DETECTION OF CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE* AMONGST NEONATES IN A REGIONAL HOSPITAL IN KWAZULU-NATAL:

Screening for Carbapenemase Production and MIC Correlation

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DECLARATION

This study represents original work by the candidate and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

All routine and experimental work (growth of isolates from storage into solid media, biochemical tests for identification, culture-based drug susceptibility testing, phenotypic screening and molecular characterisation), described in this dissertation was carried out by the candidate in the Department of Medical Microbiology and Infection Prevention and Control, College of Health Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor P. Moodley.

Kathleen Govender **Professor P. Moodley**

(Candidate) **(Supervisor)**

This dissertation is dedicated to my family and fiancé

For their endless support, love and encouragement

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LIST OF ABBREVIATIONS

CDC: Centers for Disease Control and Prevention

CLSI: Clinical and Laboratory Standards Institute

CPE: Carbapenemase-producing *Enterobacteriaceae*

DHP-1: Dehydropeptidase-1

EDTA: Ethylenediaminetetraacetic acid

ESBLs: Extended spectrum β-lactamase

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration

ICUs: Intensive Care Units

MDR: Multidrug-resistant

NaCl: Sodium Chloride

NAG: N-acetyl glucosamine -

NAM: N-acetyl muramique –

NHLS: National Health Laboratory Services

NICU: Neonatal Intensive Care Unit

OprD: Outer membrane porin protein D

PBPs: Penicillin-binding proteins

PCR: Polymerase chain reaction

PGL: Peptidoglycan layer

TE: Tris-EDTA

DEFINITIONS:

Carbapenemase-producing *Enterobacteriaceae* (CPE): include all *Enterobacteriaceae* that are resistant to the carbapenems via the production of carbapenemases only.

Carbapenem-resistant *Enterobacteriaceae* (CRE): include all *Enterobacteriaceae* that are resistant to the carbapenems via several mechanisms of resistance such as extended-spectrum β-lactamase (ESBL) production (including carbapenemases), ESBL-AmpC combination, outer-membrane impermeability and efflux pumps.

Dehydropeptidase-1 (DHP-1)**:** Dehydropeptidase is an enzyme found in the kidney and is responsible for degrading the antibiotic imipenem.

recA: a protein that is responsible for the repair and maintenance of DNA.

Spheroplasts: a cell from which the cell wall has been almost completely removed, as by the action of an antimicrobial agent. Once the microbial cell wall is digested, membrane tension causes the cell to acquire a characteristic spherical shape.

Tubular brush-border: the proximal tubule which is the part of the duct system of the nephron of the kidney leading from the Bowman's capsule

Zwitterionic molecular charge: a neutral molecule with a positive and a negative electrical charge, however multiple positive and negative charges may be present.

PREFACE

Carbapenem resistance has been linked to many mechanisms including inactivating enzyme production, outer membrane impermeability and efflux. In recent literature, carbapenemases have been reported as the primary cause for the increase in carbapenem-resistance in Gram-negative *Enterobacteriaceae*. These enzymes are β-lactamases and have the ability to hydrolyse almost all β-lactam antibiotics including carbapenems, which are used for the treatment of severe nosocomial infections. Since 2011, individual cases and outbreaks with carbapenemase-producing *Enterobacteriaceae* (CPE) have been reported in several hospitals in South Africa. Carbapenem resistance limits antibiotic choices, especially in the hospital setting, and may result in increased morbidity and mortality rates. As carbapenem resistance disseminates globally, more reports surface describing outbreaks affecting primarily more vulnerable hospital populations like neonates. Infection prevention and control (IPC) measures coupled with antibiotic stewardship policies have become paramount in the care of hospitalised patients and are crucial to addressing the problem of ever decreasing antibiotic choices. The rapid detection of carbapenem-resistant *Enterobacteriaceae* (CRE) will greatly assist in the application of IPC measures and the tailoring of antibiotic prescriptions within a facility.

ABSTRACT

Carbapenemases are the primary cause for the increase in carbapenem resistance in Gram-negative *Enterobacteriaceae*. These enzymes are β-lactamases and have the ability to hydrolyse almost all β-lactam antibiotics thereby inactivating carbapenems that are used for the treatment of severe nosocomial infections. Multiple CPE outbreaks and epidemics have been reported in several hospitals in South Africa since the year 2011. This resulted in an increase in the morbidity and mortality rates and are slowly disseminating globally among more vulnerable individuals including neonates. Therefore, the aim of the study was to determine appropriate techniques for the rapid detection of carbapenem-resistant *Enterobacteriaceae* (CRE) (including CPE) isolated from neonates from King Edward VIII Hospital as well as to determine the molecular mechanisms conferring carbapenemase production in this subset of isolates. A total of 94 *Klebsiella pneumoniae* and 41 *Enterobacter cloacae* samples were isolated in this study. Among these species 10 % (9/ 94) and 39 % (16/ 41) of *K. pneumoniae* and *E .cloacae* respectively, were resistant to the carbapenems based on the Kirby-Bauer susceptibility tests, microbroth-dilution and E-tests. However, screening for carbapenemase production using chromogenic agar (Brilliance™ CRE agar and ChromID® CARBA agar), Modified Hodge test and amoxycillin-clavulanate double disc synergy test did not correlate with these resistance patterns and exhibited false positive results possibly due to the presence of extended spectrum beta-lactamase (ESBL) production by these organisms. Due to such discrepancies in the phenotypic results, further detection for the presence of carbapenemases was performed using multiplex real-time PCR assays. This revealed the presence of the *bla*OXA-48 gene in only 1 *K. pneumoniae* isolate. Further molecular characterisation will be required to determine if alternate mechanisms of resistance are present in the resistant isolates detected in this study.

CHAPTER ONE

INTRODUCTION

The family of *Enterobacteriaceae* comprises Gram-negative, rod shaped bacteria, some of which are inclusive of *Escherichia* spp., *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Serratia* spp., *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Morganella* spp., and *Providencia* spp. (Martin *et al.,* 2013). Some of these potentially pathogenic organisms inhabit the intestinal tract and are common sources of hospital-acquired infections (Nordmann *et al.,* 2012). These organisms may cause gastrointestinal and extraintestinal infections such as urinary tract infections, respiratory tract infections, skin and soft tissue infections and infections of the central nervous system (Kurakawa *et al.,* 2013; Nordmann *et al.,* 2012; Paterson, 2006).

A number of these pathogens have become prevalent in hospitals in the form Multi-Drug resistant (MDR) strains (Kurakawa *et al.,* 2013). These MDR strains occur more frequently among patients with severe illnesses, including those in Intensive Care Units (ICUs) (Kurakawa *et al.,* 2013). Neonates are an especially vulnerable group due to their immature immune systems (Vergnano and Heath, 2013; Zaidi *et al.,* 2011). The main route of transmission of *Enterobacteriaceae* in hospitals is via direct patient-to-hospital personnel and patient-to-patient contact. Indirect contact may also occur due to contamination of inanimate objects such as equipment or medication vials. An alternate means of transmission may be a result of ingestion of contaminated food and water at the hospital (Nordmann *et al.,* 2012).

Over the past few years, the resistance of *Enterobacteriaceae* to common antimicrobial agents has increased significantly (DiPersio and Dowzicky, 2007). These antimicrobial agents include tetracycline, β-lactams, fluoroquinolones, polymyxins, aminoglycosides and co-trimoxazole, third- and fourth-generation cephalosporins (Bonelli *et al.,* 2014; DiPersio and Dowzicky, 2007; Tang *et al.,* 2014). The increase in the prevalence of ESBL producing organisms has led to an increased use of carbapenems. This has resulted in an increase in the antibiotic pressure on carbapenems which lends to the activation of bacterial resistance genes against these drugs (El-Herte *et al.,* 2012). Carbapenem resistance may result from the production of carbapenemases, outer membrane impermeability, efflux pumps, or a combination of these.

Carbapenemase-producing *Enterobacteriaceae* (CPE) is on the increase and have been reported globally (El-Herte *et al.,* 2012). In developing countries like South Africa, CPE and ESBL-producing *Enterobacteriaceae* are widespread (Martin *et al.,* 2013). Therefore, clinicians are faced with the decision to treat seriously ill patients with toxic empiric therapies such as colistin and tigercycline (Martin *et al.,* 2013).

Carbapenemase production by *Enterobacteriaceae* is viewed as the most clinically significant resistance mechanism from a public health perspective (Martin *et al.,* 2013). The genes responsible for CPE are primarily plasmid-encoded and associated with various mobile genetic elements (Gazin *et al.,* 2012; Patel *et al.,* 2009). These are able to accumulate resistance genes to a number of different antibiotics and are easily transferrable between bacterial species (Bush *et al.,* 2013; DiPersio and Dowzicky, 2007). The implementation of

strict infection control measures in order to prevent the spread of carbapenemase encoding genes to unrelated clones or to other bacterial species is paramount. It is therefore essential that patients colonised or infected with these organisms are rapidly identified and cohort nursed with strict contact precautions. The reliable detection of carbapenem-resistant organisms is also essential in outbreak detection and for the institution of appropriate treatment options (Stuart and Leverstein-Van Hall, 2010; Kaase *et al.,* 2012).

Many phenotypic tests can be used as indicators of resistance determinants, thereafter molecular techniques may be perform for confirmation of these resistance determinants. It is debatable as to which test is most effective (Stuart and Leverstein-Van Hall, 2010; Nordmann *et al.,* 2009; Thomson, 2010). However, carbapenemase production is linked to the transference of plasmid-mediated genes encoding the carbapenemases within and between species (Bush *et al.,* 2013; DiPersio and Dowzicky, 2007). These enzymes are normally produced in combination with several other β-lactamases, hence making it difficult to identify carbapenemases using simple phenotypic methods (Bush *et al.,* 2013)

The aim of this study was to screen for CRE amongst neonates at King Edward VIII Hospital, Durban, South Africa by means of susceptibility testing and minimum inhibitory concentration (MIC) determination. Additionally screening for CPE using previously described phenotypic tests was evaluated and correlated with multiplex real-time polymerase chain reaction (PCR) for the production of carbapenemases.

The objectives of this study were:

- To isolate *Enterobacteriaceae* from rectal swabs.
- To determine the carbapenem susceptibility based on the Brilliance™ CRE Agar and Kirby-Bauer tests.
- To compare the carbapenem MICs based on the microbroth dilution, E-Test and VITEK® 2 Automated System.
- To ascertain carbapenemase production based on the ChromID® CARBA agar, Modified Hodge Test and Amoxycillin-Clavulanate Double Disc Synergy Test.
- To link the results of the phenotypic tests with the results from the multiplex real-time PCR for carbapenemase production.

CHAPTER TWO

BACKGROUND

2.1. INFECTIONS IN NEONATES

The World Health Organisation estimated that about 41% (3.6 million) of under 5 year old deaths occur during the neonatal period (Hoque *et al.,* 2011). Most of these deaths occur in developing countries and approximately 1 million deaths are attributed to infections such as neonatal sepsis, meningitis and pneumonia (Zaidi *et al.*, 2011). In South Africa, the most common reasons for neonatal deaths are birth asphyxia, prematurity, neonatal bacterial infection and congenital abnormality (Pattinson, 2007). Neonates are highly susceptible to bacterial infections, and an immature immune system may lead to the rapid progression of disease. In addition, there may be a delay in recognition and treatment of such infections due to poor recognition of symptoms and constraints of healthcare resources (Zaidi *et al.*, 2011).

2.1.1 Acquisition of Neonatal Infections

Neonates are immunocompromised due to their thin skin and mucous membranes (which are weak barriers against infections) and an immature immune system with respect to specific and innate immunity (Vergnano and Heath, 2013). Infections in neonates may occur as a result of vertical transmission in utero, acquisition during the peripartum period or nosocomial infections following delivery.

In 1998, in South Africa, a rate of 21 per 1000 live births occurred. According to the Statistics South Africa, in 2009, the neonatal mortality rate decreased to 14 per 1000 live births, with a target decrease of 7 per 1000 predicted for the year 2015 (Lloyd and de Witt, 2013; Velaphi and Rhoda, 2012). The rate of mortality in under-fives has increased as a result of HIV/ AIDS and has led to numerous deaths within the first 28 days of life (Pattinson, 2009).

The TORCH group (*Toxoplasma gondii*; Others such as Parvovirus B19, Varicella-Zoster virus infection, *Treponema pallidum* infection, Hepatitis B; Rubella virus, Cytomegalovirus infection and Herpes Simplex virus infection) comprise organisms that predominantly cause congenital infection during pregnancy (van der Weiden *et al.*, 2011). The gestational age of the fetus influences the severity of such infections.

Early onset infections are referred to as infections that occur 2 to 3 days after birth, and are caused by the transmission of pathogens from the birth canal during or before birth (Fleming *et al.,* 2012). The incidence of early onset infections vary from <1 to 10 per 1000 live births (Vergnano and Heath, 2013). The most common pathogens leading to early onset infections in developed countries are Group B streptococci and *E. coli*, followed by *S. aureus* and *Listeria monocytogenes* (Capretti and Faldella, 2013).

Late-onset infections in hospitalised neonates occur after 48 hours of birth and are primarily nosocomial (Fleming *et al.,* 2012). The incidence of late-onset infection differs from 20 to 30

per 1000 neonatal admissions in western countries (Vergnano and Heath, 2013). These infections usually occur in the preterm population due to their prolonged stay and immune deficiency.

In developed countries the most common infections are caused by coagulase-negative staphylococci, followed by *S. aureus*, *E. coli*, *Klebsiella* spp., and other *Enterobacteriaceae* (Fleming *et al.*, 2012; Vergnano *et al.*, 2011). Gram-negative rods are a common cause of nosocomial infection in neonates in developing countries. *K. pneumoniae* has been shown to account for between 16-28% of blood culture positives in septic neonates (Zaidi *et al.*, 2005).

2.1.2 Factors influencing Neonatal Infections

Environmental risk factors associated with the transmission of infections are significant and related to the seasonal changes in the frequency of neonatal hospital acquired infections. Warmer climates are normally associated with increased colonisation with *Enterobacter* spp. (Fryklund *et al.*, 1993). An increase in the humidity in the nursery, propagates airborne dispersion of *Acinetobacter* spp. and is associated with bloodstream infections (Srivastava and Shetty, 2007). Hospitalisation results in the colonisation of skin and gastrointestinal tract with resistant organisms present in hospitals (Srivastava and Shetty, 2007). This in turn leads to bloodstream infections when there are abrasions on the skin or mucosa.

Nosocomial pathogens are known for causing common-source outbreaks since they flourish in multi-use containers of medication, liquid soap, antiseptics, and disinfectants, as well as inadequately disinfected or sterilised equipment (Zaidi *et al.,* 2005). The primary source of such infection is the hands of health-care personnel.

Vertically acquired pathogens have the ability to cause infection of the amniotic fluid and stillbirths. In South Africa, the pathogens associated with neonatal sepsis suggest that many neonatal infections may be primarily acquired from the environment. The organism responsible for such infections is the group B *Streptococcus* (Cutland *et al.,* 2009). Other pathogens that contribute to an increase in the mortality rate in neonates in South Africa include *E. coli*, *Klebsiella* spp., and *Candida* spp.

2.1.3. Infection, Prevention and Control

Several strategies may be implemented to prevent the spread of nosocomial pathogens in neonatal nurseries, including entry restrictions, maintenance of a clean environment outside the unit, and the promotion and practice of hand hygiene. In order to prevent or reduce the multiplication of environmental organisms, regular cleaning, disinfection and sterilisation of equipment and instruments are necessary (Shaffer, 2013). The transmission of organisms between the neonates may be further prevented by: reducing overcrowding in incubators and open cribs (Shaffer, 2013), promoting the use of disposable items and non-re-usable items, and increasing the hospital personnel-to-patient ratio by adequate staffing (Uwaezuoke and Obu, 2013). Whilst these strategies prevent colonisation and subsequent infection in

neonates, it must be partnered with antibiotic stewardship policies and practice to restrict the emergence and spread of antibiotic resistant organisms.

2.1.4. Multi-Drug Resistant Nosocomial Pathogens

There has been an increase in the resistance amongst Gram-negative and Gram-positive pathogens that cause infections in hospitals. These pathogens are referred to as 'ESKAPE' pathogens and comprise *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Boucher *et al.,* 2013). The alarming rates of MDR are driven by poor infection-control practices as well as selective pressure of incorrect and prolonged used of antimicrobials. This allows for the emergence and amplification of resistance within hospital nurseries. 'ESKAPE' pathogens have become a common occurrence in the healthcare environment (Boucher *et al.,* 2009). The development of resistance amongst this group of pathogens is related to antibiotic usage patterns (Boucher *et al.,* 2009).

K. pneumoniae is one of the most important neonatal pathogens that occur in developing countries. *Klebsiella* spp. can be observed in the normal gastrointestinal and vaginal flora. However, its detection in a MDR form in hospital-born babies suggests that highly contaminated environmental reservoirs serve as a source of infection (Newman, 2002). The prevalence of these pathogens amongst neonates is cause for concern since colonization appears to occur at rapid rates (Chandrashekar *et al.,* 1997; Newman, 2002).

2.2. β-LACTAM ANTIBIOTICS

2.2.1. Mechanisms of Action

β-lactams comprise a variety of antibiotics that are differentiated on the basis of their chemical structure. They can be divided into four groups which include penicillins, cephalosporins, monobactams and carbapenems.

The activity of β-lactam antibiotics against bacteria results in the inhibition of the synthesis of the Peptidoglycan layer (PGL) (Nordmann *et al*., 2012). The final transpeptidation step during PGL synthesis involves the transpeptidases known as peptidoglycan-binding proteins (PBPs) (Nordmann *et al.,* 2012). These PBPs differ in 2 ways: their affinity to bind to β-lactams varies, and the quantity of PBPs differ between different species of bacteria (Nordmann *et al.,* 2012). According to Nordmann *et al.* (2012), the β-lactam antibiotic structure is similar to that of D-alanyl-D-alanine, which has terminal amino acid deposits on the N-acetyl muramique (NAM)/ N-acetyl glucosamine (NAG) peptide subunits of the emerging peptidoglycan layer. This similarity allows for the binding of the β-lactams to the PBPs active site.

The β-lactam nucleus of the molecule can become permanently bound to the Ser403 residue of the PBP active site. This will prevent the crosslinking of the new peptidoglycan layer, resulting in the disruption of the cell wall synthesis. Usually the peptidoglycan precursors signal the reorganisation of the cell wall which encourages the activity of the autolytic cell

wall hydrolases. However, if the crosslinking via β-lactams is inhibited; this results in the accumulation of peptidoglycan precursors. This involves the assimilation of the existing peptidoglycans by autolytic hydrolases in the absence of nascent peptidoglycan. Subsequently, the bactericidal action of β-lactam antibiotics are enhanced to a greater extent and the structural integrity of the cell wall decreases until lysis occurs. (Nordmann *et al.*, 2012).

2.2.2. Mechanisms of Resistance

In *Enterobacteriaceae*, the primary mechanism of resistance is the production of β-lactamases which hydrolyse β-lactams, while an altered expression of efflux pumps and/ or porins is thought to play a minor role (Gazin *et al.*, 2012). Depending on the substrate, the β-lactamases constitute of four functional groups which are: penicillinases (or classic β-lactamases), ESBLs, carbapenemases, and AmpC-type cephalosporinases (Dhillon and Clark, 2009; Gazin *et al.*, 2012). These four groups of β-lactamases are defined according to their functions. Penicillinase inactivates penicillin but cannot cause degradation of cephalosporins, aztreonam or carbapenems (Dhillon and Clark 2009; Nordmann *et al.* 2012). Cephalosporinase inactivates cephalosporins and aminopenicillins, with the exception of penicillins, aztreonam or carbapenems (Dhillon and Clark 2009; Nordmann *et al.* 2012). ESBLs cause inactivation of all β-lactams excluding carbapenems but may be inhibited by clavulanic acid, tazobactam, sulbactam, Ethylenediaminetetraacetic acid (EDTA), and Sodium Chloride (NaCl). Finally, carbapenemases may selectively inactivate carbapenems based on the enzyme produced, and a variety of β-lactam antibiotics (Dhillon and Clark 2009; Nordmann *et al.* 2012).

2.2.3. β-Lactamase Inhibitors

Clavulanic acid, sulbactam and tazobactam are β-lactamases inhibitors (Nordmann *et al.*, 2012). These are derived from β-lactams and are most commonly used for clinical purposes unlike other uncommon inhibitors like EDTA and NaCl. The inhibitors have weak antimicrobial activity although they share the β-lactam ring trait from β-lactam antibiotics (Nordmann *et al.*, 2012). The similarity in the chemical structure of the inhibitory molecules allows the substrate to act in a suicidal manner. This occurs by the covalent binding of the inhibitor with the active sites of β-lactamases produced by the bacteria (Nordmann *et al.*, 2012).

