Invitro antibacterial activity of imidazole and triazole-based antimicrobials against Carbapenem Resistant *Enterobacteriaceae*

Thesis submitted in fulfilment of the requirements for the degree of

Masters of Technology

Biomedical Technology

By

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Declaration

I, Siyabonga Protus Radebe (student number: 21556512) hereby declare that this dissertation is the outcome of my own research, undertaken while registered for MTech: Biomedical Technology in the Department of Biomedical and Clinical Technology at Durban University of Technology. This dissertation has not previously formed the basis for the award of any certificate, diploma or degree of this Institute or any other university. I have duly acknowledged in the text all the sources used in the preparation of this dissertation.

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Signature of student Date of signature

Mr S P Radebe

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Signature of co-supervisor Date of signature

Ms T S Ndlovu

Abstract

Introduction

The frequency of serious bacterial infections has increased due to the high prevalence of HIV infection, contributing to the increasing rates of multi-drug organisms which include carbapenem-resistant *Enterobacteriaceae* (CRE). This trend has become a serious challenge to the health care system of South Africa, resulting in the higher use of immunosuppressive and cytotoxic drugs to treat serious bacterial infections. Optimal treatment for infections caused by CRE is yet unknown. The benefits of imidazole and triazole antimicrobials have become very topical due their diverse spectrum of pharmacological properties, but its efficacy against bacterial infections has not been tested in the South African context.

Aim

The primary aim of this study was to determine the antibacterial effects of selected imidazole and triazole-based antimicrobials against Carbapenem-Resistant *Enterobacteriaceae*.

Methodology

Different concentrations of test drugs (ketoconazole, metronidazole and fluconazole) were used to prepare sensitivity disks and four pathogenic strains of Carbapenem Resistant *Enterobacteriaceae* (*K. pneumonia*, *E. coli*, *S. marcescens* and *C. freundii*) obtained from Lancet Laboratory in Durban were used to determine the antibacterial activity of the selected test drugs, using Disk Diffusion, Modified Agar Diffusion and Minimum Inhibition Concentration (MIC) method, described by Bauer *et al*. 1966.

Results

Antimicrobial Susceptibility Testing revealed that, test drugs selected for this study have no inhibition activity against CRE test organisms and biochemical tests also showed that imidazole and triazoles antimicrobials have no adverse effects on the CRE organisms.

Conclusion

Although the results obtained in this study indicated no activity of against CRE, laboratory studies are still necessary in modification of the imidazole and triazoles to synthesize derived drugs to confirm and optimize the antibacterial potency of these compounds.

Keywords: ketoconazole; fluconazole; metronidazole; carbapenem-resistant *Enterobacteriaceae*

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List of Abbreviations

α	Alpha
Amp C	Ampicillin C
AMR	Antimicrobial Resistance
API	Analytical Profile Index
B. subtilis	*Bacillus subtilis*
ß	Beta
C. freundii	*Citrobacter freundii*
CLSI	Clinical & Laboratory Standards Institute
CRE	Carbapenem Resistant Enterobacteriaceae
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
ESBL	Extended Spectrum Beta Lactamases
G –ve	Gram negative
G +ve	Gram positive
IMP	Imipenemase
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapemases
MBC	Minimum Bactericidal Concentration
MBL	Metallo Beta Lactamases

mg	MilliGrams
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NDM	New Delhi Metallo-beta-lactamase
SA	South Africa
S. marcescens	*Serratia marcescens*
ul	Microliters
VIM	Verona Integron–encoded Metallo-beta-lactamase

Chapter one: Introduction

For several decades, antimicrobial agents have saved the human species from bacterial infections. However, since the introduction of penicillin in 1928, antimicrobial resistance has been a major concern and this has increased the frequency of infections with high rates of multi-drug resistant organisms which include Carbapenem-Resistant *Enterobacteriacea*e (CRE) (Vardakas *et al*. 2012).

