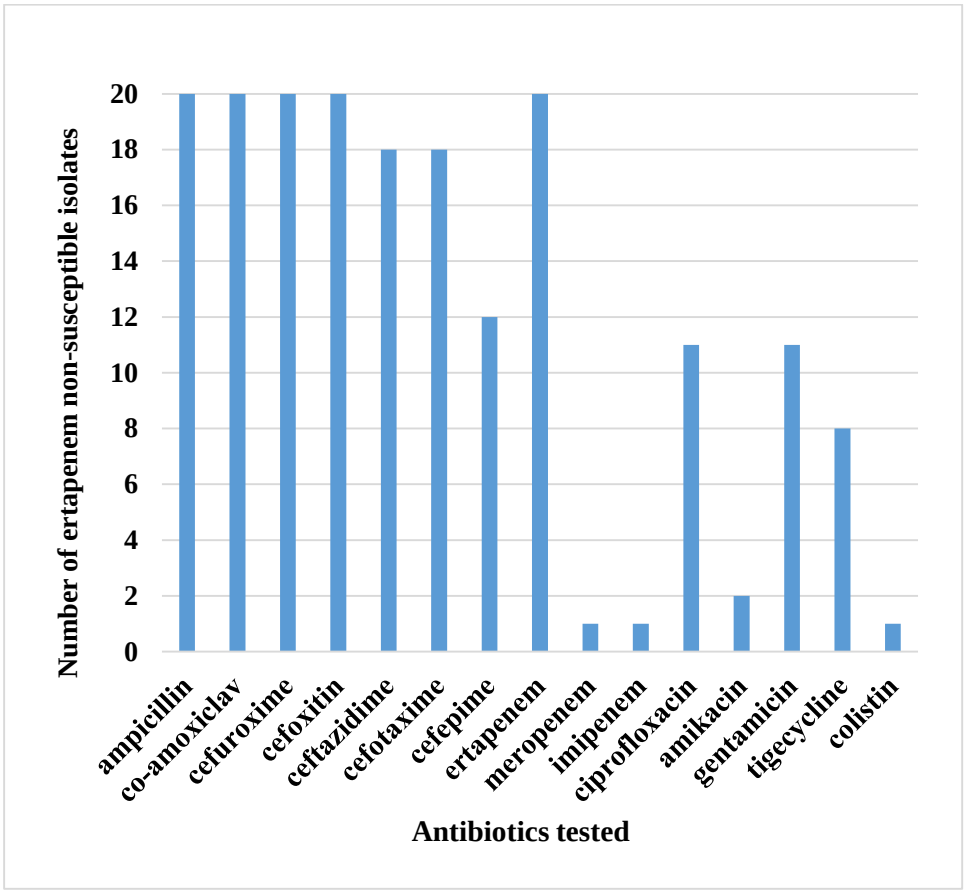

Figure 2.3: Antimicrobial profiles of the ertapenem non-susceptible isolates by the VITEK2®AES

2.4 Discussion

This chapter describes the collection of ertapenem non-susceptible *E. cloacae* study isolates, and the determination of their antimicrobial susceptibility profiles using various AST assays. The majority of these isolates originated from urine samples, with only a few from swab, sputum, pus and pleural fluid samples. This may suggests that these *Enterobacter* infections are acquired more commonly through endogenous sources as previously speculated (Flynn *et al* 1987). Eighty percent of the study isolates were from nine peripheral hospitals other than Tygerberg Hospital suggesting resistance mechanisms might have been acquired independently; however, this remains to be investigated in chapters 3 to 5.

The gradient diffusion assay identified only 6 (30%) of the study isolates as ertapenem non-susceptible, compared to 17 (85%) by broth microdilution. Major and minor error rates of 15% each were observed for the VITEK2®AES, whilst a very major error rate of 35% and minor error rate of 40% were observed for the gradient diffusion assay. Overall, there was a good correlation between the VITEK2®AES and the broth microdilution assay, but not the gradient diffusion assay in determining ertapenem MICs. However, three isolates (ERD 6, ERD 7 and ERD 14), confirmed as ertapenem non-susceptible by the VITEK2®AES, were determined to be susceptible to ertapenem by broth microdilution and gradient diffusion assay, suggesting possible overcalling of carbapenem resistance by the VITEK2®AES or undercalling by the gradient diffusion assay. Our findings support a previous study conducted by Pailhoriès *et al* (2014) where ertapenem MICs of ertapenem non-susceptible *E. cloacae* isolates determined by the VITEK2®AES were higher compared to the gradient diffusion assay. In contrast to our findings, Lee and Chung (2015) confirmed an essential agreement (greater than 90%) between both gradient diffusion and MicroScan assays, when compared to BMD, in comparison to only 30.5% agreement with the VITEK2®AES, during the susceptibility testing of ertapenem resistant Enterobacteriaceae. Some limitations that may impact our results include the fact that the AST assays were not performed in replicates due to cost and time constraints and this may influence the integrity of the results.Sixty percent of the ertapenem non-susceptible *E. cloacae* isolates identified by the VITEK2®AES were non-susceptible to tested extended-spectrum cephalosporins, suggesting that they harbor an ESBL or derepressed chromosomal *bla*AmpC β-lactamases, or both. In addition, 90% of the ertapenem non-susceptible isolates were fully susceptible to both imipenem and meropenem antibiotics, with only one isolate each non-susceptible to meropenem (ERD 4 with MIC = 1.0 µg/ml) and imipenem (ERD 20 with MIC = 1.0 µg/ml).

Similar to our findings, a previous study conducted by Yang *et al* (2012), which investigated mechanisms mediating carbapenem resistance in ertapenem resistant *E. cloacae* isolates, determined the isolates to be fully susceptible to both imipenem and meropenem antibiotics, compared to

ertapenem. Ertapenem resistance in our isolates might be attributed to the slow penetration of the Gram-negative outer membrane by ertapenem, as a result of its large and negatively-charged structure, in comparison to imipenem and meropenem (Nikaido *et al* 1983). However, one limitation of the VITEK2®AES in determining MICs of meropenem and imipenem is that the reported MIC values do not go below 0.25 µg/ml and cannot confirm if these isolates are highly sensitive to these agents.

2.5 Conclusion

Twenty *E. cloacae* isolates with reduced susceptibility to carbapenems, based on the VITEK2®AES, were identified. Although the majority of the isolates were confirmed to be ertapenem non-susceptible based on broth microdilution, the gradient diffusion assay substantially undercalled ertapenem non-susceptibility, when compared to both VITEK2®AES and BMD. The mechanisms of carbapenem resistance in these 20 isolates were investigated, as described in chapters 3 to 5.

Chapter 3: The role of β-lactamases in ertapenem resistance

3.1 Introduction

Carbapenem resistance in *Enterobacte*r spp. has been shown by several studies to be mediated through a number of mechanisms such as acquired carbapenemase production and reduced outer membrane permeability, extended-spectrum β-lactamases (ESBLs), hyper-production of chromosomal *bla*AmpC β-lactamases and over-expression of efflux pumps (Yang *et al* 2012; Szabó *et al* 2006).

A number of phenotypic assays (double-disk diffusion, Carba NP assay and the Modified Hodge test), automated systems (VITEK2®AES) and some commercialized kits (Rapidec® Carba NP kit) are used to detect β-lactamases such as ESBLs, *bla*AmpC β-lactamases and carbapenemases in several bacterial species.

The double-disk diffusion assay, which involves the use of two sets of discs, one impregnated with extended-spectrum cephalosporins and the other with the cephalosporin and a β-lactamase inhibitor, can be used to detect ESBLs and *bla*AmpC β-lactamases (Jarlier *et al* 1988). However, ESBLs can be difficult to detect in some genera, such as *Enterobacter*, which co-harbor *bla*AmpC β-lactamases on their chromosomes.

The Carba NP assay and Modified Hodge test (MHT) are both used to detect carbapenemases. The MHT can detect carbapenemases in Enterobacteriaceae which are non-susceptible to one or more carbapenems, whilst the Carba NP assay can detect carbapenemases in carbapenem non-susceptible *Acinetobacter* spp. and *P. aeruginosa* in addition to Enterobacteriaceae (CLSI, 2016). The Carba NP assay is rapid to perform, however it requires some special reagents. Inconsistency in detecting certain types of carbapenemases, such as the oxacillinases (OXAs) and chromosomally-mediated types have been reported to be associated with this assay (CLSI, 2016). The MHT is simple to perform and no special reagents are required. Some limitations to this assay are that isolates which produce ESBLs or *bla*AmpC β-lactamases in addition to reduced membrane permeability are often reported as false positives and isolates which produce New Delhi metallo β-lactamase (NDM) carbapenemases are often reported as false negatives (CLSI, 2016).

The VITEK2®AES can predict the presence of ESBLs, carbapenemases and *bla*AmpC β-lactamases (either derepressed or inducible types) using specific algorithms, based on the range of minimum inhibition concentration (MIC) values generated for the tested antibiotics, and compared to a database of expected phenotypes for different resistance mechanisms. However, some limitations associated with this system include the absence of some phenotype criteria in the database, limited antibiotic profiles on test cards and errors encountered when generating MICs, leading to incorrect prediction of resistance mechanisms in the tested isolates (Sanders *et al* 2000).

The reported discrepancies associated with these assays and lack of molecular-based methods recommended by CLSI, has led to under-detection of these enzymes during routine laboratory investigation (Singh-Moodley & Perovic, 2016). Therefore, other modern techniques such as disc diffusion using Mastdiscs (Mast Group, United Kingdom) and the Rapidec® Carba NP kit (bioMérieux, France) have been developed to identify ESBLs, hyper-production of chromosomal *bla*AmpC β-lactamases and carbapenemases phenotypically.

The Mastdiscs combination sets (D68C, D69C and D63C) are simple, reliable and low cost, and can detect ESBLs in addition to both chromosomal and hyper-produced *bla*AmpC β-lactamases in some enterobacterials including *Enterobacter* spp. (Mast Group, United Kingdom). The principle of these tests is based on the differences in sizes of the zone of inhibition (ZOI) generated in the presence of a β-lactam antibiotic alone, and / or a β-lactam with β-lactamase inhibitors (βLIs). The differences in the ZOI generated will determine the type of enzyme present in a particular isolate under investigation. For example, an ESBL-producing enterobacterial isolate is defined when the differences in ZOI between disc A (containing only cefepime at 30 µg) and disc B (containing cefepime at 30 µg and clavulanic acid at 10 µg) is greater than or equal to 5 mm, using the D63C combination set, whilst differences in ZOI of less than or equal to 4 mm will indicate a non-ESBL producer.

The Rapidec® Carba NP kit detects carbapenemases based on the principle previously described by Dortet *et al* (2012) that, *in vitro* hydrolysis of imipenem by carbapenemase-producing bacteria results in a color change of the pH indicator. The kit is rapid, low cost, with both specificity and sensitivity of 97.8% in detecting all three classes (A, B and D) of carbapenemases in Gram-negative bacilli (http://www.biomerieux-diagnostics.com/rapidec-carba-np).

By using specific target primers and probes, these enzymes can also be detected by PCR. A reverse transcription quantitative (RT-qPCR) assay can also detect and quantify the expression levels of these resistance genes. Furthermore, sequencing the PCR products can help identify mutations which might have contributed to resistance (Georgios *et al* 2014).

The aim of this chapter is to detect the presence of carbapenemases, ESBLs and both inducible and derepressed chromosomal *bla*AmpC β-lactamases in ertapenem non-susceptible clinical *E. cloacae* isolates from Tygerberg Hospital, using phenotypic and molecular-based assays.

3.2 Materials and methods

Twenty ertapenem non-susceptible *E. cloacae* isolates, four ertapenem susceptible controls and five wild-type controls as described in Chapter 2, were used for this study. One susceptible isolate (ERD 25) was excluded from the RT-qPCR assay due to loss of viability.

The VITEK2®AES prediction of whether ESBLs, hyper-production of chromosomal *bla*AmpC β-lactamases and carbapenemases were likely to be harbored by these isolates was performed as part of routine diagnostic procedures.

3.2.1 Phenotypic detection of carbapenemases using the Rapidec® Carba NP kit

Carbapenemase production was determined using the Rapidec® Carba NP kit. A *Klebsiella pneumoniae* carbapenemase (KPC-2) producing *K. pneumoniae* control strain, BAA 1705 (American Type Culture Collection), obtained from the NHLS Microbiology laboratory, was included as a positive control.

Isolates were cultured on Trypticase Soy Agar (TSA) plates [(3% tryptic soy broth (Sigma-Aldrich, Germany), 1.5% bacteriological agar (Oxoid, England)], and the test was performed according to the manufacturer's protocol (Refer to Appendix 2.1). Interpretation of results was based on a color change of the solutions in wells 'd' and 'e' of the Rapidec® Carba NP test strip. Presence of carbapenemase production is determined when the color of the solution in well 'd' is red and that in well 'e' is either orange or orange-red or yellow. Alternatively, absence of carbapenemase production is determined when the color of the solution in both wells is red (Figure 3.1).

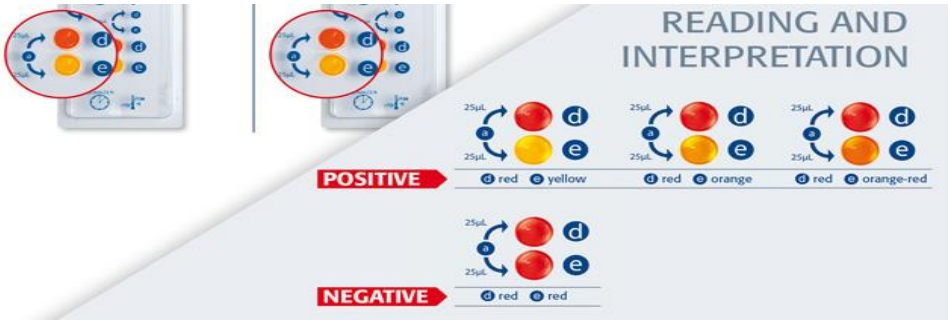

Figure 3.1: Carbapenemase detection using the Rapidec® Carba NP kit (http://www.biomerieux-diagnostics.com/rapidec-carba-np).

3.2.2 Molecular detection of carbapenemases

DNA extraction

Crude DNA was extracted by inoculation of a loop full of fresh bacterial colonies of the same morphology, after culturing twice from the Microbank (MBK) beads, in 400 µl of nuclease free water. The mixture was vortexed briefly and incubated at 95°C for 30 minutes to lyse the cells. The solution was then frozen at -20°C for 30 minutes to release the DNA, and finally centrifuged at 14000 x *g* for 10 minutes to remove any cell debris. The supernatant solution containing the DNA was aliquoted into newly labelled 1.5 ml micro-centrifuge tubes and stored at -20°C for downstream analyses.

Carbapenemase PCR

Two carbapenemase PCR reactions (A touchdown multiplex PCR and a singleplex PCR) were performed to detect the most common carbapenemase genes *bla*KPC, *bla*VIM, *bla*OXA-48, *bla*IMP, and *bla*NDM, and *bla*GES respectively. The touchdown multiplex PCR was performed using primers previously described by Zowawi *et al* (2014) (Table 3.1). An internal control, *rpoB*, as previously described by Hoffmann & Roggenkamp (2003), was included in the assay to validate the PCR run. All primers used were obtained from Integrated DNA Technologies, USA.

Table 3.1: Primers used for detecting *bla*VIM, *bla*OXA-48, *bla*KPC, *bla*IMP and *bla*NDM genes

Target gene	Primer	Sequence(5'-3')	Product size (bp)	Final Primer Concentration (µM)
*bla*IMP	IMP-F IMP-R	CTACCGCAGCAGAGTCTTTGC GAACAACCAGTTTTGCCTTACC	591	0.2
*bla*KPC	KPC-F KPC-R	ATCTGACAACAGGCATGACG GACGGCCAACACAATAGGTG	452	0.2
*bla*NDM	NDM-F NDM-R	GCAGGTTGATCTCCTGCTTG ACGGTTTGGCGATCTGGT	203	0.2
*bla*OXA-48	OXA-48-F OXA-48-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	438	0.2
*bla*VIM	VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	0.2
rpoB	rpoB-F rpoB-R	AACCAGTTCCGCGTTGGCCTGG CCTGAACAACACGCTCGGA	1088	0.08

The PCR reaction mix consisted of 2X KAPA2G Fast Multiplex PCR buffer (Kapa Biosystems, South Africa), final primer concentrations of 0.08 µM for primer sets amplifying the *rpoB* gene, 0.2 µM for primer sets amplifying *bla*OXA-48, *bla*VIM, *bla*KPC, *bla*IMP and *bla*NDM genes and DNase free water to bring the final volume of the master mix without the DNA template to 23 µl.

The KPC,VIM, OXA-48, IMP, NDM positive controls included in this assay were obtained from the National Institute for Communicable Diseases (NICD, South Africa) satellite unit at Groote Schuur Hospital. Two microlitres of crude DNA extracts were added as templates. The PCR reaction was performed on the Proflex PCR system (Applied Biosystems, Life technologies, USA) using cycling conditions outlined in Table 3.2.

Table 3.2: Cycling conditions for the carbapenemase multiplex PCR

Initial Denaturation	95˚C – 3 minutes	1 cycle
Denaturation	95˚C – 15 seconds	X 20 cycles
Annealing	68˚C - 30 seconds	
Extension	72˚C - 60 seconds	
Denaturation	95˚C – 15 seconds	X 10 cycles
Annealing	62˚C – 30 seconds	
Extension	72˚C – 60 seconds	
Final Extension	72˚C – 5 minutes	1 cycle
Final hold	4˚C	

Agarose gel electrophoresis

Five to ten microlitres of amplified PCR product as well as the KAPA Universal DNA Ladder (Kapa Biosystems, South Africa) were stained with 1 µl of Novel Juice (GeneDireX, USA), a non-mutagenic fluorescent reagent containing three tracking dyes: bromophenol blue, xylene cyanol FF, and orange G; for visually tracking the DNA migration. The PCR products were separated by horizontal gel electrophoresis through a 2% agarose gel [(2% SeaKem ® LE agarose powder (Lonza, USA) in 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer], in 1X TAE buffer at 100V for 60 minutes. Amplified DNA bands were observed under a UV Transilluminator (Alliance 2.7, UVITEC Cambridge).

*bla*GES singleplex PCR

A singleplex PCR was performed to detect the *bla*GES gene using primers previously described by Weldhagen *et al* (2004), in the presence of the internal control, *rpoB* (Table 3.3). The GES positive control included in this assay was obtained from the NICD satellite unit at Groote Schuur Hospital.

The PCR reaction mix consisted of 2X KAPA2G Fast Multiplex PCR buffer, final primer concentrations of 0.08 µM for sets amplifying the *rpoB* gene, 0.2 µM for primers amplifying the *bla*GES gene, and DNase free water to bring the final volume of the master mix without the DNA template to 23 µl. Two microlitres of crude DNA extracts was added as DNA templates. The PCR reaction was performed using cycling conditions described in Table 3.4. Five to ten microlitres of the PCR products were separated in a 2.5% agarose gel during a horizontal gel electrophoresis.

Table 3.3: Primers used for detecting *bla*GES gene

Target gene	Primer	Sequence (5'-3')	Product size (bp)	Final Primer Concentration (µM)
blaGES	GES-C	GTT TTG CAA TGT GCT CAA CG	371	0.2
	GES- D	TGC CATAGC AAT AGG CGT AG		
rpoB	rpoB-F	AACCAGTTCCGCGTTGGCCTGG	1088	0.08
	rpoB-R	CCTGAACAACACGCTCGGA		

Table 3.4: Cycling conditions for the *bla*GES singleplex PCR

Initial Denaturation	95˚C – 2 minutes	1 cycle
Denaturation	95˚C - 30 seconds	X 35 cycles
Annealing	60˚C - 1 minute	
Extension	72˚C - 1 minute	
Final Extension	72˚C - 5 minutes	1 cycle
Final Hold	4ºC	

3.2.3 Phenotypic detection of ESBL and *bla*AmpC β-lactamases using mastdiscs

The Mastdiscs D68C combination set (detects ESBL and differentiates derepressed *bla*AmpC mutants from inducible types), D63C (confirms ESBL detection), and D69C combination set (confirms chromosomal *bla*AmpC β-lactamases detection) were used for the phenotypic detection of ESBLs and increased levels of *bla*AmpC β-lactamases consistent with derepressed mutants.

Bacterial suspensions with concentrations of 0.5 McFarland (equivalent to 1 – 2 x 10^8 CFU/mL), as previously described (Section 2.2.2), were prepared from fresh colonies of isolates which had been sub-cultured twice from MBK beads.

These suspensions were then inoculated onto fresh Mueller Hinton sensitivity agar plates using clean swabs. Three plates were prepared for each isolate to test the Mastdiscs D63C, D68C and D69C combination sets separately. All these activities were performed close to the Bunsen burner to prevent contamination of the plates. The plates were incubated at 37°C for 16 - 20 hours. Interpretation of results using the different combination sets was based on the zones of inhibition (ZOI) around all of the discs used, as per the manufacturer's specifications (Table 3.5).

The Mastdiscs combination sets were only performed on the wild-type controls because characterization of the ertapenem non-susceptible and the remaining ertapenem susceptible *E. cloacae* controls, using Mastdiscs, had already been performed by a previous student in our division, Sharona de Rijk.

Table 3.5: Components of the Mastdiscs D63C, D68C and D69C combination sets, and ZOI interpretation for β-lactamase detection

Disc combination set	**Components**	**Interpretation for β-lactamase detection based on zone of inhibition (ZOI)**	**β-lactamases detected**
D63C	**Disc A:** 30 µg cefepime disc, **Disc B:** 30 µg cefepime disc with 10 µg clavulanic acid.	ZOI of **disc B** - ZOI of **disc A** ≥ 5 mm	ESBL
D68C	**Disc A:** 10 µg of cefpodoxime, **Disc B:** 10 µg of cefpodoxime with ESBL inhibitor **Disc C:** 10 µg of cefpodoxime with AmpC inhibitor **Disc D:** 10 µg of cefpodoxime with both ESBL and AmpC inhibitors	**ESBL positive** = ZOI of **disc B** - ZOI of **disc A** and ZOI of **disc D** - ZOI of **disc C** ≥ 5 mm, and ZOI of **disc B** - ZOI of **disc D and** ZOI of **disc A** - ZOI of **disc C** < 4 mm. **AmpC positive** = ZOI of **disc D** - ZOI of **disc B** and ZOI of **disc C** - ZOI of **disc A** ≥ 5 mm; and ZOI of **disc A** - ZOI of **disc B,** ZOI of **disc C** - ZOI of **disc D** < 4 mm.	ESBL and derepressed *bla*AmpC producers
D69C	**Disc A:** 10 µg of cefpodoxime with AmpC inducer **Disc B:** 10 µg of cefpodoxime with AmpC inducer and ESBL inhibitor **Disc C:** 10 µg of cefpodoxime with AmpC inducer, with both ESBL and AmpC inhibitors	ZOI of **disc C** - ZOI of **disc A** and ZOI of **disc C** - ZOI of **disc B** ≥ 5 mm	Chromosomal *bla*AmpC producers

3.2.4 Molecular detection of ESBLs

A multiplex PCR was performed to detect the ESBL family genes *bla*SHV, *bla*TEM, and *bla*CTX-M, using specific primers as previously described by Monstein *et al* (2007) (Table 3.6). An internal control, *rpoB*, was included in the assay to validate the PCR run. The PCR reaction mix consisted of 2X KAPA2G Fast Multiplex PCR buffer, final primer concentrations of 0.2 µM for primer sets amplifying the *rpoB*, *bla*TEM and *bla*CTX-M genes and 0.24 µM for the *bla*SHV primer set, and DNase free water to bring the final volume of the master mix without the DNA template to 23 µl. All primers used were obtained from Integrated DNA Technologies, USA. Two microlitres of crude DNA extract was used as template DNA.

Table 3.6: Primers used to amplify the *bla*SHV, *bla*TEM, *bla*CTX-M genes

Target gene	Primer	Sequence (5'-3')	Product size (bp)	Final primer concentration (µM)
bla_{TEM}	TEM-164.SE	TCGCCGCATACACTATTCTCAGAATGA	445	0.20
	TEM-165.SE	ACGCTCCACGGCTCCAGATTTAT		
bla_{CTX-M}	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	593	0.20
	CTX-M-U2	TGGGTRAATARGTSACCAGAAYCAGCGG		
bla_{SHV}	bla-SHV.SE	ATGCGTTATATTCGCCTGTG	747	0.24
	bla-SHV.AS	TGCTTTGTTATTCGGGCCAA		
rpoB	rpoB-F	AACCAGTTCCGCGTTGGCCTGG	1088	0.20
	rpoB-R	CCTGAACAACACGCTCGGA		

The PCR reaction was performed on the Proflex PCR system using cycling conditions as outlined in Table 3.7. TEM, SHV and CTX-M positive controls, obtained from the NICD were included in this assay. Five to ten microlitres of the PCR products were separated through a 2.5% agarose gel by horizontal gel electrophoresis, as previously described in Section 3.2.2.