The resistance of Gram-negative bacteria to antimicrobial agents is increasing rapidly. The third generation cephalosporins initially could overcome the resistance caused by β-lactamases (Paterson, 2006). Third-generation agents such as ceftriaxone, cefotaxime and ceftazidime were stable in the presence of classic β-lactamases (Paterson, 2006). However, Gram-negative bacilli obtained from hospitals, such as *K. pneumoniae,* produced mutant forms of β-lactamases that made them resistant to both third-generation cephalosporins and monobactams (Goossens and Grabein, 2005; Paterson, 2006). Most hospital acquired *Enterobacteriaceae* are resistant to third-generation cephalosporins due to the production of β-lactamases. ESBLs that act by hydrolysing both broad- and extended- spectrum cephalosporins, monobactams and penicillins, are examples of this resistance (Paterson, 2006). Therefore, ESBL-producing *Enterobacteriaceae* in most ICUs are MDR, making it difficult to treat nosocomial infections (Gazin et al., 2012; Goossens and Grabein, 2005).

Late detection and inappropriate treatment with cephalosporins for illnesses caused by ESBL producers, have led to an increase in the mortality rate (Dhillon and Clark, 2009).

The genes encoding ESBLs commonly occur in the plasmids that also have genes encoding aminoglycoside- and sulphonamide- resistance, are present in numerous *Enterobacteriaceae* (Paterson, 2006). Organisms that produce ESBLs may therefore also be resistant to non-β-lactam antibiotics such as quinolones, aminoglycosides, and trimethoprim which reduces the treatment options (Dhillon and Clark, 2009). ESBLs are transferred by means of a plasmid, making it difficult to effectively control and treat organisms that produce this enzyme. Another mechanism of resistance in Gram-negative bacteria is the hyperproduction of Bush group 1 chromosomally mediated cephalosporinases such as AmpC β-lactamases (Goossens and Grabein, 2005). This allows for the resistance to most β-lactams including third-generation cephalosporins (Goossens and Grabein, 2005). The AmpC-producing strains that undergo derepression are the organisms that are often isolated from hospitals, predominantly from the ICU (Pfaller and Jones, 2002). This is commonly due to the use of broad-spectrum β-lactams (Pfaller and Jones, 2002). The treatment for ESBL- and AmpC- producing organisms are the carbapenems. These are the only antimicrobial agents that are active against the two mechanisms of resistance described (Goossens and Grabein, 2005).

2.3. THE CARBAPENEMS

Carbapenems are a subclass of β-lactam agents and comprise four Food and Drug Administration (FDA) approved antibiotics, namely imipenem, doripenem, ertapenem and

meropenem (Patel *et al.*, 2009). These antimicrobial agents have been classified into 3 groups. Ertapenem falls within group 1, which comprises broad-spectrum carbapenems that have limited activity against non-fermentative Gram-negative bacilli. This antibiotic is more suitable for severe community acquired infections and some hospital acquired infections where *Pseudomonas* spp. and/ or *Acinetobacter* are not suspected. Group 2 comprises imipenem, meropenem and doripenem, which are broad-spectrum carbapenems. These antibiotics are active against fermentative and non-fermentative Gram-negative bacilli and are appropriate for treatment of severe nosocomial infections. The third group includes carbapenems that are clinically active against methicillin-resistant *Staphylococcus aureus* (non-licensed) (Brink *et al.*, 2004; Shah and Isaacs, 2003).

Carbapenems have broad spectrum bactericidal activity based on the inhibition of cell wall synthesis. Recently, resistance against this group of antimicrobial agents has been underscored, from the emergence and spread of ESBL producing Gram-negative bacteria in the community and healthcare settings, to an increase in the consumption of carbapenems for the treatment of infections with ESBL producers (El-Herte *et al.*, 2012; Nordmann *et al.*, 2012). In many hospitals, carbapenems have become the first line antimicrobial agents of choice for the management of nosocomial infections. These infections are caused by MDR strains of *Pseudomonas aeruginosa*, *Acinetobacter* species, and ESBL-producing *Enterobacteriaceae* (Patel *et al.*, 2009; Nordmann *et al.*, 2012; Zhang *et al.*, 2012).

Carbapenems have a zwitterionic molecular charge which allows the entry of the antibiotic through the cell wall of Gram-positive and Gram-negative bacteria (El-Hertea *et al.*, 2012).

There are three properties of carbapenems responsible for its broad spectrum of activity (El-Herte *et al.,* 2012; Parekh and Desai, 2009). Firstly, the molecules are small and have the charge characteristic allowing the utilisation of special porins in the outer membrane of Gram-negative bacteria gaining access to the PBPs. Secondly, the antibiotic structure confers resistance to cleavage by most β-lactamases including carbapenemases. Thirdly, carbapenems have an affinity for a broad range of PBPs from various bacteria. These properties allow carbapenems to gain access to the periplasm, without being inactivated by β-lactamases, and then successfully attaching to PBPs which then results in cell death (Parekh and Desai, 2009). This bactericidal activity results in the inhibition of peptidoglycan cell wall synthesis by binding to specific PBPs and inactivating the enzymes responsible for the cell wall synthesis (Parekh and Desai, 2009).

The occurrence of mutations as a result of carbapenem resistance led to a decrease in expression of the outer membrane porin protein D (OprD) which prevents the entry of imipenem into the bacterium (Knapp and English, 2001). Resistance caused by this type of mutation is uncommon in meropenem since it is readily transported through the OprD porin (Knapp and English, 2001). If the PBP affinity was modified this would provide an alternate mechanism of resistance to carbapenems (Knapp and English, 2001). Similar to other carbapenems described, doripenem derives its bactericidal action from the inhibition of PBPs which eventually results in cell death (Dong *et al.,* 2012; Gagliotti *et al.,* 2013). The enzymes that undergo inhibition due to carbapenems are the high-molecular-weight enzymes PBP1a, PBP1b, PBP2, and PBP3 (Matthews and Lancaster, 2009). The inhibition of PBP1a and PBP1b leads to the development of spheroplasts and rapid bacterial death. PBP2 enzymes

alter rod-shaped organisms by making them spherical, while PBP3 results in the formation of filamentous shaped organisms (Matthews and Lancaster, 2009).

2.3.1. Mechanisms of Action

2.3.1.1. Ertapenem

The chemical structure of ertapenem has a *trans* hydroxyethyl group (as shown in Fig. 1) of the molecule (not found in other carbapenems) which contributes to the stability of this class of anti-infectives to β-lactamases (Motyl *et al.,* 2003). Ertapenem is a long-acting 1-β-methyl carbapenem. The 1-β-methyl group protects the β-lactam carbonyl which reduces the Dehydropeptidase-1 (DHP-1) catalysed hydrolysis of the β-lactam ring and allows for the administration of ertapenem, independently (Motyl *et al.,* 2003). The meta-substituted benzoic acid complex increases the molecular weight and lipophilicity of the molecule, and the carboxylic acid, ionised at physiological pH, gives ertapenem a net negative charge (Motyl *et al.,* 2003). Therefore, protein binding in ertapenem is greater compared to that of imipenem and meropenem, resulting in an extended half-life. Similar to other β-lactam antimicrobial agents, ertapenem has the ability to inhibit the synthesis of bacterial cell wall peptidoglycan layer by binding to PBPs. In *E.coli*, it binds to PBP1a, 1b, 2, 3, 4, and 5, with a high affinity of PBP2 and 3 (Motyl *et al.,* 2003).

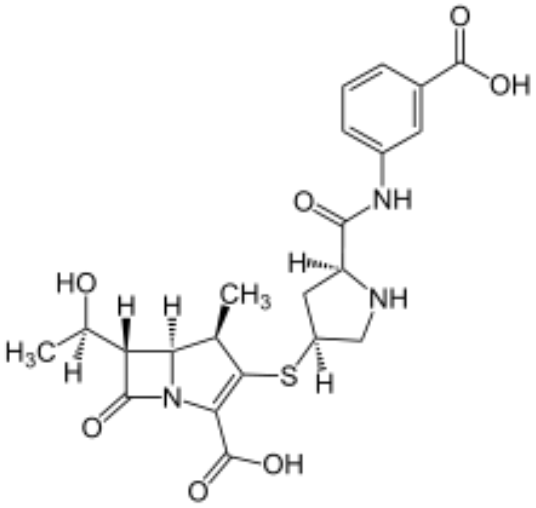

Fig. 1: Chemical structure of ertapenem (Sigma-Aldrich)

Ertapenem is not extensively hydrolysed by β-lactamases and acts against a wide variety of Gram-positive, Gram-negative, and anaerobic microorganisms, in particular *Enterobacteriaceae* (Bora *et al.,* 2012). However it provides limited activity against *P. aeruginosa*, and *Acinetobacter* spp., normally associated with nosocomial infections (Borbone *et al.,* 2006). Ertapenem is used for the treatment of infections such as intra-abdominal, skin-to-skin structure, urinary tract, acute pelvic infections and acquired pneumonia (Borbone *et al.,* 2006).

2.3.1.2. Imipenem

Imipenem, N-formimidoylthienamycin (shown in Fig. 2), was the first type of carbapenem derived from a stable and synthetic drug, known as thienamycin (Knapp and English, 2001). In an early study by Jacobs (1986), imipenem was metabolised in conjunction with the tubular brush border by the DHP-1 enzyme. This accounts for the renal tubular damage

observed in animal studies as well as low recovery of imipenem in the urine of humans (Knapp and English, 2001). In other human studies, it was found that imipenem had to be produced with cilastatin sodium (a reversible inhibitor of DHP-1) that has no intrinsic antibacterial properties (Knapp and English, 2001). Therefore, when imipenem is administered to a patient it has to be co-administered with cilastatin in order to inhibit the action of DHP-1 (Darville, 1999). Notably, the imipenem/ cilastatin combination was reported to induce seizures in humans at clinical doses. The convulsive action of this β-lactam antibiotic may be associated with their ability to inhibit the binding of the γ-aminobutyric acid receptor (Horiuchi *et al.*, 2006).

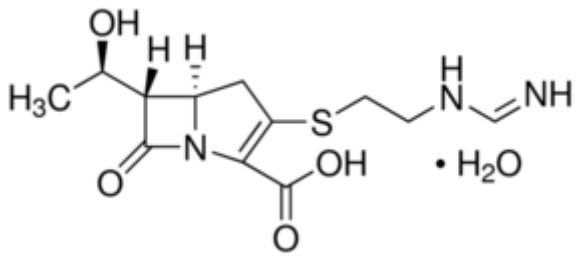

Fig. 2: Chemical structure of imipenem (Sigma-Aldrich)

Imipenem has a high binding affinity *in vitro* to PBP2 and PBP4 in *P. aeruginosa* and inhibits the enzyme activities of PBP1a, PBP1b, PBP2, PBP4 and PBP5 in *E. coli*. PBP2 is responsible for the rod-shaped morphology of the cells and is the binding site of several β-lactam antibiotics which in turn contributes to their resistance (Song *et al.*, 1998). Inhibition of PBP3 in *Pseudomonas* spp. influences a greater number of genes involved in transcription, DNA repair, transcription of pyocin genes and genes implicated in antibiotic resistance (Farra, 2008).

2.3.1.3. Meropenem

Meropenem is an ultra-broad spectrum injectable antibiotic of the carbapenem group. It was the first carbapenem antibiotic that exhibited resistance to hydrolysis by DHP-1, hence there is no need for the co-administration of a DHP-1 inhibitor (Parekh and Desai, 2009). Although the main carbapenem nucleus has a β-lactam ring, which differs from penicillin and cephalosporins, the sulphur on the side chain is substituted with a methylene group and the ring contains a double bond (shown in Fig. 3) (Parekh and Desai, 2009). These differences confer meropenem resistance to degradation by the usual β-lactamases and the ESBLs, as well as chromosomally produced AmpC β-lactamases (Parekh and Desai, 2009). This structure also has a long substitute pyrroline side chain at C-2 which enhances its activity against *P. aeruginosa* (Darville, 1999). Meropenem has the ability to readily penetrate various Gram-negative organisms, accounting for its enhanced activity against these bacteria (Knapp and English, 2001).

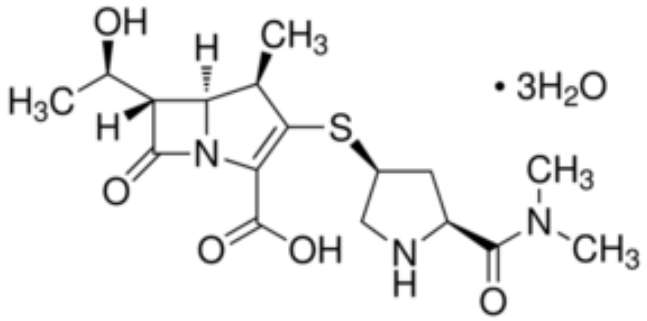

Fig. 3: Chemical structure of meropenem (Sigma-Aldrich)

2.3.1.4. Doripenem

Doripenem is a synthetic 1-β-methyl carbapenem antibiotic that has a broad-spectrum of activity (Goldstein and Citron, 2009). Its structure provides stability against DHP-1 and various other β-lactamases. Doripenem displays potent antibacterial activity against both Gram-negative and Gram-positivebacteria, whilst meropenem is active against Gram-negative bacteria (Goldstein and Citron, 2009; Horiuchi *et al.,* 2006). Doripenem has a sulfamoylaminoethyl-pyrrolidinylthio group (in its side chain) at the second position (as shown in Fig. 4) (Gales *et al.*, 2011). This enhances its activity against non-fermentative Gram-negative bacilli. According to Gales *et al.* (2011), doripenem is stable against the hydrolysis by most β-lactamases, including penicillinases and cephalosporinases, but not to carbapenem hydrolysing β-lactamases. The PBPs that are preferentially bound by doripenem vary with different organisms. In *E.coli*, doripenem prefers to bind to PBP2, followed by PBP1a, 1b, and then 3. In *P. aeruginosa*, doripenem binds to PBP2 and 3 first and then PBP1a and1b. However, with *Streptococcus pneumoniae*, doripenem has high affinity towards PBP1a, 2b and 2x (Matthews and Lancaster, 2009).

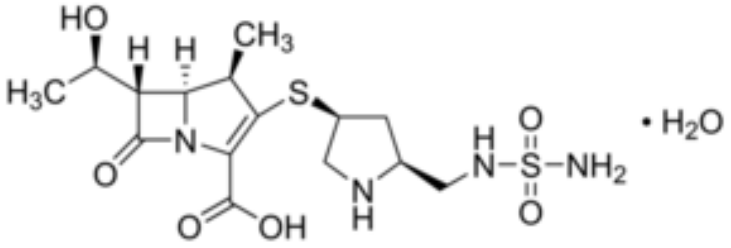

Fig. 4: Chemical structure of doripenem (Sigma-Aldrich)

Doripenem is active against ESBLs and AmpC producing *Enterobacteriaceae* (Castanheira *et al.,* 2009; Dong *et al.,* 2012). It displays the highest activity toward *P. aeruginosa* in terms of MIC values. This is possible because doripenem retains activity against strains with resistance to other carbapenems via the loss of OprD alone or in combination with efflux pumps (Castanheira *et al.*, 2009). According to Goldstein and Citron (2009), anaerobes are unable to use efflux pumps as resistance mechanisms against doripenem. *In vitro* studies proved that this antibiotic was reported as restricted in its selection for resistant strains. In general, its activity is less potent than imipenem, but more potent than meropenem, against Gram-positive bacteria (Gales *et al.,* 2011). However, the activity of doripenem against Gram-negative bacteria was reported as being similar to that of meropenem but much greater than imipenem (Gales *et al.,* 2011).

2.4. CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE* (CRE)

2.4.1. Non-Carbapenemase Mediated CRE

2.4.1.1. ESBL Production

In *Enterobacteriaceae,* resistance may develop due to a combination of ESBLs and outer membrane permeability defects being produced. In this case, genes such as bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M,}}$ that code for ESBLs, are found on a plasmid (Qin *et al.*, 2008). This mechanism of resistance was observed in almost all *Enterobacteriaceae*, including *E. coli*, *K. pneumoniae*, *Salmonella* spp., and *Enterobacter* spp. According to Nordmann *et al.* (2012), ertapenem-resistant and ESBL-producing *K. pneumoniae* (observed in Italy) carried OmpK36

porin variants which played a role in its resistance (Nordmann *et al.,* 2012). The resistance to ertapenem was related to the reduction in the susceptibility to meropenem and imipenem. Ertapenem-resistant isolates developed due to the distribution of CTX-M-producing isolates globally (Pitout and Laupland, 2008).

2.4.1.2. Outer Membrane Impermeability and Efflux

Gram-negative bacteria have a hydrophobic outer membrane which prevents external agents from entering the cell. The membrane comprises proteins known as porins which allow the hydrophilic pathways to acquire necessary nutrients and compounds, such as antibiotics (Koebnik *et al.,* 2000; Pagès *et al.,* 2008). In *Enterobacteriaceae*, the porins responsible for the uptake of antibiotics belong to outer-membrane protein F (OmpF) or outer-membrane protein C (OmpC) family. If the number or the activity of the porins are altered this could affect antibiotic resistance (Nordmann *et al.,* 2012). A reduction in the sensitivity of the antibiotics may occur as a result of mutations in the gatekeeping loop or central channel within the porin proteins, a lack of porin expression, or an alteration in the type of porins present in the outer membrane (Patel *et al.,* 2009).

Carbapenem resistance was initially observed in *Enterobacter* spp. which overexpressed a chromosomal *ampC* gene encoding an intrinsic cephalosporinase and showed altered OmpC and OmpF porins (Nordmann *et al.,* 2012). This mechanism was also detected in other species from the family of *Enterobacteriaceae* that do not express the intrinsic cephalosporinase such as *E. coli*, *K. pneumoniae* and *Salmonella* spp. In these organisms,

resistance prevailed due to the linking of plasmid-encoded AmpC expression with a decline in cell membrane permeability (Armand-Lefevre *et al.,* 2003; Chia *et al.,* 2009; Shin *et al.,* 2012). This was due to the modified OmpK35 or OmpK36 for *K. pneumoniae,* OmpF and OmpC for *E. coli*, and OmpF for *Salmonella typhimurium* (Armand-Lefevre *et al.,* 2003; Chia *et al.,* 2009; Shin *et al.,* 2012). Plasmid-mediated cephalosporinase genes are usually related to co-resistance to other antibiotics as a result of the co-localisation of antibiotic resistance genes on the same plasmid (Nordmann *et al.,* 2012). According to Patel *et al.* (2009), carbapenem resistance in organisms with porin deficiencies are unstable due to a reduction in fitness and growth ability of organisms that have a defect in terms of the permeability barrier of the outer membrane (Patel *et al.,* 2009).

Reports done on carbapenem resistance in *Klebsiella* spp. associated with porin loss in combination with SHV ESBLs, showed undetectable carbapenemase activity *in vitro*. The ability of weak carbapenemases to confer resistance in impermeable strains differs from the inability of strong carbapenemases to confer substantial resistance in highly permeable isolates. Strains that lack carbapenemase production but are resistant to carbapenems are normally susceptible to other antibiotics. This carbapenem resistance characteristic may not be transferred when compared to strains that harbour carbapenemase genes. (Livermore and Woodford, 2000).

The efflux of antibiotics to the exterior of the bacteria through the production of an efflux pump also leads to resistance to various antimicrobial agents including the carbapenems (Nordmann *et al.,* 2012). Imipenem selects strains that contain the active efflux pumps that

eject various unrelated antibiotics including quinolones, tetracycline, and chloramphenicol. The overexpression of the AcrA efflux pump component was reported in *Enterobacter aerogenes* and was responsible for imipenem resistance (Bornet *et al.,* 2003). The overexpression of marA, involved in the genetic control of membrane permeability via porin and efflux pump expression, showed that the activation of the resistance genes are forced in imipenem resistant variants (Bornet *et al.,* 2003).

2.4.1.3. AmpC Production

Another non-carbapenemase mediated resistance mechanism was evident in a few carbapenem-resistant isolates that expressed AmpC-type enzymes in combination with porin loss (Patel *et al.,* 2009; Qin *et al.,* 2008). The AmpC-type enzymes confer resistance to extended-spectrum cephalosporins and have low carbapenem activity. However, when this enzyme activity is combined with decreased cellular penetration of carbapenems as a result of porin loss then carbapenem resistance is observed (Patel *et al.,* 2009). Similar activity was observed in *Enterobacteriaceae,* where carbapenem resistance was observed in isolates that had ESBLs and porin loss (Patel *et al.,* 2009). In this case, β-lactamase had the ability to confer resistance in strains that had reduced uptake or up-regulated efflux in the absence or presence of weak carbapenemase activity (Livermore and Woodford, 2000). These organisms may be able to express susceptibility to various types of carbapenem agents (Livermore and Woodford, 2000).

Imipenem resistance related to porin loss and the hyperproduction of chromosomal AmpC β-lactamase was observed in *Enterobacter* spp. (Livermore and Woodford, 2000). However, imipenem resistance due to porin loss and the presence of plasmidic AmpC β-lactamase was evident in *K. pneumoniae* (Livermore and Woodford, 2000). Organisms that are responsible for the hyperproduction of AmpC due to mutation or plasmid encodement are usually resistant to most cephalosporins and monobactams but are not inhibited by β-lactamase inhibitors (Denton, 2007; Qin *et al.,* 2008). According to Livermore and Woodford (2000), AmpC β-lactamase displayed weak activity against imipenem, however this mechanism was more successful against meropenem which has the ability to deactivate AmpC enzymes *in vitro*.