Carbapenems have been valuable antimicrobial agents and the last class of β-lactam antibiotics retaining near-universal inhibitory activity against *Enterobacteriaceae* (Nordmann *et al.* 2011). *Enterobacteriaceae* have now emerged to be resistant to many antibiotics, including the carbapenems (Bilavsky *et al*. 2010), therefore leaving a very limited number of treatment options for patients infected with these strains (Schwaber and Carmeli 2008: Wiener *et al* 2010). These carbapenem resistant *Enterobacteriaceae* have been detected in South Africa (Brink *et al*. 2011) and this trend has become a challenge to the health care system of South Africa as it has contributed to the increased irrational use of immunosuppressive and cytotoxic drugs to treat serious these bacterial infections.

CRE may cause serious invasive infections, especially bacteremia and urosepsis, and are associated with high mortality rate (Schwaber and Carmeli, 2008). Patients diagnosed with CRE infections are treated under conditions of contact isolation and during hospitalizations, surveillance cultures are obtained and these patients are treated under identical isolation conditions (Ben-David *et al.* 2010). Currently the duration of CRE carriage following hospitalization discharge is unknown and there are no new antimicrobial agents developed to combat CRE (Huttner *et al*. 2013).

A recent study by Marchaim *et al*. showed that mortality was higher for patients infected with imipenem-resistant KPC- producing *Enterobacter* species (11 of 33 patients) than for those infected with imipenem-susceptible strains (3 of 33 patients), (Marchaim *et al.* 2008). The epidemiology of CRE varies because of diversity in the carbapenemase enzymes that these bacteria produce (Zarfel *et al*. 2011). The Australian Commission on Safety and Quality in Health Care (2013) noted that a range of enzymes with different structural characteristics have developed, seemingly independently, in different parts of the world, underlying the need for population-specific investigations in this area. Studies from the USA, Europe, Asia and the Middle East have described the detection of CRE infection in children (Dirajlal-Fargo *et al*. 2014). Age at presentation ranged between 0 to 18 years, and the main types of infection were bloodstream infection, urinary tract infection and soft-tissue infection (Dirajlal-Fargo *et al*. 2014).

Currently, the "gold standard" treatment of CRE infections has not been established in clinical trials, but combination therapy with two active drugs (Colistin, tigecycline, and intravenous fosfomycin) has been recommended (Kanj and Kanafani, 2011). Recent data from United States have explored alternate treatment options with the view to treat these highly resistant strains. Results have been largely ineffective although combination therapy which includes colistin, tigecycline, and fosfomycin remains the only promising option (Perez *et al*. 2013). However, these options are limited due to the lack of clinical data on their efficacy and colistin resistant strains have been detected, rendering infections which are virtually untreatable (Kanj and Kanafani, 2011). This calls for further investigation of combination therapy and optimization of systemically active antimicrobial extracts as an alternative means to combat this global challenge.

The main aim of this study is to determine the effects of imidazole and triazole-based antimicrobials against the CRE, by testing in-vitro antibacterial activity of these antimicrobials against these strains and therefore answer the call for a concerted global commitment in the development of alternative therapies which are desperately needed. The initiative to investigate already existing drugs as an alternative treatment for CRE is urgently needed, especially in South Africa, where pharmaceutical resources are limited and newly developed antimicrobials are usually expensive.

The goal of in-vitro antibacterial susceptibility testing in this study was to provide a reliable prediction of how CRE is likely to respond on imidazole and triazoles antimicrobials. At the time of this study, the researcher was not aware of any South African studies done to investigate alternate methods for treatment of these CRE, which therefore warrants this study.

1.1 Aim of the study

The aim of this study was to determine the antibacterial effects of imidazole and triazoles against the CRE, by testing in-vitro antibacterial activity of these antimicrobials against selected strains of CRE and therefore answer the call for a concerted global commitment in the development of alternative therapies which are desperately needed

The initiative to investigate already existing drug as an alternative treatment for CRE is urgently needed, especially in South Africa, where pharmaceutical resources are limited and newly developed antimicrobials are usually expensive. For this study, the antibacterial activity of was determined using Kirby Bauer Disk Diffusion, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) techniques, and the biochemical effects of imidazole and triazoles against CRE was observed by comparing results from biochemical reactions of CRE before and after exposure to these antimicrobials.