Table 3.7: Cycling conditions for the ESBL Multiplex PCR

Initial Denaturation	95˚C – 3 minutes	1 cycle
Denaturation	95˚C – 15 seconds	X 35 cycles
Annealing	67˚C – 30 seconds	
Extension	72˚C – 30 seconds	
Final Extension	72˚C – 3 minutes	1 cycle
Final hold	4˚C	

Differentiation of ESBL and non-ESBL *bla*TEM and *bla*SHV β-lactamases

The multiplex ESBL PCR was not able to differentiate *bla*TEM and *bla*SHV ESBL types from non-ESBL genes, hence this was further investigated by Sanger sequencing.

Two singleplex PCRs were performed to amplify the *bla*TEM and *bla*SHV family genes using the specific primers (Table 3.6). The PCR reaction mix consisted of 2X KAPA2G Fast Multiplex PCR buffer, final primer concentrations of 0.4 µM for both *bla*TEM and *bla*SHV primer sets, and DNase free water to bring the final volume of the master mix without the DNA template to 23 µl. Two microlitres of crude DNA extract were used as template. TEM and SHV positive controls obtained from the NHLS NICD were included in this assay. The PCR reaction runs and gel electrophoresis steps were carried out as described above.

Ten to fifteen microlitres of the PCR products were sent to Inqaba Biotechnologies, South Africa for Sanger sequencing. The raw sequencing files were edited using the BioEdit Sequence alignment editor version 7.2.5 (Hall, 1999). The edited *bla*TEM nucleotide sequences were aligned to TEM-1 and TEM-2 sequences retrieved online from the National Centre for Biotechnology Information (NCBI) GenBank database for identification. For *bla*SHV identification, a partial raw sequencing file was received. The incomplete sequencing data made identification difficult, hence, the *bla*SHV nucleotide sequences were converted into amino acid sequences using the online ExPASy translate tool (https://web.expasy.org/translate/). The amino acid sequences were searched against the *E. cloacae* NCBI protein database using the protein-protein Basic Local Alignment Search Tool (blastp) for identification.

3.2.5 Molecular characterization of *bla*AmpC β-lactamase hyper-production

The expression of *bla*AmpC in the ertapenem non-susceptible *E. cloacae* isolates and the susceptible controls relative to the wild-type controls was determined by reverse transcription quantitative PCR (RT-qPCR).

Total mRNA sample preparation

Total mRNA was extracted from all *E. cloacae* isolates using a modified protocol of the Quick-RNA Miniprep kit (Zymo Research, USA) (Refer to Appendix 2.2). The integrity of the mRNA extracts was determined by separating 10 µl of each extract in a 1% agarose gel in TAE buffer at 100V for 60 minutes followed by staining in 500 ml of 10 mg/ml ethidium bromide solution for 20 minutes (Refer to Appendix 3.1).

A No Reverse Transcriptase (NoRT) PCR was performed to exclude genomic DNA contamination (Refer to Appendix 3.2). When genomic DNA was detected, a clean-up step was performed using a modified protocol of the Ambion ® DNA-*free*™ DNase Treatment and Removal reagents kit (Life technologies, USA) (Refer to Appendix 2.3 for protocol). The quality and quantity of the mRNA extracts were then determined using the NanoDrop spectrophotometer (ThermoFisher Scientific, USA), and Qubit® 2.0 Fluorometer (Life technologies, USA) respectively. Ten nanograms of each mRNA extract was used as the template material for RT-qPCR analysis.

*bla*AmpC RT-qPCR

A one step SYBR Green-based RT-qPCR assay was performed using primer sets previously described by Doumith *et al* (2009) (Table 3.8) to determine the expression levels of *bla*AmpC relative to the endogenous control gene, *rpoB.* The RT-qPCR was performed using the Luna One step Universal kit (BioLabs, New England, United Kingdom). The components of the PCR reaction mix are outlined in Table 3.9. The PCR experiment was performed on a Rotor-Gene Q instrument (Qiagen, Germany) using the cycling conditions proposed by the manufacturer of the Luna One step Universal kit (Table 3.10).

Table 3.8: Primers used for *bla*AmpC RT-qPCR

Target gene	Primer	Sequence (5'-3')	Product size (bp)
rpoB	rpoB-F	AAGGCGAATCCAGCTTGTTCAGC	148
	rpoB-R	TGACGTTGCATGTTCGCACCCATCA	
*bla*AmpC	ampC-F	GCATGGCGGTGGCCGTTAT	221
	ampC-R	CTGCTTGCCCGTCAGCTGT	

Table 3.9: Components of master mix for the *bla*AmpC RT-qPCR

Reagents	Volume per sample (µl)	Final concentration
PCR-grade water	6.4	
Luna Universal One step Master Mix (2X)	10.0	1X
ampC Forward Primer (10 µM)	0.8	0.4 µM
ampC Reverse Primer (10 µM)	0.8	0.4 µM
Template (RNA)	2.0	10 ng
Total reaction volume	20.0	

Table 3.10: Cycling condition for the *bla*AmpC RT-qPCR

Step	Temperature	Time	Number of cycles
Hold (cDNA processing step)	55°C	10 minutes	1
Hold	95°C	1 minute	1
Cycling	95°C	15 seconds	X 40
	60°C (Acquiring data)	30 seconds	
Melt	Ramp from 75°C to 95°C Hold for 90 seconds on first step Hold for 5 seconds on next steps, Melt A		

RT-qPCR validation

The absolute standard curve quantification method was used to quantify the level of gene expression of the candidate genes by comparing the ratio of the copy numbers of the *bla*AmpC gene, normalized to the *rpoB* gene, in the ertapenem non-susceptible and susceptible controls to the wild-type controls. This method requires that the expression levels of the target and reference gene(s) be measured from a standard curve. Standard curves were constructed by generating a dilution series consisting of six points ranging from 100 ng to 1 pg of extracted genomic DNA (Refer to Appendix 2.4 for extraction protocol). The absolute DNA concentrations were determined using a modified template of the KAPA Library Quantification Data Analysis Template version 4.14 (https://www.kapabiosystems.com/.../KAPA-Library-Quant_Data-Analysis-Template_IL). The PCR experiments for both the target gene, *bla*AmpC, and the endogenous gene, *rpoB*, were validated using pure genomic DNA extracts from WT 4 as templates. PCR efficiencies between 80 and 110 %, and R^2 values $\geq$ 0.99 (http://gene-quantification.com/SIAL-qPCR-Technical-Guide.pdf) were required for validation of the relative quantification. All the validation experiments and samples were performed in technical triplicates. No Reverse Transcriptase (NoRT) controls were run for all samples, and qPCR for the housekeeping gene, *rpoB* was used to control for cDNA conversion as well as to normalize the qPCR results of the different experiments.

Data Analysis

The freely available web-based DNA Copy Number Calculator from ThermoFisher Scientific (https://www.thermofisher.com/za/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html) was used to generate a ratio of the absolute copy numbers of *bla*AmpC and *rpoB* mRNA, based on the comparative threshold cycles (Ct values) generated after the PCR experiments and the product sizes of both genes. The ratio of the absolute copy numbers was used to determine the relative expression levels of *bla*AmpC in the ertapenem non-susceptible *E. cloacae* isolates and the ertapenem susceptible controls, compared to the wild-type controls. The expression levels of *bla*AmpC relative to *rpoB* were compared to the average normalized expressed levels of the wild-type controls, and expressed as log10 values. The plotted log10 values are the average of the three experiments, where the error bar generated on the graphs represents the sample standard deviation. A significant change in expression was defined as a two-fold change corresponding to a log10 value of 0.3 or -0.3. Isolates with log10 values between 0.3 and -0.3 were considered to have expression levels consistent with that of the wild-type controls. However, isolates with a log10 value greater than 0.3 were considered high *bla*AmpC producers, and lower than -0.3 were considered low *bla*AmpC producers.

3.3 Results

3.3.1 Carbapenemases

The 20 ertapenem non-susceptible *E. cloacae* isolates, four susceptible controls and five wild-type controls were phenotypically screened for carbapenemase production using VITEK2®AES and the Rapidec® Carba NP kit. Seven (35%) of the ertapenem non-susceptible isolates were predicted to be carbapenemase producers by the VITEK2®AES. One ertapenem non-susceptible isolate (ERD 1) was identified as a carbapenemase producer using the Rapidec® Carba NP kit, however this isolate was not predicted to be a carbapenemase producer by the VITEK2®AES.

PCR was used to screen for the *bla*OXA-48, *bla*NDM, *bla*VIM, *bla*KPC, *bla*GES and *bla*IMP carbapenemase genes, but no carbapenemase genes were detected in any of the isolates (Figures 3.2 and 3.3).

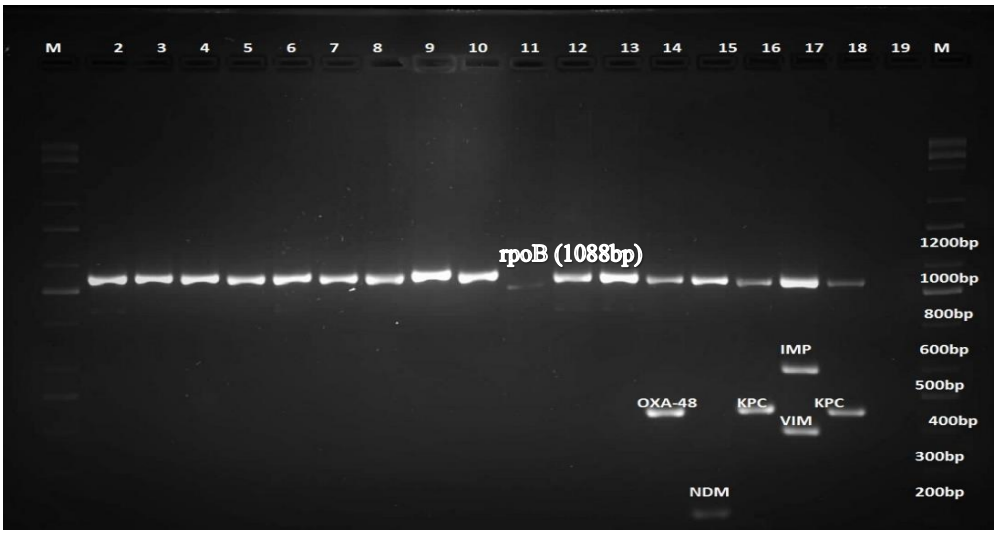

Figure 3.2: Carbapenemase multiplex PCR. 2.5% agarose gel showing common carbapenemase genes *bla*OXA-48, *bla*IMP, *bla*NDM, *bla*KPC and *bla*VIM with an internal control (*rpoB* gene). Lane M- Kapa Universal DNA Marker, Lanes 2 to 13 – represent isolates showing the internal control band of 1088bp, Lanes 14 to 18- represent the amplified positive control bands for OXA-48, NDM, KPC, VIM and IMP, and the KPC-2 control strain BAA 1705. Lane 19 – represents a no template control

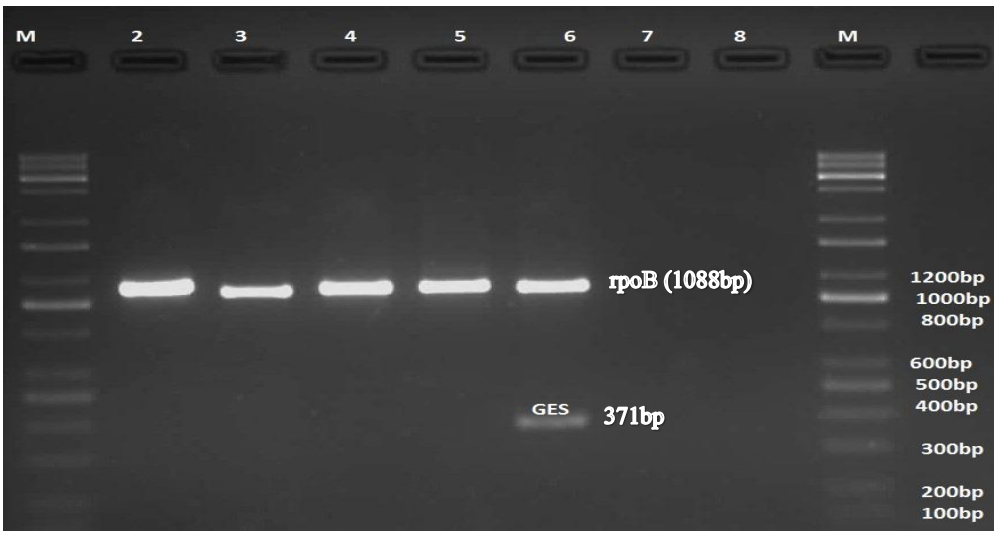

Figure 3.3: *bla*GES singleplex carbapenemase PCR. 2.5% agarose gel showing carbapenemase gene, *bla*GES with an internal control (*rpoB* gene). Lane M- KAPA Universal DNA Marker, Lanes 2 to 5 - represent test samples showing the internal control band, (*rpoB* band – 1088bp), Lane 6 represents the amplified band for the positive GES control. Lanes 7 & 8 represent no template controls.

3.3.2 ESBLs

Nineteen of the twenty ertapenem non-susceptible *E. cloacae* isolates were predicted to be ESBL producers by the VITEK2®AES. The Mastdiscs D68C combination set identified 40% (8/20) of the ertapenem non-susceptible isolates as ESBL producers, whereas 55% (11/20) of isolates were identified as ESBL producers by the Mastdiscs D63C combination set. Three of the four ertapenem susceptible controls were predicted to be ESBL producers by VITEK2®AES and this was confirmed by the D68C combination set. However, none of the five wild-type controls were identified as ESBL producers by any of the phenotypic assays.

The ESBL multiplex PCR identified one or more genes in thirteen ertapenem non-susceptible isolates and three of the four ertapenem susceptible controls (Figure 3.4); while 35% (7/20) ertapenem non-susceptible isolates and one ertapenem susceptible control harbored no ESBL genes. Different combinations of the ESBL family genes, *bla*SHV, *bla*CTX-M and *bla*TEM were identified. Eight of the thirteen ertapenem non-susceptible isolates mentioned above harboured both *bla*CTX-M and *bla*TEM genes (Figure 3.5).

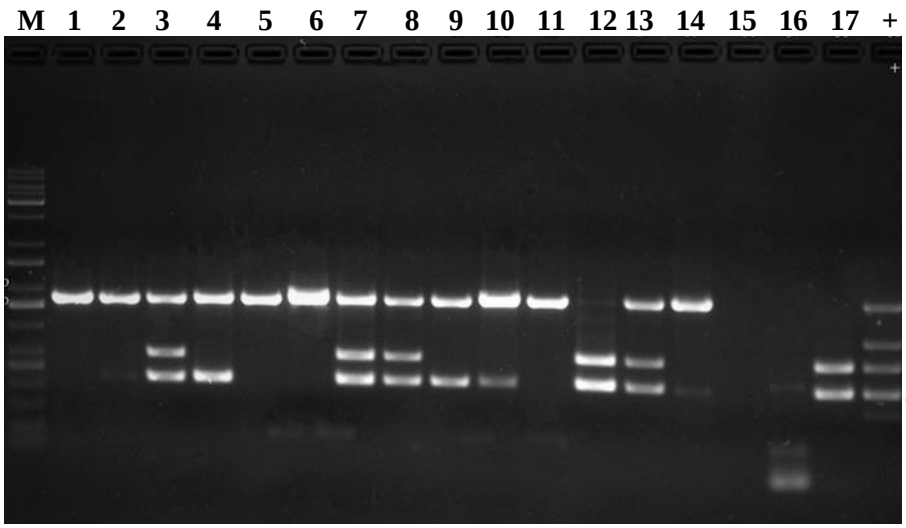

Figure 3.4: ESBL multiplex PCR. 2% agarose gel showing ESBL family genes *bla*TEM, *bla*SHV and *bla*CTX-M) with an internal control (*rpoB*) gene. Lane M - KAPA Universal DNA Ladder; lanes 1 - 17 represent test isolates; lane 18 represents a positive control showing all the three ESBL family genes (SHV, TEM & CTX-M) with an internal control (*rpoB*).

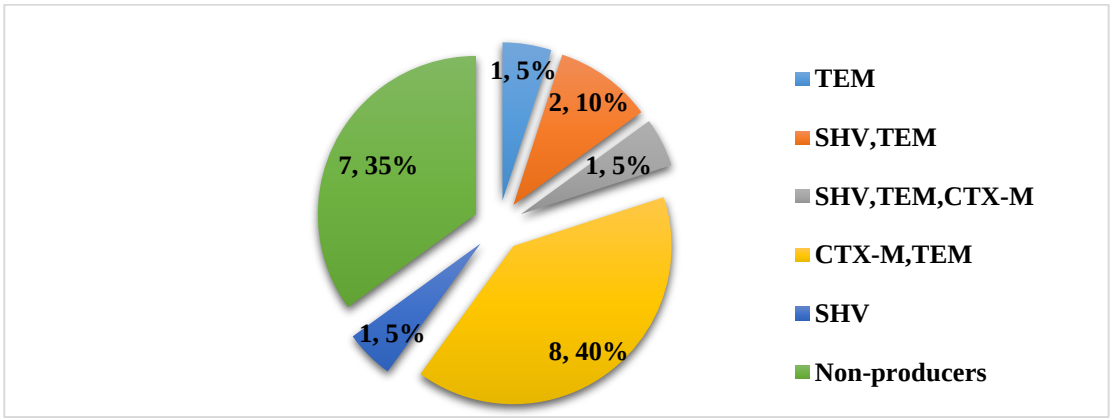

Figure 3.5: Distribution of ESBL family genes *bla*SHV, *bla*TEM and *bla*CTX-M in ertapenem non-susceptible *E. cloacae* isolates

The ESBL multiplex PCR does not differentiate between *bla*TEM-1, *bla*TEM-2 and *bla*SHV-1 genes, which are β-lactamases without extended-spectrum activity, and the ESBL *bla*TEM and *bla*SHV genes; therefore the *bla*TEM PCR products from fifteen isolates (combining both ertapenem susceptible and ertapenem non-susceptible isolates) and the *bla*SHV PCR products from four isolates were sequenced to differentiate non-ESBL β-lactamases from ESBL types. All *bla*TEM genes were identified as *bla*TEM-1 β-lactamases. All four of the *bla*SHV genes were identified as *bla*SHV-12 ESBL type with 98% identity and 97% level of coverage between *bla*SHV-12 sequence in the *E. cloacae* database with accession number ABV82949.1 and our sequences (Figure 3.6).

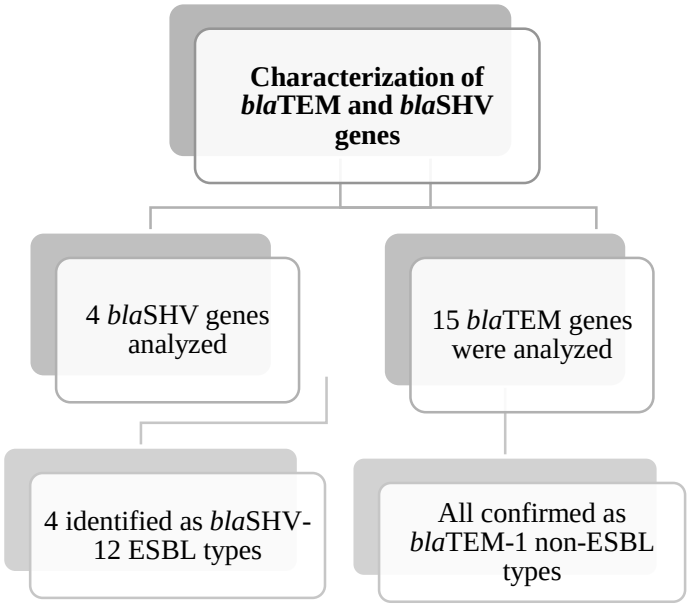

Figure 3.6: Identification of *bla*TEM and *bla*SHV genes. All *bla*TEM genes identified as *bla*TEM-1 types, 4 *bla*SHV genes as *bla*SHV-12 ESBL types.

Overall, twelve ertapenem non-susceptible isolates were confirmed to be ESBL producers by PCR and sequencing (eight isolates harboring the *bla*CTX-M gene only, three isolates with the *bla*SHV-12 gene only, and one isolate with both the *bla*SHV-12 and *bla*CTX-M genes). Amongst these, nine ESBL producers (all harboring *bla*CTX-M enzymes) were identified by the Mastdiscs D63C combination set, whereas seven ESBL producers (five harboring *bla*CTX-M and two harboring *bla*SHV-12 genes) were identified by the Mastdiscs D68C combination set. Five ESBL-producing ertapenem non-susceptible isolates harbouring only *bla*CTX-M gene were identified by both Mastdiscs D63C and D68C combination sets. Three ertapenem susceptible controls were identified as ESBL producers, all harboring the *bla*CTX-M gene, but none of the wild-type controls were identified as ESBL producers by any of the detection methods (Table 3.11).

Table 3.11: ESBL analysis using VITEK2®AES, Mastdiscs, PCR and sequencing

Isolates	VITEK2®AES	D68C discs	D63C discs	ESBL PCR
ERD 1	ESBL	Negative	Negative	Negative
ERD 2	ESBL	unint	ESBL	CTX-M
ERD 4	ESBL	unint	ESBL	CTX-M
ERD 5	Negative	Negative	Negative	Negative
ERD 6	ESBL	Negative	Negative	Negative
ERD 7	ESBL	ESBL	ESBL	Negative
ERD 8	ESBL	unint	ESBL	Negative
ERD 9	ESBL	ESBL	ESBL	CTX-M
ERD 10	ESBL	unint	ESBL	CTX-M
ERD 11	ESBL	ESBL	ESBL	CTX-M
ERD 12	ESBL	unint	Negative	Negative
ERD 13	ESBL	Negative	Negative	Negative
ERD 14	ESBL	Negative	Negative	SHV-12
ERD 16	ESBL	ESBL	ESBL	CTX-M
ERD 17	ESBL	ESBL	Negative	SHV-12
ERD 18	ESBL	ESBL	Negative	SHV-12
ERD 20	ESBL	ESBL	ESBL	CTX-M
ERD 29	ESBL	Negative	Negative	Negative
ERD 34	ESBL	ESBL	ESBL	CTX-M
ERD 42	ESBL	unint	ESBL	CTX-M & SHV-12
Ertapenem susceptible controls				
ERD 23	ESBL	ESBL	ESBL	Negative
ERD 25	ESBL	ESBL	ESBL	CTX-M
ERD 35	Negative	Negative	ESBL	CTX-M
ERD 39	ESBL	ESBL	ESBL	CTX-M
Wild-type controls				
WT1	Negative	Negative	Negative	Negative
WT2	Negative	Negative	Negative	Negative
WT3	Negative	Negative	Negative	Negative
WT4	Negative	Negative	Negative	Negative
WT5	Negative	Negative	Negative	Negative

unint = uninterpretable result

3.3.3 *bla*AmpC β-lactamases

Twelve of the ertapenem non-susceptible *E. cloacae* isolates were predicted to be derepressed *bla*AmpC producers by VITEK2®AES. Seventeen of the ertapenem non-susceptible isolates were identified as chromosomal *bla*AmpC producers by the Mastdiscs D69C combination set. The Mastdiscs D68C combination set identified 13 derepressed *bla*AmpC producers among the ertapenem non-susceptible isolates. Two ertapenem susceptible controls (ERD 39 and ERD 23) were predicted to be derepressed *bla*AmpC producers;with one isolate each identified by the VITEK2®AES and the Mastdiscs D68C combination set. Three of the ertapenem susceptible controls were found to harbor chromosomal *bla*AmpC enzymes using the Mastdiscs D69C combination set (Table 3.12). One of the three isolates (ERD 35), is likely to be an inducible *bla*AmpC producer as neither the VITEK2®AES nor the Mastdiscs D68C combination set predicted this isolate to be a derepressed *bla*AmpC mutant. The expression of *bla*AmpC, relative to the endogenous control gene *rpoB*, was used to confirm *bla*AmpC hyper-production in the ertapenem non-susceptible isolates and the ertapenem susceptible controls, relative to wild-type controls. The RNA quality and qPCR validation results are described in Appendix 3.