El-Hertea *et al.* (2012) undertook a study involving dual resistance mechanisms which involved the outer membrane permeability defect with the production of β-lactamases, such as AmpC, cephalosporinases and ESBLs, predominantly in the presence of CTX-M variants. It is not necessary to detect for the production of AmpC organisms that produce an inducible chromosomal AmpC β-lactamase. This is because the organisms that are assumed to be carriers of the AmpC enzymes are known and include 100% of *Enterobacter cloacae*, *E. aerogenes*, *Citrobacter freundii*, *Serratia marcescens*, *Providencia* spp., *Morganella morganii*, *Aeromonas* spp. and *P. aeruginosa* (Thomson, 2010). These organisms possess the ability to readily mutate in order to confer resistance during treatment with β-lactam antibiotics other than carbapenems, penems and fourth-generation cephalosporins (Thomson, 2010).

2.4.2. Carbapenemase Production

Carbapenem-resistant bacteria in the healthcare setting restrict treatment of nosocomial infections. The emergence of carbapenem-inactivating β-lactamases (i.e. carbapenemases) has recently emerged and was reported in pathogenic *Enterobacteriaceae* in various parts of the world (Patel *et al.,* 2009). Carbapenemases confer resistance to β-lactam agents and are usually found in mobile genetic elements that allow for the transfer of resistance mechanisms from one isolate to the next. However, organisms that produce this enzyme may also spread among patients (Patel *et al.,* 2009). The coexistence of ESBL and carbapenemase genes with other antibiotic resistance genes on mobile genetic elements is a major concern. This, coupled with the plasticity of *Enterobacteriaceae,* results in the rapid acquisition of resistance to new classes of antibiotics. The uncontrollable transfer of intra- and inter-species of mobile genetic elements has also contributed to the emergence of organisms with resistance to almost all antibiotics (Gazin *et al.,* 2012). There are three crucial factors that distinguish the emerging carbapenemase associated resistance from early ESBL resistance (Livermore, 2012). Firstly there is no apparent next line antibiotic to use against carbapenemase-producers (the way carbapenems were used for ESBL-producers). Secondly, detection is difficult because the problem has reduced since the prevalence of carbapenemases are lower compared to other ESBLs and the rapid detection methods still require optimisation. Finally, carbapenemase-producers are more diverse in terms of enzymes, species and epidemiology, which complicates the development of inhibitor-based responses by the pharmaceutical industry (Livermore, 2012).

The first type of carbapenemases discovered was SME-1 found in *Enterobacteriaceae* in London in 1982 and IMI-1 was found in the US in 1984 (Rasmussen *et al.,* 1996). Serine-based carbapenemases, originated from the chromosomally-encoded NmcA from the clinical isolate *E. cloacae* (the first to be reported in literature) (Rasmussen *et al.,* 1996). Since then, the dissemination of carbapenem resistance has increased significantly, primarily due to the triggering of carbapenemase production. The new classes of carbapenemases were first discovered in Gram-negative bacteria in Japan in the 1990's and the first clinically significant isolate was found in Europe, Italy in 1999 (Zimmerman *et al.,* 2013).

The emergence of carbapenemases in *Enterobacteriaceae* is of great concern since these pathogens occur frequently and the diversity of enzymes is rapidly emerging. This makes recognition, treatment and response problematic (Livermore, 2012). In *Enterobacteriaceae*, carbapenemases are divided into three main molecular classes of β-lactamases, these include: Ambler class A, which comprises *Klebsiella pneumoniae* carbapenemase (KPC); class B metallo-β-lactamases, which comprises Verona integron-encoded metallo-β-lactamases (VIM), imipenemase (IMP), and New-Delhi metallo-β-lactamases (NDM); and class D carbapenemase, which comprises Oxacillinase (OXA-48) (Dhillon and Clark, 2009; Livermore, 2012; Nordmann *et al.,* 2012). The Ambler class C (AmpC) may also fall within these classes however it is a rare chromosome-encoded cephalosporinase.

Resistance mechanisms of metallo-β-lactamases of VIM and IMP families were described in Europe. In the USA, a carbapenemase-producing organism that was resistant to serine-based carbapenemases was first described in *K. pneumoniae* in 2001 **(**Zimmerman *et al.,* 2013).

This resistance mechanism was transmitted to other *Enterobacteriaceae* via *K. pneumoniae*. In Israel in 2007, the CRE triggered were similar to that found in USA, primarily due to KPC in *K. pneumoniae* (Zimmerman *et al.,* 2013). According to Nordmann *et al.* (2012), there are two main mechanisms responsible for the production of CREs. The first mechanism entails the acquisition of carbapenemase genes that encode carbapenem degrading enzymes. The second mechanism involves the reduction in antibiotic uptake by a qualitative and/ or quantitative absence of porin expression associated with over expression of β-lactamases with a weak affinity for carbapenems.

2.4.2.1. Ambler Class A Carbapenemases

Class A serine-based carbapenemases can be subdivided into three major types of chromosomally encoded enzymes including NmcA/ IMI, SME, and KPC (Livermore and Woodford, 2000; Nordmann *et al.,* 2012; Patel *et al.,* 2009). These enzymes have the ability to hydrolyse various β-lactams such as penicillins, cephalosporins, carbapenems, and aztreonam (Nordmann *et al.,* 2012; Patel *et al.,* 2009). They are susceptible to different types of inhibitors. Whilst all enzymes are weakly inhibited by sulbactam, the IMI and NmcA enzymes are inhibited strongly by clavulanate but the SME carbapenemases are not inhibited (Rasmussen and Bush, 1997). This hydrolytic mechanism involves the active serine site at position 70 (Ambler numbering of class A β-lactamases) and the activity is inhibited *in vitro* by clavulanic acid and tazobactam (Ambler *et al.,* 1991). The SME enzymes have been identified exclusively in *S. marcescens* (Patel *et al.,* 2009). This family comprises three variants (SME-1, SME-2, and SME-3) that are chromosomally-encoded and are found infrequently in USA (Naas *et al.,* 1994; Nordmann *et al.,* 2012).

IMI and NmcA enzymes were recovered from rare isolates of *Enterobacter* spp. in the UK, France, and Argentina (Nordmann *et al.,* 2012; Patel *et al.,* 2009). The gene responsible for encoding the IMI-2 variant was located in the plasmid of *Enterobacter asburiae* strains found in a river in the USA, as well as one strain of *E. cloacae* in China (Nordmann *et al.,* 2012). The genes encoding SME, IMI, and NmcA enzymes that are located in the chromosome and the lack of association with mobile genetic elements have resulted in their limited distribution (Queenan *et al.,* 2000; Rasmussen *et al.,* 1996). Another type of carbapenemase that belongs to this class is known as the Guiana extended-spectrum β-lactamase (GES) (Brink *et al.,* 2012a). According to Brink *et al.* (2012a), GES was identified in various isolates in several hospitals in South Africa: *K. pneumoniae* in Cape Town, *K. oxytoca* in Bloemfontein, *E. cloacae* in Witbank, and *S. marcescens* in Port Elizabeth.

In class A β-lactamases, the most clinically significant enzyme is KPC since it is most frequently encountered (Arnold *et al.,* 2012; Patel *et al.,* 2009). KPC was first described in the USA and is now found globally. Its variant, KPC-2, was identified in *K. pneumoniae* in 1996 in Eastern USA (Bulik *et al.,* 2010). Ever since, 11 KPC variants were observed, ranging from KPC-2 to KPC-12 (Nordmann *et al.,* 2012). KPC-2 was observed in *K. pneumoniae* in Johannesburg, South Africa in 2012 (Brink *et al.,* 2012a). These enzymes were primarily acquired from nosocomial isolates of *K. pneumoniae* and others from *Enterobacteriaceae* species (Bulik *et al.,* 2010). The subtype KPC-1 also exists which harbours genes that are identical to that of KPC-2 (Bulik *et al.,* 2010; Patel *et al.,* 2009). KPC-4 was initially discovered in *Enterobacter* spp. in Scotland in 2004 and was reported in *K. pneumoniae* in Puerto Rico (Patel *et al.,* 2009). The hydrolytic profiles of both KPC-2 and KPC-3 are similar since they both possess a higher affinity for meropenem when compared to

imipenem. However, in KPC-3 there is evidence of higher hydrolysis of ceftazidime (Patel *et al.,* 2009).

According to Livermore (2012) recently, non-clonal *K. pneumoniae* were responsible for the spread of plasmids encoding KPC enzymes in the UK. The mortality rate, as a result of the spread of infection by KPC producing organisms, increased to over 50%. A possible reason for this occurrence may be due to MDR organisms harbouring the KPC enzymes (Nordmann *et al.,* 2012). This may result in first line therapy failing and limits the therapeutic options (Nordmann *et al.,* 2012). KPC-producers have varying susceptibility patterns, but many studies have proved that when bla_{KPC} (an enzyme-encoding gene) is transferred to a susceptible *E. coli* then the enzyme will confer resistance to all β-lactam agents (Patel *et al.,* 2009). A clone of *K. pneumoniae* strain ST258 expressing the bla_{KPC-2} was identified globally and was the cause for the geographical dissemination of the bla_{KPC} (Cuzon *et al.,* 2010). However, various other KPC clones may have spread in the same region but differ in multilocus sequence type, β-lactamase content, and plasmid size (Nordmann *et al.,* 2012). β-lactamase inhibitors are active against KPC enzymes but this activity is low, allowing the KPC-producers to confer resistance to all β-lactam-β-lactamase inhibitor combinations (Patel *et al.,* 2009; Villegas *et al.,* 2006). This includes ampicillin-sulbactam, amoxicillin-clavulanic acid, and piperacillin-tazobactam combinations (Patel *et al.,* 2009).

The spread of the bla_{KPC} may be partially due to their presence on the plasmid. KPC encoding plasmids (some of which are conjugative) harbour alternate mechanisms of resistance including: β-lactamases, plasmid-mediated flouroquinolone resistance, and aminoglycoside

resistance (Patel *et al.,* 2009). A 95 kb plasmid carrying the bla_{KPC} was found in 2 different species of *Enterobacteriaceae,* namely *C. freundii,* and *Klebsiella oxytoca* (Rasheed *et al.,* 2008). These strains were isolated from two different patients in the same hospital (Rasheed *et al.,* 2008). The transfer of this plasmid may have occurred due to interspecies transfer within the hospital. According to Nordmann *et al.* (2012), there is evidence that the bla_{KPC} are associated with the genetic element otherwise known as the transposons Tn4401. This transposon is Tn3-like and was found in isolates of various geogFraphical origins and differ in terms of sequence types in *Enterobacteriaceae* and *P. aeruginosa* (Yang and Bush, 1996). Tn4401 is usually introduced at different loci as well as on the plasmids of different sizes and incompatibility group (Arnold *et al.,* 2011; Patel *et al.,* 2009). This transposon undergoes transposition which results in the formation of a duplicate 5-bp target site which has a non-specific target site (Arnold *et al.,* 2011; Patel *et al.,* 2009).

2.4.2.2. Class B Metallo-β-Lactamases

The class B metallo-β-lactamases display hydrolytic activity against penicillins, cephalosporins and carbapenems yet it is not inhibited by β-lactamase inhibitors (Nordmann *et al.,* 2012). This hydrolytic activity depends on the interaction of β-lactams with Zn^{2+} ions as a cofactor in the active site and is therefore inhibited by EDTA (Patel *et al.,* 2009). The first metallo-β-lactamase (MBL) was identified in environmental and opportunistic bacteria such as *Bacillus cereus*, *Aeromonas* spp., and *Stenotrophomonas maltophilia*. The MBL genes within these pathogenic organisms are intrinsically and chromosomally-encoded (Iaconis and Sanders, 1990; Kuwabara and Abraham, 1967; Lim *et al.,* 1988; Yang and Bush, 1996). Since the 1990's, a drastic increase in the acquisition or transference of MBL genes

were reported in *Enterobacteriaceae*, which included IMP, VIM and NDM groups (Iaconis and Sanders, 1990; Yang and Bush, 1996)).

IMP was one of the first MBLs identified and detected in *Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacteriaceae* (Patel *et al.*, 2009). In Japan in 1991, the IMP-1 variant was the first of these types of genes to be reported in *S. marcescens* as well as a single variant in *K. pneumoniae* (Ito *et al.*, 1995). Since the 1990's, 33 IMP variants have emerged and have spread worldwide (Nordmann *et al.*, 2012). The selection of numerous IMP-type genes in Japan may have been possible due to the over use of carbapenems. IMP-1, -2, and -3 enzymes have broad activity against β-lactams with the exception of monobactams (Livermore and Woodford, 2000). IMP-1 may be relatively more active against carbenicillin than ampicillin, while IMP-2 and IMP-3 display similar activity against both these antibiotics (Livermore and Woodford, 2000). Isolates that have IMP-type β-lactamases express the bla_{IMP} which reveal features of class 1 integrons (Zhao and Hu, 2011).

The class 1 integrons are structures of DNA that have antibiotic resistance genes called gene cassettes that are co-expressed from a single promoter. These resistance genes code for reduced susceptibility to dissimilar antibiotic molecules such as β-lactams, aminoglycosides, sulphonamides, and chloramphenicols. The genes from the integrons containing the bla_{IMP} are known for encoding aminoglycoside resistance (*aacA4*, *aadA1*, and *aadB*), chloramphenicols (*catB*), and class D β-lactamases (bla_{OXA}). Class 1 integrons normally occur within the transposon structure allowing their dispersion (Zhao and Hu, 2011). However, it may integrate resistance genes that are unrelated but are mobile on their own

(Zhao and Hu, 2011). The relationship between *bla*$_{IMP}$ alleles and carbapenem resistance is flawed, and some *bla*$_{IMP}$ hosts are susceptible (Livermore and Woodford, 2000). This may be possible due to *bla*$_{IMP}$ not being expressed frequently, or because substantive resistance may require reduced uptake of carbapenems and the presence of β-lactamase enzymes (Livermore and Woodford, 2000).

The variants of the VIM enzymes is the next type of MBL, from which VIM-1 originated in Italy in 1997 and sometime later, VIM-2 emerged in France from *P. aeruginosa* isolates. VIM-2 enzymes have a 90% amino acid homology to VIM-1 (Takahashi, 2000). The VIM group of enzymes eventually made up 33 variants, majority of which were observed in *P. aeruginosa* and the remainder in *Enterobacteriaceae.* Similar to IMP-type genes, these genes correspond to the gene cassettes found inside the class 1 integrons. VIM-2 was associated with 45 kb non-conjugative plasmid, whereas VIM-1 was not associated with extra chromosomal DNA (Livermore and Woodford, 2000). VIM-2 is the most commonly reported MBL found worldwide, with the greatest prevalence in Southern Europe and Southeast Asia. The clonal spread of *K. pneumoniae* encoding VIM carbapenemases occurred around the UK, which explains the prevalence of KPC enzymes between the year 2010 and 2011 in this region (Livermore, 2012). VIM was also detected in *K. pneumoniae* in South Africa, Johannesburg (Brink *et al.*, 2012a).

The final family of MBLs is NDM, from which NDM-1-positive *Enterobacteriaceae* variants emerged. NDM-1 was originally identified in an Indian patient in Sweden in 2008 (previously hospitalised in New Delhi). Since then, NDM-1-producers have also spread

throughout the world (Livermore, 2012). India is referred to as the reservoir of the *bla*$_{\text{NDM-1}}$, since the majority of the patients that have acquired the NDM-1 producers have either travelled to the Indian subcontinent or have been in contact with India, Pakistan or Bangladesh (Livermore, 2012; Potron *et al.*, 2011b). Many other *Enterobacteriaceae* isolates that produce NDM variants were identified in patients that had relationships with Balkan states or the Middle East (Livermore, 2012). This implies that this region could be the secondary reservoir of NDM-1 producers. An *E. cloacae* producing NDM-1 strain was recently detected in Pretoria, South Africa (Brink *et al.*, 2012a). The transmission of NDM-1 enzyme differs from other carbapenemases. The plasmids that encode the NDM-1 gene have a broad range of hosts which allows for the easy dissemination among other *Enterobacteriaceae* and unrelated species (Coetzee and Brink, 2011). According to Govind *et al.* (2013), organisms that produce *bla*$_{\text{NDM-1}}$ are able to colonise the gastrointestinal tract of humans for an extended duration. These are normally spread via contaminated water and environmental surfaces which was similar to that observed in the current surveillance.

Studies reported that the spread of *bla*$_{\text{NDM-1}}$-producing *Enterobacteriacea*e was not associated with any of the following: transmission of specific clones, the transfer of specific plasmids, or the distribution of a given genetic structure (Poirel *et al.*, 2011). Therefore it may be identified from different Gram-negative organisms carried by a variety of plasmids (Nordmann *et al.*, 2012). Plasmids that harbour the *bla*$_{\text{NDM-1}}$ are diverse in size, incompatibility group, and its association with resistance genes (Nordmann *et al.*, 2012). NDM normally have plasmid AmpC-β-lactamases and ESBLs in combination with the ArmA and/ or RmtC methylases that alter the 16S rRNA in order to block the binding of most aminoglycosides (Livermore, 2012).

All NDM-1 producers possess the ability to express various unrelated resistance genes like those encoding carbapenemases (VIM-type, OXA-48-type), AmpC, cephalosporinases, ESBLs; and resistance to aminoglycosides (16S RNA methylases), macrolides (esterases), rifampicin (rifampicin-modifying enzymes) and sulfamethoxazole (Nordmann *et al.,* 2012). This association with such a large number of plasmids and chromosomes that code for resistance genes in single isolates are uncommonly reported, but is the primary reason for the emergence of MDR isolates. Several NDM-1 producers are susceptible to fosfomycin, colistin, and tigecycline. However, the spectrum and side effect profile of these agents limit its utility in the management of infections with MDR isolates. In addition, there have been recent reports of infection with organisms that are resistant to all available antibiotics (Nordmann *et al.,* 2012).

In recent years, an association with $bla_{\text{NDM-1}}$ and ble_{MBL} (encodes functional bleomycin resistance protein) was reported (Dortet *et al.,* 2012). These two genes share the same operon and are co-expressed by the same promoter, with ble_{MBL} being partially developed by the 3' end of the insertion sequence ISAba125 (Dortet *et al.,* 2012). The production of bleomycin resistance proteins reduces the rate of mutations of the *recA*, which in turn stabilises the $bla_{\text{NDM-1}}$ positive isolates (Nordmann *et al.,* 2012). This means that the emergence of carbapenem-resistant NDM-1 may be chosen by bleomycin type molecules produced by the environmental organism *Streptomyces* spp. These strains have proteins that encode bleomycin resistance due to the development of the NDM genes. This may result in its persistence in the environment due to the selection by bleomycin-related molecules developed in nature (Nordmann *et al.,* 2012).

2.4.2.3. Class D Carbapenemases

OXA is a type of class D β-lactamase enzyme that is classified phenotypically because of its oxacillinase activity (Livermore and Woodford, 2000; Nordmann *et al.,* 2012; Poirel *et al.,* 2012a). This enzyme comprises 232 variants that have carbapenemase resistance (Livermore and Woodford, 2000; Nordmann *et al.,* 2012; Poirel *et al.,* 2012a). OXA was found in Mediterranean countries and is currently circulating to other geographical areas. The first organism that produced OXA-48 was identified in a *K. pneumoniae* isolate in Turkey in 2003 (Livermore, 2012). OXA-48 producers were sourced from nosocomial infections which spread throughout Turkey and were prevalent in Southern Europe and North Africa. Organisms producing OXA-48 such as *K. pneumoniae*, *E. coli*, or *E. cloacae* were reported in nosocomial transmission amongst patients in France, Germany, Spain, Netherlands, and the UK (Poirel *et al.,* 2012a). OXA-48 and its derivatives were also reported among *Enterobacteriaceae* in hospitalised patients in Johannesburg, Cape Town and Port Elizabeth (Brink *et al.,* 2012a).

Carbapenem-hydrolysing class D β-lactamases (CHDLs) are unable to hydrolyse extended-spectrum cephalosporins, with the exception of the OXA-163 variant which has weak carbapenemase activity (Nordmann *et al.,* 2012; Poirel *et al.,* 2012a). OXA-2 and OXA-10 have the ability to yield mutants that attack oxyimino-aminothiazolyl cephalosporins but lack carbapenemase activity (Livermore and Woodford, 2000). In general, the carbapenemase activity of CHDLs is weak and can be inhibited by NaCl but not clavulanic acid or EDTA (Nordmann *et al.,* 2012). Majority of the CHDLs variants were observed in *Acinetobacter* spp. but OXA-48 was found in *Enterobacteriaceae* only (Poirel *et al.,* 2012a).

The *bla*$_{OXA-48}$ is spread among *Enterobacteriaceae* via a single plasmid which is 62 kb in size (Potron *et al.,* 2011a). This plasmid is self-conjugative and has an IncL/ M type backbone that lacks other genes required for antibiotic resistance (Poirel *et al.,* 2012a). The *bla*$_{OXA-48}$ is bracketed by two IS1999 elements in order to form a functional composite transposon (Poirel *et al.,* 2012b). Another type of gene responsible for resistance (identical to the *bla*$_{OXA-48}$ in terms of hydrolytic properties) is the *bla*$_{OXA-181}$ (Poirel *et al.,* 2012b). This gene is a point-mutant derivative of the *bla*$_{OXA-48}$ but differs completely in genetic composition (Poirel *et al.,* 2012b).