1.2 Research Objectives

1.2.1 First objective

To impregnated blank discs with test antimicrobials and determine the *in vitro* antibacterial activity on carbapenem-resistant *Enterobacteriaceae* (*Klebsiella pneumoniae, Escherichia coli, Serratia marcescens* and *Citrobacter freundii*) using Kirby Bauer Disc diffusion method.

1.2.2 Second Objective

To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of each test antimicrobial on the selected test organisms, using tube dilution method.

1.2.3 Third objective

To evaluate adverse effects of imidazole and triazoles on the selected CRE by comparing the results of cultural changes and biochemical reactions of the test organisms before and after exposure to selected test antimicrobials.

1.3 Hypothesis

A compound that interferes with DNA synthesis in a fungal cell will have adverse effects on the DNA activity of the bacteria.

Chapter two: Literature Review

2.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is a global healthcare challenge that limits the therapeutic options for infectious diseases caused by a variety of organisms including Gram-negative, Gram-positive bacteria, fungi or mycobacteria (Egglestone *et al.* 2010). The increased use of antimicrobial agents in recent years has resulted in the development of resistance to these agents and this has heightened the interest in the study of antimicrobial resistance (Bilavsky *et al*. 2010). Bacterial strains acquire resistance mostly due to antibiotic misuse and inadequate access to drugs especially in financially disadvantaged countries, consequentially yielding an ineffective therapy of severe bacterial infections (Oboho, 1984) as this is also an economic burden on the healthcare system since resistant infections not only cost more to treat, but also can prolong healthcare use.

Currently, antimicrobial resistance is a serious threat to infectious disease management local and globally. Increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world including developing countries (Byarugaba, 2005). Many pathogenic organisms have developed some degree of resistance to antimicrobials and they confer resistance through different modes of action, with a negative impact on available treatment options (Murray, 1991: Levy, 1992). The significant clinical implication of this resistance has led to heightened interest in the study of antimicrobial resistance from all possible aspects.

Resistance may occur through one of four processes: immunity and bypass; target modification; efflux; and enzyme-catalyzed destruction (Wright, 2010). The human gut flora can normally transfer resistance genes among themselves (Davison, 1999: Finlay and Falkow, 1997). However, this type of gene transfer becomes a significant threat when these harmless normal floras transform into pathogens (Manges *et al* 2001). The mechanisms of resistance to antimicrobials have been studied extensively at a molecular level (figure 1.1) and have involved investigations of the genetics and biochemistry of many different aspects of bacterial cell function (Alekshun and Levy, 2007).

Antimicrobial use is the most significant factor responsible for increased antimicrobial resistance (Byarugaba, 2005) and other reasons for increasing resistance levels include the following:

- Prolonged hospitalization, increased number and duration of intensive care unit stays
- Increased use of invasive medical devices and catheters
- Grouping of colonized patients in long-term-care facilities
- Noncompliance with infection control principles