Amplification of the *bla*AmpC gene failed in four ertapenem non-susceptible isolates (ERD 4, ERD 9, ERD 10 and ERD 14) and all of the ertapenem susceptible controls, even after the experiment was repeated three times; and despite the successful amplification of the endogenous gene, *rpoB*. Of the 16 ertapenem non-susceptible isolates which were successfully amplified, six isolates (ERD 1, ERD 2, ERD 5, ERD 12, ERD 18 and ERD 42) showed high *bla*AmpC expression (≥ 2 fold higher expression), with extremely high expression (approximately 100-fold greater) observed in isolate ERD 5, compared to the wild-type controls (Figure 3.7). Five (25%) showed similar expression to wild-type controls and five (25%) had reduced expression of *bla*AmpC.

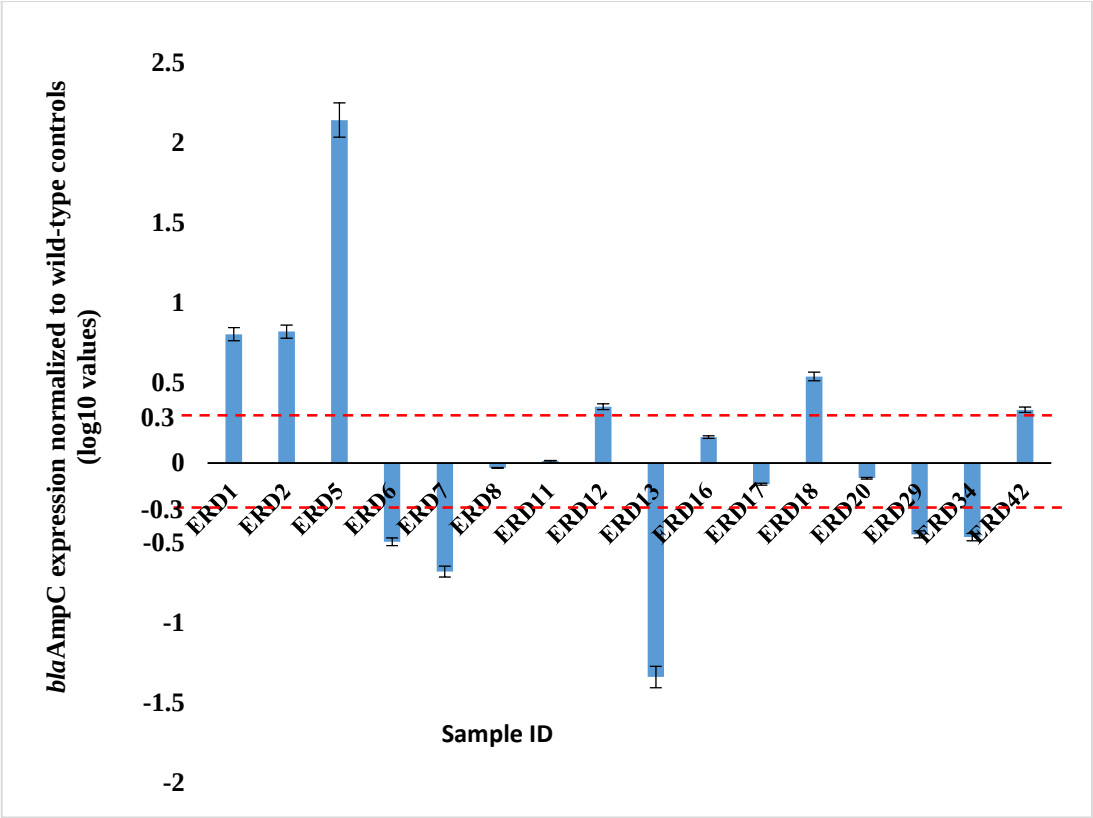

Figure 3.7: *bla*AmpC expression levels in ertapenem non-susceptible isolates normalized to wild-type controls. Diagram shows data for ertapenem non-susceptible isolates. Ertapenem non-susceptible isolates (ERD 4, ERD 9, ERD 10, and ERD 14) and three of the ertapenem susceptible controls (ERD 23, ERD 35 and ERD 39) were excluded from the graph due to failed PCR amplification. The horizontal line (in a red color) at log 0.3 / -0.3 indicates a two-fold threshold of significance.

Four of the six isolates which were identified as high *bla*AmpC producers based on RT-qPCR were also identified as derepressed *bla*AmpC mutants by both the VITEK2®AES and the Mastdiscs D68C combination set (Table 3.12).

Table 3.12: *bla*AmpC β-lactamases analysis using VITEK2®AES, Mastdiscs D68C & D69C and RT-qPCR

Isolates	VITEK2®AES	D68C discs	D69C discs	*bla*AmpC expression	
				log10 value (expression relative to wild-type controls)	Level of expression
ERD 1	Derepressed	Derepressed	Chromosomal *bla*AmpC	0.806	high
ERD 2	negative	unint	Chromosomal *bla*AmpC	0.822	high
ERD 4	Derepressed	negative	negative	no amplification*	
ERD 5	Derepressed	Derepressed	Chromosomal *bla*AmpC	2.144	Extremely high
ERD 6	negative	Derepressed	Chromosomal *bla*AmpC	-0.492	low
ERD 7	negative	Derepressed	Chromosomal *bla*AmpC	-0.678	low
ERD 8	Derepressed	unint	Chromosomal *bla*AmpC	-0.030	normal
ERD 9	Derepressed	Derepressed	Chromosomal *bla*AmpC	no amplification*	
ERD 10	negative	unint	Chromosomal *bla*AmpC	no amplification*	
ERD 11	negative	negative	Chromosomal *bla*AmpC	0.015	normal
ERD 12	Derepressed	Derepressed	Chromosomal *bla*AmpC	0.353	high
ERD 13	Derepressed	Derepressed	Chromosomal *bla*AmpC	-1.337	Extremely low
ERD 14	Derepressed	negative	negative	no amplification*	
ERD 16	negative	Derepressed	Chromosomal *bla*AmpC	0.163	normal
ERD 17	Derepressed	Derepressed	Chromosomal *bla*AmpC	-0.132	normal
ERD 18	Derepressed	Derepressed	Chromosomal *bla*AmpC	0.541	high
ERD 20	Derepressed	Derepressed	Chromosomal *blaAmpC*	-0.094	normal
ERD 29	Derepressed	Derepressed	Chromosomal *bla*AmpC	-0.446	low
ERD 34	negative	Derepressed	Chromosomal *bla*AmpC	-0.463	low
ERD 42	negative	unint	negative	0.333	high
Ertapenem susceptible controls					
ERD 23	negative	Derepressed	Chromosomal *bla*AmpC	no amplification	
ERD 25	negative	negative	unint	not tested	
ERD 35	negative	negative	Chromosomal *blaAmpC*	no amplification	
ERD 39	Derepressed	negative	Chromosomal *bla*AmpC	no amplification	
Wild-type controls					
WT1	negative	negative	Chromosomal *bla*AmpC	normal	
WT2	negative	negative	negative	normal	
WT3	negative	negative	Chromosomal *bla*AmpC	normal	
WT4	negative	negative	negative	normal	
WT5	negative	negative	Chromosomal *bla*AmpC	normal	

unint = uninterpretable results, ERD 25- not tested due to loss of viability

3.4 Discussion

A number of resistance mechanisms, including carbapenemases and / or reduced membrane permeability, in addition to ESBLs, hyper-production of chromosomal *bla*AmpC β-lactamases and / or over-expression of efflux pumps, have been shown to be responsible for carbapenem resistance in *Enterobacter* species (Szabó *et al* 2006; Yang *et al* 2012). This chapter involved the characterization of the β-lactamase enzymes (ESBLs, carbapenemases and expression of chromosomal *bla*AmpC β-lactamases) in order to understand the role they may play in mediating carbapenem resistance in our study population, using different phenotypic and molecular-based assays.

One ertapenem non-susceptible isolate (ERD 1), susceptible to both imipenem and meropenem was identified as a carbapenemase producer by the Rapidec® Carba NP kit, but was negative by both the VITEK2®AES and PCR. One limitation associated with the Rapidec® Carba NP kit is that it can identify false positives in enterobacterials harboring chromosomal *bla*AmpC β-lactamases, and other mechanisms such as reduced membrane permeability (http://www.biomerieux-diagnostics.com/rapidec-carba-np). All *Enterobacter* spp. isolates harbor *bla*AmpC genes on their chromosomes, and ERD 1 was shown to be an *bla*AmpC hyperproducer based on VITEK2®AES, Mastdiscs D68C combination set and RT-qPCR assays; implying that this might be a false positive isolate. Further analysis of membrane permeability is described in Chapter 4. It is also possible that this isolate might harbor a rare carbapenemase type such as *bla*NMC-A (Nordmann *et al* 1993), *bla*SPM (Toleman *et al* 2002), *bla*GIM (Castanheira *et al* 2004), *bla*SIM (Lee *et al* 2005), or *bla*AIM (Yong *et al* 2007) which were not screened for in this study. This could be confirmed using other methods such as Ultra Violet Spectrophotometry (Dortet *et al*, 2012), Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (Hrabák *et al* 2012) or whole-genome sequencing (Van El *et al* 2013). With reference to the Rapidec® Carba NP kit, no negative control was used nor were experiments repeated because, low prevalence of carbapenemase production in South Africa is expected as previously reported (Perovic *et al.*, 2014). Also, the assay was properly functioning and could detect carbapenemase producing isolates. However, a positive control was rather included in the study to assist in identifying potential carbapenemase producing isolates.

The VITEK2®AES predicted seven ‘carbapenem non-susceptible’ isolates to be carbapenemase producers, none of which were confirmed by PCR or the Rapidec® Carba NP kit, suggesting that the VITEK2®AES overcalled carbapenemase production in these isolates. The VITEK2®AES might predict the presence of carbapenemase phenotypes when isolates are resistant to cephalosporins and non-susceptible to two or more carbapenems. All of the seven predicted ertapenem non-susceptible isolates were resistant to cephalosporins; however, only two isolates (ERD 4 and ERD 20) were non-

susceptible to either meropenem or imipenem antibiotics in addition to ertapenem (Refer to Appendix 1 for antimicrobial susceptibility profiles).

Based on this, only two isolates (ERD 4 and ERD 20) should have been predicted to be carbapenemase producers by the VITEK2®AES. This result suggests that the VITEK2®AES might have used other unknown parameters in predicting carbapenemase production in the remaining five isolates; and that the prediction of carbapenemase production by the VITEK2®AES in *Enterobacter* spp. isolates should be interpreted with caution. Alternatively, uncommon carbapenemase types, not investigated in this study, could be present in all seven carbapenemase-producing isolates predicted by the VITEK2®AES.

Since all but two ertapenem non-susceptible isolates were susceptible to both imipenem and meropenem, the absence of carbapenemases in these isolates was not surprising, as these enzymes also confer resistance to these antibiotics. Most often, the level of carbapenem resistance is dependent on the carbapenemase type harbored by an isolate, as the presence of enzymes such as oxacillinases often result in very low imipenem and meropenem MICs, and their detection is enhanced when other mechanisms such as reduced membrane permeability or other carbapenemases are present (Walther-Rasmussen & Høiby, 2006). This suggests that other resistance mechanisms besides carbapenemase production mediate carbapenem resistance in these isolates. A previous study conducted by Woodford *et al* (2007) produced similar findings amongst *Klebsiella* spp. and *Enterobacter* spp. isolates in the United Kingdom. Carbapenemase production has also been shown to be rare among *Klebsiella* spp. from South Africa (Perovic *et al* 2014) and some *E. coli* blood-culture isolates from Europe (European Centre for Disease Prevention and Control, 2015).

Eleven ertapenem non-susceptible isolates were confirmed to be ESBL producers byMastdiscs D63C combination set whilst twelve producers were confirmed by PCR and sequencing. Eleven ESBL producers identified by both methods were resistant to ceftazidime, cefepime, and cefotaxime antibiotics based on VITEK2®AES (Refer to Appendix 1 for antimicrobial susceptibility profile). The VITEK2®AES and the Mastdiscs D68C combination set identified 19 and 8 ESBL producers respectively. Of the 11 ESBL-producing isolates identified by the Mastdiscs D63C combination set, 9 isolates were PCR positive for ESBLs. This suggests a good correlation between PCR and the Mastdiscs D63C combination set assay. However, the VITEK2®AES appears to have overcalled ESBL production and no other study has validated the VITEK2®AES for ESBL production.

The *bla*CTX-M ESBL gene was harbored by most of the ESBL-positive isolates, and these enzymes are considered to be highly disseminated among different bacterial species found in communities and in hospital settings (Bonnet, 2004). This poses a high transmission risk for resistance as plasmids

conveying these genes may harbor other resistance genes, leading to a high selective pressure by antibiotics used in treating infections caused by these isolates.

The only *bla*SHV ESBL type amongst our isolates was *bla*SHV-12 which has been identified by previous studies to be the most common SHV type in *E. coli* and *K. pneumoniae* isolates from Korea, and *E. cloacae* isolates from Taiwan (Ryoo *et al* 2005; Yu *et al* 2006). In South Africa, this gene has been identified among *Salmonella* spp. and *E. cloacae* species isolated from Durban, in the KwaZulu Natal province (Govinden *et al* 2008; Rubin *et al* 2014). However, our findings indicate the first detection of *bla*SHV-12 in *E. cloacae* isolates from the Western Cape province.

Six of the ertapenem non-susceptible isolates (ERD 1, ERD 2, ERD 5, ERD 12, ERD 18 and ERD 42) were shown to be derepressed *bla*AmpC mutants based on high *bla*AmpC mRNA levels. Three of these isolates (ERD 2, ERD 18 and ERD 42), were shown to harbor ESBLs. Both the VITEK2®AES and the Mastdiscs D68C combination set identified four of these isolates as derepressed *bla*AmpC mutants. Nine additional derepressed *bla*AmpC mutants were identified by the Mastdiscs D68C combination, whereas the VITEK2®AES identified seven additional derepressed *bla*AmpC mutants. This suggests that both the VITEK2®AES and the Mastdiscs D68C combination set overcalled *bla*AmpC hyper-production in these isolates, and that these methods may not be reliable for the detection of derepressed *bla*AmpC mutants. On the contrary, these two phenotypic assays could be considered when detecting *bla*AmpC mutants despite limitations associated when identifying *bla*AmpC hyper-producers. No other studies have been found to validate these assays for characterization of these enzymes. While alternative methods such as the quantitative PCR assay may be beneficial in confirming the results of the phenotypic assays, the molecular assay is technically challenging, labor intensive and expensive, and probably not suitable for use in a routine diagnostic environment.

Four ertapenem non-susceptible isolates and the ertapenem susceptible control strains failed *bla*AmpC PCR amplification. This was unexpected as all *Enterobacter* species harbor chromosomal *bla*AmpC β-lactamases. Three of the ertapenem non-susceptible isolates were predicted to be derepressed *bla*AmpC producers by the VITEK2®AES, one by the Mastdiscs D68C combination set, and two as harboring chromosomal *bla*AmpC genes by the Mastdiscs D69C combination set. One possible reason for *bla*AmpC PCR failure is the presence of mutations in the primer binding sites in the *bla*AmpC gene. Future studies could include sequencing of this region to confirm the presence of mutations in these isolates. These results suggest that some derepressed *bla*AmpC mutants may have been missed by the RT-qPCR assay; and that further optimization of the methodology is required before the test can be applied routinely. Some limitations of the RT-qPCR results include the lack of biological duplicates, and that only basal expression levels were investigated. The expression analysis

was performed at basal levels because this study focussed on identifying constitutive *bla*AmpC hyperproduction; however this methodology precluded detection of *bla*AmpC inducers and further characterization of the isolates by future studies would be valuable.

Overall, the VITEK2®AES overcalled the presence of all enzymes characterized in this study. This is of great concern as this system is widely used in most laboratories as part of routine diagnostics. The algorithms used by the VITEK2®AES in predicting ESBL and derepressed *bla*AmpC mutants are unable to differentiate between the phenotypes in *Enterobacter* spp. Hence, most often, it will assign both phenotypes to these isolates. For example, an *Enterobacter* spp. having antimicrobial susceptibilty profiles of co-amoxiclav MIC = 32 µg/ml; cefuroxime MIC= 64 µg/ml; cefotaxime MIC = 64 µg/ml; ceftazidime MIC = 32 µg/ml; cefepime MIC= 16 µg/ml and ertapenem MIC = 0.5 µg/ml will be defined as having one or other, or both phenotypes (ESBL and derepressed *bla*AmpC mutants) by the VITEK2®AES.

In order to overcome future problems, several improvements in its database could be considered. These include: a greater range of possible phenotypes and the MIC distributions of all tested antibiotics, the number of antibiotics on the test cards should be increased, and finally, algorithms used by the system should be specific for a particular phenotype regardless of the organisms tested (Sanders *et al*, 2000).

The Mastdiscs D63C, D68C and D69C combination sets are promising assays for characterizing both ESBLs and *bla*AmpC β-lactamases at the phenotypic level; however, some limitations are associated with these discs. For the ESBL gene characterization, the Mastdiscs D63C combination set confirmed ESBL production in isolates harboring either only *bla*CTX-M gene or in combination with other ESBL family genes. On the contrary, the Mastdiscs D68C combination set was able to confirm ESBL production in all isolates harboring *bla*SHV-12 gene and some with the *bla*CTX-M gene; however, some of the results were uninterpretable resulting in undefined phenotypes for those isolates. Furthermore, no controls and replicates were used during the mastdisc assay due to cost and time constraints and this might impact the integrity of the results.For the *bla*AmpC gene characterization, the Mastdiscs D69C combination set (capable of detecting chromosomal *bla*AmpC β-lactamases) failed to detect this enzyme in two wild-type controls (WT 2 and WT 4). Although the Mastdiscs D68C and D69C combination sets might be associated with some limitations in characterizing these enzymes, a broader range of isolates may be needed to assess this assay as our findings are based on a small set of ertapenem non-susceptible isolates with limited controls.

3.5 Conclusion

Carbapenemase production may have contributed to carbapenem resistance in only one of the ertapenem non-susceptible isolates, although the type of carbapenemase could not be identified by PCR. This observation suggests that other mechanisms are likely to be involved in carbapenem resistance in these isolates. Among the isolates, 60% were ESBL producers by PCR, 30% were confirmed *bla*AmpC hyper-producers by RT-qPCR and 15% were both *bla*AmpC hyper-producers and ESBL producers. Additional mechanisms involved in carbapenem resistance such as reduced membrane permeability and over-expression of the AcrAB/TolC efflux pump, are investigated in Chapters 4 and 5.

Chapter 4: The role of reduced outer membrane permeability in ertapenem resistance

4.1 Introduction

Reduced membrane permeability, in combination with other mechanisms such as carbapenemase and / or ESBL production, hyper-production of chromosomal *bla*AmpC β-lactamases or over-expression of efflux pumps, has been shown to promote carbapenem resistance in *Enterobacter* spp. (Szabó *et al* 2006; Yang *et al* 2012). Reduced permeability can be due to the loss or down-regulation of some major porins, caused by mutations in their promoter regions, hence reducing antibiotic uptake and leading to antimicrobial resistance (Koebnik *et al* 2000). In *E. cloacae* isolates, two major osmoporins, OmpF and OmpC, have been speculated to mediate ertapenem resistance (Doumith *et al* 2009; Yang *et al* 2012).

Various techniques have been utilized to investigate the role of reduced outer membrane permeability in antimicrobial resistance. The relative abundance of these porins and the expression levels of the major porin genes associated with carbapenem resistance have been investigated.

Relative abundance of these porins can be investigated by extracting the outer membrane proteins (OMPs) using the rapid outer membrane protein (ROMP) extraction procedure by Carlone *et al* (1986). The OMPs are then separated based on their molecular weights using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Shapiro *et al* 1967; Weber & Osborn, 1969). This assay uses an anionic detergent (SDS) which denatures proteins conferring a negative charge to their polypeptide backbone in proportion to their length. Other methods include isoelectric focusing, where proteins are separated based on their charges (pIs). The SDS-PAGE assay has been utilized for OmpF and OmpC quantification by both Doumith *et al* (2009) and Yang *et al* (2012), whilst isoelectric focusing was used by Szabó *et al* (2006). The proteins can be identified using a sensitive and specific method such as Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), which is suitable for the analysis of complex samples (Fenn *et al* 1989). LC-MS/MS was used by Yang *et al* (2012) to identify the two major porins associated with ertapenem resistance in *E. cloacae* isolates from Taiwan. Other types of outer membrane proteins in different species have also been identified in this manner.

Gene expression analyses of porin genes in resistant bacterial populations after exposure to certain types of antibiotics play an important role in antimicrobial resistance research. Several methods used include northern blots, DNA microarrays, reverse transcription quantitative polymerase chain reaction (RT-qPCR), comparative expressed sequence tag (EST) assay and serial analysis of gene expression (SAGE) (Fryer *et al* 2002). Among these assays, RT-qPCR is most commonly used.

RT-qPCR involves fluorescence-based detection of amplification products through the use of a DNA-binding dye (SYBR Green) or probes. It is a semi-quantitative, sensitive method for detecting low-abundance messenger RNA (mRNA) and can be used as a validation method to confirm the expression levels of a small number of genes in biological samples. However, this assay is relatively expensive to perform if many different transcripts in many samples are to be analyzed (Wang *et al* 1989). The expression levels of genes under investigation are often normalized to housekeeping or endogenous control genes. Housekeeping genes are synthesized in both nucleated and non-nucleated cell types because they are important for cell survival. These genes are considered to show a constant level of expression among different tissues of an organism at all stages of development, unaffected by any experimental treatment and roughly expressed at the same level as the RNA under study (Thellin *et al* 1999).

Amplification and sequencing of the promoter regions and coding sequences of the porin genes to identify mutations which may influence the expression levels and functions of these genes have also been utilized in several studies which investigated reduced permeability associated with carbapenem resistance (Doumith *et al* 2009; Yang *et al* 2012).

The aim of this chapter is to determine whether reduced membrane permeability contributes to ertapenem resistance in the clinical ertapenem non-susceptible *E. cloacae* isolates, by investigating the expression of OmpC and OmpF proteins using SDS-PAGE analysis of the outer membrane protein fraction, and RT-qPCR of the *ompC* and *ompF* transcripts.

4.2 Materials and methods

The expression of OmpC and OmpF was investigated in all 20 ertapenem non-susceptible *E. cloacae* isolates and four ertapenem susceptible controls, in comparison to the wild-type controls (as described in Chapter 2), using SDS-PAGE, and *ompC* and *ompF* RT-qPCR. One susceptible control isolate (ERD 25) was excluded from both the OmpC and OmpF expression and the transcription level analyses due to loss of viability.

4.2.1 Characterization of outer membrane porins using SDS-PAGE

Extraction of the outer membrane proteins

E. cloacae isolates were inoculated once from Microbank (MBK) beads onto fresh TBA plates, and incubated for 18 – 20 hours at 37°C under ambient air. A single colony from each culture was inoculated in 10 ml of brain heart infusion (BHI) broth (NHLS Media Laboratory, Greenpoint, South Africa) and incubated for 18 hours at 37°C while shaking at 180rpm. The bacterial cultures were diluted (one part of bacterial culture in four parts of BHI broth) in sterile cuvettes to obtain optical densities of 0.5 - 0.6 nm at a wavelength of 600 nm, using the Ultrospec 10 cell density meter (Amersham Biosciences, England). Ten millilitres of the diluted bacterial suspension was centrifuged at 4640 x *g* for 10 minutes at 4°C in a Heraeus multifuge 4KR centrifuge (Thermo Scientific, USA). The supernatant was decanted, and bacterial cells were washed once by resuspending the cells in 1.5 ml of cold 10 mM HEPES buffer (Amresco, USA) at pH 7.4, and centrifuging at 15,600 x *g* for 2 minutes at 4°C. The supernatant was decanted and cell pellets were used immediately or stored at -70°C. The outer membrane proteins were extracted from the cell pellets using a modified protocol of the rapid outer membrane protein (ROMP) extraction method previously described by Carlone *et al* (1986) (Appendix 2.5), where the cell pellets were suspended in 10 mM HEPES buffer containing proteinase inhibitor, cOmplete (Roche Diagnostics, Germany) at the beginning of extracting the outer membrane proteins. All steps were carried out on ice to preserve the proteins. The concentrations of the extracted outer membrane protein fractions were determined using the Biorad RC-DC method according to manufacturer's instructions (Biorad, USA) (Appendix 2.6).

SDS-PAGE gel electrophoresis

Ten micrograms of outer membrane protein from each isolate was separated on a 12% SDS-PAGE gel, as described in Appendix 2.7. A pre-stained broad range protein marker (Bio Labs, New England, UK) was loaded into the first and last lanes of each gel, and each gel included a wild-type strain. Gel electrophoresis was performed using the Biorad electrophoresis set-up (Biorad, USA) at 200V for at least an hour, or until the dye had reached the end of the gel. The SDS-PAGE gels were then washed with deionised water and stained overnight in 10 ml of AcquaStain buffer (Bulldog, England), visualized and stored at 4°C in deionised water.