The *bla*$_{OXA-181}$ is positioned on a non-conjugative ColE2 type plasmid (7 kb in size) when associated with the insertion sequence ISEcp1 (Potron *et al.,* 2011c). This ISEcp1 is responsible for acquiring the gene by means of one-ended transposition mechanism (Potron *et al.,* 2011c). *Shewanella oneidensis* harbours the *bla*$_{OXA-48}$ while *Shewanella xiamenensis* holds the *bla*$_{OXA-181}$ (Poirel *et al.,* 2004; Potron *et al.,* 2011d). The transfer of the resistance gene among *Shewanella* spp. and *Enterobacteriaceae* may be a result of these organisms residing in the same water-borne environment. The organisms that produce OXA-48 and OXA-181 normally do not display high levels of resistance to the carbapenems. This may be problematic for detecting the OXA-48-like enzymes unless those organisms have mechanisms that are associated with resistance such as ESBL production and/ or permeability defects (Nordmann *et al.,* 2012). Numerous strains that harbour the OXA-48 enzyme produce resistance through co-production of ESBLs and tend to lack activity against oxyimino-cephalosporins (Livermore, 2012).

2.5. JUSTIFICATION OF THE STUDY

Carbapenemases are the primary cause for the increase in carbapenem-resistance in Gram-negative *Enterobacteriaceae* (Bush *et al.,* 2013). Carbapenems are frequently used to treat nosocomial infections caused by microorganisms that are resistant to all other classes of antibiotics. Carbapenemases not only have the ability to render the potent carbapenem group of antibiotics inactive, but are also able to inactivate unrelated groups of antibiotics. The bacteria that produce carbapenemases have become a major threat in hospitals globally, and present a management dilemma in selecting appropriate and effective antibiotics (Bush *et al.,* 2013). This adversely impacts the morbidity and mortality in patients with nosocomial infections.

Carbapenem-resistance in South Africa has only been reported in the context of CPEs (Coetzee and Brink, 2011). *K. pneumoniae* and *E. cloacae* harbouring the NDM and KPC enzymes were initially isolated (Brink *et al.,* 2012b). To date the most common enzymes in South Africa includes GES, KPC, MBLs, NDM-1, and OXA-48 (Brink *et al.,* 2012b). In the private sector, the use of imipenem, meropenem and ertapenem has exceeded its monthly units sold by more than 50%, between January 2009 and June 2011 (Coetzee and Brink, 2011). The excessive consumption of these antibiotics, through selective pressure, has led to the development of carbapenem resistance among *Enterobacteriaceae* and in turn CPEs. In South Africa multiple outbreaks and epidemics have been reported with regard to these CPEs (Brink *et al.,* 2012b; Coetzee and Brink, 2011).

The focus on efforts to control the spread of these organisms centre around the rapid detection of the organisms and isolation of patients. The genes responsible for the transfer of these enzymes may become endemic in hospitals, with eradication becoming unattainable, regardless of the intensive infection control measures. As a result, the timeous detection of CPEs and appropriate isolation of patients is considered to be of critical importance in stemming the spread of these highly resistant organisms. However, the enzymes conferring carbapenemase resistance are usually produced together with other enzymes of the β-lactamase group, making individual carbapenemase detection difficult with simple phenotypic tests. Therefore further research is required to determine the appropriate techniques for the rapid detection of CPEs and the mechanisms of action used by these organisms to develop antibiotic resistance. Such resistance may be alleviated through aggressive infection control measures and optimisation of antibiotic therapy, which could result in the subsequent reduction in the mortality.

CHAPTER THREE

MATERIALS AND METHODS

3.1. ETHICS APPROVAL

This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal under the ethics number BE222/13 and the KZN Provincial Research Committee (Appendix A).

3.2. STUDY SITE AND PATIENTS

The study was conducted in the neonatal nursery at King Edward VIII Hospital, Durban, KwaZulu-Natal, South Africa. King Edward VIII hospital is a regional hospital that delivers approximately 670 babies per month. Of these around 300 are admitted to the nursery for care.

The nursery at King Edward VIII has 3 Neonatal Intensive Care Unit (NICU) beds, 4 high care beds and 10 servo-cribs and 15 incubators. The clinical indication for admission to the unit is varied and includes prematurity and infection.

3.3. SPECIMEN COLLECTION

Specimens were collected from neonates in the following wards: Observation 1, Old premature nursery and New premature nursery (Appendix B, Table 1). All specimens were collected by a professional nurse. Cotton tipped swabs were used to sample the rectum of neonates. Three swabs were collected from each neonate and the swabs were then placed into 10 ml sterile white screw capped tubes (Whitehead Scientific, Cape Town) and transported to the Infection Prevention and Control Laboratory on the premises. Each neonate was allocated a unique 'R' number given by the laboratory (Appendix B, Table 2).

3.4. LABORATORY INVESTIGATIONS

3.4.1. Bacterial Isolation and Detection of Carbapenemase producing *Enterobacteriaceae*

The 3 rectal swab specimens were used to inoculate each of the following media:

- MacConkey agar (Oxoid, United Kingdom) for the isolation of Gram-negative bacilli (preparation as per Appendix C, 1.1).
- Brilliance™ CRE agar (Oxoid, United Kingdom) for the detection of carbapenem-resistant Gram-negative bacilli.
- ChromID® CARBA agar plates (Biomerieux, France) for the detection of carbapenemase producing Gram-negative bacteria.

A four-way streak method was used to inoculate the plates to obtain single colonies (Shankel and Arnold, 1968). The plates were incubated at 37 °C, aerobically for 24 h.

The plates were then read and an initial phenotypic assessment was made based on variation in colour (Table 1), from both the chromogenic agar plates, and based on lactose fermentation (yellow colonies) from the MacConkey plates.

Based on the different morphologies colonies were picked off each of the MacConkey agar, Brilliance™ CRE and ChromID® CARBA agar plates. The selected colonies were plated out onto blood agar plates (preparation as per Appendix C, 1.4) to ensure purity. These plates were then incubated at 37 °C for 24 h. A 0.5 McFarland standard was prepared by adding a single colony from the purity plate into 5 ml of distilled water. This was vortexed for 10-15 seconds or until a homogenous suspension was obtained.

Table 1: Colour of *Enterobacteriaceae* Colonies on MacConkey and Chromogenic Agar

Organisms	MacConkey agar	Brilliance™ CRE agar	ChromID® CARBA agar
K. pneumoniae	Lactose-fermenters	Blue	Green-blue
Enterobacter spp.	Lactose-fermenters	Blue	-
E. coli	Lactose-fermenters	Pink	Orange-red
Citrobacter spp.	Weak Lactose-fermenters	Blue	-
Serratia spp.	Weak lactose-fermenter	Blue	-
Proteus mirabilis	Non-lactose fermenter	-	-

The controls used throughout this study were: *E. coli* ATCC 25922 (negative control); and *K. pneumoniae* POZ KPC-3, *K. pneumoniae* DIH VIM-1, *K. pneumoniae* CHE GES-5, *K. pneumoniae* BIC OXA-48, *K. pneumoniae* 6852-IMP-1, *K. pneumoniae* NDM-1 (positive controls).

3.4.2. Identification of Gram-negative Bacilli

Identification of each isolate was performed using the API 20E (Biomerieux, France) according to the instruction manual. The standardised bacterial suspension (equivalent to a 0.5 MacFarland) was used for inoculation of the API 20E strips. Results were interpreted according to instruction manual (refer to Appendix D, Table 3a and 3b). These were incubated at 37 °C for 24 h.

3.4.3. Kirby-Bauer Disc Diffusion Susceptibility Test

The standardised inoculums were seeded onto Mueller-Hinton agar (MHA) (Oxoid, United Kingdom), (preparation as per Appendix C, 1.2) using a cotton swab. The susceptibility test was performed using the Kirby-Bauer disc diffusion technique using the following antibiotic impregnated discs: ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), doripenem (10 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and chloramphenicol (30 µg) (Oxoid, United Kingdom). The susceptibility plates were incubated at 37 °C for 24 h. The diameter of the inhibition zones were measured and compared to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (2013).

3.4.4. Storage of Isolates

All isolates were stored for future testing. The colonies of a 24 h culture were picked off and added to cryovials containing 500 µl storage media with glass beads (preparation as per Appendix C, 1.5). This was vortexed and stored at -80 °C.

3.5. PHENOTYPIC DETECTION OF CARBAPENEMASE PRODUCING *ENTEROBACTERIACEAE*

3.5.1. Modified Hodge Test (MHT)

The MHT was prepared according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (2012). A single colony of *E .coli* ATCC 25922 was added into 5 ml of Mueller-Hinton broth (MHB) (preparation as per Appendix C, 1.3) and standardised to a 0.5 McFarland. This inoculum was spread onto a MHA plate using a cotton swab and dried for 5 min. Antibiotic discs (10 µg) for meropenem, imipenem, doripenem and ertapenem were placed at equal distances on the lawn 2 cm from the edge of the plate. The test isolate was streaked in a straight line from the edge of the discs to the centre of the plate (as shown in Fig. 5). These plates were incubated at 37 °C for 24 h.

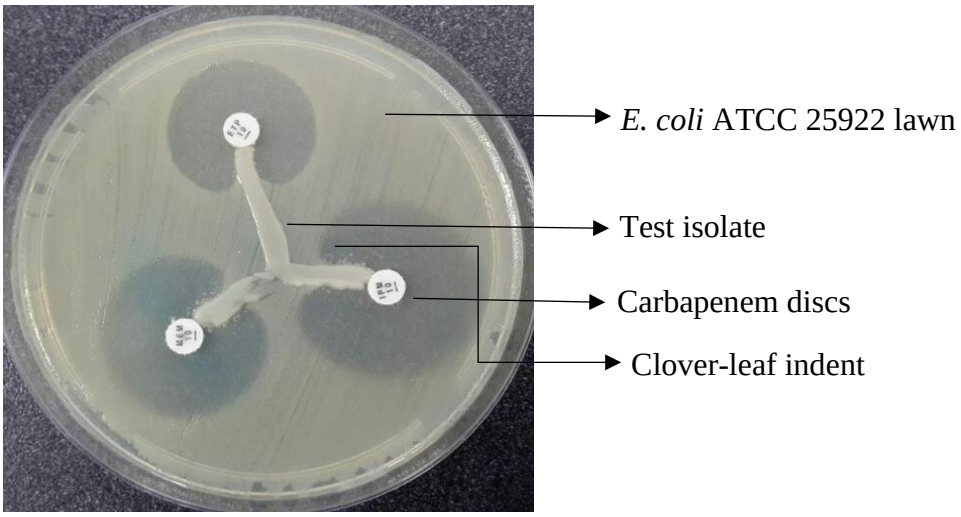

Fig. 5: Image of the Modified Hodge Test

A clover leaf-type indentation at the intersection of the test isolate and *E.coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disc, should appear after incubation. Results were interpreted according to CLSI recommendations (2012) as follows: if there was no distortion of the inhibition zone around the disc then the test was negative, if there was distortion of the *E. coli* ATCC 25922 inhibition zone around the disc then the test was positive, and the test was indeterminate if the inhibition of *E. coli* ATCC 25922 growth around the streaked test isolates are evident by a clear area. A positive MHT was an indication that the test isolates produced carbapenemases.

3.5.2. Amoxycillin-Clavulanate Double Disc Synergy Test

A 0.5 McFarland standard was prepared for each test isolate. The standardised suspensions were spread onto MHA plates and left to dry for 5 min. Amoxycillin-clavulanate (AMC) discs were placed at the centre of the lawn, with meropenem, imipenem, doripenem and ertapenem discs (10 µg) placed at 2 cm away from the inhibitor disc (AMC), (as shown in Fig. 6). The plates were incubated at 37 °C for 24 h. This method was performed using an in-house technique.

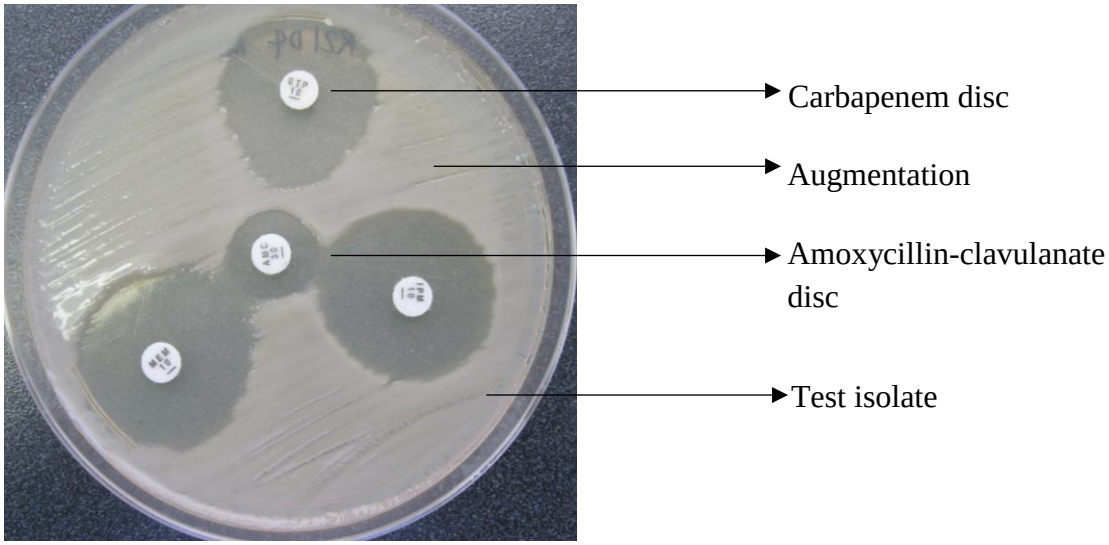

Fig. 6: Amoxycillin-clavulanate inhibitor test

3.6. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

3.6.1. Microbroth-dilution

3.6.1.1. Preparation of antibiotic stock

MICs for the 4 carbapenems were performed on all *K. pneumoniae* and *E. cloacae* isolates. The MIC breakpoint of the specific antibiotics used is shown in Table 2. The range of the antibiotic concentration tested was 4 dilutions above and 4 below the known breakpoint for resistance. These tests were performed in triplicate. The appropriate amount of antibiotic powder was weighed out using the calculations shown in appendix E (1.1). The antibiotic stocks were prepared as follows: 0.010 g of ertapenem dissolved in 10 ml distilled water, 0.013 g of doripenem dissolved in 10 ml distilled water, 0.027 g of imipenem dissolved in 10 ml distilled water and 0.030 g of meropenem dissolved in 10 ml of distilled water. All

antibiotic stock solutions were filter sterilised, aliquoted into cryovials and stored at -20 °C until needed.

3.6.1.2. Preparation of bacterial suspension

A 0.5 McFarland standard was prepared as described in 3.4.2.

3.6.1.3. Minimum Inhibitory Concentration using Microbroth-dilution method

MICs were performed in a 96 well microtitre plate. Two hundred microlitres of antibiotic stock was dispensed into well 1 only and 100 µl was dispensed into well 10. One hundred microlitres of MHB was then dispensed into wells 2 to 12. Two-fold dilutions were performed by transferring 100µl from well 1 to well 2, consecutively until well 9 (the final 100µl from well 9 was discarded). After dilutions were made, 100µl of bacterial suspension was dispensed into wells 1-9 and well 12. Well 10 was used as the antibiotic control, well 11 was used as the broth control and well 12 was used as the bacterial growth control. The plates were incubated at 37 ºC for 18-24 h. MIC results were then read as the lowest concentration of antimicrobial agent that completely inhibited the growth of the organisms in micro-dilution wells. Results were interpreted according to EUCAST and CLSI breakpoints as updated in 2013 and 2012, respectively (shown in table 2a and 2b).

3.6.2. E-Test

The stored isolates were revived. A bacterial suspension was prepared and standardised to a 0.5 McFarland. This was streaked out onto blood agar plates and incubated at 37 °C for 24 h. The E-tests were performed for imipenem, ertapenem, meropenem and doripenem (Biomerieux, France). The bacterial inoculums were spread onto MHA plates and dried for 5 min. The E-test strips were placed on the lawns using sterile forceps and the plates were incubated for 24 h at 37 °C. An elliptical zone of inhibition will form and the MICs are read directly from the graduated E-test strips at the point of intersection of the inhibition zone with the strip. Results were interpreted according to the EUCAST and CLSI breakpoints (shown in table 2a and 2b).

3.6.3. VITEK® 2 Automated System

Antimicrobial susceptibility testing using VITEK® 2 (Biomerieux, France) was performed by the National Health Laboratory Services (NHLS) at Mahatma Gandhi Memorial Hospital, KwaZulu-Natal, South Africa according to the instruction manual. Twenty-four hour cultures were prepared as previously described. These cultures were standardised according to a 0.5 McFarland standard. The VITEK® 2 automated system (Biomerieux, France) was used to determine the MICs of ertapenem, meropenem and imipenem using the AST-N255 susceptibility cards (Biomerieux, France). Doripenem was not available on test system. The results were interpreted according to the EUCAST and CLSI breakpoints as updated in 2013 and 2012, .respectively (shown in table 2a and 2b). Further interpretation was performed by the VITEK® 2 automated system with the use of the Advanced Expert System (AES). The

AES is a database that comprises of information pertaining to the specific organism and its specific MIC results based on previous findings.

Table 2a: MIC interpretive standards for *Enterobacteriaceae* according to EUCAST guidelines

Antimicrobial agent	MIC breakpoint (mg/L)	
	Susceptible ≤	Resistant >
Ertapenem	0.5	1
Doripenem	1	4
Imipenem	2	8
Meropenem	2	8

Table 2b: MIC interpretive standards for *Enterobacteriaceae* according to CLSI guidelines

Antimicrobial agent	MIC breakpoint (mg/L)	
	Susceptible ≤	Resistant ≥
Ertapenem	0.5	2
Doripenem	1	4
Imipenem	1	4
Meropenem	1	4

3.7. GENE DETECTION FOR CARBAPENEMASES

3.7.1. DNA extraction

The isolates that had MICs ranging from intermediate to resistant were used. These were grown on MacConkey agar and incubated for 18-24 h at 37 °C. Crude DNA was extracted

using a boiling method. This was done by picking off 5 to 6 pure colonies from different sections of the plate. The colonies were inoculated in 400 µl of 1x Tris-EDTA (TE) buffer (preparation as per Appendix F, 1.1.) and vortexed for 10-15 seconds or until suspension is emulsified. This was placed on the heating block for 25 min at 95 °C. After which it was centrifuged for 3 min at 12 000 rpm. A 1:10 dilution of the supernatant was prepared by adding 2 µl of the supernatant into 8 µl of PCR-grade water (Roche, Switzerland).

3.7.2. Multiplex Real-Time PCR

Multiplex real-time PCR was performed using the Lightcycler® 480 II real-time instrument (Roche, Switzerland). The TIB Mobiol PCR kits (Roche, Switzerland) were used to determine the presence or absence of the carbapenemase genes (refer to table 3). The mastermix that was used was the Lightcycler® 480 Probes Master kit (Roche, Switzerland). These kits were used as per instruction manuals. Briefly, 2 multiplex real-time PCR reactions were set up. The first PCR targeted the bla_{KPC}, $bla_{\text{NDM-1/2}}$ and $bla_{\text{OXA-48}}$, for which the mastermix was prepared as described in Appendix F, table 4a. The second PCR targeted bla_{VIM}, bla_{IMP} and bla_{GES}, for which the mastermix was prepared as described in Appendix F, Table 4b.

Table 3: Lightmix Primer/ Probe Kits for the Detection of Genes encoding Carbapenemases

Carbapenemase genes	TIB Mobiol PCR Lightmix kits
bla_{KPC}	Lightmix Modular KPC (ESBL)
bla_{IMP}	Lightmix Modular IMP (ESBL)
bla_{OXA-48}	Lightmix Modular OXA-48 (ESBL)
bla_{NDM-1}	Lightmix Modular NDM-1/2 (ESBL)
bla_{VIM}	Lightmix Modular VIM (ESBL)
bla_{GES}	Lightmix Modular GES (ESBL)
Internal control	Lightmix Modular PhHV internal control

A Lightmix Modular PhHV internal control (Roche, Switzerland) was used throughout the run and produced positive results for all isolates. Positive kit controls for KPC, NDM-1, GES, IMP, VIM and OXA-48 were used in parallel with each run performed. These controls were run to ensure that valid results are produced.

The cycle conditions that were used are shown below (Appendix F, Table 4).

Results were interepreted as follows: If the amplified product produced a crossing point (CP) value of less than 37 (between cycles 10-40) in the relevent channels then the result is taken as positive. If the amplification curve occurs from cycle 41-45 then the result is recorded as low positive. If no amplification curves are produced or if it is amplified beyond the 46th cycle and has a CP value > 45 then this is taken as a negative result. If a diluted sample is run and an amplification curve is produced after the 46th cycle, this may lead to a low positive result.