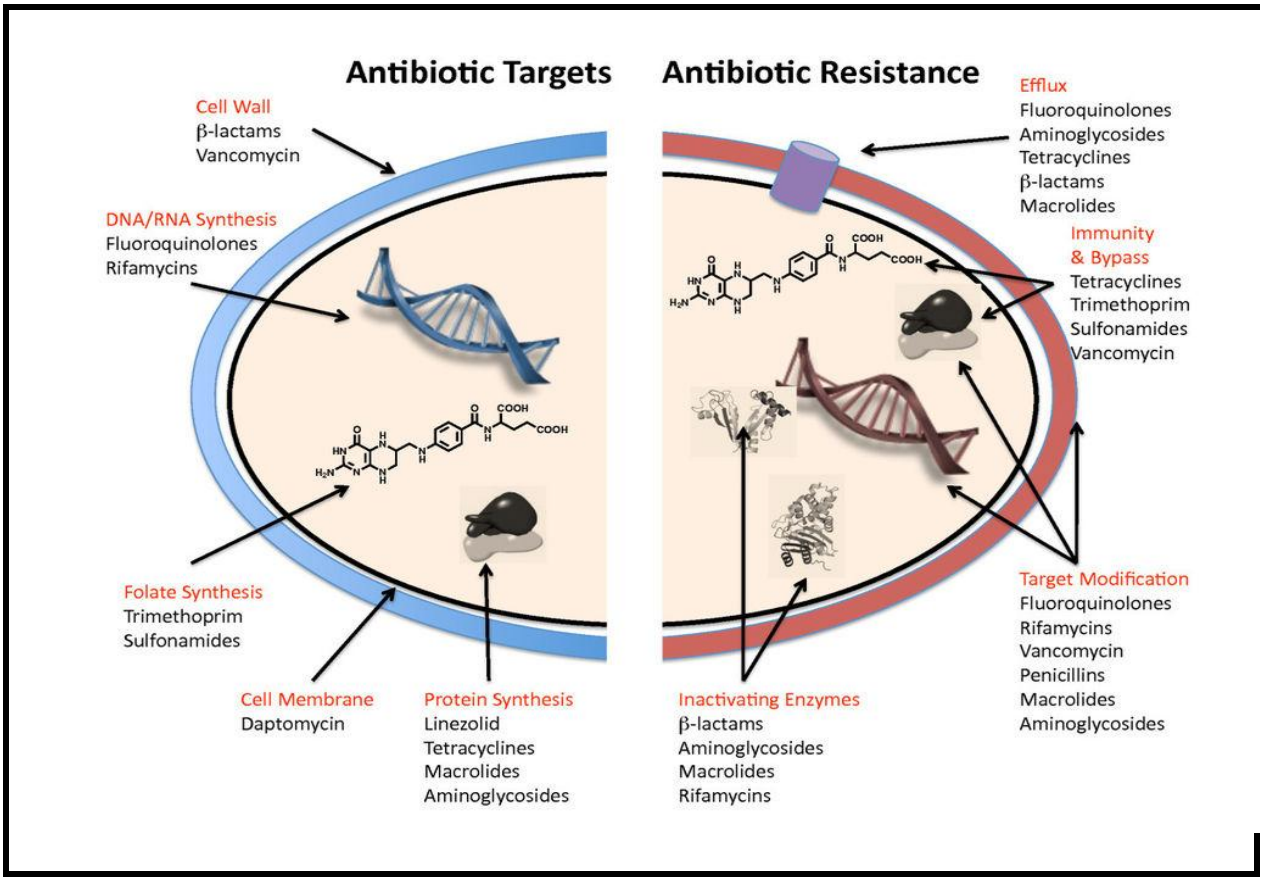

Figure 1.1 Antibiotic Resistance Mechanisms (Wright, 2010), indicating the different modes of antibacterial action and resistant development mechanisms by different classes of antibiotics.

2.2 Extended Spectrum Beta lactamases (ESBL)

Enterobacteriaceae resistance has developed from the production of beta-lactamases, which was first described in the early 1960s (TEM-1) (Datta and Kontomichalou, 1965), then extended spectrum beta-lactamases (ESBLs) producers, which was later detected in 1979 (Sanders and Sanders, 1979) and the latest, commonly referred to as Carbapenem Resistant *Enterobacteriaceae* (CRE) which was first reported in the late 1990s. These enzymes open the beta-lactam ring, inactivating the antibiotic.

Since the discovery of the first ESBL-producing *K. pneumoniae,* Gram-negative bacteria expressing ESBLs have posed a very serious therapeutic challenge (Knothe *et al*. 1983). The ESBL demonstrate plasmid and integron-mediated propagation, which has now increased the resistance rates of nosocomial pathogens (Paterson, 2005). The plasmid-mediated beta-lactamases in Gram-negative bacteria was first discovered in Greece in the 1960s and they confer resistance to most beta-lactam antibiotics, including penicillins, cephalosporins, and the monobactam aztreonam. Infections with ESBL-producing organisms have been associated with poor outcomes (Bradford, 2001).