The quantification of OmpC and OmpF proteins as intense, faint or absence of a porin band was based on visual inspection of the SDS-PAGE gels.

Protein identification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Selected protein bands were excised from the gel, cut into 1 x 1 mm slices on a sterile glass surface with the aid of sterile surgical blade, and placed into sterile, labelled 1.5 ml micro-centrifuge tubes. One hundred microlitres of wash solution [80 mg/ml ammonium bicarbonate (Sigma-Aldrich, Germany)] was distributed into each labelled tube. The tubes were incubated at room temperature for 20 minutes and mixed intermittently. The supernatant was removed and the washing process repeated. The gel pieces were dehydrated by the addition of 50 µl of acetonitrile (ACN, HPLC grade, Sigma-Aldrich, Germany), mixed intermittently for 2 minutes at room temperature. This washing process was repeated at least 3 times; including an overnight wash step in ACN. After the overnight wash, the ACN solution was removed and the gel pieces were submitted to the Proteomics Unit of the Central Analytical Facility (CAF), Faculty of Medicine and Health Sciences, Stellenbosch University for identification. The proteins were reduced, thiomethylated and trypsin digested, after which the peptides were desalted and analyzed using the Orbitrap Fusion Mass Spectrometer (Thermo Scientific, USA). The raw files from the LC-MS/MS analysis were interpreted using the MaxQuant software version 1.5.3.17 against an *E. cloacae* proteome database (http://www.uniprot.org/proteomes/UP000017834) with assistance from Dr. Nastassja Kriel (Division of Molecular Biology and Human Genetics, Stellenbosch University). Proteins were identified based on the presence of at least two unique tryptic peptides.

4.2.2 *ompF* and *ompC* RT-qPCR

A one step SYBR Green-based RT-qPCR was performed following procedures outlined in Section 3.2.5, to quantify the expression levels of *ompC* and *ompF* genes relative to the endogenous control, *rpoB*, using a previously described set of primers by Doumith *et al* (2009) (Table 4.1) The relative expression levels of both the *ompF* and *ompC* transcripts in the ertapenem non-susceptible *E. cloacae* isolates and the ertapenem susceptible controls were compared to the wild-type controls.

Table 4.1: Primers used for the *ompF* and *ompC* RT-qPCR

Target gene	Primer	Sequence (5'- 3')	Product size (bp)
rpoB	rpoB-F	AAGGCGAATCCAGCTTGTTCAGC	148
	rpoB-R	TGACGTTGCATGTTCGCACCCATCA	
ompF	ompF-F	TCCCTGCCCTGCTGGTAG	139
	ompF-R	TAAGTGTTGTCGCCATCGTTG	
ompC	ompC-F	GCGACCAGACCTACATGCGT	113
	ompC-R	TTCGTTCTCACCAGAGTTACCCT	

The PCR experiments for both of the target genes, *ompF* and *ompC,* and the endogenous gene, *rpoB* were validated (Appendix 3.3), using criteria as outlined in Section 3.2.5. Data from the *ompC* and *ompF* RT-qPCR experiments were analyzed as previously outlined in Section 3.2.5.

4.3 Results

4.3.1 OmpF and OmpC protein abundance

In total, five distinct protein bands of the outer membrane protein were detected by SDS-PAGE. Two single protein bands (labelled D and E) were observed at positions between 32 and 46kDa on a 12% SDS-PAGE gel. These bands correlated with the previously published sizes of 39 – 40 kDa and 37 kDa which have been predicted to be OmpC and OmpF proteins respectively (Kaneko *et al* 1984).

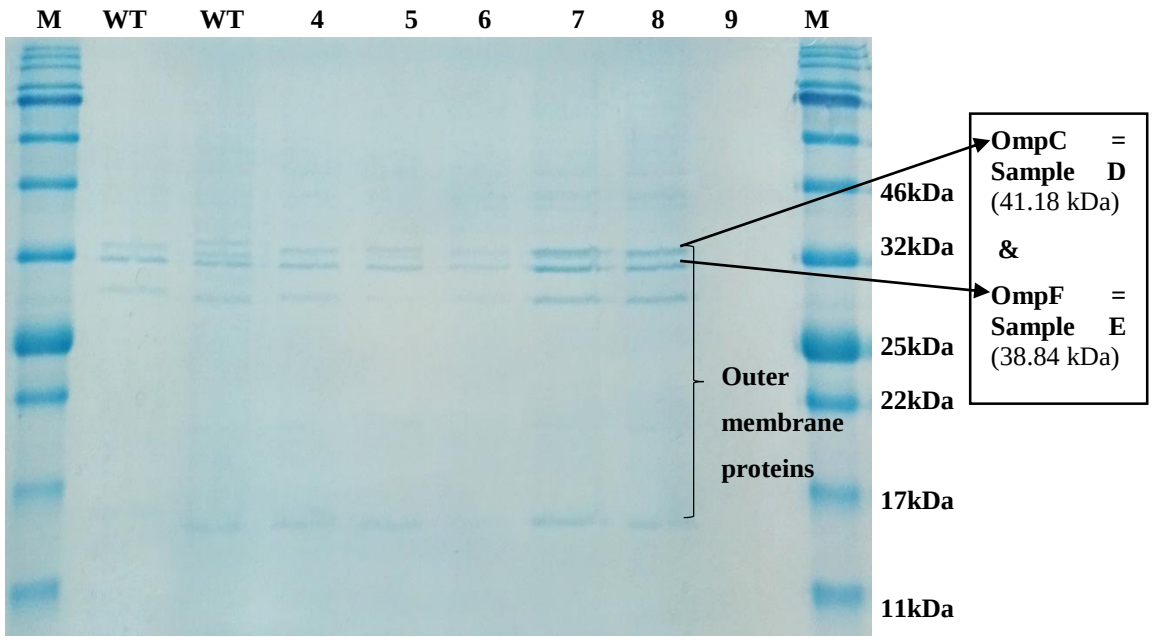

Figure 4.1: OMP profiles of selected ertapenem non-susceptible & wild-type *E. cloacae* isolates. 12% SDS PAGE gel showing outer membrane proteins profiles in selected wild-type and ertapenem non-susceptible isolates. Lane M: Protein markers. Lane WT: Wild-type controls. Lanes 4 - 6: ertapenem non-susceptible *E. cloacae* isolates with faint bands, Lanes 7 & 8: ertapenem non-susceptible *E. cloacae* isolates with intense bands, and lane 9: ertapenem non-susceptible *E. cloacae* isolate with no band.

LC-MS/MS was performed on protein bands labelled D and E, and based on the MaxQuant analysis, they were confirmed to be OmpC and OmpF, with molecular weights of 41.18 kDa and 38.84 kDa respectively (Table 4.2).

Table 4.2: LC-MS/MS Identification of OmpF and OmpC porins

Sample	Unique peptides	Molecular weight (kDa)	Majority Protein ID	Protein
D	2	41.18	V5CTF0	OmpC
E	2	38.84	V5A844	OmpF

The OmpC and OmpF porins were detected in 15 (75%) of the ertapenem non-susceptible isolates, while both porin bands were absent in the remaining five ertapenem non-susceptible isolates. The two protein bands were also observed in all ertapenem susceptible control isolates and the wild-type controls, except isolate ERD 23, a susceptible control. The porin bands were visually defined as intense, faint or absent. In all of the isolates the two porin bands showed similar band intensities to each other; in 4 (20%) the bands were faint and 11 (55%) had intense bands.

4.3.2 Expression levels of *ompC* and *ompF* genes

The *ompC* and *ompF* RT-qPCR experiments were validated as described in Appendix 3.3. The PCR efficiencies for *rpoB*, *ompC* and *ompF* PCR assays were 94%, 98% and 90% respectively; with R^2 values of ≥ 0.998.

The majority of the ertapenem non-susceptible isolates (13, 65%) showed lower *ompF* expression levels relative to the wild-type controls (Figure 4.2), with isolates ERD 2 and ERD 4 showing extremely low levels with log10 values of -3.02 and -3.13 respectively, indicating an approximately 1000-fold lower expression in each. Three ertapenem non-susceptible isolates (ERD 8, ERD 9 and ERD 10) failed *ompF* amplification, even after the PCR experiment was repeated three times. Two ertapenem susceptible controls (ERD 23 and ERD 39) showed lower *ompF* expression levels with log10 values of -4.17 and -0.64 respectively indicating a 10^4 and a 4-fold lower expression compared to the wild-type controls. One susceptible control (ERD 35) and four of the ertapenem non-susceptible isolates (ERD 5, ERD 13, ERD 14 and ERD 20) were observed to have a similar expression relative to the wild-type controls.

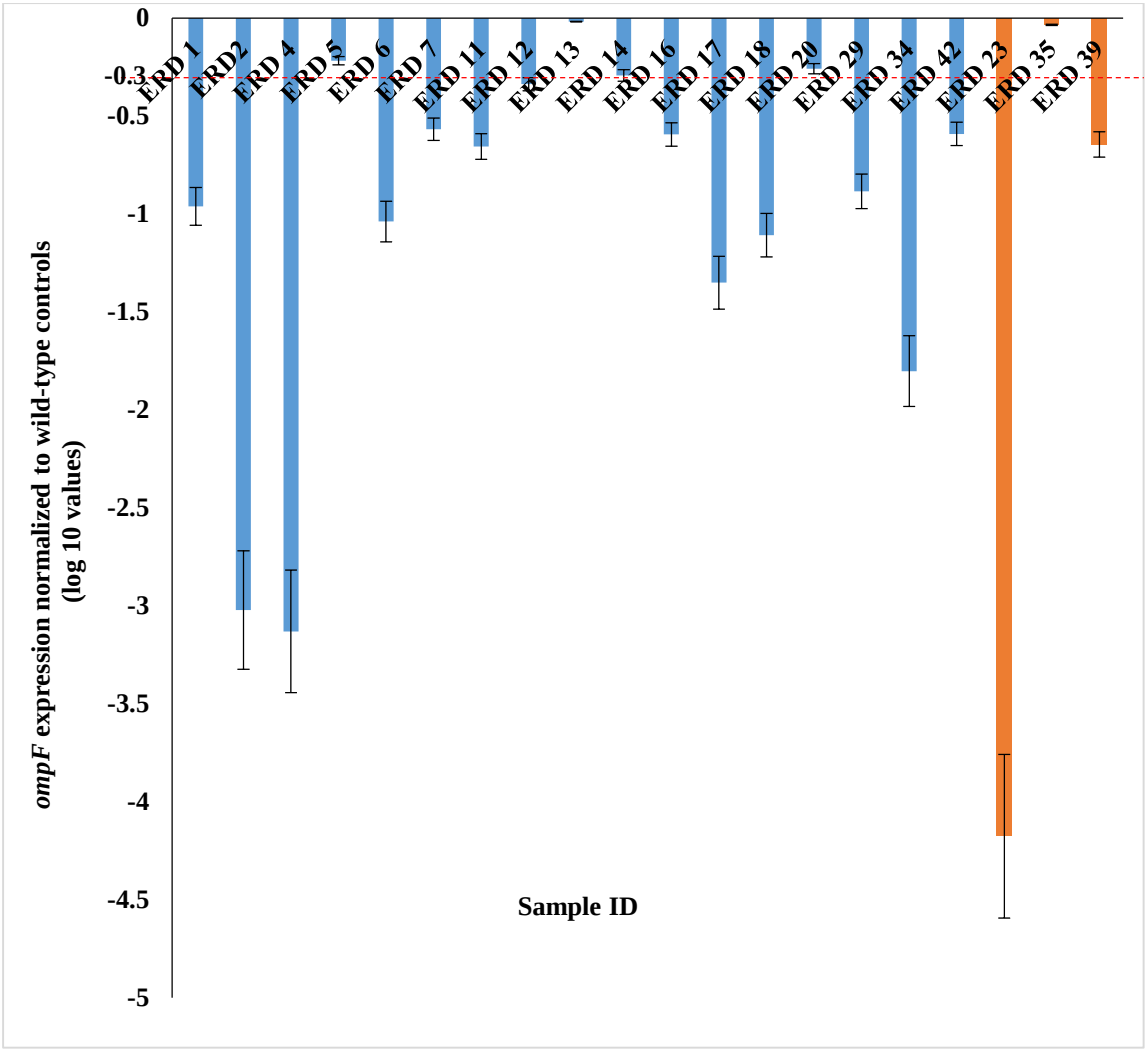

Figure 4.2: *ompF* expression levels of ertapenem non-susceptible isolates and ertapenem susceptible controls normalized to wild-type controls. Diagram represents data for ertapenem non-susceptible isolates (shown in a blue) and ertapenem susceptible *E. cloacae* controls (shown in an orange). Ertapenem non-susceptible isolates (ERD 8, ERD and ERD 10) were excluded from the graph due to failed PCR amplification. The horizontal line (**in a red**) at log 0.3 / -0.3 indicates a two-fold threshold of significance.

Eleven ertapenem non-susceptible isolates showed lower *ompC* expression levels (Figure 4.3) relative to wild-type controls. One isolate (ERD 1) showed extremely low expression with log10 value of -1.921, indicating approximately 100-fold reduced expression compared to wild-type controls. Four ertapenem non-susceptible isolates (ERD 14, ERD 18, ERD 20 and ERD 34) showed increased expression levels of *ompC*, with ERD 34 showing the highest log10 value of 1.42, indicating a 25-fold change. One susceptible control (ERD 23) had extremely low expression of *ompC* (log10 value of -5.765, 10^6 fold difference), whereas the remaining susceptible controls (ERD 35 and ERD 39) showed high expression levels of the *ompC* gene.

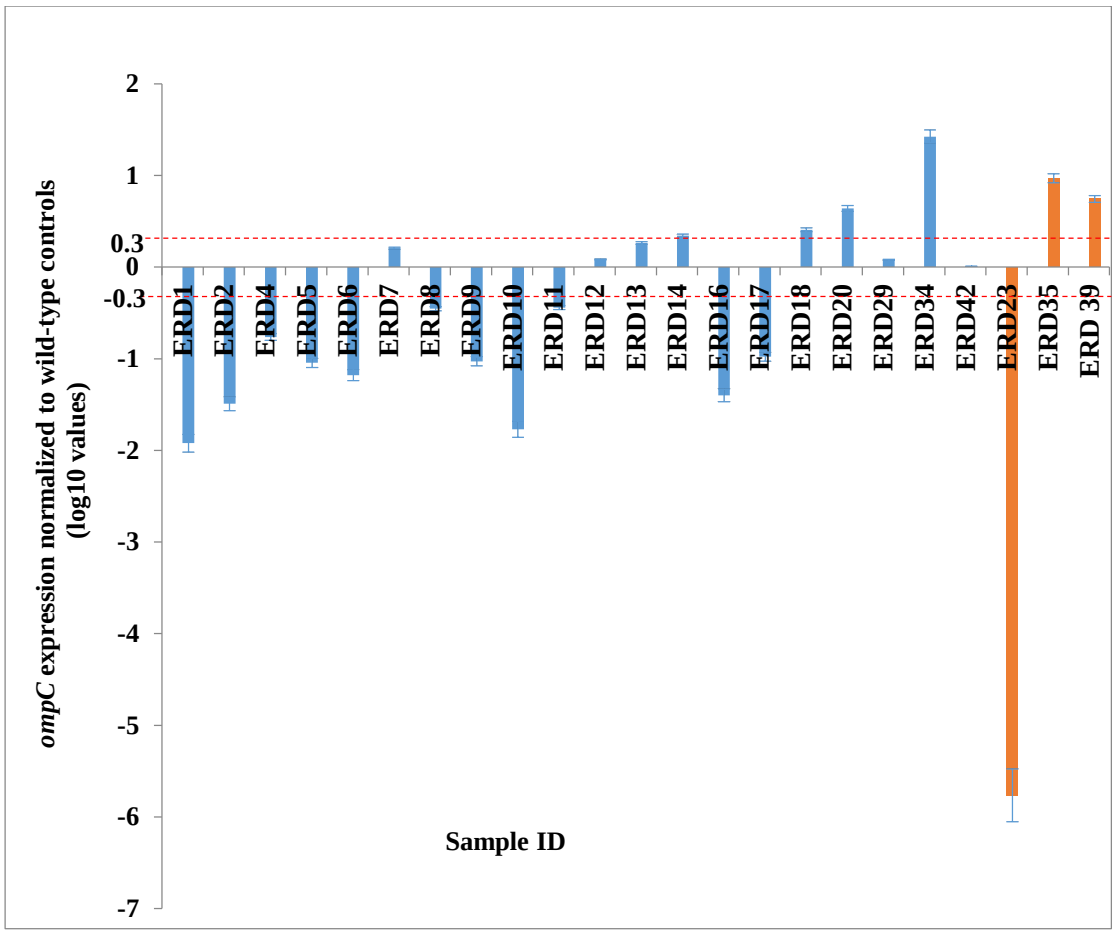

Figure 4.3: *ompC* expression levels of ertapenem non-susceptible isolates and ertapenem susceptible controls normalized to wild-type controls. Diagram represents data for ertapenem non-susceptible isolates (shown in a blue color) and *ertapenem susceptible E. cloacae* controls (shown in an orange color). The horizontal line (in a red color) at log 0.3 / -0.3 indicates a two-fold threshold of significance.

Overall, seven (35%) of the ertapenem non-susceptible isolates showed lower mRNA expression levels of both the *ompF* and *ompC* genes. One susceptible control (ERD 23) also showed lower expression of both genes, while the remaining susceptible controls (ERD 35 and ERD 39) showed high expression of the *ompC* gene and lower or normal expression of *ompF*. All three ertapenem non-susceptible isolates with failed *ompF* PCR amplification showed lower expression levels of *ompC*. Although there was high *ompC* expression in some of the non-susceptible isolates, lower expression of *ompF* or both *ompC* and *ompF* was observed in all of the ertapenem non-susceptible isolates.

The results of the SDS-PAGE and mRNA expression analyses did not correlate well, with only one ertapenem non-susceptible isolate (ERD 1) and one ertapenem susceptible control (ERD 23) showing lower expression levels of both porin genes and faint OmpC and OmpF protein bands. For the remaining isolates, the SDS-PAGE results did not correlate with the mRNA expression levels (Table 4.3).

Due to the discrepancies associated with the SDS-PAGE analysis, and the more subjective nature of the assessment of the SDS-PAGE results, characterization of reduced membrane permeability was based on the mRNA expression levels of *ompF* and *ompC* genes.

Table 4.3: Outer membrane proteins analysis based on SDS-PAGE, LC-MS/MS and, *ompC* and *ompF* RT-qPCR

Sample IDs	OmpF and OmpC band intensities	Relative expression levels of *ompF* and *ompC* to wild-type controls			
		log10 value (*ompC* expression)	*ompC* expression	log10 value (*ompF* expression)	*ompF* expression
ERD 1	faint	-1.921	low*	-0.961	low
ERD 2	intense	-1.492	low	-3.02	low**
ERD 4	intense	-0.763	low	-3.130	low**
ERD 5	intense	-1.045	low	-0.218	normal
ERD 6	intense	-1.179	low	-1.039	low
ERD 7	intense	0.207	normal	-0.568	low
ERD 8	intense	-0.454	low	No amplification	
ERD 9	intense	-1.028	low	No amplification	
ERD 10	intense	-1.770	low*	No amplification	
ERD 11	intense	-0.443	low	-0.655	low
ERD 12	absent	0.082	normal	-0.338	low
ERD 13	faint	0.263	normal	-0.019	normal
ERD 14	faint	0.344	high	-0.293	normal
ERD 16	absent	-1.401	low	-0.594	low
ERD 17	absent	-0.977	low	-1.350	low
ERD 18	absent	0.405	high	-1.109	low
ERD 20	absent	0.640	high	-0.259	normal
ERD 29	intense	0.079	normal	-0.884	low
ERD 34	faint	1.422	high	-1.802	low*
ERD 42	intense	0.007	normal	-0.591	low
Ertapenem susceptible controls					
ERD 23	absent	-5.765	low***	-4.175	low***
ERD 35	very faint	0.968	high	-0.035	normal
ERD 39	faint	0.744	high	-0.644	low
Wild-type controls					
WT 1	intense	reference		reference	
WT 2	intense	reference		reference	
WT 3	intense	reference		reference	
WT 4	intense	reference		reference	
WT 5	intense	reference		reference	

Low expression without * indicates expression < a 100-fold change, * = low / high expression levels (indicating approximately a 100-fold change); ****** = low / high expression levels (indicating approximately a 1000-fold change); ******* = extremely low / high expression (indicating approximately a 10^4 - 10^6 fold change).

4.4 Discussion

Reduced outer membrane permeability attributed to decreased expression or loss of one or both of the major outer membrane porins (OmpF and OmpC), due to mutations in their promoter regions, in combination with other mechanisms such as acquired ESBLs or carbapenemase production and / or chromosomal *bla*AmpC hyper-production and over-expression of efflux pumps have been speculated to promote carbapenem resistance in *E. cloacae* isolates (Doumith *et al* 2009; Yang *et al* 2012). In this study, we investigated the relative abundance of OmpF and OmpC, and the expression levels of *ompF* and *ompC* transcripts using SDS-PAGE analysis and RT-qPCR respectively.

The two major porins in *E. cloacae*, known to be involved in carbapenem resistance, were successfully extracted from the outer membrane and identified probably as OmpF and OmpC, with molecular weights of 38.84 kDa and 41.18 kDa respectively, using LC-MS Mass Spectrometry. Previously, most studies have identified these porins based only on their sizes on SDS-PAGE gels. Several types of outer membrane proteins in different bacterial species have been identified using LC-MS Mass Spectrometry. For example in *Enterobacter* spp. (Yang *et al* 2012); *Acinetobacter* spp. (Dupont *et al* 2005); *Yersinia pestis* and *E. coli* (Jabbour *et al* 2010). In our study, the outer membrane proteins were identified based on an *E. cloacae* database (http://www.uniprot.org/proteomes/UP000017834) which has not been validated, as at the time of analysis the validated protein database contained only 73 *Enterobacter* spp. proteins.

The OmpC and OmpF bands were described as intense, faint or absent based on visual assessment. This method was also used by Doumith *et al* 2009 to determine the presence of OMP bands on SDS PAGE gels. However, some limitations associated with this assessment method were observed. Some lanes on the gels had faint and others strong bands; likely due to unequal protein loading caused by interference of other OMP components during protein quantification. Also, the intensity of other visible OMP bands correlated with the intensities of both OmpC and OmpF for a particular isolate, suggesting that SDS-PAGE may not be a reliable method for quantifying the OmpC and OmpF proteins. This is highlighted by the complete absence of proteins for some isolates despite normalization of the input protein. Due to these inconsistencies, the outer membrane protein expression results were based on the RT-qPCR rather than the protein results. SDS-PAGE has some limitations, is not always reproducible and certain proteins types are difficult to analyze (Gygi *et al* 2000). To improve upon protein analysis using SDS-PAGE, a number of factors could be considered for further studies. Fluorescent dyes used for a 2D- Difference Gel electrophoresis (2D-DIGE) could be used instead of classical protein staining methods such as Coomassie blue and silver staining, as these methods provide less sensitivity and lack quantitative parameters, respectively. 2D-DIGE is

sensitive, provides good quantitation, reproducibility and is also compatible with mass spectrometry analysis (Unlu *et al* 1997).

Eleven (55%) of the ertapenem non-susceptible isolates had low *ompC* expression whilst thirteen (65%) showed low *ompF* expression. Reduced expression of both *ompC* and *ompF* genes was observed in seven (35%) of the ertapenem non-susceptible isolates. Among these isolates, extremely low expression of *ompF* was detected in isolate ERD 4 (showing a 1000-fold lower expression than wild-type controls), and *ompC* in isolate ERD 1 (showing a 100-fold lower expression). This observation suggests that, down-regulation of both o*mpC* and *ompF* may have played a role in ertapenem resistance in our isolates. One may expect that reduced expression of the OmpC protein channel would be the main contributor to ertapenem resistance due to its large pore size and weak selectivity for solutes based on their electric charges, compared to OmpF with its smaller pore size and a preference for positively charged antibiotics and not negatively charged ones such as ertapenem (Kaneko *et al* 1984; Nikaido *et al* 1983); however, this was not apparent from the results.