CHAPTER FOUR

RESULTS

4.1. GRAM-NEGATIVE BACILLI ISOLATED

There was no growth from the rectal swabs of 8 neonates. A total of 241 *Enterobacteriaceae* were isolated from the remaining 22 neonates. One or more isolates per species were identified per neonate. The 3 most prevalent species isolated were *K. pneumoniae* (94), *E. coli* (80), and *E. cloacae* (41), (Table 4). Of these, 97 grew on the Brilliance™ CRE agar: *K. pneumonia* (n=38), *E. cloacae* (n=22), *E. aerogenes* (n=2), *E. coli* (n=34), and *P. mirabilis* (n=1), (Table 5). None of the isolates grew on the ChromID® CARBA plates.

Table 4: *Enterobacteriaceae* spp. Isolated from each Neonate

Patients No.	*Klebsiella pneumoniae*	*Enterobacter cloacae*	*Enterobacter aerogenes*	*E. coli*	*Proteus mirabilis*	*Serratia liquefaciens*	*Serratia marcescens*	*Morganella morganii*
R1		✓						
R2	✓			✓				
R3				✓				
R4	✓							
R6	✓			✓	✓			
R8				✓				
R9	✓	✓						
R11						✓		
R12				✓				
R14		✓		✓				
R16	✓		✓	✓				
R17	✓	✓		✓			✓	✓
R18	✓	✓		✓		✓		
R19	✓			✓			✓	
R20	✓			✓				
R21	✓	✓		✓				
R23	✓	✓		✓	✓			
R24	✓				✓			
R25				✓	✓			
R28	✓	✓		✓	✓			
R29	✓			✓	✓			
R30				✓				
Total no. of isolates	94	41	2	80	15	3	5	1

Table 5: Growth of *Enterobacteriaceae* on Brilliance™ CRE agar and MacConkey agar

Isolates	MacConkey agar (Carbapenem-resistant and carbapenem-susceptible)	Brilliance™ CRE agar (Carbapenem-resistant)
K. pneumoniae	94	38
E. cloacae	41	22
E. aerogenes	2	2
E. coli	80	34
P. mirabilis	15	1
S. liquefaciens	3	0
S. marcescens	5	0
M. morganii	1	0

4.2. KIRBY-BAUER SUSCEPTIBILITY TEST

Of the *Enterobacteriaceae* that grew on the MacConkey agar plates, only *K. pneumoniae* and *E. cloacae* were resistant to carbapenems based on the Kirby-Bauer susceptibility testing (Table 6). Therefore only *K. pneumoniae* and *E. cloacae* isolates were selected for further phenotypic studies.

Based on the EUCAST criteria (2013), 9 *K. pneumoniae* isolates were resistant to carbapenems. These isolates had zone sizes of ≤22 mm for ertapenem, and ≤18 mm for doripenem, and ≤16 mm for imipenem and meropenem (Table 6). Fifty-eight *K. pneumoniae* isolates were resistant to all the cephalosporins and ciprofloxacin. Sixteen *E. cloacae* isolates were resistant to the carbapenems. Seven of these also grew on Brilliance™ CRE agar. All *E. cloacae* isolates were resistant to the cephalosporins, ciprofloxacin, chloramphenicol, nalidixic acid and gentamycin (Table 6).

Table 6: Kirby-Bauer Susceptibility Patterns for *Enterobacteriaceae* using EUCAST Criteria

Isolates (n)	Zone of inhibition (mm)										
	ERT	IMP	MEM	DOR	CTX	FOX	CAZ	C	CIP	*NA	CN
***K. pneumoniae* (2)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***K. pneumoniae* (51)**	≥25	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≥17
***K. pneumoniae* (24)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≤14
***K. pneumoniae* (8)**	≥25	17-21	≥22	≥24	≥20	≤19	18-20	≤17	≤19	≤13	≤14
***K. pneumoniae* (2)**	≥25	≥22	≤16	≥24	≤17	≤19	≥21	≤17	≥22	14-18	≥17
***K. pneumoniae* (7)**	≤22	≥22	≥22	≤18	≤17	≤19	≤17	≤17	≤19	≤13	≤14
***E. coli* (18)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***E. coli* (56)**	≥25	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≥17
***E. coli* (3)**	≥25	≥22	17-21	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≥17
***E. coli* (2)**	21-24	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≤14
***E. coli* (1)**	≥25	17-21	≥22	≥24	18-19	≤19	≥21	≤17	≥22	≥19	≥17
***E. cloacae* (17)**	≥25	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≤14
***E. cloacae* (16)**	21-24	≤16	≤16	≤18	≤17	≤19	≤17	≤17	≤19	≤13	≤14
***E. cloacae* (8)**	21-24	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	≤14
***E. aerogenes* (1)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***E. aerogenes* (1)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***P. mirabilis* (8)**	≥25	≥22	≥22	≥24	≥20	≤19	≥21	≤17	≥22	≥19	≤14
***P. mirabilis* (5)**	≥25	≥22	≥22	≥24	≤17	≤19	≤17	≤17	≤19	≤13	13-16
***P. mirabilis* (1)**	≥25	20-22	≥22	19-23	≥20	≤19	≥21	≤17	≥22	≥19	13-16
***P. mirabilis* (1)**	≥25	≥22	≥22	19-23	≥20	≤19	≥21	≤17	≤19	≤13	≤14
***S. liquefaciens* (1)**	≥25	≥22	≥22	≥23	≥20	≤19	≥21	≤17	≤19	≤13	≥17
***S. liquefaciens* (2)**	≥25	≥22	≥22	≥23	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***S. marcescens* (5)**	≥25	≥22	≥22	≥23	≥20	≤19	≥21	≤17	≥22	≥19	≥17
***M. morganii* (1)**	≥25	17-21	≥22	≥24	≥20	≤19	≥21	≤17	≤19	≥19	≤14

This table represents the organisms identified in this study and the specific susceptibility patterns of these organisms for several antibiotics. The figures in green represent the intermediate diameter; figures in red represent the resistant diameter. ERT: ertapenem, IMP: imipenem, MEM: meropenem, DOR: doripenem, CTX: cefotaxime, FOX: cefoxitin, CAZ: ceftazidime, C: gentamycin, CIP: ciprofloxacin, CN: chloramphenicol. * NA: naladixic acid (present in CLSI guidelines only).

When comparing the susceptibility criteria of the CLSI (2012) and the EUCAST (2013) guidelines, differences were noted (Tables 7a and 7b). Thirty-eight *K. pneumoniae* isolates grew on Brilliance™ CRE agar. Based on the CLSI criteria, 3 were resistant and 3 were intermediate. However, based on the EUCAST criteria, 6 were resistant and 6 were intermediate as per the Kirby-Bauer susceptibility testing. Twenty-two *E. cloacae* isolates grew on Brilliance™ CRE agar. Based on the CLSI criteria, 7 were resistant, and based on the EUCAST criteria 9 were resistant as per the Kirby- Bauer susceptibility testing.

Table 7a: Comparison of the Kirby-Bauer Susceptibility Patterns based on the CLSI and EUCAST Criteria amongst isolates that Grew by Brilliance™ CRE Agar

Isolates	Growth on Brilliance™ CRE agar (carbapenem-resistant)	Kirby-Bauer susceptibility					
		CLSI			EUCAST		
		R	I	S	R	I	S
K. pneumoniae	38	3	3	32	6	6	26
E. cloacae	22	7	7	8	9	6	7

The comparison of the CLSI and EUCAST criteria for the susceptibilities for organisms that grew from MacConkey agar plates, but not on Brilliance™ CRE is shown in Table 7b. Fifty-six *K. pneumoniae* and 19 *E. cloacae* isolates did not grow on the Brilliance™ CRE agar. However, 7 *E. cloacae* isolates were resistant to the carbapenems based on the EUCAST criteria, and 4 were resistant based on the CLSI criteria. Among the *K. pneumoniae* isolates, 2 and 3 isolates were resistant based on the CLSI and EUCAST criteria, respectively.

Table 7b: Comparison of the Kirby-Bauer Susceptibility Patterns based on the CLSI and EUCAST Criteria amongst isolates that were Inhibited by Brilliance™ CRE Agar

Isolates	Isolates that were inhibited by Brilliance™ CRE	Kirby-Bauer susceptibility					
		CLSI			EUCAST		
		R	I	S	R	I	S
K. pneumoniae	56	2	1	53	3	2	51
E. cloacae	19	4	4	11	7	2	10

4.3. MODIFIED HODGE TEST (MHT) AND AMOXYCILLIN-CLAVULANATE (AMC) DOUBLE DISC SYNERGY TEST

Seventy nine percent (30/38) of the *K. pneumoniae* isolates that grew on Brilliance™ CRE and 64% (36/56) that did not grow on the Brilliance™ CRE had a positive MHT result (p = 012), (Table 8a). Among the *E. cloacae* isolates, 91% (20/22) that grew on the Brilliance™ CRE and 79% (15/19) that were inhibited by the Brilliance™ CRE produced a positive MHT result (p=0.27), (Table 8b).

Table 8a: MHT Results for *Klebsiella pneumoniae* (n=94) Stratified by Growth on Brilliance™ CRE Agar

	Modified Hodge Test	
Brilliance™ CRE	**Positive**	**Negative**
Growth	30	8
Growth Inhibited	36	20

Table 8b: MHT Results for *Enterobacter cloacae* (n=41) Stratified by Growth on Brilliance™ CRE Agar

	Modified Hodge Test	
Brilliance™ CRE	**Positive**	**Negative**
Growth	20	2
Growth Inhibited	15	4

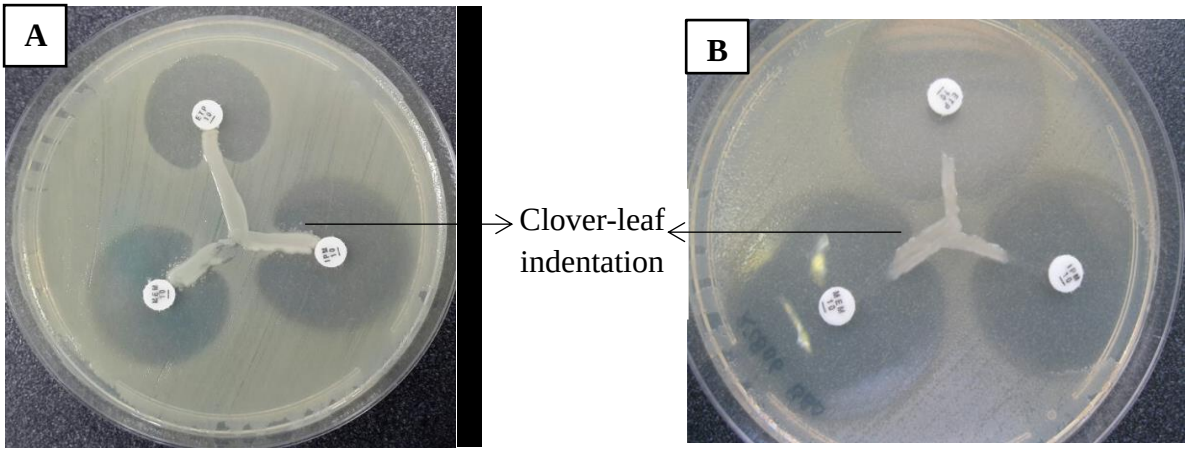

Fig. 7: MHT test results for (A) *E. cloacae* harbouring carbapenemase (positive control) and (B) positive result for test isolate *K. pneumoniae* KP37.

Among the *K. pneumoniae* isolates, a positive AMC double disc synergy test result was significantly higher amongst isolates that grew on the Brilliance™ CRE agar than those that did not [79% (30/38) vs 57% (32/56)] (p = 0.02). For *E. cloacae*, there was no significant difference [55% (12/22) vs 32% (6/19)] (p=0.14), (Tables 9a and 9b).

Table 9a: AMC Double Disc Synergy Test for *Klebsiella pneumoniae* (n=94) Stratified by the Growth on Brilliance™ CRE Agar

	AMC Double Disc Synergy Test	
Brilliance™ CRE	**Positive**	**Negative**
Growth	30	8
Growth Inhibited	32	24

Table 9b: AMC Double Disc Synergy Test for *Enterobacter cloacae* (n=41) Stratified by the Growth on Brilliance™ CRE Agar

	AMC Double Disc Synergy Test	
Brilliance™ CRE	**Positive**	**Negative**
Growth	12	10
Growth Inhibited	6	13

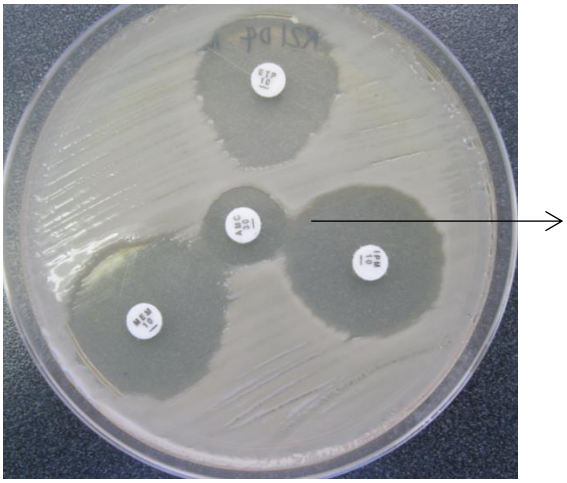

Fig. 8: Positive result for AMC double disc synergy test for test isolate *K. pneumoniae* R21

4.4. MINIMUM-INHIBITORY CONCENTRATION (MIC)

According to the MIC results from the 3 different techniques (microbroth-dilution, E-test and VITEK® 2) performed, the Brilliance™ CRE agar appeared to support the growth of *K. pneumoniae* and *E. cloacae* isolates that produced both resistant and susceptible carbapenem MICs. When comparing these results to the MIC results of the *K. pneumoniae* and *E. cloacae*

isolates that grew on MacConkey agar, some isolates had resistant MICs for the carbapenems, but these isolates did not grow on the Brilliance™ CRE agar (Table 10).

When comparing the CLSI and EUCAST results for the *K. pneumoniae* isolates that grew on the Brilliance™ CRE agar, it was observed that the difference was not as significant. The microbroth-dilution MICs showed that none of the *K. pneumoniae* isolates were resistant based on the EUCAST and CLSI breakpoint criteria's. However, only 1 *K. pneumoniae* had an intermediate MIC based on the CLSI breakpoints but this was not evident based on the EUCAST breakpoints. The E-test results showed that 1 *K. pneumoniae* isolate had a resistant MIC using EUCAST breakpoints and 1 isolate had an intermediate MIC using this breakpoint. However, the latter was not evident based on the CLSI breakpoints, since there was no *K. pneumoniae* isolate that had resistant or intermediate results.

When comparing the EUCAST and CLSI breakpoints for *E. cloacae*, there appeared to be a slight difference in the resistance patterns for the carbapenems. Most of the isolates had intermediate MICs based on the EUCAST breakpoints, but these isolates were resistant based on the CLSI breakpoints. It was observed that the *E. cloacae* isolates that grew from Brilliance™ CRE agar differed significantly when comparing the EUCAST and CLSI breakpoint criteria for all 3 MIC methods. The microbroth-dilution results showed that only 2 *E. cloacae* isolates had resistant MICs based on the EUCAST and CLSI breakpoints. However, 2 *E. cloacae* isolates also had intermediate MICs based on the EUCAST criteria but 8 were intermediate based on the CLSI criteria (Table 10). The E-test results for these organisms showed that 7 had resistant MICs based on the EUCAST breakpoints but 4 were

resistant based on the CLSI breakpoints. The VITEK® 2 results showed that 12 *E. cloacae* isolates were resistant based on both the EUCAST and CLSI breakpoint criteria. However, 5 *E. cloacae* isolates that were intermediate based on the CLSI breakpoints were not resistant based on the EUCAST breakpoints (Table 10).

Table 10: MIC determination for *K. pneumoniae* and *E. cloacae* grown from MacConkey and Brilliance™ CRE agar plate, for the carbapenems

Type of agar	Isolates	Microbroth-dilution						E-Test						VITEK® 2					
		EUCAST			CLSI			EUCAST			CLSI			EUCAST			CLSI		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Isolates inhibited by Brilliance™ CRE agar	*Klebsiella pneumoniae (n=56)*	2	1	53	1	6	49	0	1	55	1	0	55	1	0	55	1	1	54
	Enterobacter cloacae (n=19)	1	2	16	0	3	16	1	4	17	0	5	14	3	0	16	3	2	14
Isolates grown on Brilliance™ CRE agar	*Klebsiella pneumoniae (38)*	0	0	38	0	1	37	1	1	36	0	0	38	1	0	37	1	0	37
	Enterobacter cloacae (n= 22)	2	2	18	2	8	12	7	2	15	4	6	12	12	0	10	12	5	5

Table 11a to 11e show the MICs results for *K. pneumoniae* and *E. cloacae* from the techniques used. All figures in red represent the resistant MICs and those in green represent the intermediate MICs. Only two *K. pneumoniae* isolates had resistant MIC values from the microbroth-dilution method (Table 11a). Isolate KP5 had a resistant MIC value of 1 mg/L for ertapenem and KP37 had resistant MIC values of 4 mg/L for ertapenem, 4 mg/L for doripenem, 16 mg/L for meropenem and 8 mg/L for imipenem.

Table 11a: MIC results from the Microbroth-Dilution Method for 94 *K. pneumoniae* test isolates based on the EUCAST breakpoints

Antibiotics	Dilutions (mg/L)											
	≤0.0625	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**
ERT	85	4	3	0	1	0	1	0	0			
DOR			70	9	13	1	1	0	0	0	0	
MEM				89	1	3	0	0	1	0	0	0
IMP				84	7	2	0	1	0	0	0	0

Four *E. cloacae* isolates were resistant to the carbapenems from the microbroth-dilution (Table 11b). Isolates EC5 and EC6 had the similar results with an intermediate MIC value of 4 mg/L for imipenem. Isolate EC9 had resistant MIC values of 2 mg/L for ertapenem and intermediate MIC values for imipenem (4 mg/L) and doripenem (2 mg/L). Isolate EC12 was resistant to ertapenem with a MIC value of 1 mg/L and an intermediate MIC value of 4 mg/L for imipenem and 2 mg/L for doripenem. Isolate EC21 produced intermediate results for meropenem with a MIC value of 4 mg/L, a resistant MIC value of 2 mg/L for ertapenem and 4 mg/L for doripenem but was susceptible to imipenem.

Table 11b: MIC results from the Microbroth-Dilution Method for 41 *E. cloacae* test isolates based on the EUCAST breakpoints

Antibiotics	Dilutions (mg/L)											
	≤0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128
ERT	27	3	3	5	1	2	0	0	0			
DOR			27	3	7	3	1	0	0	0	0	
MEM				31	7	2	1	0	0	0	0	0
IMP				25	4	8	4	0	0	0	0	0

Based on the E-test method, all *K. pneumoniae* isolates were susceptible with the exception of KP37 (Table 11c). The MIC values for the resistant isolate KP37 had a resistant MIC value of 6 mg/L for ertapenem, 6 mg/ L for doripenem and 8 mg/L for imipenem. Isolate KP5 and KP30 had an intermediate MIC value of 0.75 mg/L for ertapenem.

Eight *E. cloacae* isolates were resistant to carbapenems from the E-tests (Table 11c). Isolates EC5 and EC6 had a resistant MIC value of 1.5 mg/L and 2 mg/L for ertapenem, respectively. Isolate EC9 had an intermediate MIC value of 0.75 mg/L for ertapenem and were susceptible to imipenem and doripenem. Isolate EC12 was resistant to ertapenem with a MIC value of 1 mg/L and an intermediate MIC value of 3 mg/L for imipenem. Isolate EC21 was resistant to ertapenem and doripenem with a MIC of 3 mg/L and 4 mg/L, respectively, and an intermediate MIC value of 3 mg/L for imipenem.

Table 11c: MIC results from the E-Test strips based on the EUCAST breakpoints

Dilutions (mg/L)	*K. pneumoniae* (94)			*E. cloacae* (41)		
	Antibiotics			Antibiotics		
	ERT	DOR	IMP	ERT	DOR	IMP
>32	0	0	0	0	0	0
24	0	0	0	0	0	0
16	0	0	0	0	0	0
12	0	0	0	0	0	0
8	0	0	1	0	0	0
6	1	1	0	0	0	0
4	0	0	0	0	1	0
3	0	0	0	1	0	2
2	0	0	2	3	0	6
1.5	0	0	0	3	1	4
1	0	1	0	1	0	3
0.75	2	0	1	6	0	0
0.5	2	3	0	5	5	4
0.38	3	0	3	2	3	4
0.25	15	1	9	4	6	9
0.19	10	8	22	2	4	9
0.125	6	10	21	3	5	0
0.094	0	21	2	3	5	0
0.064	0	0	0	1	6	0
0.047	2	11	8	0	5	0
0.032	9	8	20	0	0	0
0.023	9	0	0	0	0	0
0.016	7	30	5	7	0	0
0.012	20	0	0	0	0	0
0.008	8	0	0	0	0	0

The VITEK® 2 AES showed that of the 94 *K. pneumoniae* strains isolated, 39 were assumed to harbour the OXA enzyme, 37 were ESBL-positive strains, and 18 harboured both ESBLs and were impermeable. Results from the VITEK® 2 showed that only 2 *K. pneumoniae* isolates were resistant (Table 11d). Isolate KP29 had a resistant MIC of 4 mg/L for ertapenem, but produced susceptible MIC values of 1 mg/L for both meropenem and imipenem. This isolate was assumed to be positive for OXA enzyme according to the AES interpretation. The second isolate that was resistant was the KP37. This isolate had a resistant MIC value of 8 mg/L for ertapenem and susceptible MIC values (2 mg/L) for both meropenem and imipenem. According to the AES interpretation, isolate KP37 was inconsistent with the results from the database. All other *K. pneumoniae* isolates were completely susceptible to meropenem, ertapenem and imipenem.