2.3 Carbapenem Resistant *Enterobacteriaceae*

Carbapenems are the last class of β-lactam antibiotics developed in the early 1980's and nearly all *Enterobacteriaceae* were susceptible to carbapenems (Papp-Wallace *et al*. 2011). They diffuse easily into bacteria and have a very wide spectrum of activity and they are active against many Gram-negative, Gram-positive as well as anaerobic bacteria (Kesado *et al*. 1980). These antibiotics act on the bacterial cell by targeting the penicillin-binding proteins (PBPs) (Yang *et al*. 1995) and they are recommended first-line treatment for severe infections caused by *Enterobacteriaceae* producing extended-spectrum b-lactamases (ESBLs) (Paterson, 2000).

Carbapenems have been agents of last resort in treating life-threatening infections caused by drug-resistant bacteria (Nordmann *et al*. 2011; Schwaber *et al*. 2011). *Enterobacteriaceae* have now emerged that are resistant to many antibiotics, including the carbapenems and these organisms are referred to as Carbapenem Resistant *Enterobacteriaceae* (CRE) (Bilavsky *et al*. 2010). The b-lactamases are considered as the primary cause of bacterial resistance to these drugs (Davies, 1994).

The emergence of carbapenem-hydrolysing beta-lactamases has threatened the clinical utility of the carbapenem antibiotic class and this results in the global challenge of "extreme drug resistance" in Gram-negative bacilli (Doi and Paterson, 2007). Recently, the widespread outbreaks of CRE have been increasingly reported (Vardakas *et al*. 2012). In South Africa, the first case of CRE was reported in 2011 (Brink *et al*: 2012). This global spread of CRE has become a major challenge in clinical and public health settings, as these organisms cause infections which are extremely difficult to treat. Severe infections with CRE are associated with significant morbidity, mortality, and health-care costs (Bilavsky *et al*. 2010). The genes encoding carbapenemases are usually found on plasmids or other mobile genetic elements which allow the organism to acquire genes conferring resistance to other classes of antimicrobials, such as aminoglycoside-modifying enzymes and fluoroquinolone-resistance determinants, and beta-lactamases (Endimiani *et al*. 2009). Bacterial genes that code for this type of resistance can be easily transferred between bacteria, rapidly spreading and evolving the antimicrobial resistance (Canton *et al*. 2012). Therefore, resulting in CRE isolates being increasingly multidrug-resistant (resistant to three or more classes of antimicrobials), extensively drug-resistant (resistant to all but one or two classes) and or pan drug-resistant (resistant to all available classes of antibiotics) (Magiorakos *et al*. 2012). Outbreaks and epidemics of infections related to these organisms have been reported, and a high mortality rate has been ascribed to these infections (Nordmann *et al*. 2011).

The bacteria possessing enzyme β-lactamases emerged in the late 1960s, and reduced the efficacy of penicillin, and thereafter an intensive search for β-lactamase inhibitors began. By 1976 the first such inhibitors had been discovered - these olivanic acids were metabolites produced by the Gram-positive bacterium *Streptomyces clavuligerus* (Papp-Wallace *et al*. 2011).

Carbapenemases from three relevant classes, class A*; Klebsiella pneumoniae* carbapenemases (KPC), class B; OXA-type and class B; metallo-beta-lactamases (MBL), have been isolated in South Africa (figure 1.2) (Brink *et al*. 2011). The first CRE invasive infections in South Africa were reported in adult patients from Gauteng Province in 2011, caused by New Delhi metallo-β-lactamases (NDM-1) and KPC-2-expressing *K. pneumoniae* isolates (Brink *et al.* 2012). The emergence of this high resistance among *Enterobacteriaceae* is of major concern in South Africa, especially with *Klebsiella pneumoniae*, which has been reported in both public sector (55-74%) and (55-60%) in the private sector (Bamford *et al*. 2011; Brink *et al*. 2008).