There was up-regulation of *ompC* gene expression in four (20%) of the ertapenem non-susceptible isolates (ERD 14, ERD 18, ERD 20 and ERD 34), with isolate ERD 34 showing the highest expression level of approximately 25-fold higher expression compared to the wild-type controls. Two of the ertapenem susceptible controls (ERD 35 and ERD 39) also showed high expression levels of *ompC* while one susceptible isolate (ERD 23) showed lower expression of both *ompF* and *ompC* genes, with 10^4 and 10^6 fold reduction respectively. The increased expression of OmpC in some resistant and susceptible isolates might be attributed to mutations in the porin gene itself. The porin (although expressed) may be unable to transport solutes into the bacterial cell, as efficiently as it should. Hence, the cell may upregulate expression of the ‘’non-functional’’ porin to compensate for the lack of uptake of various solutes. Carbapenem resistance is attributed to reduced membrane permeability in combination with other mechanisms such as hydrolyzing enzymes, over-expression of efflux pump; therefore low porin expression alone may be insufficient to cause ertapenem resistance.

ompF failed to amplify in 3 (15%) of the ertapenem non-susceptible isolates. All these isolates showed intense OmpC and OmpF protein bands on SDS-PAGE, which means the gene is present and the proteins are expressed. The failed amplification may therefore be due to technical error and not due to the complete absence of the *ompF* transcript. A possible reason for *ompF* PCR failure in these isolates includes the presence of mutations in the primer binding sites. Future studies should consider sequencing the promoter and coding regions of these genes to identify mutations which may influence expression levels, primer binding and or porin functionality. In addition, to confirm whether the loss of these porins promotes ertapenem resistance in these isolates, restoration experiments could be

conducted. This would involve cloning and expressing the *ompF* and *ompC* genes in isolates that show reduced OmpF and OmpC expression, and determination of ertapenem MICs. Restoration of susceptibility to ertapenem antibiotics would confirm that loss of these porins contributed to ertapenem resistance.

There was poor correlation between the results of the SDS-PAGE analysis investigating the abundance of the OmpF and OmpC proteins and the RT-qPCR analysis describing the expression of the *ompC* and *ompF* transcripts. Both outer membrane proteins were detected in several isolates which showed low *ompF* and / or *ompC* expression; and both outer membrane proteins were absent in some isolates which showed normal or high *ompC* and *ompF* transcript levels. Our findings do not support those of a previous study which confirmed a good correlation between the patterns of both OmpC and OmpF proteins on a 12.5% SDS-PAGE gel and the mRNA expression levels of these porins in a resistant *E. cloacae* isolate (Doumith *et al* 2009). Possible reasons for our findings include some of the previously mentioned technical issues associated with SDS-PAGE and some parameters associated with biological processes in the cell (Maier *et al* 2009). Most studies have shown a 40% correlation between cellular concentrations of proteins with abundance of their corresponding mRNA, in both bacteria and eukaryotes (Abreu *et al* 2009). Certain factors contributing to the 60% variation include the different lifetimes of both mRNA and proteins (Vogel & Marcotte, 2013). For example, bacterial mRNAs are short-lived and few copies per cell are produced at a time leading to fluctuations in their concentrations, in comparison to proteins, which have longer half-lives (Selinger *et al* 2003). Other important factors contributing to this variation are regulation post-transcriptionally, and during translation and protein degradation. Based on this, one would expect the protein results (from the SDS-PAGE analysis) to be more representative of the actual abundance of the proteins and therefore impact the permeability of the outer membrane. Therefore, the major confounder for this variation may likely be due to technical issues associated with the SDS-PAGE. Due to the technical issues associated with SDS-PAGE in this study, the RT-qPCR method appeared to be more robust for investigating the role of reduced membrane permeability to carbapenem resistance in our setting, however the results generated by the former method could improve after some of the modifications explained previously have been considered.

4.5 Conclusion

Reduced membrane permeability due to the reduced expression of both OmpF and OmpC, or either porin alone, may be a contributor to ertapenem resistance in the clinical *E. cloacae* isolates from Tygerberg Hospital, with 85% of the ertapenem non-susceptible isolates exhibiting reduced expression of at least one porin gene. Additional mechanisms which may contribute to ertapenem resistance in these isolates are discussed in Chapters 3 and 5.

Chapter 5: The contribution of efflux to ertapenem resistance

5.1 Introduction

Bacterial efflux pumps, especially the Resistance-Nodulation-Division (RND)-type, are one of the major contributors to antibiotic resistance in Gram-negative bacteria. These pumps have a broad substrate specificity, and hence, over-expression results in decreased susceptibility to a range of antibacterial agents and biocides (Nikaido & Pagès, 2012).

RND efflux pumps such as AcrAB/TolC cause resistance to many antimicrobials, including β-lactams, in GNB. It is a tripartite system consisting of AcrB (outer membrane channel), AcrA (inner membrane channel) and TolC (membrane fusion protein). The expression of most efflux pumps is influenced by multiple levels of regulation through a number of factors. These factors include local and global regulators (such as AcrR and MarA), environmental factors (bile, fatty acids, oxidative stress and salicylate), and post-translational and post-transcriptional factors (Weston *et al* 2017).

To combat efflux-mediated activity, several efflux pump inhibitors (EPIs), which are molecules capable of increasing the susceptibility to most administered drugs, have been developed. Among these, phenylalanine-arginine β-naphthylamide (PaβN) (Renau *et al* 1999) was the first to be developed. PaβN is considered a broad spectrum EPI as it reduced levofloxacin MICs in resistant *P. aeruginosa* isolates and reduced resistance to quinolones and phenicols in Gram-negative bacteria (Pagès *et al* 2005).

Ertapenem resistance in *Enterobacter* spp. has been speculated in several studies to be mediated by AcrAB/TolC, due to over-expression of the main transporter gene, *acrB,* in combination with carbapenemase production, reduced outer membrane permeability, ESBL production and / or hyper-production of chromosomal *bla*AmpC β-lactamases (Szabó *et al* 2006; Pérez *et al* 2007; Yang *et al* 2012). Therefore, the aim of this chapter is to investigate the contribution of efflux, mediated by the AcrAB/TolC efflux pump system, to ertapenem resistance in clinical *E. cloacae* isolates from Tygerberg Hospital, in the Western Cape province of South Africa.

5.2 Materials and methods

Synergy studies involving the use of disc diffusion in the presence or absence of an EPI, PaβN, was used to phenotypically assess the impact of efflux on antibiotic resistance, while a molecular-based RT-qPCR assay was used to quantify the mRNA expression levels of the *acrB* gene.

Two ertapenem resistant isolates (ERD 4 and ERD 34) and one ertapenem susceptible control (ERD 39) were used for the synergy study.

All twenty ertapenem non-susceptible *E. cloacae* isolates, three susceptible controls and five wild-type controls (described in Chapter 2) were used for *acrB* expression level analysis. One susceptible control (ERD 25) was excluded from the expression level analysis due to loss of viability.

5.2.1 Disc diffusion synergy assay

Disc diffusion was performed on standard Mueller Hinton (MH) agar plates. MH agar plates were prepared using MH agar powder (Sigma-Aldrich, Germany) with or without PaβN (Sigma-Aldrich, Germany) at 25 µg/ml, as previously used by Yang *et al* (2012). The inoculation of the plates was performed as previously described for the gradient diffusion assay in Section 2.3. Antibiotic discs containing ceftazidime, ertapenem, ciprofloxacin and gentamicin (Mast Diagnostics, UK) and imipenem, meropenem and cefepime (Oxoid, UK) were placed on the plates. The antibiotic content of each disc is shown in Table 5.1. The zones of inhibition (ZOI) were measured with a straight ruler and interpreted based on CLSI 2016 breakpoints (Table 5.1). If the ZOI on the MH agar plate with PaβN was greater than the ZOI on plain MH agar plate, this would imply inhibition of an efflux pump, thus rendering the organism more susceptible. This experiment was repeated as least twice.

Table 5. 1: Breakpoints of antibiotics used for the disc diffusion assay (CLSI, 2016)

Antibiotic	**Concentrations of antibiotic discs**	**ZOI (mm) and interpretation**		
ertapenem	10 µg	≥22 (S)	19 – 21 (I)	≤18 (R
imipenem	10 µg	≥23 (S)	20 – 22 (I)	≤19 (R)
meropenem	10 µg	≥23 (S)	20 – 22 (I)	≤19 (R)
ceftazidime	30 µg	21 (S)	18 – 20 (I)	≤17 (R)
cefepime	30 µg	25 (S)	19 - 24 (I)	≤18 (R)
gentamicin	10 µg	≥15 (S)	13 -14 (I)	≤12 (R)
ciprofloxacin	5 µg	≥21 (S)	16 - 20 (I)	≤ 15 (R)

R – resistant, S – susceptible, I - intermediate

5.2.2 *acrB* RT-qPCR

A one step SYBR Green-based RT-qPCR assay was performed following procedures outlined in Section 3.2.5, to quantify the expression levels of the efflux pump gene, *acrB*, relative to an endogenous control gene, *rpoB*, using a previously described set of primers by Doumith *et al* (2009) (Table 5.2). The relative expression levels of *acrB* in the ertapenem non-susceptible *E. cloacae* isolates and the ertapenem susceptible controls were compared to the wild-type controls.

Table 5.2: Primers used for *acrB* RT-qPCR

Target gene	**Primer**	**Sequence (5'-3')**	**Product size (bp)**
rpoB	rpoB-F	AAGGCGAATCCAGCTTGTTCAGC	**148**
	rpoB-R	TGACGTTGCATGTTCGCACCCATCA	
acrB	*acrB*-F	CGATAACCTGATGTACATGTCC	
	acrB-R	CGATAACCTGATGTACATGTCC	**207**

The PCR experiments for both the target gene, *acrB* and the endogenous gene, *rpoB* were validated (Appendix 3.3), using criteria as outlined in Section 3.2.5. Data from the *acrB* RT-qPCR experiment was analyzed as previously outlined in Section 3.2.5.

5.3 Results

5.3.1 Disc Diffusion synergy assay

The susceptibility of selected isolates to various antibiotics in the presence or absence of the EPI, PaβN, was compared using disc diffusion to determine if efflux pumps contribute to antibiotic resistance through active pumping of antibiotics out of the cell to reduce the intra-cellular concentrations of administered antibiotics, leading to reduced susceptibility of the bacterial species.

Two ertapenem resistant isolates (ERD 4 and ERD 34) and one ertapenem susceptible control isolate (ERD 39) were used. It was expected that the presence of the EPI, PaβN, would increase the susceptibility of these isolates by reducing the efflux of antibiotics and thereby increasing the accumulation of the antibiotic in the cell. For the susceptible control ERD 39, no change in the ZOI in the presence of PaβN was observed for any of the antibiotics tested, except ciprofloxacin, where a 5 mm increase (16 mm to 21 mm) in ZOI was observed indicating increased susceptibility (Table 5.3). For the ertapenem non-susceptible isolates (ERD 4 and ERD 34), the presence of EPI decreased the ZOI of all three carbapenems in contrast to what was anticipated (Tables 5.3). No effect on the ZOI of the cephalosporin antibiotics was observed for ERD 4, however an increase in the ZOI was observed for ERD 34. Due to these unexpected results, the expression levels of the efflux pump gene, *acrB*, as determined by RT-qPCR, was used to assess the potential impact of increased efflux on ertapenem resistance.

Table 5.3: Zones of inhibition of tested antibiotics with and without PaβN

Isolates	**ERD 39 (S)**		**ERD 4 (R)**		**ERD 34 (R)**	
Antibiotic	-PaβN	+PaβN	-PaβN	+PaβN	-PaβN	+PaβN
ertapenem	28 (S)	28 (S)	16 (R)	11 (R)	16 (R)	11 (R)
imipenem	27 (S)	27 (S)	28 (S)	22 (I)	25 (S)	21 (I)
meropenem	30 (S)	31 (S)	28 (S)	17 (R)	25 (S)	19 (R)
ceftazidime	14 (R)	16 (R)	No zone	No zone	No zone	No zone
cefepime	15 (R)	16 (R)	9 (R)	9 (R)	9 (R)	10 (R)
gentamicin	9 (R)	8 (R)	8 (R)	No zone	7 (R)	No zone
ciprofloxacin	16 (I)	21 (S)	No zone	No zone	11 (R)	15 (R)

R= resistant, S= susceptible and I= intermediate, No zone = Isolates were highly resistant to tested antibiotics

5.3.2 *acrB* RT-qPCR analysis

The *acrB* RT-qPCR was successfully validated, with PCR efficiencies of 94% and 95% for *rpoB* and *acrB* respectively, and R^2 value of 0.999 each (Refer to Appendix 2.3).

High *acrB* expression was observed in only two ertapenem non-susceptible *E. cloacae* isolates (ERD 9 and ERD 11) with log10 values of approximately 0.6 and 0.4 respectively (Figure 5.1), indicating 4-fold and 2.5-fold higher expression relative to the wild-type controls. Four of the ertapenem non-susceptible isolates (ERD 2, ERD 4, ERD 7 and ERD 10) had *acrB* expression levels similar to that seen in the wild-type controls. The remaining non-susceptible isolates had *acrB* expression levels that were lower than seen in the wild-type controls, with extremely low levels detected in ERD 18 (log10 value of -1.8, 60-fold lower expression). With reference to the ertapenem susceptible controls, low expression of *acrB* gene was observed in all isolates, with extreme low levels expressed by isolate ERD 39 (Table 5.4).

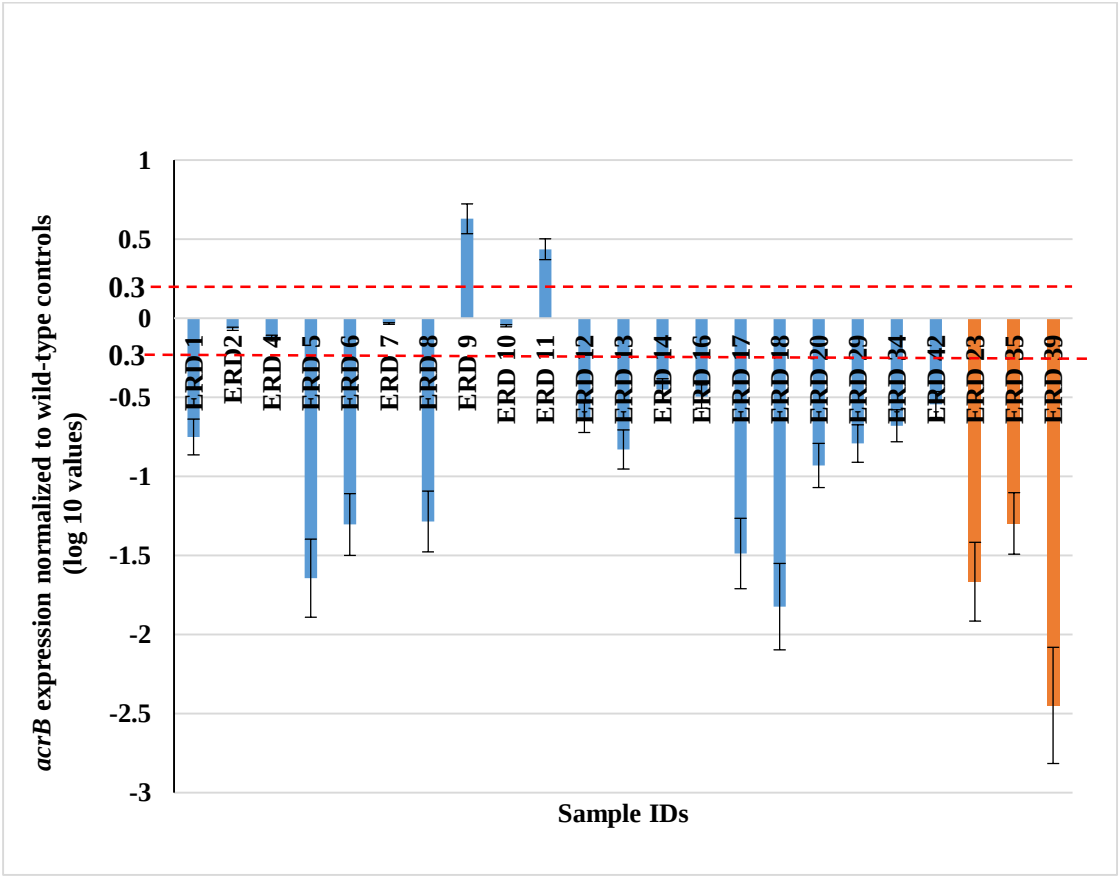

Figure 5.1: *acrB* expression levels of ertapenem non-susceptible isolates and ertapenem susceptible controls normalized to wild-type controls. Graph represents expression levels of *acrB* normalized levels in both ertapenem non-susceptible isolates (indicated in blue) and ertapenem susceptible controls (indicated in an orange). The horizontal line (in a red) at log 0.3 / -0.3 indicates a two-fold threshold of significance.

Table 5.4: Summary of *acrB* expression level analysis

Isolate	***acrB* expression analysis (relative to wild-type isolates)**	
	log10 value (relative expression to wild-type controls)	**Level of expression**
ERD 1	-0.75059	Low
ERD 2	-0.0661	Normal
ERD 4	-0.12519	Normal
ERD 5	-1.64387	Low**
ERD 6	-1.30356	Low
ERD 7	-0.03282	Normal
ERD 8	-1.28541	Low
ERD 9	0.629322	High
ERD 10	-0.04628	Normal
ERD 11	0.436651	High
ERD 12	-0.62764	Low
ERD 13	-0.82947	Low
ERD 14	-0.44997	Low
ERD 16	-0.49508	Low
ERD 17	-1.48779	Low
ERD 18	-1.82358	Low**
ERD 20	-0.93152	Low*
ERD 29	-0.79153	Low
ERD 34	-0.67938	Low
ERD 42	-0.54493	Low
Ertapenem susceptible controls		
ERD 23	-1.6655	Low**
ERD 25	not tested	
ERD 35	-1.29644	Low *
ERD 39	-2.44768	Low**

** = extremely low / high expression level (log10 value approximately 2 / -2, showing a 100-fold difference in expression), *= low / high expression level (log10 value approximately 1 / -1, showing a 10-fold difference in expression). Isolate ERD 25 not tested due to loss of viability

5.4 Discussion

Increased efflux, mediated by over-expression of efflux pumps, in addition to reduced outer membrane penetration, hyper-production of chromosomal *bla*AmpC and / or acquisition of plasmid-mediated β-lactamases has been speculated to promote ertapenem resistance in *Enterobacter* spp. (Szabó *et al* 2006; Yang *et al* 2012).

This study investigated the contribution of efflux to ertapenem resistance in *E. cloacae* isolates using two approaches. A disc diffusion synergy assay was performed to study changes in the susceptibility of selected isolates to various antibiotics when the efflux pump inhibitor, PaβN was included in the culture medium. Two ertapenem resistant isolates and one susceptible control were used to validate this assay. The presence of PaβN resulted in no changes to the ZOI for the susceptible isolate except for ciprofloxacin, where an increased ZOI was observed. However, for the ertapenem resistant isolates, a reduction in the ZOI was observed for all tested carbapenems when PaβN was added, indicating increased resistance. No changes in ZOI were observed for any of the tested cephalosporins and gentamicin antibiotics in either the susceptible or resistant isolates in the presence of PaβN. Our findings were the opposite of what was anticipated, implying that the presence of the EPI decreased the susceptibility to the antibiotics. Similarly, no change in ertapenem MICs of clinical *E. cloacae* isolates was observed after the addition of PaβN at a concentration of 100 µg/ml in a previous study by Doumith *et al* (2009), suggesting that efflux did not promote ertapenem resistance in these isolates. However, our findings contradict those of Szabó *et al* (2006) and Yang *et al* (2012), who showed a reduction in ertapenem MICs by ≥ 3-fold and ≥ 2-fold in the presence of PaβN at concentrations of 40 µg/ml and 25 µg/ml respectively. In addition, PaβN has been shown to potentiate levofloxacin activity in resistant *Pseudomonas aeruginosa* strains, reduce the level of resistance to phenicols and quinolones in Gram-negative bacteria including *E. aerogenes*, and enhance the activity of arylpiperazine derivatives, which reversed multi-drug resistance in *E. coli* cells that overexpressed efflux pumps (Pagès *et al* 2005).

It has been suggested that PaβN might not be the most effective EPI against the AcrAB/TolC efflux pump system (Nakashima *et al*, 2013). In a kinetic study of the AcrAB/TolC efflux system, the EPI MBX2319, a pyranopyridine, but not PaβN, was shown to completely inhibit nitrocefin efflux, suggesting that PaβN does not overcome efflux of some antimicrobial agents at low concentrations. It was deduced that MBX2319 was effective because it was able to compete with nitrocefin for the same binding site in the binding pocket of AcrB, or to decrease the access of nitrocefin to the binding site (Opperman *et al* 2014).

Therefore, other EPIs such as MBX2319 and D13-9001 (a potent inhibitor of AcrB in *E. coli* and MexB in *P. aeruginosa*) could be used to investigate the impact of AcrB-related efflux on ertapenem. For future studies, concentrations of PaβN could be varied and alternative EPIs investigated to further optimize and validate the synergy experiment.

With reference to the research conducted by Lamer *et al*, 2013, the impact of PaβN on some intracellular responses in our study would be difficult to address due to the following reasons. Firstly, the levels of hydrolyzing enzymes in the whole cell lysates were not determined before and after PaβN treatment to determine intra-cellular effects. Furthermore, based on the RT-qPCR results, repeating the synergy assay on isolates that showed high or low AcrB expression may have been prudent. Hence, further studies involving the use of appropriate controls and replicates could investigate this effect which may have impacted the results.

Due to the counterintuitive results of the phenotypic experiments, the expression levels of the *acrB* gene were analyzed using RT-qPCR; to investigate whether the expression of the efflux pump was increased in the ertapenem non-susceptible *E. cloacae* isolates. Two ertapenem non-susceptible isolates (ERD 9 and ERD 11) showed high expression levels of the *acrB* gene. No difference in expression, compared to the wild-types, was observed in four other resistant isolates, whilst the remaining non-susceptible isolates had lower expression levels. All of the ertapenem susceptible isolates also showed low *acrB* expression. This observation suggests that efflux may contribute to ertapenem resistance in only a small proportion of the isolates.

AcrB is not the only protein associated with efflux in the cell. The local repressor AcrR, has been shown to be a secondary modulator to control the over-expression of the *acrAB* transcript in *E. coli* (Ma *et al* 1996). Mutations in the *acrR* gene have been shown to prohibit *acrAB* repression, leading to over-expression of the AcrAB/TolC system in clinical and veterinary *E. coli* isolates (Webber *et al* 2005). Other regulators such as AcrS and EnvR have also been shown to repress *acrAB* expression in *E. coli* (Hirakawa *et al* 2008). Furthermore, some knock-out experiments involving the deletion of *acrA* and *tolC* genes restored MICs of oxacillin antibiotics in *E. cloacae*, indicating the roles of these genes in efflux activity (Pérez *et al* 2012). Other components of the AcrAB/TolC efflux pump system (such as AcrA and TolC), and these regulators, AcrR, AcrS and EnvR, may therefore also contribute to efflux-mediated ertapenem resistance, instead of the *acrB* gene directly, and future studies could utilize various approaches to investigate additional efflux mechanisms. Knowledge about all these regulatory factors will help identify possible targets for novel drug discoveries, to control efflux activity. Furthermore, ertapenem non-susceptible and or ertapenem susceptible isolates which showed extremely high and low *acrB* expression levels could also be used to further validate the synergy experiment.

5.5 Conclusion

High *acrB* expression was detected in two ertapenem non-susceptible *E. cloacae* isolates, suggesting that efflux may promote ertapenem resistance in these isolates, in combination with additional mechanisms such as reduced outer membrane permeability, chromosomal *bla*AmpC β-lactamase hyper-production and / or the production of ESBLs. While *acrB* mediated efflux does not appear to be involved in efflux activity in the remaining isolates, other efflux mechanisms should be investigated.

Chapter 6: General Discussion and Conclusion

6.1: Discussion

Enterobacter cloacae is a recognized cause of both nosocomial and community-related infections. In addition, *E. cloacae* isolates resistant to almost all current broad spectrum antibiotics, including carbapenems (last resort drugs), have been isolated. Resistance to carbapenems in *Enterobacter* spp. has been speculated by previous studies to be mediated by the possession of acquired carbapenemases, or by the production of ESBLs and / or hyper-production of chromosomal *bla*AmpC β-lactamases, in combination with reduced outer membrane permeability and over-expression of efflux pumps (Doumith *et al* 2009; Yang *et al* 2012).

This study aimed to determine the mechanisms mediating carbapenem resistance in clinical *E. cloacae* isolates from Tygerberg Hospital, in the Western Cape province of South Africa. Twenty ertapenem non-susceptible isolates, four ertapenem susceptible control and five wild-type controls were collected based on VITEK2®AES antibiotic susceptibility testing performed as part of routine diagnostics. BMD confirmed ertapenem non-susceptibility in 17 (85%) of the ertapenem non-susceptible isolates, while only 6 (30%) were shown to be non-susceptible by gradient diffusion. BMD is considered to be the 'gold standard assay' for determining antimicrobial susceptibility, therefore the VITEK2®AES appears to overcall ertapenem resistance and the gradient diffusion assay appears to undercall ertapenem resistance in clinical *E. cloacae* isolates in this setting. Ertapenem resistance, defined by the VITEK2®AES as part of routine diagnostics, should be interpreted with caution with regard to its impact on treatment, in order to preserve the activities of the broad spectrum antibiotics used in most settings.