Table 11d: MIC results from the VITEK® 2 method for 94 *K. pneumoniae* test isolates based on the EUCAST breakpoints

	Dilutions (mg/L)								
Antibiotics	**≤0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**
ERT		92	0	0	1	1	0	0	0
MEM	92	0	1	1	0	0	0	0	
IMP	84	5	4	1	0	0	0	0	

According to the VITEK® 2 AES interpretation, of the 41 *E. cloacae* isolates, 23 harboured MBLs and/ or KPC, 6 were ESBL-positive, and 12 harboured both the ESBLs and AmpC. The VITEK® 2 results showed that 15 *E. cloacae* isolates had resistant MICs. All 15 isolates were only resistant to ertapenem (Table 11e). Fourteen of which had an MIC value of 4 mg/L

and 1 (EC9) had an MIC value ≥8 mg/L. All resistant isolates were assumed to carry the MBL or KPC enzymes according to the AES.

Table 11e: MIC results from the VITEK® 2 method for 41 *E. cloacae* test isolates based on the EUCAST breakpoints

	Dilutions (mg/L)								
Antibiotics	**≤0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**≥64**
ERT		22	4	0	14	1	0	0	0
MEM	21	1	16	3	0	0	0	0	
IMP	8	5	25	3	0	0	0	0	

4.5. GENE DETECTION: MULTIPLEX REAL-TIME PCR

Multiplex real-time PCR were performed on all *K. pneumoniae* and *E. cloacae* isolates that were either intermediate or resistant to the carbapenems from the Kirby-Bauer susceptibilty tests, Microbroth-dilution, E-test and VITEK® 2. A total of 41 test isolates were run, from which only 1 *K. pneumoniae* isolate (KP37) was positive for the $bla_{\text{OXA-48}}$ with a CP value of 20.70 (as shown in Fig. 9). All other isolates from this study were negative for all 6 carbapenemases and their variants (Appendix F, 2).

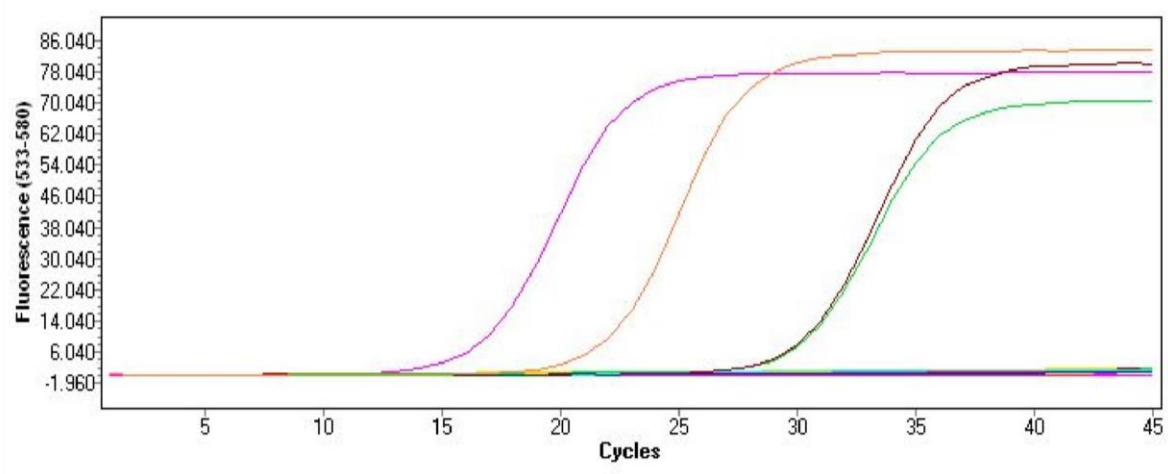

Fig. 9: Amplification curves from Real-time PCR results for *bla*$_{\text{OXA-48}}$: Green and Red amplification curves: OXA-48 positive kit control. Orange amplification curve: NICDs in-house *bla*$_{\text{OXA-48}}$ positive control (*K. pneumoniae* NCTC 13442). Pink amplification curve: test isolate KP37. Flat lines: represent all isolates that were negative for *bla*$_{\text{OXA-48}}$.

CHAPTER FIVE

DISCUSSION

In the past 10 years, the emergence of CRE has spread rapidly worldwide (Drew *et al.*, 2013). The primary mechanisms of carbapenem resistance includes: the development of resistance due to the production of carbapenemases, and impermeability in combination with AmpC or other ESBL activity. CRE poses a serious threat in healthcare settings by causing infections that are virtually untreatable. Carbapenem-producing *Enterobacteriaceae* (CPE) have been implicated in the transmission of resistance genes to various pathogens within the hospital setting, as well as in large mono- or multi-clonal hospital outbreaks (Vrioni *et al.,* 2012). The need for the rapid detection of CPE from colonised patients is imperative. This will assist in the timeous implementation of infection control policies, in an attempt to prevent further dissemination of such resistant pathogens within the hospital setting (Bracco *et al.,* 2013; Vrioni *et al.*, 2012).

Rectal swabs collected from neonates at the KEH VIII hospital were inoculated directly onto the Brilliance™ CRE and ChromID® CARBA agar to evaluate these commercial screening methods for CRE. The Brilliance™ CRE agar plates are used for the presumptive detection of carbapenem-resistant organisms (regardless of mechanism of resistance), and the ChromID® CARBA agar plates are used for the presumptive detection of carbapenemase-producing organisms. The ChromID® CARBA agar are supplemented with specific ingredients that allow for the inhibition of Gram-positive and non-carbapenemase producers, whilst the Brilliance™ CRE agar are supplemented with ingredients that assist in inhibiting Gram-positive organisms only (Vrioni *et al.,* 2012). Both the chromogenic agar constitutes of a

combination of peptones, carbohydrates, several antibiotics and a mixture of chromogenic substrates. The antibiotics favour the selective growth of CREs and CPEs (on the Brilliance™ CRE and ChromID® CARBA agar, respectively) and the chromogenic substrates are indicators of the activity of specific metabolic enzymes that may be used to differentiate *Enterobacteriaceae*.

The Brilliance™ CRE agar contains a modified carbapenem that is compatible with EUCAST and CLSI criteria for the detection of resistance. The organisms that grow on this agar should therefore be resistant to the carbapenems. In this study, a total of 38 *K. pneumoniae* isolates grew on Brilliance™ CRE agar. However, only 6 were resistant to carbapenems based on the Kirby-Bauer susceptibility test and 2 were resistant as per all MIC tests performed.

In comparison, the ChromID® CARBA agar did not appear to support the growth of any organisms. Our results are in contrast to the study by Vrioni *et al.* (2012), who showed that 73 rectal swab specimens were collected, from which 89 isolates grew as greenish blue colonies on ChromID® CARBA agar and were presumed to be CPE. The molecular tests performed on the variety of organisms that grew on the chromogenic media were identified as follows: 54 were KPC-positive *K. pneumoniae,* 25 were VIM-positive *K. pneumoniae* and 6 were KPC-positive *E. aerogenes* strains (Vrioni *et al.*, 2012). The study showed that the ChromID® CARBA agar displayed 97% specificity for the detection of CPE. However, the study was performed in an area where the prevalence of bla_{KPC} and bla_{VIM} producing isolates was high, as compared to our study where CPE production amongst *Enterobacteriaceae* is

uncommon. Since all control strains grew as expected, the results suggested that none of the isolates were carbapenemase producers. The production of carbapenemases are infrequent in South Africa. This was evident from the results reported by NICD-NHLS in 2014, where only a total of 34 *Enterobacteriaceae* were screened for carbapenemase production, of which only 22 were detected as carbapenemase-positive. Ten isolates were positive for NDM, of which only 6 were from a private hospital in KwaZulu-Natal and 4 from public hospitals in the Free-State and Western Cape. Seven isolates harboured the OXA-48 enzyme, of which 6 identified in Gauteng and KwaZulu-Natal and 1 was from a public hospital in Gauteng. Five VIM-positive isolates were detected from public hospitals in Gauteng.

When comparing the growth of carbapenem-resistant isolates on the Brilliance™ CRE agar to MacConkey agar, it appeared that some isolates that grew on MacConkey agar were resistant to the carbapenems but did not grow on the Brilliance™ CRE agar. In a study by Wilkinson *et al.* (2012), when comparing ChromID® CARBA agar and Brilliance™ CRE agar it was observed that the ChromID® CARBA agar was highly sensitive in comparison to the Brilliance™ CRE agar in terms of CPE detection. According to Bracco *et al.* (2013), the Brilliance™ CRE agar was able to support the growth of various carbapenem-resistant and carbapenemase-producing isolates, particularly strains that harboured the KPC, VIM and IMP β-lactamases. However, these isolates were previously characterised on a molecular level before growing them on the chromogenic agar, therefore they were known carbapenemase producers (Bracco *et al.*, 2013).

The results from our study showed that both the chromogenic agars were unreliable for the detection of CREs and CPEs. It may appear that the Brilliance™ CRE agar does allow easy differentiation of bacterial isolates based on the colour of each species. However, *K. pneumoniae* and *Enterobacter* spp. both grew as dark blue, light blue and brown colonies which made it difficult to differentiate one organism from the other. It may be postulated that the chromogenic substrates are not specific for each of these species. According to a study by Day *et al.* (2013), 153 *Enterobacteriaceae* isolates grew on Brilliance™ CRE but did not produce carbapenemases and were not resistant to meropenem. However, 55% of these isolates produced other ESBLs and 12% produced AmpC β-lactamases (Day *et al.,* 2013). It was concluded that the presence of other ESBL producers as well as AmpC non-carbapenemase producers may grow on Brilliance™ CRE agar. This supports our results where, despite the 38 *K. pneumoniae* and 22 *E. cloacae* isolates that grew on Brilliance™ CRE agar, only 3 *K. pneumoniae* and 7 *E. cloacae* were resistant to the carbapenems by the MIC tests.

Carbapenem antimicrobial agents have been used extensively in clinical settings as a result of the emergence and spread of MDR strains that produce ESBLs (Drew *et al.,* 2013). In our study, 9 *K. pneumoniae* isolates were resistant to carbapenems based on the Kirby-Bauer susceptibility test: 7 to ertapenem and doripenem, and 2 were resistant to meropenem only (Table 6). The latter isolates were also resistant to the cephalosporins, ciprofloxacin, chloramphenicol and naladixic acid. These results are comparable to a study performed by Day *et al.* (2013), where 37 *Enterobacteriaceae* harbouring the *bla*NDM-1 were resistant to ertapenem, cefotaxime, ciprofloxacin and other drugs used in the study, but were susceptible

to meropenem and imipenem. However, doripenem could not be compared since it was not used for susceptibility testing in the latter study.

Sixteen *E. cloacae* isolates in this study were resistant to meropenem, imipenem and doripenem; and 8 were intermediate resistance to ertapenem (Table 6). The current study was similar to a study performed by Drew *et al.* (2013), who reported on the isolation of 7 carbapenemase producing *Klebsiella* sp., and 11 *Enterobacteriaceae* producing AmpC/ ESBL in combination with decreased permeability to the outer membrane. The carbapenemase producers were more resistant to cephalosporins and carbapenems in comparison to those that harboured the AmpC/ ESBL and had decreased permeability as a resistance mechanism (Drew *et al.*, 2013). Drew *et al.* (2013) also showed that the prevalence of carbapenemases was limited in comparison to the production of the AmpC/ ESBL activity and impermeability mechanisms by *Enterobacteriaceae*. This was evident since, 71% of the patients that harboured 76% *Enterobacter* spp., 12% *Klebsiella* spp., and 12% *Serratia* spp., were resistant to carbapenems due to the presence of AmpC/ ESBL activity and impermeability whereas only 29% *Klebsiella* sp. harboured bla_{NDM} and bla_{KPC} carbapenemases (Drew *et al.*, 2013).

The susceptibility profiles of *K. pneumoniae* and *E. cloacae* isolates, when comparing the EUCAST with CLSI guidelines, did not always concur. According to the CLSI guidelines, a zone diameter of ≤18 mm for ertapenem, and ≤22 mm for meropenem, imipenem and doripenem, should be used to classify an organism as being resistant. However, according to the EUCAST guidelines a zone of inhibition diameter of ≤22 mm for ertapenem, ≤16 mm for

meropenem and imipenem, and ≤18 mm for doripenem, is classified as resistant. Therefore, some isolates in this study that were intermediate or even susceptible based on the CLSI criteria, were classified as resistant according to the EUCAST guidelines. Due to such differences in the inhibitory zone sizes when comparing the EUCAST and CLSI criteria for susceptibility testing, the detection of carbapenem resistant isolates could go unnoticed, therefore the possible detection of carbapenemases will be overlooked.

The MHT and AMC double disc synergy test have been reported to be used for the detection of carbapenemase production. In our study the MHT was used to determine the presence of various carbapenemases produced by the test isolates, evident by a clover-leaf indentation pattern (as shown in Fig, 7). It should also be stated that the isolates that produced carbapenemases, and in turn were positive for the MHT, should have been resistant to at least 1 carbapenem agent. However, in this study the results obtained from the MHT showed that the clover-leaf indentation appeared for isolates that had diverse susceptibility patterns for the carbapenems based on the Kirby-Bauer susceptibility test. These results were similar to a study by Cury *et al.* (2012), where the MHT was only used for the detection of KPC carbapenemases among *Enterobacteriaceae*. In the latter study, 7 isolates showed positive MHT results but were negative for *bla*KPC. It was therefore postulated that these isolates harboured other types of carbapenemases, produced CTX-M or hyperproduced AmpC (Cury *et al.,* 2012). This is in contrast to results observed in a study by Castanheira *et al.* (2011), where 11 of 15 NDM-1 producing isolates were negative and weakly positive for the MHT. All other carbapenemase producers showed positive results for the MHT. The MHT may therefore be problematic for the detection of the NDM-1 strains due to false-negative results. It is unreliable as a screening test for CPE and will not always be able to guide the

implementation of strict infection control measures. False-negative results will allow the CPE to go unnoticed and will allow the continuous spread of these organisms (Castanheira *et al.,* 2011).

In other literature, the MHT was used for the specific detection of KPC and NDM β-lactamases from pre-defined and characterised bacterial species (Cardile *et al.,* 2014; Cury *et al.,* 2012; Girlich *et al.,* 2012; Ribeiro *et al.,* 2014). This might be the reason for the high level of agreement with the molecular detection techniques (Cardile *et al.,* 2014; Cury *et al.,* 2012; Girlich *et al.,* 2012; Ribeiro *et al.,* 2014). However, according to Thomson (2010), the MHT does not have the ability to differentiate between carbapenemase types and lacks sensitivity for the detection of MBLs. Furthermore, Ribeiro *et al.* (2014), explained that even though the MHT has >90% sensitivity, it was difficult to interpret and is prone to subjectivity. This was evident in the present study, since all test isolates that produced even the slightest indentation was taken as positive. However, according to Centers for Disease Control and Prevention (CDC), some isolates may produce a slight indentation but still lack carbapenemases. This was further proven by Ribeiro *et al.* (2014), in a study were the sensitivity and specificity of the MHT was performed for the detection of KPC. Positive results from the MHT was dependent on the enzyme activity, and the results showed that a larger and enhanced growth intensity (indentation) would occur with a stronger hydrolytic activity of the enzyme produced by the test organisms (Ribeiro *et al.,* 2014). According to Thomson (2010), accurate results may have been observed if this test was performed according to the 'true' MHT method on MacConkey agar or carbapenem discs impregnated with zinc. False-positive results for the MHT normally arise due to high-level AmpC producers, and more specifically with an imipenem rather than the other carbapenems. In

screening tests it is uncertain which carbapenem disc will provide optimum results for the MHT. However, imipenem is the least specific agent but is the most sensitive for the detection of OXA enzymes (Thomson, 2010).

A positive result from the AMC double disc synergy test has been reported to indicate the presence of the *bla*$_{KPC}$. An augmented zone of inhibition around the carbapenem disc indicated resistance to the carbapenems as a result of carbapenemase activity (as shown in Fig. 8). However, similar to the results obtained from the MHT, isolates that were both resistant and susceptible to the carbapenems appeared positive for the AMC double disc synergy test. This test should assist in the elimination of isolates that produce AmpC β-lactamases, since isolates with this enzyme usually confer resistance to clavulanic acid (β-lactamase inhibitor) which is present in the AMC disc (Kocsis and Szabó, 2013). Although this test may not present false-positive results, the presence of AmpC interference may still occur due to the organism harbouring other ESBLs. This is because other ESBLs (apart from carbapenemases) like SHV, TEM or CTX-M, are inhibited by clavulanic acid (Kocsis and Szabó, 2013).

K. pneumoniae and *E. cloacae* isolates were selected for MICs based on their prevalence as well as the susceptibility patterns for all antibiotics tested. MICs are considered the 'gold-standard' for determining the susceptibility of microorganisms to antimicrobial agents, and is therefore used to evaluate the performance of other susceptibility tests (Andrews, 2001). These methods provide definitive results if a borderline result is obtained from other phenotypic methods of testing (Andrews, 2001).

When comparing the CLSI breakpoints to the EUCAST breakpoints it was evident that the CLSI breakpoints reported resistance at a lower level than the EUCAST breakpoints. The resistant CLSI breakpoints for the carbapenems were as follows: ≤8 mg/L for ertapenem, ≤16 mg/L for imipenem and ≤16 mg/L for meropenem. There was no CLSI breakpoint for doripenem. The EUCAST resistant breakpoints for the carbapenems were as follows: ≥1 mg/L for ertapenem, ≥4 mg/L for doripenem, and ≥8 mg/L for imipenem and meropenem. A total of 12 isolates were resistant to the carbapenems based on the EUCAST breakpoints. However, based on the CLSI breakpoints, it was observed that only one isolate, *E. cloacae* EC9 (≥8 mg/L for ertapenem from VITEK® 2) was resistant from all the *K. pneumoniae* and *E. cloacae* isolates tested. This implied that the difference in the breakpoints were significant. It was evident that if the CLSI guidelines were used, isolates that may have been resistant to the carbapenems would not have been identified.

The E-test was performed for ertapenem, imipenem and doripenem (E-tests for meropenem were not performed due to the suppliers (Biomerieux, France) experiencing quality control problems and the batches produced being recalled. The new batches will only be available in 2015). Only meropenem, imipenem and ertapenem MICs were performed based on the VITEK® 2 automated system, since doripenem is unavailable for this system. Among the *K. pneumoniae* isolates, only KP37 was resistant to all carbapenems based on the microbroth-dilution and E-test methods, but was only resistant to ertapenem based on the VITEK® 2. The E-test and the VITEK® 2 produced the same MIC for ertapenem. However, the E-test showed greater resistance for ertapenem and doripenem since the MIC increased from 4

mg/L to 6 mg/L for both the antibiotics, in comparison to the microbroth-dilution method. The MICs for ertapenem for isolate KP5 differed when comparing the 3 methods. The MIC values for this isolate was 1 mg/L, 0.75 mg/L and 0.5 mg/L for the microbroth-dilution, E-test and VITEK® 2, respectively. Only one other *K. pneumoniae* isolate (KP29) was resistant to ertapenem based on the VITEK® 2 results but was susceptible based on the microbroth-dilution and E-test. In a study performed by Brink *et al.* (2013), the MICs for most OXA-48 producing *K. pneumoniae* fell within the range of 16 to 32 mg/L for all 4 carbapenems which was slightly greater than that observed in the present study for KP37. However, isolate KP37 produced much higher levels of resistance in comparison isolates KP5 and KP29, which was probably due to these organisms producing chromosomally encoded resistance mechanisms.

The *E. cloacae* isolates appeared to show more resistance than *K. pneumoniae*, since a total of 11 isolates had resistant MIC values. The MICs of the *E. cloacae* isolates differed significantly when comparing the VITEK results to the microbroth-dilution and E-test results. The MIC for ertapenem for isolate EC5 was 1.5 mg/L and 1 mg/L based on the VITEK® 2 and E-test, respectively. The MIC for ertapenem for isolate EC6 was 2 mg/L and 4 mg/L based on the VITEK and E-test, respectively. However, both these isolates were susceptible to ertapenem, with a similar MIC value of 0.5 mg/L, based on the microbroth-dilution test. Isolate EC9 was only resistant to ertapenem based on the microbroth-dilution and VITEK® 2 but was intermediate based on the E-test results. The VITEK® results showed that isolate EC9 had a MIC value ≥8 mg/L for ertapenem, which was 6 mg/L and 7.25 mg/L greater than the microbroth-dilution and E-test, respectively. Several other *E. cloacae* isolates were resistant based on the VITEK® 2, but were susceptible based on the microbroth-dilution and E-test. However, from the VITEK® 2 AES it was assumed that all these resistant isolates

produced carbapenemases, which was not the case. The MIC results from this study were much lower than MIC values produced in a study by Sianou *et al.* (2012). In the latter study the microbroth-dilution was used to determine the MICs of isolates that harboured the VIM β-lactamase. The MICs for the carbapenemase positive *K. pneumoniae* were 16 mg/L, 16 mg/L and 4 mg/L for imipenem, meropenem and ertapenem respectively; and 4 mg/L, 4 mg/L, and 2 mg/L for these carbapenems for carbapenemase positive *E. cloacae* (Sianou *et al.*, 2012).