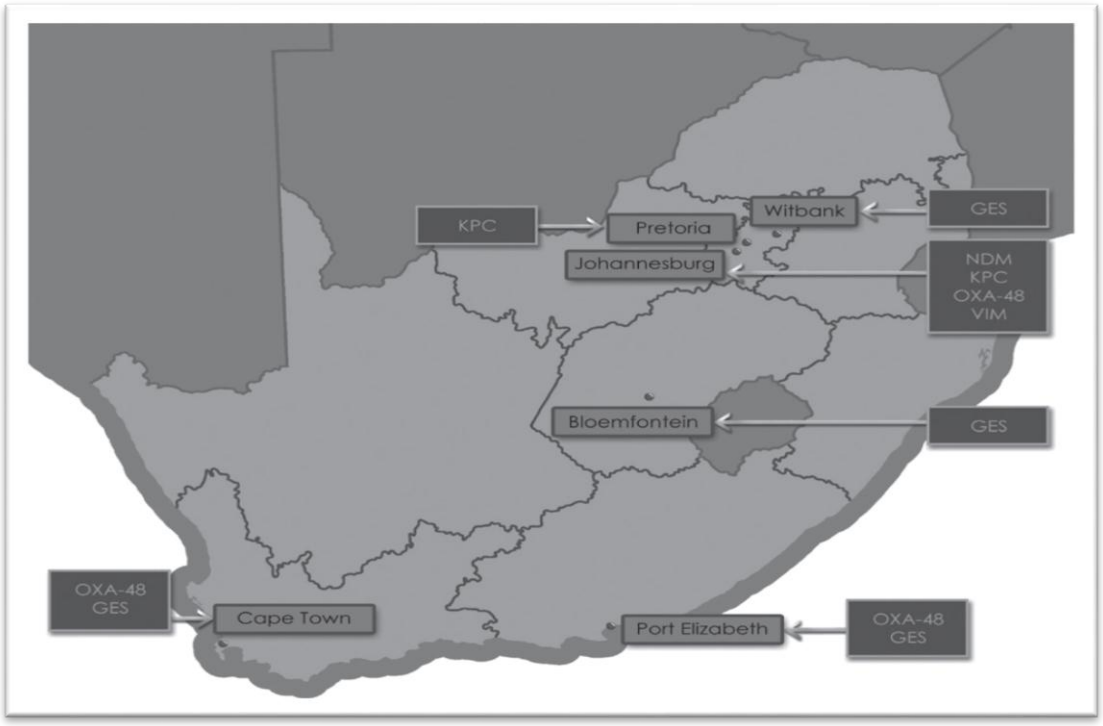

Figure 1.2 Spread of carbapenemase genes among *Enterobacteriaceae* in SA (KPC = *Klebsiella pneumoniae* carbapenemases; GES = Guiana extended-spectrum β-lactamases; NDM = New Delhi metallo-β-lactamases; OXA-48 = oxacillinase-type carbapenemases; VIM = Verona integron-encoded metallo-β-lactamases) (Brink *et al*. 2012).

Resistance may be related to association of a decrease in bacterial outer-membrane permeability, with an overexpression of β-lactamases with no carbapenemase activity or to expression of carbapenemases (Nordmann *et al.* 2011: Martinez, 2008), modifications to outer membrane permeability and up-regulation of efflux system associated with hyper production of AmpC β lactamases , extended spectrum beta lactamases (ESBL) and or production of specific carbapenem-hydrolyzing β lactamases, (Ambler *et al*.1991).

The enzyme carbapenemases first developed as a result of a mutation in the *Klebsiella pneumoniae* that had been exposed to wide range of antibiotics and these carbapenemases have the ability to hydrolyze many antibiotics, including penicillins, cephalosporins, monobactams and carbapenems (Australian Commission on Safety and Quality in Health Care, 2013). Carbapenemases have been reported in *Enterobacteriaceae*, such as: *Klebsiella pneumoniae* carbapenemase (KPC; Ambler class A); Verona integron–encoded metallo-β-lactamase (VIM), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM) (all Ambler class B); and oxacillinase-48 (OXA-48; Ambler class D), (Nordmann, Naas and Poirel, 2011).