Carbapenemases are considered the primary cause of carbapenem resistance (Queenan & Bush, 2007). All of the isolates were screened for carbapenemase activity using the Rapidec® Carba NP kit, and the presence of the common carbapenemase genes: *bla*OXA-48, *bla*VIM, *bla*NDM, *bla*IMP and *bla*GES was determined by PCR. Although one of the ertapenem non-susceptible isolates (ERD 1) was positive for carbapenemase activity, no carbapenemase genes were detected by PCR. This isolate might harbor another carbapenemase genes such as *bla*NMC-A (Nordmann *et al* 1993), *bla*SPM (Toleman *et al* 2002), *bla*GIM (Castanheira *et al* 2004), *bla*SIM (Lee *et al* 2005), *bla*AIM (Yong *et al* 2007) or *bla*KHM-1 (Sekiguchi *et al* 2008) which were not included in the carbapenemase PCRs.

Alternatively, it may have been a false-positive isolate, as false-positive results have been reported in *Enterobacter* spp. possessing additional resistance mechanisms such as hyper-production of chromosomal *bla*AmpC β-lactamases and reduced outer membrane permeability (http://www.biomerieux-diagnostics.com/rapidec-carba-np). Both of these mechanisms were

identified in this isolate (ERD 1), supporting speculation of this being false-positive Carba-NP result. Further characterization of this isolate using an advanced method such as whole genome sequencing (WGS) could confirm whether a carbapenemase is present, and enable the identification of the carbapenemase gene. Of the seven carbapenemase producers predicted by the VITEK2®AES, none were confirmed by PCR or the Rapidec® Carba NP kit. This suggests that VITEK2®AES overcalled carbapenemase production in these isolates. Overall, no true carbapenemase production was observed to promote carbapenem resistance in these isolates. Similarly, Woodford *et al*, (2007) did not identify any carbapenemase mediated ertapenem resistance in both *Klebsiella* spp. and *Enterobacter* spp. isolates from the United Kingdom. Other studies have confirmed carbapenem resistance to be rare among *Klebsiella* spp. from South Africa (Perovic *et al* 2014) and some *E. coli* blood-culture isolates from Europe (European Centre for Disease Prevention and Control, 2015). The low prevalence of carbapenemase production in our setting is an advantage as these enzymes are acquired on plasmids, which harbor additional resistance genes and can be transferred between bacteria of the same and different species, posing a significant threat to public health.

Extended-spectrum β-lactamases (ESBLs) were identified in twelve of the ertapenem non-susceptible isolates based on PCR and sequencing. Molecular methods were used to define the presence of ESBLs as the VITEK2®AES was observed to overcall ESBL production and various discrepancies were associated with the Mastdiscs assay. Three of the ertapenem susceptible controls were also shown to harbor ESBLs. All of the ESBL producers were non-susceptible to extended-spectrum cephalosporins. The commonest ESBL gene identified was *bla*CTX-M, considered to be common both in communities and healthcare settings (Poirel, 2002). The ESBL gene *bla*SHV-12 was also identified in a smaller number of isolates, and has previously been detected in *Salmonella* spp. isolates from Durban, in the KwaZulu Natal province and *E. cloacae* in South Africa (Govinden *et al* 2008; Rubin *et al* 2014). However, this is the first detection of *bla*SHV-12 in *E. cloacae* isolates from the Western Cape province. Detection of ESBL genes in the majority of the study population is worrisome as these enzymes are also acquired on plasmids, and have a strong activity against extended-spectrum cephalosporins which are frequently used in treating bacterial infections (Bradford, 2001).

Hyper-production of chromosomal *bla*AmpC β-lactamases was detected in six ertapenem non-susceptible isolates, based on *bla*AmpC mRNA expression levels. Again, the VITEK2®AES appeared to overcall the presence of derepressed *bla*AmpC producers and some discrepancies were observed with the Mastdiscs assay, influencing its reliability. Three of the ertapenem non-susceptible isolates and all of the susceptible isolates failed *bla*AmpC PCR amplification, therefore no

conclusions could be drawn regarding *bla*AmpC expression in these isolates and additional work is required to investigate this further, as described in Chapter 3.

Reduced outer membrane permeability has been associated with ertapenem resistance in Gram-negative bacteria. The two major outer membrane proteins, OmpC and OmpF, have been speculated to contribute to this resistance in *E. cloacae* isolates. Reduced membrane permeability was investigated using SDS-PAGE to determine the abundances of OmpF and OmpC porins of the outer membrane protein fraction, and *ompC* and *ompF* RT-qPCR. Although OmpC and OmpF porins were detected and identified by LC/MS Mass Spectrometry, similar band intensities were observed across all proteins detected in an isolate, despite normalization of the input protein, precluding the reliable determination of differences in OmpC and OmpF abundance by visual inspection of the SDS-PAGE gels. Therefore, the *ompC* and *ompF* mRNA expression levels were determined. Of the 17 true ertapenem non-susceptible isolates, as determined by BMD, ten isolates showed lower expression levels of the *ompC* gene, whilst 12 showed lower expression of the *ompF* gene. Six of the isolates showed lower expression levels of both *ompF* and *ompC* genes. Two isolates (ERD 6 and ERD 7), defined as ertapenem susceptible by BMD, and two ertapenem susceptible controls (ERD 23 and ERD 39) also showed reduced membrane permeability due to lower expression of one or both porin genes. Therefore, although reduced membrane permeability may have contributed to ertapenem resistance in the majority of the ertapenem non-susceptible isolates, this mechanism alone is not sufficient to cause resistance.

The AcrAB/TolC efflux pump is the main transporter system found in *Enterobacter* species, and is known to confer resistance to a number of antibiotics and other solutes by actively extruding these substances out of the intra-cellular component of the bacterial cell (Nikaido & Pagès, 2012). The expression levels of the efflux transporter gene, *acrB*, was used as a marker of increased efflux as there were some discrepancies associated with the phenotypic synergy test using the EPI PaβN. Two ertapenem non-susceptible isolates showed increased expression of *acrB*, suggestive of increased efflux activity. Based on our results, efflux may not be a major contributing factor to carbapenem resistance in these isolates.

The above-mentioned mechanisms have been proposed to work synergistically to promote carbapenem resistance. Reduced membrane permeability will allow limited amounts of carbapenem antibiotics to enter the intra-cellular space of the bacterial cell. The efflux pumps can actively pump the antibiotics out of the bacteria cell in order to maintain a reduced concentration of the antibiotic in the cell. Although ESBLs and *bla*AmpC β-lactamases do not have high activity against carbapenems (Bradford, 2001; Jacoby, 2009), they may be effective in hydrolyzing low concentrations of the antibiotics resulting from reduced permeability or increased efflux.

Based on our findings, ESBL production and reduced membrane permeability were the major contributors to carbapenem resistance in these isolates. Eleven out of the twelve ESBL producers confirmed to be non-susceptible to ertapenem by BMD also showed reduced membrane permeability, due to lower expression levels of either or both of the *ompC* and *ompF* porin genes. This is consistent with the findings of previous studies which identified these as the main contributors to carbapenem resistance in *E. cloacae* isolates (Lee *et al* 1991; Szabó *et al* 2006; Doumith *et al* 2009; Yang *et al* 2012). However, one ertapenem susceptible control, ERD 39, was also shown to harbor an ESBL in addition to reduced membrane permeability, suggesting these two mechanisms alone might not be sufficient to cause carbapenem resistance, and additional mechanisms may be involved.

Hyper-production of chromosomal *bla*AmpC in addition to ESBL production and reduced membrane permeability mechanisms were detected in three of the ertapenem non-susceptible isolates (ERD 2, ERD 18 and ERD 42). Increased efflux in addition to both ESBL and reduced permeability was also identified in two ertapenem non-susceptible isolates (ERD 9 and ERD 11). One ertapenem non-susceptible isolate (ERD 1) showed carbapenemase production in addition to hyper-production of chromosomal *bla*AmpC and reduced membrane permeability, but as discussed previously, it is unclear whether the isolate is a true carbapenemase producer.

One ertapenem non-susceptible isolate (ERD 5) showed hyper-production of chromosomal *bla*AmpC and reduced membrane permeability, while two isolates (ERD 8 and ERD 29) showed only reduced membrane permeability, and, one isolate (ERD 12) showed only hyper-production of chromosomal *bla*AmpC (Table 6.1).

In one ertapenem non-susceptible isolate (ERD 13), none of the investigated mechanisms were identified, suggesting that additional unknown mechanisms, possibly a rare ESBL type and or additional efflux pumps may mediate carbapenem resistance in this isolate.

Carbapenem resistance amongst these *E. cloacae* isolates appears to be mediated by a combination of complex and diverse mechanisms including those previously associated with carbapenem resistance. However, carbapenem resistance in these isolates cannot be fully explained based on the presence of these mechanisms, as many of these mechanisms are also present in ertapenem susceptible isolates. Future studies involving whole genome sequencing, proteomics and transcriptomics may assist in identifying additional mechanisms which contribute to carbapenem resistance in these isolates.

6.2: Conclusion

Ertapenem resistance in these clinical *E. cloacae* isolates from Tygerberg Hospital is mediated by complex and diverse mechanisms. No certain carbapenemase production was shown to promote resistance, as no carbapenemase gene was detected in the suspected carbapenemase-producing isolate. Acquired ESBL production and reduced membrane permeability appear to be the major mechanisms promoting ertapenem resistance in clinical *E. cloacae* isolates from Tygerberg Hospital. However, these two mechanisms were also identified in a subset of ertapenem susceptible controls. Additional unknown mechanisms may be involved in ertapenem resistance, as suggested by one ertapenem non-susceptible isolate with no confirmed resistance mechanisms, hence further investigation using advanced methods such as whole genome sequencing will be useful in identifying novel resistance mechanisms.

Table 6.1: Characterization of the ertapenem non-susceptible, ertapenem susceptible *E. cloacae* controls and wild-type controls

Sample ID	Ertapenem MIC[1]	ESBL detection by PCR & Sequencing	Carbapenemase	*bla*AmpC expression[3]	*ompF* expression[3]	*ompC* expression[3]	*acrB* expression[3]
Ertapenem non-susceptible isolates (as determined by VITEK2®AES)							
ERD 1	4 (R)	-	Positive[2]	+	-	-	0
ERD 2	4 (R)	CTX-M	negative	+	-	-	0
ERD 4	4 (R)	CTX-M	negative	NA	-	-	0
ERD 5	1 (I)	-	negative	+**	0	-	0
ERD 8	4 (R)	-	negative	-	NA	-	-
ERD 9	4 (R)	CTX-M	negative	NA	NA	-	+
ERD 10	4 (R)	CTX-M	negative	NA	NA	-	0
ERD 11	2 (R)	CTX-M	negative	0	-	-	+
ERD 12	2 (R)	-	negative	+	-	0	-
ERD 13*	1 (I)	-	negative	-	0	0	-
ERD 16	I (I)	CTX-M	negative	0	-	-	-
ERD 17	2 (R)	SHV-12	negative	0	-	-	-
ERD 18	2 (R)	SHV-12	negative	+	-	+	-
ERD 20	2 (R)	CTX-M	negative	0	-	+	-
ERD 29	1 (I)	-	negative	-	-	0	-
ERD 34	4 (R)	CTX-M	negative	0	-	+	-
ERD 42	4 (R)	CTX-M & SHV-12	negative	+	-	0	-
Ertapenem susceptible controls (as determined by VITEK2®AES)							
ERD 6[4]	0.25 (S)	-	-	-	-	-	-
ERD 7[4]	0.25 (S)	-	-	-	-	0	0
ERD 14[4]	≤ 0.06 (S)	SHV-12	-	NA	0	+	-
ERD 23	1 (I)	-	negative	NA	-	-	-
ERD 25	0.5 (S)	CTX-M	negative	Not tested			
ERD 35	1 (I)	CTX-M	negative	NA	0	+	-
ERD 39	≤ 0.06 (S)	CTX-M	negative	NA	-	+	-
Wild-type controls							
WT 1	≤ 0.5 (S)	-	negative	0	0	0	0
WT 2	≤ 0.5 (S)	-	negative	0	0	0	0
WT 3	≤ 0.5 (S)	-	negative	0	0	0	0
WT 4	≤ 0.5 (S)	-	negative	0	0	0	0
WT 5	≤ 0.5 (S)	-	negative	0	0	0	0

R = Resistant, I = intermediate, S = susceptible, NA = no amplification, 0 = normal expression levels compared to wild-types, - = low expression, + = high expression. [1]Ertapenem MIC determined by BMD, [2]Isolate was positive using the Rapidec® CarbaNP kit, but negative by PCR, [3] *bla*AmpC, *ompF*, *ompC* and *acrB* expression as determined by RT-qPCR relative to wild-type controls.[4]ERD 6, ERD 7 and ERD 14 susceptible by BMD. ERD 6, ERD 7 and ERD 14 identified as susceptible isolates by BMD were included under susceptible isolates category for easy comparison of observed mechanisms with the resistant isolates.

Appendices

Appendix 1: Antimicrobial profiles of study isolates using the VITEK2®AES, Gradient diffusion assay and Broth Microdilution (BMD)

Sample IDs	Ertapenem resistance (µg/ml)			Ceftazidime resistance (µg/ml)	Cefotaxime (µg/ml)	Cefepime (µg/ml)
	Vitek MIC	Gradient diffusion assay MIC	BMD MIC	VITEK MIC	VITEK MIC	VITEK MIC
ERD 1	4 (R)	2 (R)	2 (R)	≥ 64 (R)	≥ 64 (R)	2 (S)
ERD 2	8 (R)	0.5 (S)	4 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 4	8 (R)	2 (R)	4 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 5	2 (R)	0.25 (S)	1 (I)	≥ 64 (R)	≥ 64(R)	2 (S)
ERD 6	2 (R)	0.19 (S)	0.25 (S)	2 (S)	≤ 1 (S)	≤ 1 (S)
ERD 7	4 (R)	0.125 (S)	0.25 (S)	NR	NR	NR
ERD 8	1 (I)	0.5 (S)	4 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 9	4 (R)	1 (I)	4 (R)	≥ 64 (R)	≥ 64(R)	≥ 64 (R)
ERD 10	4 (R)	0.5 (S)	4 (R)	≥ 64 (R)	≥ 64(R)	≥ 64 (R)
ERD 11	2 (R)	0.5 (S)	2 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 12	2 (R)	0.38 (S)	2 (R)	≥ 64 (R)	≥ 64(R)	4 (I)
ERD 13	1 (I)	0.25 (S)	1 (I)	≥ 64 (R)	≥ 64 (R)	≤ 1 (S)
ERD 14	4 (R)	0.003 (S)	≤ 0.06 (S)	≥ 64 (R)	≥ 64 (R)	2 (S)
ERD 16	2 (R)	0.25 (S)	1 (I)	≥ 64 (R)	≥ 64 (R)	16 (R)
ERD 17	4 (R)	0.25 (S)	2 (R)	≥ 64 (R)	≥ 64 (R)	2 (S)
ERD 18	4 (R)	0.25 (S)	2 (R)	≥ 64 (R)	≥ 64 (R)	32 (R)
ERD 20	2 (R)	1 (I)	2 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 29	1 (I)	0.19	1 (I)	≥ 64 (R)	≥ 64 (R)	≤ 1 (S)
ERD 34	4 (R)	1 (I)	4 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 42	4 (R)	1 (I)	4 (R)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
Ertapenem susceptible controls						
ERD 23	0.5 (S)	0.38 (S)	1 (I)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 25	0.5 (S)	0.064 (S)	0.5 (S)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 35	0.5 (S)	0.75 (I)	1 (I)	≥ 64 (R)	≥ 64 (R)	≥ 64 (R)
ERD 39	0.5 (S)	0.016 (S)	≤ 0.06 (S)	≥ 64 (R)	≥ 64 (R)	16 (R)
Wild-type controls						
WT 1	≤ 0.5 (S)	0.064 (S)	NT	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)
WT 2	≤ 0.5 (S)	0.012 (S)	NT	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)
WT 3	≤ 0.5 (S)	0.023 (S)	NT	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)
WT 4	≤ 0.5 (S)	0.025 (S)	NT	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)
WT 5	≤ 0.5 (S)	0.016 (S)	NT	≤ 1 (S)	≤ 1 (S)	≤ 1 (S)

WT= wild-type control, S – susceptible; R- resistant; I – intermediate; NT – not tested; NR – no result generated

Appendix 2: Protocols

Appendix 2.1: Carbapenemase detection using the Rapidec® Carba NP kit

Sample preparation

All the isolates were sub-cultured twice from MBK beads to obtain pure bacterial colonies. A single colony from each isolate was cultured on Trypticase Soy Agar (TSA) plates [(3% Tryptic soy broth (Fluka Analytical, Germany) and 1.5% bacteriological agar (Oxoid, UK)] and incubated at 37 °C for 18 to 24 hours.

Performing the test

The test strips used for the assay were labelled with assigned sample IDs. A volume of 100 µl of the API suspension was dispensed into wells a, b and c on the test strips. These strips were incubated at room temperature for 10 minutes with the strips covered with the incubation lids. After incubation, the contents of well 'b' were mixed gently using the stirring sticks.

The prepared samples were deposited in well 'c', mixed using the stirring stick until a turbidity equivalent to that of well 'b' was obtained, and incubated at room temperature for 30 minutes. Twenty-five microlitres of solutions in wells 'a' and 'c' were transferred to each of wells 'd' and 'e' respectively. The test strips were incubated at 37°C, followed by reading imperatively after 30 – 40 minutes. In cases of doubtful reaction, the test strips were re-incubated and read after one and a half hours.

After incubation, the strips were placed on the 2-colored (black and white) support to facilitate reading of results. The color of the solutions in wells 'd' and 'e' were compared. A test is positive when the color of the solution in well 'd' is red whilst that of well 'e' is either orange or orange-red or yellow, indicating carbapenemase production. However, the type of carbapenemase harbored can only be determined at the molecular level.

Appendix 2.2: Total mRNA extraction using the Quick-RNA Miniprep kit (Zymo Research, USA)

Cell Harvesting

The extraction process for the RNA templates was preceded by streaking isolates once from the MBK beads. A single colony from an overnight culture was inoculated in 10 ml Brain Heart infusion (BHI) broth (NHLS Media Laboratory, Greenpoint, South Africa) and incubated for 18 hours at 37°C with shaking at 180rpm. The bacterial cultures were transferred into DNase-free 10 ml falcon tubes, labelled and centrifuged at 4640 x *g* for 10 minutes at 4°C using the Heraeus multifuge 4KR centrifuge (Thermo Scientific, South Africa). The cells were used during the total mRNA extraction step.

Extracting the total mRNA samples

1. The cells were suspended in 600 µl of RNA Lysis Buffer, vortexed briefly and centrifuged at 10,000 x *g* for 30 seconds.

2. The debris was removed and supernatant was transferred unto the Spin-Away Filter (yellow) in a collection tube. The tubes were centrifuged at 10,000 x *g* for 30 seconds.

3. One volume of absolute ethanol (95 - 100%) was added to the flow-through from step 2 and mixed by pipetting.

4. The mixture was transferred onto the Zymo-Spin™ III CG Column (green) in a collection tube. Centrifuged at 10,000 x *g* for 30 seconds. Flow-through was discarded.

5. 400 µl of RNA Wash Buffer was added to each column, and centrifuged at 10,000 x *g* for 30 seconds.

On-column genomic DNA removal step

DNAse1 enzyme (50U) was reconstituted in 55 µl of DNase/RNase-Free water. A genomic DNA digestion buffer was prepared by adding an amount of 75 µl of DNA Digestion Buffer to 5 µl of reconstituted DNAse1 enzyme (per sample).

80 µl of solution was added directly onto the column matrix and incubated at room temperature for 15 minutes, followed by step 6 of the extraction procedure.

6. 400 µl of RNA Prep Buffer was added to columns and centrifuged at 10,000 x *g* for 30 seconds.

7. 700 µl of RNA Wash Buffer was added to columns and centrifuged at 10,000 x *g* for 30 seconds.

8. 400 µl of RNA Wash Buffer was added to columns and centrifuged at 10,000 x *g* for 30 seconds. Flow-through was discarded. The spin columns were centrifuged again at 10,000 x *g* for 30 seconds

to get rid of excess RNA Wash Buffer. The columns were transferred into RNase-free micro-centrifuge tubes.

9. 100 µl of DNase/RNase-Free water was added directly to the column matrix and centrifuged at 10,000 x *g* for 30 seconds to elute total mRNA. Aliquots of 20 µl were used to determine the integrity, purity and concentration of the extracted RNA samples. The rest of the samples were stored at -80°C for downstream analyses.

Appendix 2.3: RNA clean-up using the Ambion ® DNA-*free*™ DNase kit (Life Technologies, USA)

Procedure

1. 0.1 volume of 10X DNase1 Buffer and 3 µl of rDNase1 enzyme were added to the RNA samples (mass ≥ 10 µg), mixed gently to bring the total final volume of the reaction mix to 40 µl in fresh and labelled 0.2 ml micro-centrifuge tubes.

2. The mixture was then incubated at 37°C for an hour in a Proflex PCR system (Applied Biosystems, Life Technologies, USA).

3. 0.2 volumes of resuspended DNase Inactivation reagent was added to incubated reaction mix, after the inactivation reagent tube was vortexed. The mixture was incubated at room temperature for 2 – 3 minutes, mixing intermittently at least 2 – 3 times.

4. The solution was then centrifuged at 10,000 x *g* for 1.5 minutes. The supernatant (RNA in solution) was transferred into fresh and labelled 0.5 ml micro-centrifuge tubes, and stored immediately at -80°C for downstream analyses.

Appendix 2.4: Genomic DNA extraction from a wild-type control using the Qiagen QIAmp mini kit (Qiagen, Germany)

Pre-extraction step

Pure colonies of *E. cloacae* isolates were obtained after culturing twice from the Microbank (MBK) beads. One to three colonies of the same morphology were used for subsequent extraction steps.

Extraction steps

1. A loop full of 1 to 3 colonies was added to a micro-centrifuge tube containing 20 µl proteinase K and 180 µl buffer ATL (both buffers provided in kit). The mixture was vortexed briefly and then incubated at 56 °C for 2 hours using a heating block.

2. A volume of 200 µl of Buffer AL was added to the mixture after incubation, the micro-centrifuge tube was vortexed briefly for 15 seconds before incubating at 70°C for 10 minutes.

3. 200 µl of absolute ethanol (Sigma-Aldrich, USA) was then added to the mixture and vortexed briefly for 15 seconds.

4. The mixture was then added to the labelled spin column, and centrifuged for 1 minute at 6000 x *g* using the 1-15 Centrifuge (Sigma, Germany).

5. The filtrate was then discarded and the column was washed with 500 µl of AW1 buffer. The spin column was then centrifuged for 1 minute at 6000 x *g*.

6. Additional wash step was carried out using 500 µl of Buffer AW2 and centrifuged for 3 minutes at 18000 x *g*. The spin column was then placed in labelled, clean micro-centrifuge tube. The spin column was then dried by centrifugation at 18000 x *g* for 1 minute.

7. At the elution step, 80 µl of pre-warmed (~50°C) elution buffer was added to the dried spin column, incubated at room temperature on bench for 5 minutes, followed by centrifugation at 18000 x *g* for 1 minute. An ethanol precipitation step was carried out using a modified version of the Lamitina laboratory protocol to purify and concentrate the DNA samples (http://docs.wixstatic.com/ugd/803ab9_1cd1cb09279649b388391953899ae1f9.pdf).

Extraction steps continued: Ethanol precipitation

8. 0.1 volumes of 3 M of sodium acetate, pH= 5.2 (Sigma-Aldrich, Germany), 2.5 volumes of cold 100% ethanol and 1 µl of 5 mg/ml of glycogen solution (Sigma-Aldrich, Germany) were added to DNA extracts. The micro-centrifuge tubes were vortexed briefly and the mixture was incubated at -20°C overnight.

9. The DNA samples were then pelleted by centrifugation at 13000 x *g* at 4°C for 30 minutes. The supernatant was discarded without disturbing the pellet.

10. The pellet was washed twice with 3 volumes of cold 80% ethanol solution, and centrifuged at 13000 x *g* for 5 minutes.