The E-test appeared to be more accurate for the lower dilutions since this went down to a MIC value of 0.008 mg/L. The majority of *K. pneumoniae* and *E. cloacae* isolates in this study were susceptible and fell within the range of 0.5 mg/L to 0.008 mg/L. In all 3 MIC methods used, it was evident that the isolates were more frequently resistant to ertapenem than to imipenem, meropenem or doripenem. The opposite result was obtained in a study by Viau *et al.* (2012), who found that the MICs for ertapenem are more sensitive to *K. pneumoniae*, however this antibiotic lacks specificity and therefore further testing is required. According to Brink *et al.* (2013b), the OXA-48-like producers in South Africa were non-susceptible to ertapenem and had a MIC value of 2 mg/L. These isolates may confer only a slight increase in carbapenem MICs due to weak hydrolytic activity in the absence of related mechanisms (Brink *et al.*, 2013b).

In literature, Livermore *et al.* (2012), stated that the MIC values obtained from the E-tests frequently differed from the automated VITEK® 2 system. In a study by McGettigan *et al.* (2009), the VITEK® 2 was 65% and 48% sensitive to imipenem and meropenem,

respectively. This proved to be insensitive for the aforementioned antimicrobial agents, which was similar to the current study. Similarly, Brink *et al.* (2012a), showed that the automated system had low sensitivity (74%) and specificity (38%) for the identification of carbapenemases. In the current study, the MICs from the VITEK® 2 were similar in comparison to the microbroth-dilution and E-tests for the *K. pneumoniae* isolates. Isolate KP37 was the only strain that had similar resistant results for all 3 MIC methods for ertapenem.

The VITEK® 2 is an automated system that is used as an ESBL confirmatory test and is referred to as an expert system which should distinguish ESBLs from other β-lactamases (Thomson *et al.*, 2007). According to Brink *et al.* (2012b), this system is normally used for routine diagnostic testing since it should provide interpretations that do not require further laboratory screening, which was evident in the current study. However in a study by Thomson *et al.* (2007), this was not the case since the VITEK® 2 system seemed to be more complicated as the mechanism of resistance needs to be identified prior to set-up. In addition, some isolates may not be detected accurately by the machine resulting in continuous looping and repetition of the test and results obtained are usually inconclusive (Thomson *et al.*, 2007). According to the results from our study, it was evident that the VITEK® 2 interpretation of MICs is inaccurate in comparison to the other MIC tests used. This is because the AES compares the test isolates to its database. This database comprises of previously described reports that had various mechanisms of resistance for specific isolates with specific MICs. Therefore if the results from a test isolate differs from that found in the database, the AES will interpret the result as inconsistent, which was similar to the result for *K. pneumoniae* KP37 in this study.

The phenotypic tests in this study produced unreliable results, possibly due to the presence of other ESBLs. None of the phenotypic tests performed had the ability to determine if the isolates harboured a carbapenemase-type enzyme or had the ability to detect which type of carbapenemase was present. Therefore, molecular detection may be the only rapid and reliable means for the detection of carbapenemases. In this study, from the 94 *K. pneumoniae* and 41 *E. cloacae* isolates and of the six carbapenemases tested, only a *K. pneumoniae* strain (KP37) was a carbapenemase producing isolate, which harboured a $bla_{\text{OXA-48}}$ enzyme. The strain was isolated from MacConkey agar and did not grow on either of the chromogenic plates. Similarly, in a study performed by Wilkinson *et al.* (2012), Brilliance™ CRE agar and ChromID® CARBA agar were unable to support the growth of OXA-48 producers.

The KP37 was resistant to ertapenem, ciprofloxacin, naladixic acid, cefoxitin, cefotaxime, ceftazidime and chloramphenicol based on the Kirby-Bauer susceptibility test. According to Poirel *et al.*, (2012b), isolates that produce the OXA-48 enzymes vary in terms of β-lactam resistance patterns: some isolates are susceptible to broad-spectrum cephalosporins and carbapenems, some are susceptible to broad-spectrum cephalosporins but are resistant to carbapenems, and others are resistant to broad-spectrum cephalosporins and carbapenems. The MHT results for EC37 were positive for all 4 carbapenems and negative for the AMC double disc synergy test, which was expected since the AMC inhibitor is presumably positive only for KPC production. The KP37 strain was also resistant to all carbapenems from the microbroth-dilution and E-tests which seemed reliable. However, according to Wilkinson *et al.* (2012) and Nordmann *et al.* (2012), the detection of these strains are difficult since they normally produce low carbapenem MICs and/ or are susceptible to cephalosporins when there is no coproduction of ESBL or AmpC enzymes. Furthermore, Poirel *et al.* (2012a) reported

that the production of carbapenemases should be suspected among *Enterobacteriaceae* that have MIC values of ≥ 0.5 mg/L for ertapenem, and ≥ 1 mg/L for imipenem or meropenem.

The OXA-type enzymes are predominantly observed among *Acinetobacter* sp., and has only recently been described in *K. pneumoniae* and *E. coli* (Kocsis and Szabó, 2013). Recently in South Africa, 38 *Enterobacteriaceae* were screened, of which 25 were positive for carbapenemases. These CPE were most commonly detected in *K. pneumoniae* and *E. cloacae*. Among these CPE, 19 were NDM-1 producers: 3 were from a private hospital in KwaZulu-Natal, and 16 were from public hospitals in Gauteng. The β-lactamase OXA-48 producers were also detected: 1 from a private hospital in Gauteng and 3 from public hospitals in Gauteng and Eastern Cape (NICD-NHLS, 2014).

The search for CPEs that have reduced susceptibility to carbapenems is imperative due to the paucity of therapeutic options for the treatment of infections caused by such isolates (Poirel *et al.,* 2012a). In addition, there appears to be inadequate knowledge of the spread of *in vivo* mutants with an enhanced level of resistance to carbapenems by means of other resistance mechanisms (Poirel *et al.,* 2012a). Several *K. pneumoniae* and *E. cloacae* isolates in this study appear to be resistant to the carbapenems but were negative for carbapenemase production. These isolates could harbour other variants of carbapenemases that were not detected based on ESBL RT-PCR kits or may have developed alternate mechanisms of resistance such as the production of plasmid mediated AmpC enzymes, efflux pumps or outer-membrane proteins. In literature, isolates that overexpress the plasmid mediated AmpC enzymes confer resistance to extended-spectrum cephalosporins including cefotaxime,

ceftazidime, and ceftriaxone, which were similar resistance patterns observed in the non-carbapenemase producers in our study. Several Gram-negative bacilli have the ability to produce chromosomally mediated AmpC which when hyperproduced, may lead to resistance against penicillin, aztreonam, and cephamycins (Thomson, 2010). Isolates that have chromosomes and plasmids that encode the AmpC enzyme, have the ability to hydrolyse broad-spectrum cephalosporins more efficiently (Kocsis and Szabó, 2013).

The rapid detection of carbapenemases, more specifically KPC, NDM-1 and OXA-48 enzymes as a result of the increased prevalence in South Africa, is crucial in order to introduce appropriate treatment, and implement suitable infection control measures (Coetzee and Brink, 2011). To our knowledge, the *bla*$_{\text{OXA-48}}$ detected among neonates in this study was the first to be detected in KwaZulu-Natal. This is a cause for concern since OXA-48 is one of the most prevalent carbapenemases transmitted and appears to be increasing rapidly worldwide. The screening of colonised neonates is imperative for the identification of patients that may be a potential threat to others via horizontal gene transfer (Lowman *et al.*, 2014). In order to do so, there needs to be a significant microbial load, in combination with the mode of transmission (Lowman *et al.*, 2014). It is possible that the primary reason for the transmission of CPE is due to contact between hospital personnel and neonates as a result of inappropriate hand hygiene practices. Therefore the implementation and adherence to basic infection control measures are necessary to reduce and eventually prevent the spread of infections caused by CPE production among neonates.

CHAPTER SIX

CONCLUSION

Based on South African publications, it is evident that colonised patients that have not been screened for CPE, unknowingly allow for the transmission of these organisms. Therefore the recognition of these colonised patients is essential for the implementation of correct infection control measures in order to reduce the risk of transmission. Various risk factors associated with the dissemination of CPE in NICU need to be taken into account, this includes low birth weight, prematurity, duration of hospitalization, use of invasive devices and prolonged administration of antibiotics (Bonura *et al.*, 2013).

Controlling the transmission and reducing the impact of CPE in South Africa will require serious efforts in public and private healthcare sectors in order to move forward. This case differs from one in South Africa, where a patient inhabited with an OXA-48 producing *K. pneumoniae* isolate was colonised with this strain throughout hospitalisation. This encouraged the screening for genes among *Enterobacteriaceae* that were resistant to carbapenems. In 2013, 240 ertapenem-non-susceptible isolates were screened, 33 of which were positive for $bla_{\text{OXA-48}}$ isolated from clinical cultures and rectal carriages (Govind *et al.*, 2013). Although the current study only revealed a single neonate colonised with an OXA-48 carbapenemase, this showed that CPE are in fact in the nursery environment at King Edward VIII Hospital. CPEs are a substantial threat to neonates and are associated with high mortality rates.

The microbroth-dilution test using EUCAST criteria appears to be the best test for MIC determination. However, more robust tests are required for the rapid detection of CPEs directly from the specimen of patients.

CHAPTER SEVEN

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APPENDICES

APPENDIX A: BREC approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 August 2013

Prof P Moodley
719 Umbilo Road
Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley

PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and Outbreak Investigation. REF: BE222/13.

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 03 May 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 31 July 2013 to queries raised on 19 July 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 07 August 2013.

This approval is valid for one year from **07 August 2013.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **10 September 2013.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

APPENDIX B:

Table 1: The duration of specimen collection from 30 neonates at KEH VIII hospital

Ward	Laboratory number
Observation 1	R1 to R15
New Premature nursery	R16 to R23
Old Premature nursery	R24 to R30

Table 2: The duration of specimen collection from 30 neonates at KEH VIII hospital

Ward	Lab No.	Date of collection (2013)																					
		28/08	20/08	30/08	13/08	1/09	2/09	3/09	4/09	5/09	6/09	9/09	10/09	11/09	12/09	16/09	17/09	18/09	19/09	20/09	23/09	25/09	26/09
Observation 1	R 1	X	D																				
	R 2	X	D																				
	R 3	X	D																				
	R 4	X	D																				
	R 5	X	X	D																			
	R 6	X	X	X	X	X	X	X	X	X	X	D											
	R 7	X	D																				
	R 8	X	D																				
	R 9	X	X	D																			
	R 10	X	D																				
	R 11	X	X	D																			
	R 12	X	X	D																			
	R 13	X	D																				
	R 14	X	X	X	X	X	X	D															
	R 15	X	D																				
New Prem Nursery	R 16	X	X	X	X	X	X	X	X	X	X	X	D										
	R 17	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	D				
	R 18	X	X	X	X	X	X	D															
	R 19	X	X	X	X	X	X	X	X	X	X	D											
	R 20	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	D
	R 21	X	X	X	X	X	X	X	X	X	X	X	X	D									
	R 22	X	-																				
	R 23	X	X	X	X	X	X	X	X	X	X	X	D										
Old prem Nursery	R 24	X	X	D																			
	R 25	X	D																				
	R 26	X	X	D																			
	R 27	X	X	D																			
	R 28	X	X	X	X	X	X	X	X	X	X	D											
	R 29	X	D																				
	R 30	X	X	X	X	X	X	X	X	X	X	X	D										

*'X' refers to the days the neonate stayed in hospital. 'D' refers to the day on which the neonate was discharged.

APPENDIX C:

1. Preparation of media used in this study:

1.1. MacConkey agar (CM0007)

MacConkey agar powder	52 g
Distilled water	1 L

Prepared by adding 52 g of powder into 1 L of distilled water. This was mixed until powder was thoroughly dissolved and autoclaved at 121 °C for 15 min.

1.2. Mueller-Hinton agar (CM0405)

Mueller-Hinton powder	21 g
Distilled water	1 L

Prepared by adding 21 g of powder into 1 L of distilled water. This was mixed until powder was thoroughly dissolved and autoclaved at 121 °C for 15 min.

1.3. Mueller-Hinton broth (CM0337)

Mueller-Hinton broth powder	38 g
Distilled water	1 L

Prepared by adding 38 g of powder into 1 L of distilled water. This was mixed until powder was thoroughly dissolved and autoclaved at 121 °C for 15 min.

1.4. Blood agar

Columbia Blood agar base powder	39 g
Distilled water	1 L
Horse blood	50 ml

Thirty nine grams of Columbia Blood agar base (CM0331) powder was dissolved into 1 L of distilled water. This was mixed until powder was thoroughly dissolved and autoclaved at 121 °C for 15 min. After autoclaving, agar was cooled down to 55 °C in a water bath. Only when agar reached this temperature was the horse blood (50 ml/L) added to the agar and mixed.

1.5. Storage media

BHI (Brain Heart Infusion) broth	12 g
Distilled water	400 ml
Glycerol	100 ml
Glass beads	

Twelve grams of BHI was dissolved into 400 ml of distilled water. Thereafter, 100 ml of glycerol was added and autoclaved at 121 °C for 15 min. Three quarter of the cryovials was filled with polysterene beads. Five hundred microlitres of the storage media was dispensed into the cryovials with beads.

APPENDIX D:

Table 3a: Biochemical Test Reactions and results using the API 20E Kit

Biochemical tests	Active ingredients	Reaction or enzyme	Results	
			Negative	Positive
ONGP	2-nitrophenyl-βD-galactopyranoside	β-galactosidase (Ortho NitroPhenyl-βD-Galactopyranosidase)	Colourless	yellow[1]
ADH	L-arginine	Arginine Dihydrolase	Yellow	red/ orange[2]
LDC	L-lysine	Lysine Decarboxylase	Yellow	red/ orange[2]
ODC	L-ornithine	Orthinine Decarboxyase	Yellow	red/ orange[2]
CIT	trisodium citrate	Citrate utilisation	pale green/ yellow	blue-green/ blue[3]
H_2S	sodium thiosulfate	H2S production	colourless/ greyish	black deposit/ thin line
URE	urea	Urease	Yellow	red/ orange[2]
TDA	L-tryptohane	Tryptophane Deaminase	Yellow	reddish brown
IND	L-tryptohane	Indole production	colourless/ pale green/ yellow	pink
VP	sodium pyruvate	acetoin production (Voges Proskauer)	colourless	pink/ red[4]
GEL	gelatine (bovine origin)	Gelatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	*fermentation/ oxidation (glucose)	blue/ blue-green	yellow
MAN	D-mannitol	*fermentation/ oxidation (mannitol)	blue/ blue-green	yellow
INO	inositol	*fermentation/ oxidation (inositol)	blue/ blue-green	yellow
SOR	D-sorbitol	*fermentation/ oxidation (sorbitol)	blue/ blue-green	yellow
RHA	L-rhamnose	*fermentation/ oxidation (rhamnose)	blue/ blue-green	yellow
SAC	D-sucrose	*fermentation/ oxidation (saccharose)	blue/ blue-green	yellow
MEL	D-melibiose	*fermentation/ oxidation (melibiose)	blue/ blue-green	yellow
AMY	amygdalin	*fermentation/ oxidation (amygdalin)	blue/ blue-green	yellow
ARA	L-arabinose	*fermentation/ oxidation (arabinose)	blue/ blue-green	yellow

* Fermentation starts in the lower portion of the tubes, oxidation starts in the cupule

[1]Avery pale yellow is considered a positive reaction

[2]An orange colour after incubation for 35-48 h is considered negative

[3]Reading made in the cupule (aerobic)

[4]A slightly pink colour after 10 min is considered negative

Table 3b: Biochemical Characterisation of *Enterobacteriaceae* using the API 20E Kit

Biochemical tests	*Klebsiella pneumoniae*	*Enterobacter cloacae*	*Enterobacter aerogenes*	*E. coli*	*Proteus mirabilis*	*Serratia liquefaciens*	*Serratia marcescens*	*Morganella morganii*
ONGP	+	+	+	+	–	+	+	–
ADH	–	+	–	–	–	+	–	–
LDC	+	–	+	+	–	+	+	–
ODC	–	+	+	–	+	+	+	+
CIT	+	+	+	–	–	+	+	–
H_2S	–	–	–	–	+	–	–	–
URE	+	–	–	–	+	–	–	+
TDA	–	–	–	–	+	–	–	+
IND	–	–	–	+	–	–	–	+
VP	+	+	+	–	–	+	+	–
GEL	–	–	–	–	+	+	+	–
GLU	+	+	+	+	+	+	+	+
MAN	+	+	–	+	–	+	+	–
INO	+	–	–	–	–	+	+	–
SOR	+	+	–	–	–	+	+	–
RHA	+	+	–	+	–	+	–	–
SAC	+	+	–	–	–	+	+	–
MEL	+	+	–	+	–	+	+	–
AMY	+	+	–	–	–	+	+	–
ARA	+	+	–	+	–	+	–	–

APPENDIX E:

Microbroth-dilution:

1.1. Calculations of antibiotic stock solutions:

Weight of powder = [highest concentration (µg/ml)] x [volume (ml)]

(potency µg/mg)

= powder weight (µg/ml)

Note: increase by x10 concentration if non-toxic or by x100 concentration if toxic.

Diluent required = [weight of powder (µg/ml)] x [volume of diluent (ml)]

Note: A 1:10 dilution was prepared from the antibiotic stock to bring the concentration back to a 1x concentration.

APPENDIX F:

1. DNA Extraction Solutions

1.1. 10x TE Buffer

Trizma base (Sigma-Aldrich, USA) 1.21g

EDTA (Sigma-Aldrich, USA) 0.37g

Weigh out the required amounts of reagent powders and dissolve the trizma base in 80ml of distilled water, pH to 8 using concentrated HCl (Merck). Add the EDTA and dissolve. Check the final pH and adjust to a final volume of 100ml. Autoclave at 121°C for 15 minutes.

Preparation of 1x TE Buffer:

Dilute 10ml of 10x TE Buffer into 90ml of triple distilled water. Store at room temperature.