Klebsiella with plasmid-mediated carbapenem resistance is a major risk to hospitalized patients, and spread of these plasmids into *Escherichia coli* poses an even worse public health threat because resistant *Escherichia coli* may become part of the normal flora in the gut and therefore be an underling source of infections among sick and the healthy people in healthcare settings and in the community (Schwaber and Carmeli, 2008). KPC-associated enterobacterial infections are not specific to sites, organs, or tissues and most infections are systemic, occurring in patients with multiple invasive devices or urinary tract infections without an indwelling catheter, particularly in immuno-compromised patients (Bratu *et al* 2005: Marchaim *et al.* 2008). Consequently, severe infections with CRE are associated with notable health care costs and significant morbidity and mortality, (Bilavsky *et al*. 2010).

The epidemiology of CRE varies because of diversity in the carbapenemase enzymes that these bacteria produce (Zarfel *et al*. 2011). A number of classes of antibiotics have been associated with colonization of infection by CRE, including cephalosporins, fluoroquinolones and carbapenems (Gupta *et al*. 2011). There are currently no new antimicrobial agents developed to combat CRE (Huttner *et al*. 2013). The optimization of already existing compounds will provide the most effective approaches to reduce the spread of this resistance and preserve the activity of available antimicrobials. According to a recent study, none of the antimicrobials currently in development demonstrate activity against the entire spectrum of CRE (Yamomoto and Pop-Vicas, 2014).

Enterobacteriaceae are a common cause of community infections and they are among the leading causes of nosocomial infections (Hidron *et al.* 2008). CRE have the potential to move from their current niche among health-care–exposed patients into the community (Nicolas-Chanoine *et al.* 2012). Some antimicrobial classes are more likely to drive emergence and spread multi-drug resistant pathogens than others. In the case of multi drug resistant *Enterobacteriaceae* like CRE, reports strongly implicate fluoroquinolones, extended-spectrum cephalosporins and carbapenems (Canton and Bryan, 2012: Ginn *et al.* 2012).

Carbapenem-resistant strains frequently possess additional resistance mechanisms that render them resistant to most available antimicrobials; pan-resistant CRE have been reported (Elemam *et al*. 2009). However, resistance levels to carbapenems of KPC producers may differ markedly (Table 2.2.1), (Yigit *et al.* 2001 and Nordmann *et al.* 2009). This raises serious problem in treating and preventing nosocomial outbreaks and community spread of these strains. The emergence of carbapenem resistance among *Enterobacteriaceae* in South Africa poses a formidable challenge to clinical microbiologists, infection control and prevention practitioners, and treating clinicians.

Currently, there is very little data with regards to the antibacterial drug response of these carbapenemases in South Africa, but in view of the dramatic shortage in the availability of therapeutic options to combat the issue of drug-resistance, the information from this study becomes extremely valuable.

2.3.1 **Screening and Detection of Carbapenem Resistant Enterobacteriaceae**

There is a need for clinical laboratories to screen for carbapenemase producers (Livermore *et al*. 2012). Laboratory tests for the detection of carbapenemases are still evolving, and are complicated by the heterogeneity of both enzymes and hosts (Thomson, 2010). For phenotypic identification of CRE, Modified Hodge test (MHT) is one of the tests used to confirm carbapenemase production in isolates of *Enterobacteriaceae* (figure 1.3) (Anderson *et al*. 2007). Currently, potential carbapenemase producers are initially screened by susceptibility testing, using breakpoint ranges for carbapenems (Nordmann *et al*. 2011). Susceptibility testing provides qualitative results by categorizing bacteria as susceptible, intermediate or resistant to the antimicrobial agent (Jorgensen and Ferraro, 2009). In 2011, the Clinical and Laboratory Standards Institute (CLSI) breakpoints of carbapenems were lowered for a better detection of carbapenem resistant isolates (Table 1.1).