11. The micro-centrifuge tube was then incubated at 37°C for about 5 to 10 minutes by inverting the tubes sealed with parafilm to evaporate residual ethanol. 40 µl of pre-warmed (~50°C) RNase free water was added to micro-centrifuge tubes to re-suspend the pellet. The mixture was then vortexed, incubated for about 10 minutes on ice and stored at -20°C for further downstream analyses.

Appendix 2.5: A modified version of the rapid outer membrane protein (ROMP) extraction method (Carlone *et al* 1986)

1. The cell pellets were resuspended in 1 ml of cold 10 mM HEPES buffer (pH=7.4) with proteinase inhibitor, cOmplete (Roche Diagnostics, Germany) by repeated pipetting.

2. The solution was sonicated for 6 X 10 seconds with a minute incubation interval on ice, to break down the cells to release the cell membranes, using the VirSonic 100 sonifier (VIRTIS, USA).

3. The unbroken cells and debris were removed by centrifugation at 15,600 x *g* for 2 minutes at 4°C using a refrigerated micro-centrifuge (Sigma-Aldrich, Germany). Centrifugation was repeated for 1 minute at the same speed.

4. The supernatant fluid was then transferred into new 1.5 ml micro-centrifuge tubes. The cell membranes (pellets) were sedimented from the supernatant fluid by centrifuging at 15,600 x *g* for 30 minutes at 4°C.

5. The supernatant fluid was decanted and the cell membranes were thoroughly suspended in 0.2 ml of 10 mM HEPES buffer (pH=7.4) by repeated pipetting.

6. The cytoplasmic membranes were solubilized by the addition of 0.2 ml 2% Sarkosyl in 10 mM HEPES buffer, incubation at room temperature for 30 minutes and by mixing intermittently.

7. The outer membrane was pelleted by centrifugation at 15,600 x *g* for 30 minutes at 4°C. The supernatant fluid was decanted and the membranes were washed once (without resuspending the pellet) with 0.5 ml of 10 mM HEPES buffer, without mixing, followed by centrifugation at 15,600 *x g* for 10 minutes at 4°C. The supernatant fluid was carefully discarded, and cell pellets were stored immediately at -80°C.

Appendix 2.6: Determination of the protein concentrations

Protein concentration was determined using the bovine serum albumin (BSA) (Biorad, USA) standard dilutions to generate a standard curve. The Biorad RC-DC method is based on the reaction of protein with an alkaline copper tartrate solution (DC reagent A) and a Folin reagent (DC reagent B). Color development is primarily due to the effect of proteins in reducing the Folin reagent by loss of some oxygen atoms resulting in a characteristic blue color, with maximum absorbance at 750 nm and, minimum absorbance at 405 nm (Biorad, USA).

The RC-DC method

1. Protein pellets were thawed on ice, reconstituted in 30 µl of cold 10 mM HEPES buffer with a proteinase inhibitor at room temperature and vortexed briefly.
2. A 100 µl solution of BSA standards from 0 mg/ml to 1.0 mg/ml was prepared and kept on ice.
3. 25 µl of standards was distributed into sterile micro-centrifuge tubes. All standards were prepared in triplicate.
4. 20 µl of 10 mM HEPES Buffer was distributed into sterile micro-centrifuge tubes. 5 µl each of the reconstituted protein samples was added, vortexed and centrifuged briefly.
5. 125 µl of RC Reagent I was distributed into each tube, vortexed briefly and incubated for 1 minute at room temperature.
6. 125 µl of RC Reagent II was pipetted into each tube, vortexed briefly and centrifuged at 18,000 x *g* for 5 minutes. The supernatant was discarded, and tubes inverted on clean, absorbent tissue paper to drain the supernatant fluid from the tubes.
7. 127 µl of DC Reagent A was added, vortexed thoroughly to dissolve the protein pellet, and incubated at room temperature for 5 minutes. DC reagent A buffer was prepared by adding 5 µl of Reagent S to every 250 µl volume of DC Reagent A.
8. 1 ml of DC Reagent B was added, vortexed briefly and the solution then transferred into labelled sterile 1ml cuvettes. Cuvettes were incubated at room temperature for 15 minutes followed by absorbance readings at 750 nm within one hour using the Ultrospec 4051 Spectrophotometer (LKB Biochrom, USA).

9. Absorbance readings were imported into a Microsoft Excel Spreadsheet. A standard curve plot of BSA (standards) concentrations against the average absorbances was generated.

Appendix 2.7: SDS-PAGE gel electrophoresis

The gel set-up apparatus (Biorad, USA) was cleaned with a tissue paper soaked in 70% ethanol, and assembled as per manufacturer's instructions. Deionised water was used to fill the space between the two glass slabs using a sterile Pasteur pipette to check for leakage. When no leakage was observed, the deionised water was discarded and the glass slabs were dried.

12% separating gel solution [4 ml of Acrylamide/bisacrylamide (Amresco, USA), 2.5 ml of 1.5 M Tris-HCl buffer, pH 8.8 (Amresco, USA), 100 µl 10% SDS solution (Amresco, USA), 3.35 ml of deionized water, 10 µl Tetramethylethylenediamine (TEMED) solution (Amresco, USA) and 50 µl of 10% Ammonium persulphate buffer] was prepared.

The separating gel solution was distributed between the two glass slabs to fill about three-fourths of the length of the slab using a Pasteur pipette. The remaining one-fourth space was filled with isopropanol to smoothen the surface of the gel. The separating gels were allowed to set for at least an hour at room temperature. The isopropanol was then discarded, and the remaining solution soaked up with clean tissue paper to get rid of excess isopropanol. A few drops of 1 M Tris-HCl, pH=6.8 was used to wash off the excess isopropanol solution.

3% stacking gel solution [containing 1.3 ml of acrylamide/bisacrylamide, 1.25 ml of 1 M Tris-HCl pH 6.8, 100 µl of 10% SDS solution, 7.4 ml of deionized water, 20 µl of TEMED solution and 50 µl of 10% ammonium persulphate buffer] was prepared and poured onto the separating gels using a fresh Pasteur pipette. 10-well combs were inserted, carefully avoiding the creation of bubbles. The stacking gels were allowed to set for at least one hour at room temperature.

The extracted protein pellets were thawed on ice. Sample solutions of a total volume of 16 µl containing 3 parts protein (total mass of 10 µg) and 1 part of 4X Reducing SDS Sample Buffer [made up of 4 ml of 100% glycerol (Radchem Laboratory Supplies, South Africa), 2.4 ml of 1M Tris-HCl, 0.8 g of SDS powder, 4 mg of Bromophenol blue (Sigma-Aldrich, Germany), 500 µl of β-mercaptoethanol (Sigma-Aldrich, Germany), and 3.1 ml of deionized water] were prepared and mixed by pipetting in sterile micro-centrifuge tubes. The 4X reducing sample buffer was used to make the protein samples dense for easy loading into the wells, to solubilize the protein samples, and act as a tracking dye during the vertical SDS-PAGE gel electrophoresis. The sample solutions were then

incubated at 95°C on a heat block (OMEG Scientific, South Africa) for 5 minutes, and centrifuged briefly to collect any droplets on the lid and sides of the tube.

Separation of OMP samples on 12% SDS-PAGE gels

The running chamber of the gel apparatus was filled with running buffer [made up of 6 g Tris powder, 28.8 g glycine (Sigma-Aldrich, Germany), 20 ml of 10% SDS solution and deionized water to bring the final volume to two litres] to the level specified by the manufacturer. The glass slabs containing the 12% SDS-PAGE gels were inserted into the running chamber. The running buffer was used to fill the spaces between the glass slabs, and to cover the surface of the gels as well as the two electrodes. The sample solutions were loaded into the wells with the aid of a loading guide provided by the manufacturer after the combs were carefully removed. Five microlitres of warmed, pre-stained broad range protein marker (BioLabs, New England, UK) was loaded into the first and last lanes of each gel. Gel electrophoresis was performed using the electrophoresis set-up (Biorad, USA) at 200V for at least an hour, or until the dye reached the edge of the gel. After the gel run, the SDS-PAGE gels were carefully removed from the glass slabs with the aid of a plastic remover (provided in kit), washed with deionised water and stained overnight in AcquaStain buffer (Bulldog, South Africa) enough to cover the entire surfaces of the gels. AcquaStain buffer was used instead of Coomassie Blue stain because no pre- or post-treatment of gels is required, protein bands can be visualized within 15 minutes of staining and stained gels are 100% compatible with Mass Spectrometry analysis (http://www.bulldog-bio.com/acquastain.html, AcquaStain, Bulldog, South Africa). The overnight stained SDS-PAGE gels were visualized and stored at 4°C in deionised water.

Appendix 3: RT-qPCR Validation Experiments

Appendix 3.1: Determining the integrity of total mRNA extracts

RNA integrity was assessed by separating 10 µl of each total mRNA extract on a 1% agarose gel at 100V for 60 minutes, and stained in ethidium bromide solution [made up of 50 µl of 10 mg/ml ethidium bromide stock in 500 ml of distilled water] for 20 minutes. Two ribosomal RNA bands (28SrRNA and 18SrRNA) were visualized under a UV Transilluminator (Figure 1). These two bands were identified in most isolates.

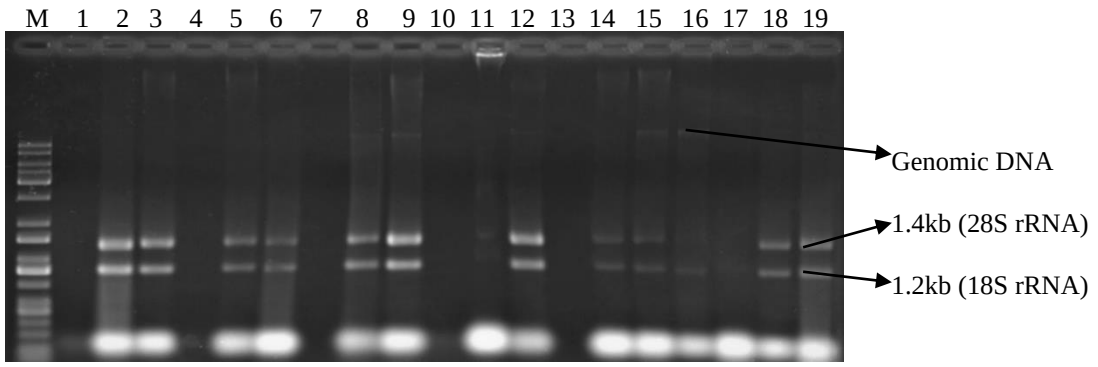

Figure 1: Extracted RNA visualized on a 1% agarose gel. Lanes M – Kapa Universal DNA ladder, Lanes 1 to 19 represents mRNA extracts of some study isolates.

Appendix 3.2: The No Reverse Transcriptase Polymerase Chain Reaction

A No Reverse Transcriptase (NoRT) PCR was performed on all extracted RNA samples to check for genomic DNA (gDNA) contamination, using the specified master mix components (Table 1). This step was performed since a previous study suggested that mRNA extracted from tissues or cells using column-based assays may still be contaminated with gDNA to about 20 to 50 % (Bustin, 2002).

A one step SYBR Green-based RT-qPCR assay using previously designed primers by Doumith *et al* (2009) was performed using the Rotor-Gene Q instrument (Qiagen, South Africa). Cycling conditions proposed by the manufacturer of the Luna One step Universal kit (BioLabs, New England, UK) (Table 2) was used. Additional parameters selected on the Rotor-Gene Q Analyzer software version 2.3.1 (Qiagen, South Africa) included: 72-well rotor plate map; data acquisition on melt from 75 °C to 95 °C.

All items used during the master mix preparation, and in the working area were sterilized under UV light. Master mix preparation was performed in the dark to avoid exposure of the SYBR Green buffer to direct light.

Table 1: Components of Master mix for NoRT PCR

Reagents	**Volume per sample (µl)**	**Final concentration**
PCR-grade water	6.4	
Luna Universal One step Master Mix (2X)	10.0	1X
rpoB Forward Primer (10 µM)	0.8	0.4 µM
rpoB Reverse Primer (10 µM)	0.8	0.4 µM
Template (RNA)	2.0	10 ng
Total reaction volume	20.0	

Table 2: Cycling conditions for NoRT PCR

Step	**Temperature**	**Time**	**Cycles**
Hold (cDNA processing step)	55°C	10 minutes	1
Hold	95°C	1 minute	1
Cycling	95°C	15 seconds	X 40
	60°C (Acquiring data)	30 seconds	
Melt	Ramp from 75°C to 95°C Hold for 90 seconds on 1st step Hold for 5 seconds on next steps, Melt A		

From the NoRT runs, it was confirmed that all mRNA extracts were contaminated with genomic DNA. Interpretation was based on amplification plots (Figure 2a) and melting curves (Figure 2b) from the *rpoB* PCR run. A genomic removal treatment step was carried out on all extracts using modified protocol for the Ambion ® DNA-*free*™ DNase Treatment and removal reagents kit (Invitrogen, USA) (Refer to Appendix 1.3). The NoRT experiment was repeated and confirmed the absence of genomic DNA following genomic DNase treatment (Figure 3a and Figure 3b).

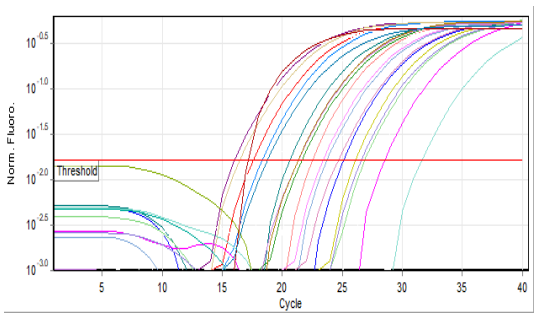

Figure 2a: Amplification plots after NoRT run

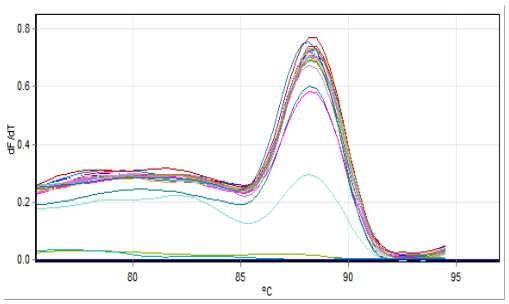

Figure 2b: Melting curve plots after NoRT run

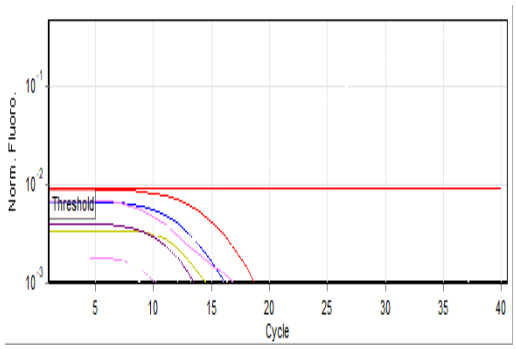

Figure 3a: Amplification plots after treatment

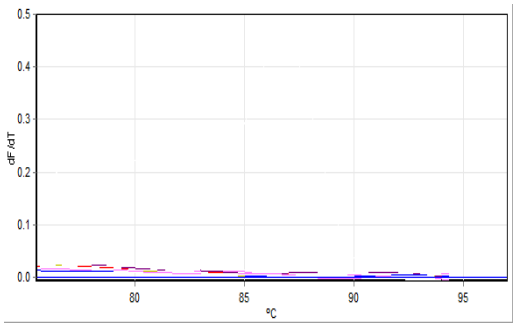

Figure 3b: Melting plots after treatment

Checking the purity levels of mRNA samples after gDNA treatment

The purity levels of the total mRNA extracts were determined using the NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific, USA) according to manufacturer's protocols. The values of the ratio A_{260}/A_{280} were assessed. This is because, nucleic acids (including RNA samples) and proteins have maximum absorbance at 260 and 280 nm. A_{260}/A_{280} ratios of ~2.0 are generally accepted as "pure" for RNA samples (https://tools.thermofisher.com/content/sfs/brochures/TN52646-E-0215M-NucleicAcid.pdf). The A_{260}/A_{280} ratios of the mRNA extracts after genomic treatment ranged from 1.71 – 2.67.

Appendix 3.3: RT-qPCR validation

A one step SYBR Green-based RT-qPCR was performed for each target gene (*bla*AmpC, *ompF*, *ompC* and *acrB*) and reference gene (*rpoB*) using primers previously described by Doumith *et al* (2009) (Table 3). The components of the master mixes for all target genes and endogenous control are outlined in Table 4. The templates used were purified genomic DNA (gDNA) extracts from a wild-type control (WT 4). Standard curves were constructed by generating a dilution series consisting of six points ranging from 1 pg to 100 ng of the extracted genomic DNA, to determine the PCR efficiencies of the various genes. All amplifications were performed in triplicate. Negative controls used include PCR grade water and master mix with no added template to check for contamination. The cycling conditions are outlined in Table 2. The PCR efficiencies were determined from the standard curves (Table 5) and compared to that of the endogenous gene (*rpoB*) (Table 5). The efficiencies of the target genes ranged from 86% to 98%, which were comparable to that of the reference gene, *rpoB*, (94%) and R^2 values ≥ 0.998 were obtained for all standard curves. The results of each PCR validation are outlined in appendices .3.3.1 to 3.3.5.

Table 3: Primers used for the validation experiment

Target gene	Primer	Sequence (5'-3')	Product size (bp)
rpoB	rpoB-F	AAGGCGAATCCAGCTTGTTCAGC	148
	rpoB-R	TGACGTTGCATGTTCGCACCCATCA	
*bla*AmpC	ampC-F	GCATGGCGGTGGCCGTTAT	221
	ampC-R	CTGCTTGCCCGTCAGCTGT	
ompC	ompC-F	GCGACCAGACCTACATGCGT	113
	ompC-R	TTCGTTCTCACCAGAGTTACCCT	
ompF	ompF-F	TCCCTGCCCTGCTGGTAG	139
	ompF-R	TAAGTGTTGTCGCCATCGTTG	
acrB	acrB-F	CGATAACCTGATGTACATGTCC	207
	acrB-R	CCGACAACCATCAGGAAGCT	

Table 4: Components of Master mixes used for validation experiments

Reagents	Volume per sample (µl)	Final concentration
PCR-grade water	5.4	
Luna Universal One step Master mix (2X)	10.0	1X
Luna RT Enzyme	1.0	
rpoB-F/ompC-F/ompF-F/ampC-F/acrB-F (10 µM) = Forward primer	0.8	0.4 µM
rpoB-R/ompC-R/ompF-R/ampC-R/acrB-R (10 µM) = Reverse primer	0.8	0.4 µM
Template (genomic DNA)	2.0	
Total reaction volume	**20.0**	

Table 5: PCR efficiencies of *ompF*, *ompC*, *bla*AmpC, *acrB* and *rpoB* genes

Target gene	PCR Efficiency	R^2 value
rpoB	94%	0.999
*bla*AmpC	86%	0.999
ompC	98%	0.999
ompF	90%	0.998
acrB	95%	0.999

Appendix 3.3.1: *rpoB* PCR

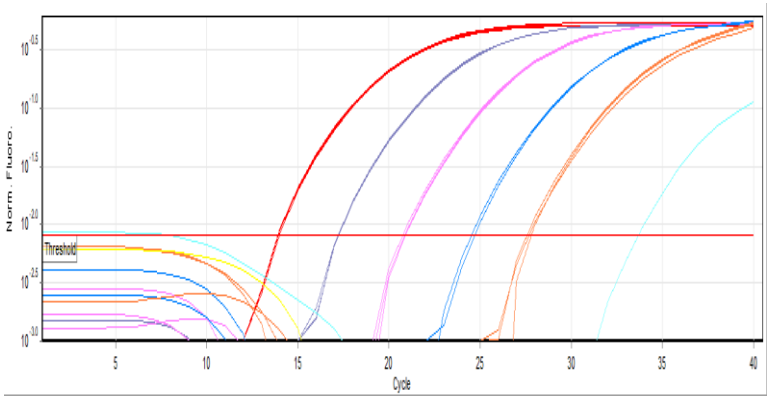

Figure 4a: Amplification plots of standards (*rpoB*)

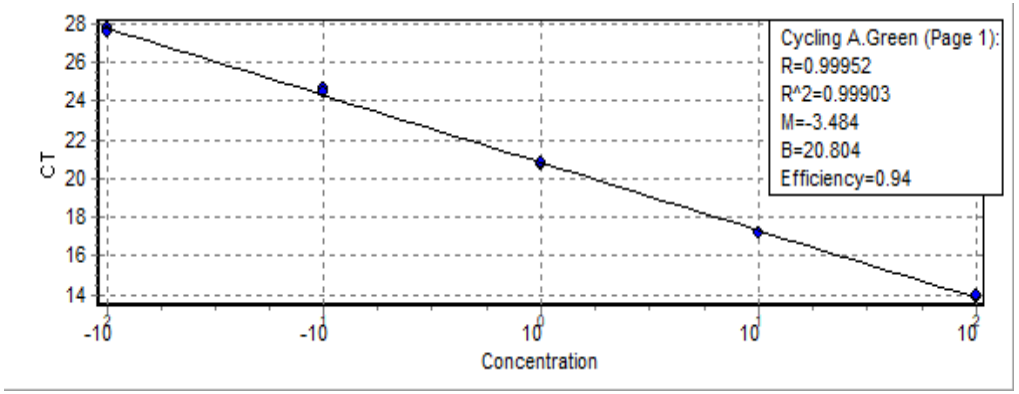

Figure 4b: *rpoB* Standard curve

Name	Type	Comparative Threshold cycle (Ct)	Given Concentration (copies/ml)	Calculated concentration (copies/ml)	% Variation	Repetitive Ct	Repetitive Ct Standard deviation
1	Standard	13.88	100.000	97.213	2.8%	13.89	0.04
1	Standard	13.86	100.000	98.444	1.6%		
1	Standard	13.94	100.000	93.318	6.7%		
2	Standard	17.17	10.000	11.004	10.0%	17.18	0.02
2	Standard	17.20	10.000	10.815	8.2%		
2	Standard	17.17	10.000	11.048	10.5%		
3	Standard	20.83	1.000	.983	1.7%	20.80	0.08
3	Standard	20.71	1.000	1.063	6.3%		
3	Standard	20.85	1.000	.967	3.3%		
4	Standard	24.67	.100	.078	22.4%	24.58	0.13
4	Standard	24.49	.100	.087	12.7%		
5	Standard	27.66	.010	.011	7.4%	27.66	0.13
5	Standard	27.79	.010	.010	1.1%		
5	Standard	27.53	.010	.012	17.1%		

Appendix 3.3.2: *acrB* PCR

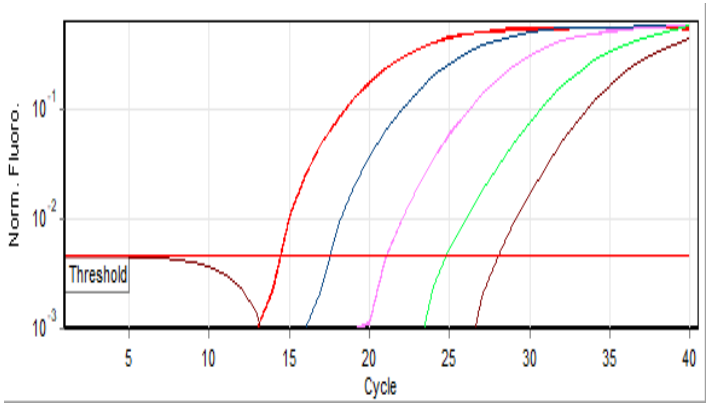

Figure 5a: Amplification plots of standards (*acrB*)

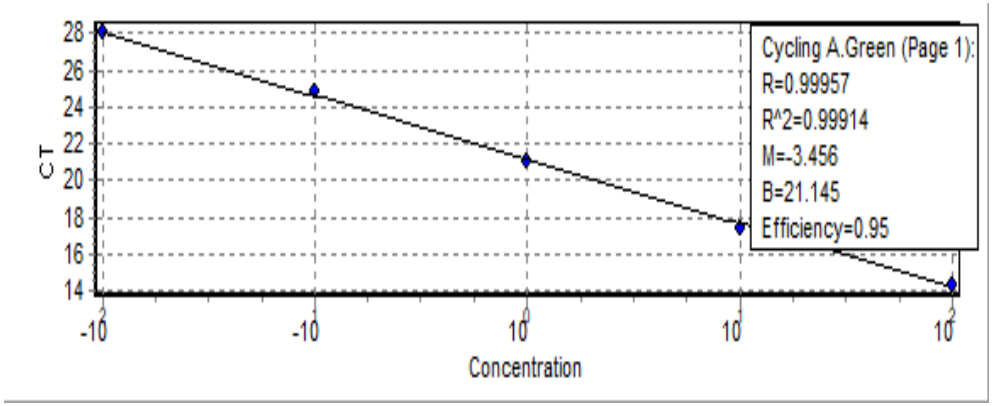

Figure 5b: *acrB* Standard curve

Name	Type	Ct	Given Concentration (copies/ml)	Calculated Concentration (copies/ml)	% Variation	Rep. Ct	Rep. Ct Standard deviation
1	Standard	14.36	100.000	92.010	8.0%	14.34	0.03
1	Standard	14.32	100.000	94.226	5.8%		
2	Standard	17.43	10.000	11.901	19.0%	17.43	
3	Standard	21.07	1.000	1.050	5.0%	21.08	0.01
3	Standard	21.09	1.000	1.040	4.0%		
4	Standard	24.79	.100	.088	12.1%	24.79	
5	Standard	28.04	.010	.010	0.9%	28.04	