1.2.Preparation of Mastermix

Table 4a: RT-PCR reaction for KPC, NDM and OXA-48 and variants

Mastermix components	**Quantity**
Probes Master	10.0 µl
Lightmix Modular NDM-1 (ESBL)	0.5 µl
Lightmix Modular OXA-48(ESBL)	0.5 µl
Lightmix Modular KPC (ESBL)	0.5 µl
Lightmix Modular PhHV internal control	0.5 µl
PCR-grade water	3.0 µl
Total Mastermix volume	**15.0 µl**

Table 4b: RT-PCR reaction for IMP, VIM and GES and variants

Mastermix components	**Quantity**
Probes Master	10.0 µl
Lightmix Modular IMP (ESBL)	0.5 µl
Lightmix Modular VIM (ESBL)	0.5 µl
Lightmix Modular GES (ESBL)	0.5 µl
Lightmix Modular PhHV internal control	0.5 µl
PCR-grade water	3.0 µl
Total Mastermix volume	**15.0 µl**

Table 4c: Components required in the final multiplex real-time PCR reaction

Mastermix volume	15.0 µl
Volume of DNA	5.0 µl
Total reaction volume	**20.0 µl**

2. Cycle conditions for multiplex real-time PCR using the Light Cycler 480 II

Table 5: Cycle Conditions for carbapenemases

	Temperature (°C)	Acquisition mode	Time (min)	Ramp rate (°C/s)	Cycles
Denaturation	95	None	5	4.4	1
Quantification	95	None	15	4.4	45
	60	None	30	2.2	
	72	Single	2	4.4	
Cooling	40	None	30	2.2	1

3. Remaining illustrations for multiplex real-time PCR:

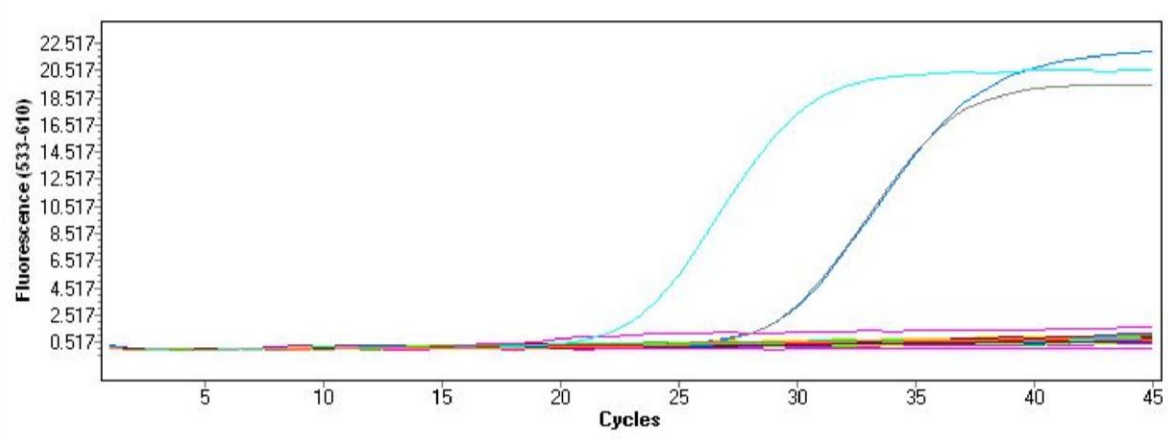

Fig. 1a: Real-time PCR results for *bla*$_{KPC}$: test isolates 1 to 24. Blue amplification curve: NICDs in-house KPC positive control (*K. pneumoniae* ATCC BAA1705). Grey and Dark Blue amplification curve: KPC positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{KPC.}$

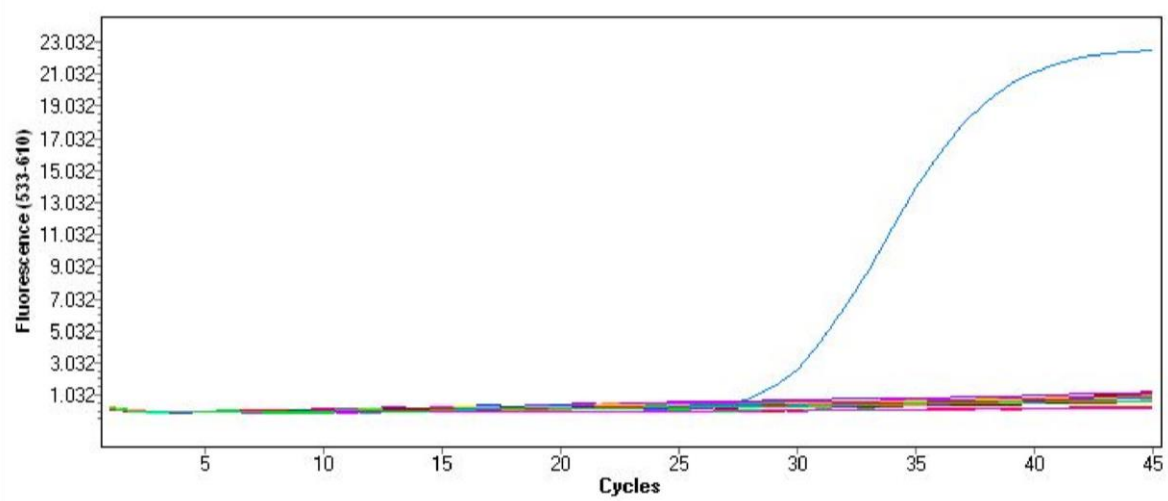

Fig. 1b: Real-time PCR results for *bla*$_{KPC}$: test isolates 25 to 47. Blue amplification curve: KPC positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{KPC.}$

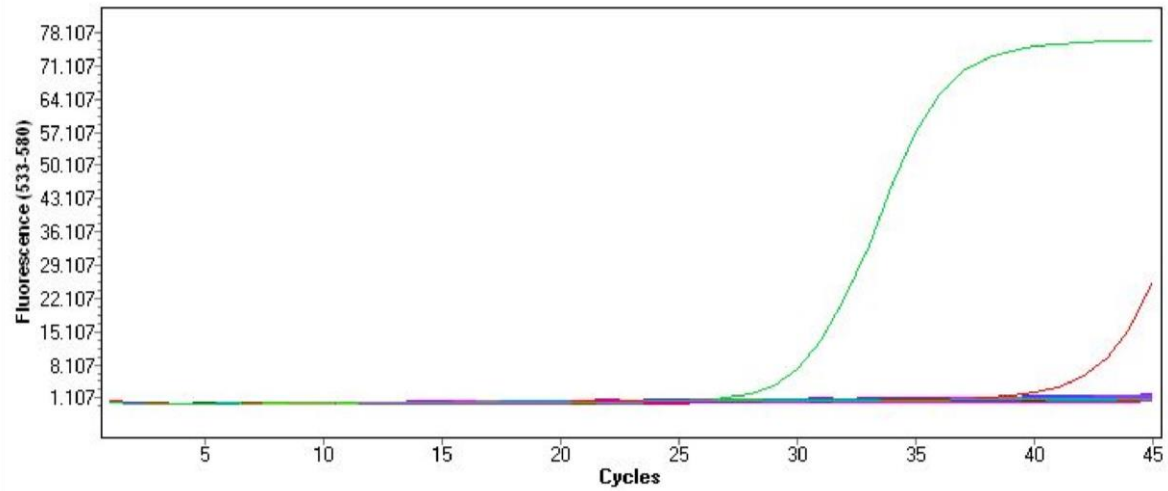

Fig. 2a: Real-time PCR results for *bla*$_{OXA-48}$ for 23 to 47: Green amplification curve: OXA-48 positive kit control. Red amplification curve: test isolate 46 which is negative (CP >40.00). Flat lines: represent all isolates that were negative for *bla*$_{OXA-48.}$

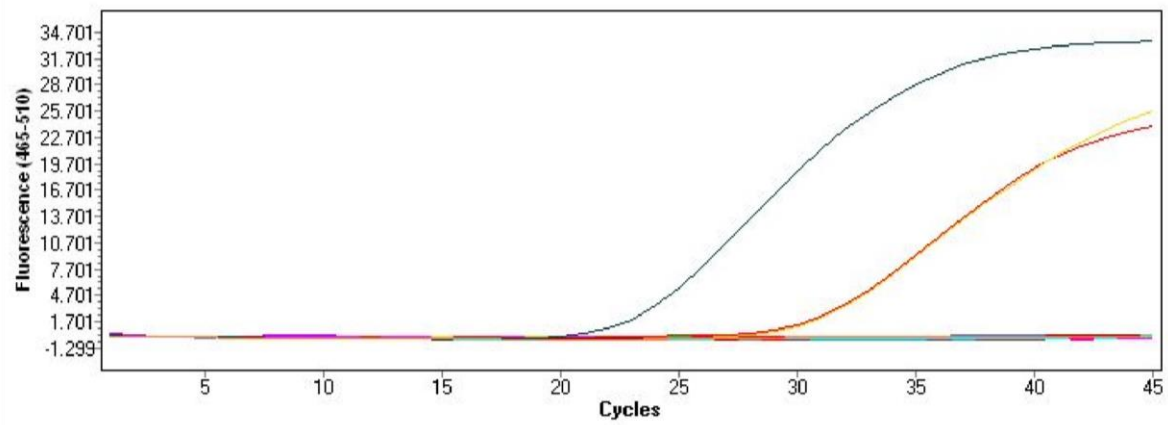

Fig. 3a: Real-time PCR results for *bla*$_{\text{NDM-1}}$: test isolates 1 to 24. Grey amplification curve: NICDs in-house KPC positive control (*K. pneumoniae* ATCC2146). Yellow and Red amplification curve: NDM-1 positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{\text{NDM-1..}}$

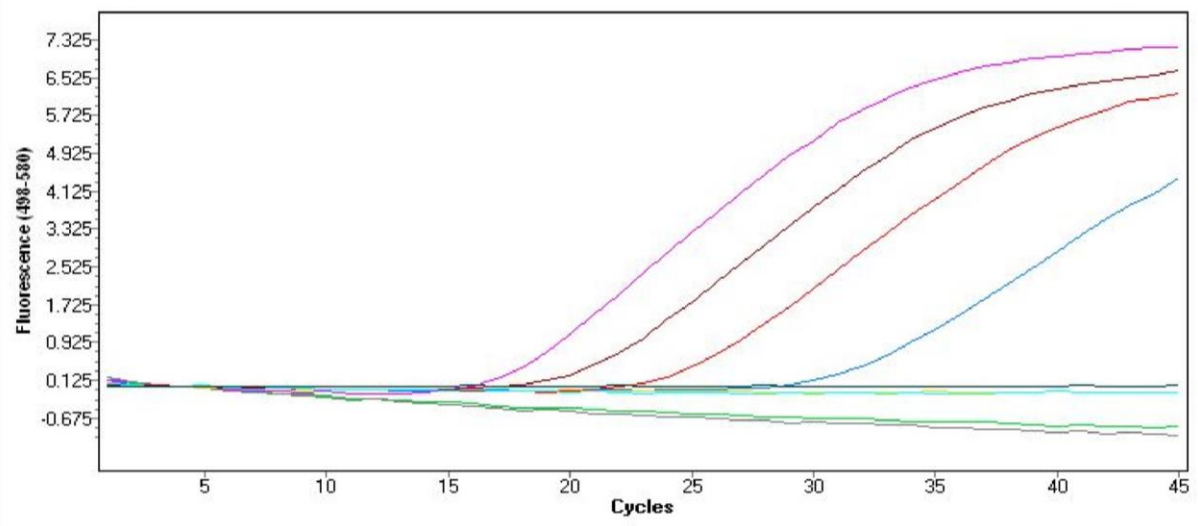

Fig. 3b: Real-time PCR results for *bla*$_{\text{NDM-1}}$: Bright red amplification curves: NDM-1 positive kit control. Blue amplification curve: NICDs in-house *bla*$_{\text{NDM-1}}$ *K. pneumoniae* ATCC2146). Dark red amplification curve: environmental test isolate 43 (Bucket A). Flat lines: represent all isolates that were negative for *bla*$_{\text{NDM-1..}}$

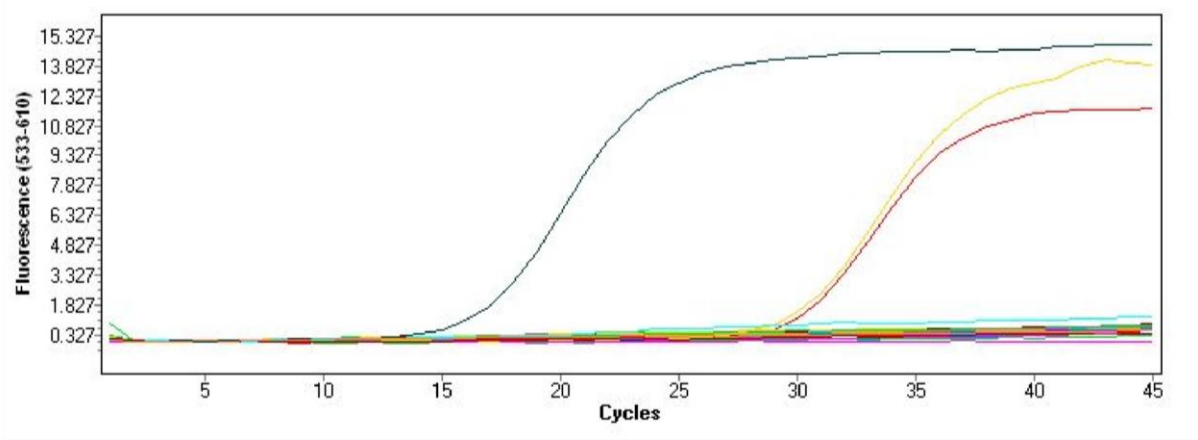

Fig. 4a: Real-time PCR results for *bla*$_{VIM}$: test isolates 1 to 24. Dark blue amplification curve: NICDs in-house VIM positive control (*Pseudomonas aeruginosa* clinical strain). Yellow and Red amplification curve: VIM positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{VIM.}$

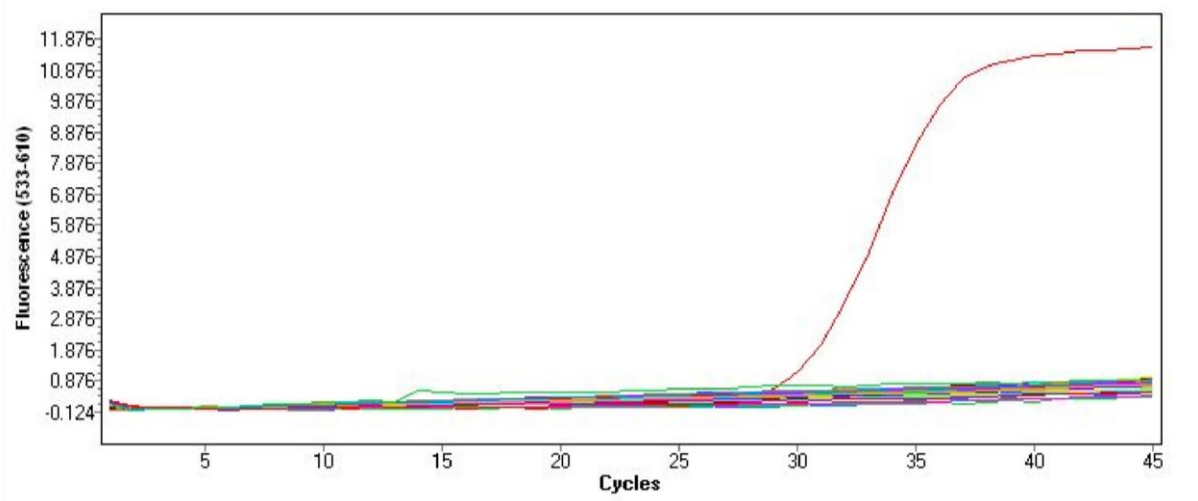

Fig. 4b: Real-time PCR results for *bla*$_{VIM}$: test isolates 25 to 47. Red amplification curve: VIM positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{VIM.}$

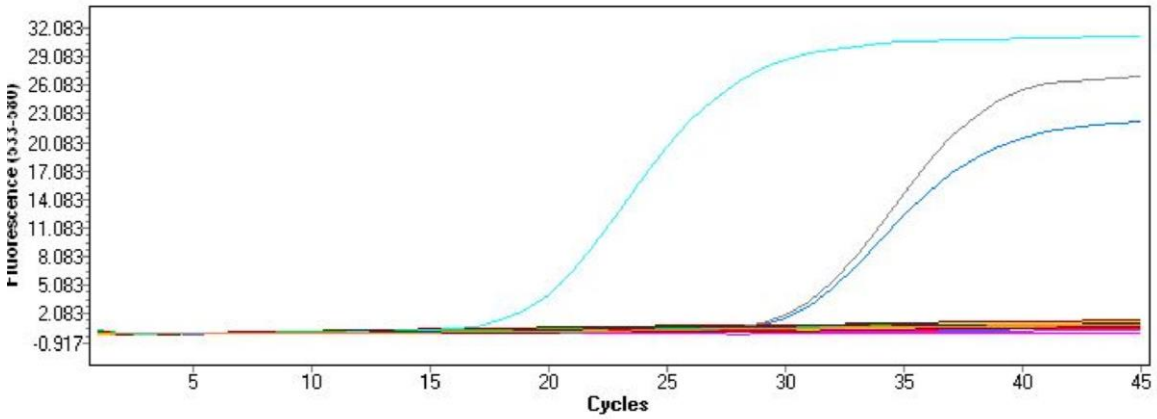

Fig. 5a: Real-time PCR results for *bla*$_{IMP}$: test isolates 1 to 24. Light Blue amplification curve: NICDs in-house IMP positive control (*E. coli* NCTC 13476). Dark Blue and Grey amplification curve: IMP positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{IMP.}$

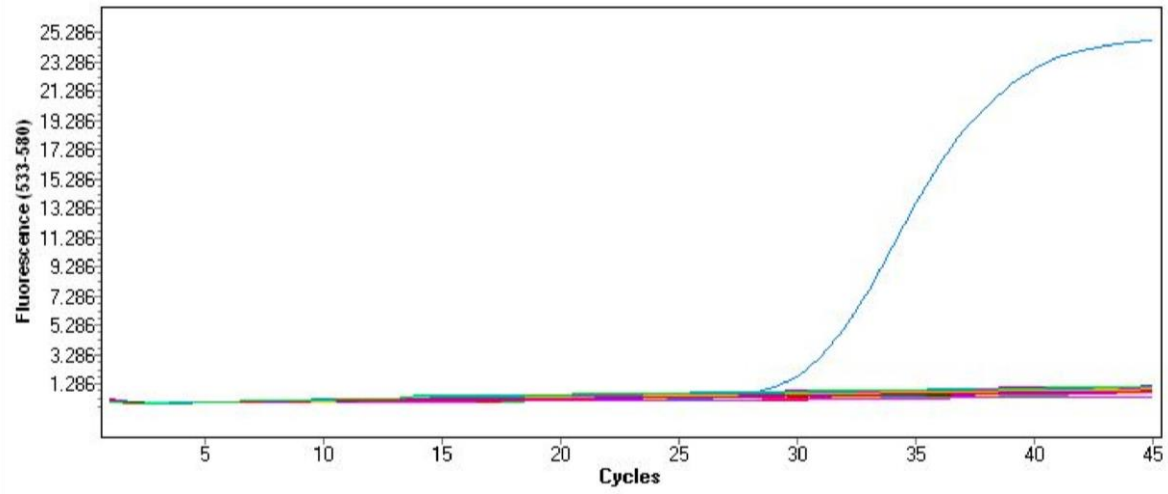

Fig. 6a: Real-time PCR results for *bla*$_{IMP}$: test isolates 25 to 47. Blue amplification curve: IMP positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{IMP.}$

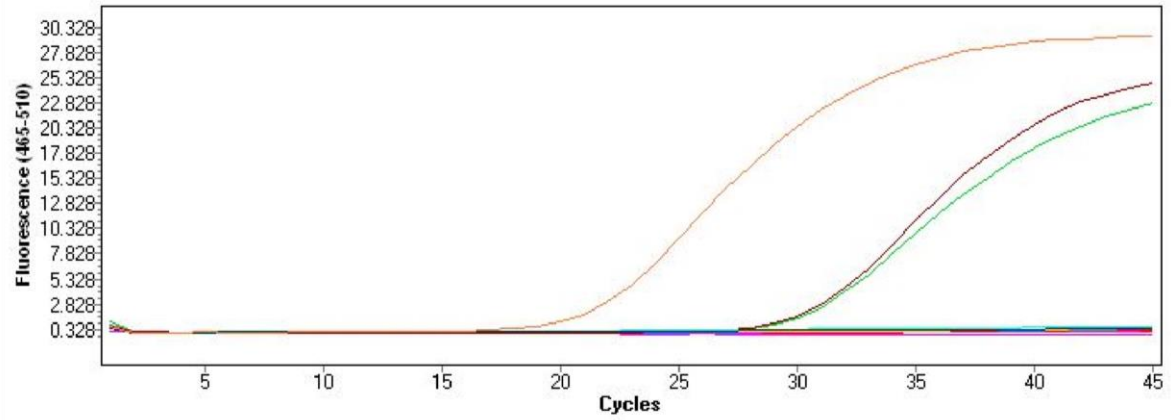

Fig. 6b: Real-time PCR results for *bla*$_{GES}$: test isolates 1 to 24. Orange amplification curve: NICDs in-house GES positive control (*K. pneumoniae* clinical strain). Red and Green amplification curve: GES positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{GES.}$

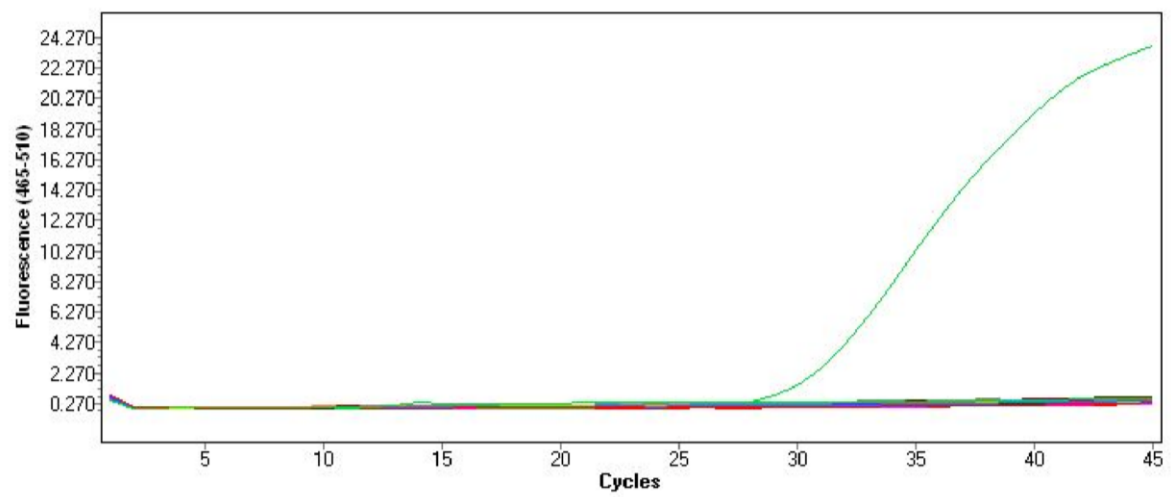

Fig. 7a: Real-time PCR results for *bla*$_{GES}$: test isolates 25 to 47. Green amplification curve: IMP positive kit control (Roche, Switzerland). Flat lines: represent all isolates that were negative for *bla*$_{GES..}$