Appendix 3.3.3: *ompF* PCR

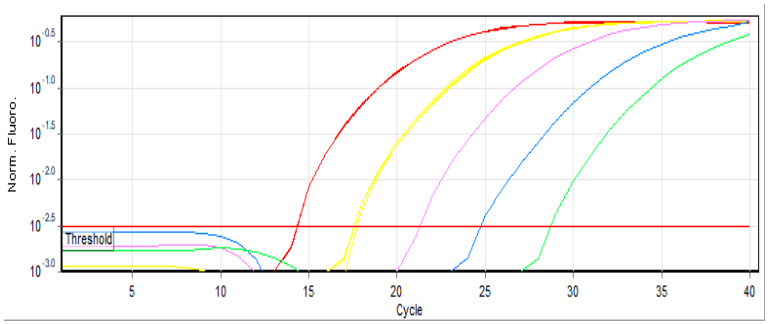

Figure 6a: Amplification plots of standards (*ompF*)

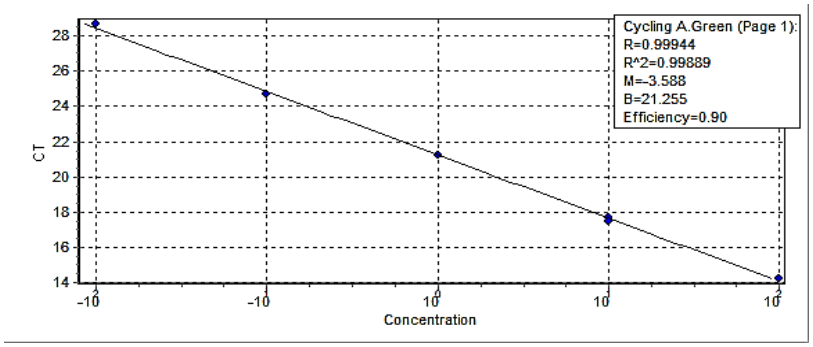

Figure 6b: *ompF* Standard curve

Name	Type	Ct	Given Conc (copies/ml)	Calc. Conc (copies/ml)	% Var.	Rep. Ct
1	Standard	14.25	100	89.492	10.5%	14.24
1	Standard	14.23	100	90.662	9.3%	
2	Standard	17.49	10	11.209	12.1%	17.55
2	Standard	17.66	10	10.011	0.1%	
2	Standard	17.50	10	11.158	11.6%	
3	Standard	21.21	1	1.027	2.7%	21.21
4	Standard	24.67	.100	.112	11.5%	24.67
5	Standard	28.67	.010	.009	14.0%	28.67

Appendix 3.3.4: *ompC* PCR

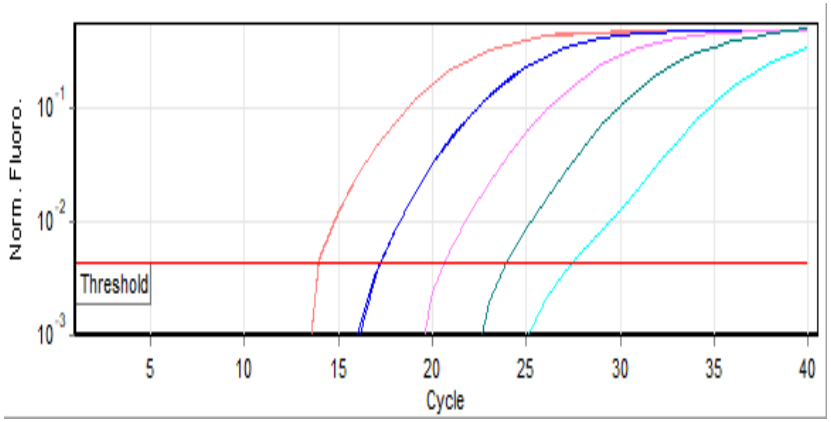

Figure 7a: Amplification plots of standards (*ompC*)

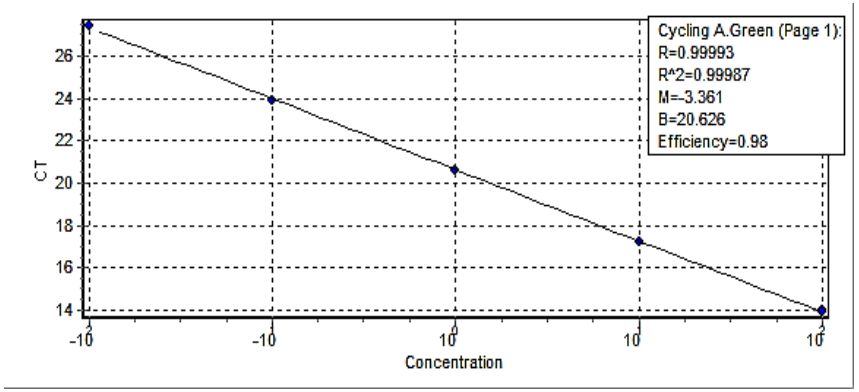

Figure 7b: *ompC* Standard curve

Name	Type	Ct	Given Conc (copies/ml)	Calc. Conc. (copies/ml)	% Var.	Rep. Ct	Rep. Ct Std. Dev.
1	Standard	13.97	100	95.668	4.3%	13.95	0.03
1	Standard	13.93	100	98.437	1.6%		
2	Standard	17.20	10	10.482	4.8%	17.22	0.04
2	Standard	17.25	10	10.097	1.0%		
3	Standard	20.61	1	1.013	1.3%	20.61	
4	Standard	23.92	.1	.105	4.5%	23.92	
5	Standard	27.43	.01	.009	5.2%	27.43	

Appendix 3.3.5: *bla*AmpC PCR

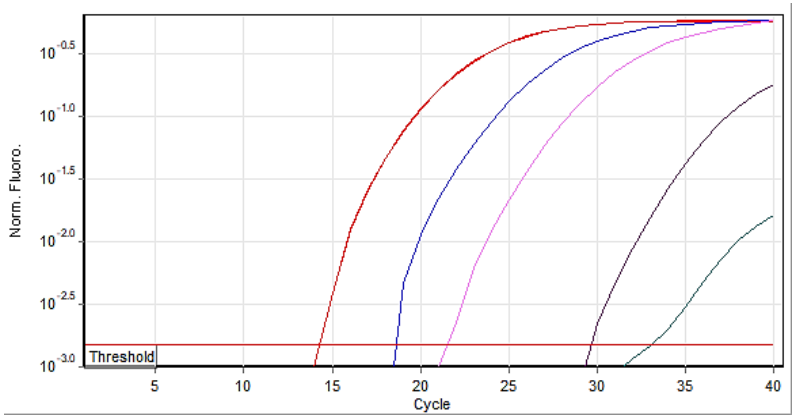

Figure 8a: Amplification plots for standards (*ampC*)

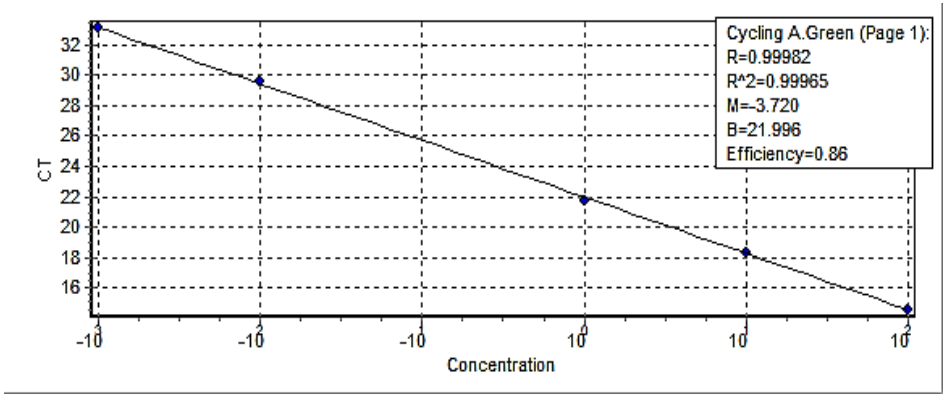

Figure 8b: *bla*AmpC Standard curve

Name	Type	Ct	Given Conc (copies/ml)	Calc. Conc. (copies/ml)	% Var.	Rep. Ct	Rep. Ct Std. Dev.
1	Standard	14.57	100	99.138	0.9%	14.59	0.03
1	Standard	14.61	100	96.685	3.3%		
2	Standard	18.34	10	9.608	3.9%	18.34	
3	Standard	21.74	1	1.174	17.4%	21.74	
5	Standard	29.61	.010	.009	10.3%	29.61	
6	Standard	33.11	.001	.001	3.1%	33.11	
6	Standard	33.11	.001	.001	3.1%	33.11	

Appendix 4: Data Analysis for expression levels of target and endogenous genes

The absolute cDNA concentration for each gene was determined after PCR to generate the comparative threshold cycles (Ct) (Table 6). Ratios of the absolute copy numbers of all target genes and the endogenous gene, *rpoB* mRNA were also generated (Table 7). The target gene expression levels were normalized to the *rpoB*, and the relative expression levels, normalized to the wild-type controls, were generated (Tables 8 and 9).

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After the generation of the absolute concentrations as shown in Table 6, the absolute copy number (shown in Table 7) was determined using the Thermo Scientific calculator based on formula stated below.

Copy Number Calculation Formula:

$$\text{Number of copies} = \frac{\text{amount} \times (6.022 \times 10^{23})}{\text{length} \times (1 \times 10^{9}) \times 660}$$

$$\text{Number of copies} = \frac{\text{ng} \times \frac{\text{number}}{\text{mole}}}{\text{bp} \times \frac{\text{ng}}{\text{g}} \times \frac{\text{g}}{\text{mole of bp}}}$$

Table 6: <u>Absolute cDNA concentrations.</u> Quantification of DNA performed using a modified template of the KAPA Library Quantification Data Analysis Template v 4.14 (https://www.kapabiosystems.com/…/KAPA-Library-Quant_Data-Analysis-Template_IL). The exact concentration of any DNA or RNA sample can be determined using the Ct value generated by RTqPCR, taking into consideration the dilution factor of each sample and the size of the expected PCR product, and determining the exact concentration of neat samples from validated standard curve (for *rpoB* gene).

Sample IDs	Dilution	Cq	fragment length (bp)	Outliers/ outside curve	Ave. Cq	Difference	log (concentration)	Average conc. (ng)	Size-adjusted conc. (ng)	Conc of undiluted sample (ng)	Conc of undiluted library (nM)	Conc of undiluted library (ng/µL)
ERD 1	20	25.5	148		25.60	-0.10	**0.20**	1.56897	4.79171	95.83422	0.09583	0.00876
		25.65				0.05						
		25.65				0.05						
ERD 2	20	26.02	148		25.98	0.04	**0.08**	1.19999	3.66483	73.29659	0.07330	0.00670
		26.1				0.12						
		25.83				-0.15						
ERD 4	20	27.45	148		27.19	0.26	**-0.29**	0.51480	1.57224	31.44477	0.03144	0.00288
		27.04				-0.15						
		27.09				-0.10						
ERD 5	20	24	148		24.31	-0.31	**0.59**	3.85866	11.78456	235.69119	0.23569	0.02155
		24.38				0.07						
		24.56				0.25						
ERD 6	20	26.66	148		26.77	-0.11	**-0.16**	0.69381	2.11893	42.37870	0.04238	0.00388
		26.87				0.10						
		26.77				0.00						
ERD 7	20	25.72	148		25.64	0.08	**0.18**	1.52213	4.64866	92.97328	0.09297	0.00850
		25.61				-0.03						
		25.6				-0.04						
ERD 8	20	20.06	148		20.24	-0.18	**1.82**	66.63532	203.50788	4070.15763	4.07016	0.37221
		20.35				0.11						
		20.31				0.07						
ERD 9	20	30.84	148		31.10	-0.26	**-1.48**	0.03350	0.10230	2.04600	0.00205	0.00019
		31.12				0.02						
		31.34				0.24						

Sample IDs	Dilution	Cq	fragment length (bp)	Outliers/ outside curve	Ave. Cq	Difference	log (concentration)	Average conc. (ng)	Size-adjusted conc. (ng)	Conc of undiluted sample (ng)	Conc of undiluted library (nM)	Conc of undiluted library (ng/µL)
ERD 10	20	31.89	148		32.03	-0.14	**-1.76**	0.01752	0.05351	1.07012	0.00107	0.00010
		31.87				-0.16						
		32.32				0.29						
ERD 11	20	21.17	148		21.15	0.02	**1.55**	35.17881	107.43798	2148.75957	2.14876	0.19650
		21.14				-0.01						
		21.15				0.00						
ERD 12	20	25.82	148		25.54	0.28	**0.21**	1.63621	4.99707	99.94141	0.09994	0.00914
		25.35				-0.19						
		25.45				-0.09						
ERD 13	2	26.55	148		26.77	-0.22	**-0.16**	0.69381	2.11893	4.23787	0.00424	0.00039
		26.89				0.12						
		26.86				0.09						
ERD 14	2	27.14	148		27.03	0.11	**-0.24**	0.57710	1.76251	3.52501	0.00353	0.00032
		26.92				-0.11						
		27.03				0.00						
ERD 16	2	26.07	148		26.00	0.07	**0.07**	1.18608	3.62236	7.24471	0.00724	0.00066
		25.87				-0.13						
		26.06				0.06						
ERD 17	2	21.83	148		21.96	-0.13	**1.30**	20.01048	61.11309	122.22618	0.12223	0.01118
		21.99				0.03						
		22.06				0.10						
ERD 18	2	21.02	148		21.26	-0.24	**1.51**	32.57383	99.48223	198.96446	0.19896	0.01820
		21.36				0.10						
		21.41				0.15						
ERD 20	2	23.95	148		23.90	0.05	**0.71**	5.14012	15.69821	31.39642	0.03140	0.00287
		23.74				-0.16						
		24.02				0.12						
ERD 29	2	21.89	148		21.82	0.07	**1.34**	22.12049	67.55718	135.11435	0.13511	0.01236
		21.82				0.00						
		21.74				-0.08						

Sample IDs	Dilution	Cq	fragment length (bp)	Outliers/ outside curve	Ave. Cq	Difference	log (concentration)	Average conc. (ng)	Size-adjusted conc. (ng)	Conc of undiluted sample (ng)	Conc of undiluted library (nM)	Conc of undiluted library (ng/µL)
ERD 34	2	28.55	148		28.64	-0.09	**-0.73**	0.18673	0.57027	1.14055	0.00114	0.00010
		28.74				0.10						
		28.64				0.00						
ERD 42	2	20.69	148		20.71	-0.02	**1.68**	48.07899	146.83583	293.67166	0.29367	0.02686
		20.71				0.00						
		20.72				0.01						
ERD 23	2	21.73	148		21.68	0.05	**1.39**	24.33924	74.33336	148.66671	0.14867	0.01360
		21.76				0.08						
		21.55				-0.13						
ERD 35	2	28.04	148		28.11	-0.07	**-0.57**	0.27115	0.82810	1.65620	0.00166	0.00015
		28.18				0.07						
		28.11				0.00						
ERD 39	2	28.66	148		28.53	0.13	**-0.70**	0.20166	0.61588	1.23176	0.00123	0.00011
		28.48				-0.05						
		28.46				-0.07						
WT 1	2	24.61	148		24.58	0.03	**0.50**	3.19466	9.75666	19.51333	0.01951	0.00178
		24.56				-0.02						
		24.58				0.00						
WT 2	2	20.62	148		20.69	-0.07	**1.69**	48.52944	148.21154	296.42308	0.29642	0.02711
		20.99				0.30						
		20.47				-0.22						
WT 3	2	20.96	148		21.05	-0.09	**1.58**	37.72732	115.22127	230.44254	0.23044	0.02107
		21.11				0.06						
		21.09				0.04						
WT 4	2	20.50	148		20.60	-0.10	**1.71**	51.80303	158.20925	316.41849	0.31642	0.02894
		20.38				-0.22						
		20.92				0.32						
WT 5	2	23.22	148		23.34	-0.12	**0.88**	7.60453	23.22465	46.44930	0.04645	0.00425
		23.36				0.02						
		23.45				0.11						

Table 7: <u>Absolute copy numbers of target genes</u>. Copy numbers are calculated based on the comparative threshold cycles (Ct values) and the product sizes, using the standard curves generated.

Sample IDs	*rpoB* (*10^8)	*ompC*	*ompF*	*acrB*	*blaAmpC*
ERD 1	98.2	220.4	13.5	3.88	6.28
ERD 2	75.1	453.1	0.09	14.35	4.99
ERD 4	32.2	1041.3	0.03	5.37	NA
ERD 5	241.5	4072.7	183.8	1.22	336.4
ERD 6	43.4	537.1	4.99	0.48	0.14
ERD 7	95.3	28730.9	32.4	19.66	0.2
ERD 8	4171	274437.6	NA	48.1	39
ERD 9	2.1	36.86	NA	1.99	NA
ERD 10	1.1	3.5	NA	0.22	NA
ERD 11	2202	148529.8	612.2	1339	22.8
ERD 12	102.4	23166.2	59.1	5.37	2.31
ERD 13	43.4	14875.8	52.2	1.43	0.02
ERD 14	36.1	14910.6	23.08	2.85	0
ERD 16	74.2	552.3	23.74	5.28	1.08
ERD 17	1252	24728.9	70.2	9.06	9.25
ERD 18	2039	970222.4	199.48	6.81	70.87
ERD 20	321.7	262950.8	222.6	8.38	2.59
ERD 29	1385	311009.8	227.3	49.8	4.96
ERD 34	11.6	57508	0.23	0.54	0.04
ERD 42	3009	572858.8	969	190.9	64.86
ERD 23	1523	0.49	0.128	7.32	NA
ERD 35	16.9	29385.2	19.6	0.19	NA
ERD 39	12.6	13085.6	3.59	0.01	NA
WT 1	199.9	81773.9	84.97	4.11	1.42
WT 2	3037	44.1	6.4	646.2	0.55
WT 3	2361	290000.9	987.3	523.9	30.5
WT 4	3242	493455.5	17236.3	592.8	44.47
WT 5	476	119857.2	56.78	225.8	7.67

WT = wild-type control; NA = No amplification of target gene

Table 8: <u>Expression levels of the target genes.</u> The generated values for the absolute copy numbers (Table 7) were normalized against the copy numbers of the housekeeping gene (*rpoB*) to determine their expression levels (indicated in the columns labelled absolute below).

Sample IDs	*ompC*		*ompF*		*acrB*		*blaAmpC*	
	absolute	**log of absolute**	**absolute**	**log of absolute**	**absolute**	**log of absolute**	**absolute**	**log of absolute**
ERD 1	2.244399	0.3511	-0.86178	0.137474542	0.039511202	-1.40328	0.06395112	-1.19415
ERD 2	6.033289	0.780554	-2.9214	0.001198402	0.191078562	-0.71879	0.06644474	-1.17754
ERD 4	32.33851	1.50972	-3.03073	0.000931677	0.166770186	-0.77788	NA	
ERD 5	16.86418	1.226965	-0.11857	0.761076605	0.00505176	-2.29656	1.392960663	0.143939
ERD 6	12.37558	1.092565	-0.93939	0.114976959	0.011059908	-1.95625	0.003225806	-2.49136
ERD 7	301.4785	2.479256	-0.46855	0.339979014	0.206295908	-0.68551	0.002098636	-2.67806
ERD 8	65.7966	1.818203	NA	NA	0.011532007	-1.9381	0.009350276	-2.02918
ERD 9	17.55238	1.244336	NA	NA	0.947619048	-0.02337	NA	
ERD 10	3.181818	0.502675	NA	NA	0.2	-0.69897	NA	
ERD 11	67.45223	1.828996	-0.55592	0.278019982	0.60808356	-0.21604	0.010354223	-1.98488
ERD 12	226.2324	2.354555	-0.23871	0.577148438	0.052441406	-1.28033	0.022558594	-1.64669
ERD 13	342.7604	2.534991	0.080181	1.202764977	0.032949309	-1.48215	0.000460829	-3.33646
ERD 14	413.036	2.615988	-0.19427	0.63933518	0.078947368	-1.10266	NA	
ERD 16	7.443396	0.871771	-0.49492	0.319946092	0.07115903	-1.14777	0.014555256	-1.83698
ERD 17	19.75152	1.2956	-1.25127	0.056070288	0.007236422	-2.14048	0.007388179	-2.13146
ERD 18	475.8325	2.677454	-1.00952	0.097832271	0.003339872	-2.47627	0.034757234	-1.45895
ERD 20	817.3789	2.912423	-0.15993	0.691949021	0.026049114	-1.58421	0.008050979	-2.09415
ERD 29	224.5558	2.351324	-0.78485	0.164115523	0.035956679	-1.44422	0.003581227	-2.44597
ERD 34	4957.586	3.69527	-1.70273	0.019827586	0.046551724	-1.33206	0.003448276	-2.4624
ERD 42	190.3818	2.279625	-0.4921	0.322033898	0.063443004	-1.19762	0.021555334	-1.66645
Ertapenem susceptible controls								
ERD 23	0.000322	-3.4925	-4.07549	8.40446E-05	0.004806303	-2.31819	NA	
ERD 35	1738.769	3.240242	0.064369	1.159763314	0.011242604	-1.94913	NA	
ERD 39	1038.54	3.016423	-0.54528	0.284920635	0.000793651	-3.10037	NA	
Wild-type controls								
WT I	409.074	2.611802	-0.37155	0.425062531	0.02056028	-1.68697	0.007103552	-2.14852
WT 2	0.014521	-1.83801	-2.67626	0.002107343	0.212775766	-0.67208	0.0001811	-3.74208
WT 3	122.8297	2.089303	-0.37865	0.418170267	0.221897501	-0.65385	0.012918255	-1.8888
WT 4	152.2071	2.182435	0.725631	5.316563849	0.182850093	-0.7379	0.013716841	-1.86275
WT 5	251.8008	2.401057	-0.92341	0.119285714	0.474369748	-0.32388	0.016113445	-1.79281

NA = No amplification of target gene

Table 9: <u>Expression of the target genes in the ertapenem non-susceptible isolates relative to the wild-type controls.</u> Relative expression of target genes normalized to wild-type controls are expressed as log10 values which were used to construct graphs to illustrate the expression ratio difference for each gene.

Sample IDs	*ompC*	*ompF*	*acrB*	*blaAmpC*
ERD 1	-1.9211715	-0.96085	-0.75059	0.805559938
ERD 2	-1.4917175	-3.02047	-0.0661	0.82217239
ERD 4	-0.7625516	-3.12981	-0.12519	NA
ERD 5	-1.0453063	-0.21764	-1.64387	2.143650634
ERD 6	-1.1797062	-1.03846	-1.30356	-0.491649912
ERD 7	0.20698472	-0.56762	-0.03282	-0.678351123
ERD 8	-0.4540682	NA	-1.28541	-0.029463801
ERD 9	-1.0279356	NA	0.629322	NA
ERD 10	-1.7695962	NA	-0.04628	NA
ERD 11	-0.4432753	-0.655	0.436651	0.014829314
ERD 12	0.08228324	-0.33778	-0.62764	0.353023805
ERD 13	0.26271899	-0.01889	-0.82947	-1.336747952
ERD 14	0.34371631	-0.29334	-0.44997	NA
ERD 16	-1.4005005	-0.594	-0.49508	0.162731632
ERD 17	-0.9766711	-1.35034	-1.48779	-0.131750814
ERD 18	0.40518246	-1.10859	-1.82358	0.540756989
ERD 20	0.64015183	-0.259	-0.93152	-0.094439515
ERD 29	0.07905269	-0.88392	-0.79153	-0.446256315
ERD 34	1.42299867	-1.8018	-0.67938	-0.462686216
ERD 42	0.00735379	-0.59117	-0.54493	0.333266538
Ertapenem susceptible isolates				
ERD 23	-5.7647754	-4.17456	-1.6655	NA
ERD 35	0.96797034	-0.0347	-1.29644	NA
ERD 39	0.74415149	-0.64435	-2.44768	NA

NA = No amplification of target gene

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