CRE are continuously evolving and thus becoming more randomly complex and diverse – it is anticipated that the detection will become a serious challenge for clinical microbiology laboratories. Early studies have reported false resistance to imipenem due to degradation of the drug, resulting in difficulties in obtaining accurate susceptibility testing results for carbapenems (White *et al*. 1991).

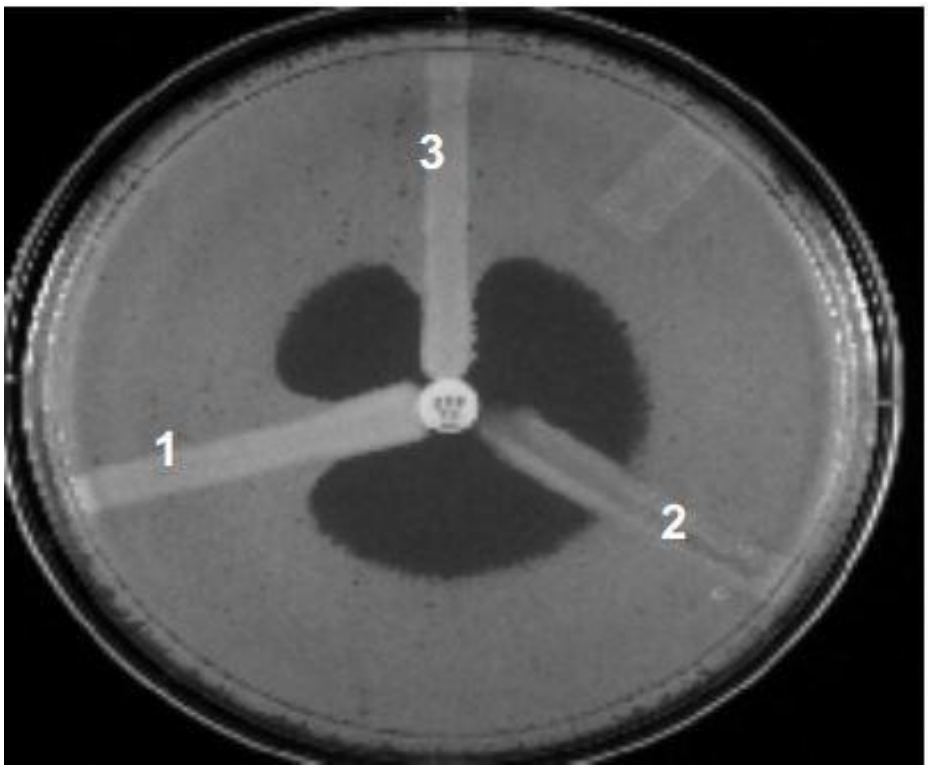

Figure 1.3 Modified Hodge Test (MHT), showing the cloverleaf-like indentation of the inhibition zone which is regarded as a positive result for carbapenemases producing organism (CLSI M100-S22). (1) MHT Positive test has a clover leaf-like indentation of the *Klebsiella pneumoniae* (ATCC BAA-1705) growing along the test organism growth streak within the disk diffusion zone. (2) MHT Negative test has no growth of the *Klebsiella pneumoniae* (ATCC BAA-1706) along the test organism growth streak within the disc diffusion. (3) MHT positive clinical isolate.

Table 1.1: Different carbapenem susceptibility breakpoints interpretations: Imipenem, Meropenem, Ertapenem and Doripenem (CLSI, 2011).

Carbapenem	Disc diffusion break points (zone diameters mm)			MIC break points (ug/ml)		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Imipenem 10ug	≥23	20-22	≤19	≤1	2	≥4
Meropenem 10ug	≥23	20-22	≤19	≤1	2	≥4
Ertapenem 10ug	≥23	20-22	≤19	≤0.25	0.5	≥1
Doripenem 10ug	≥23	20-22	≤19	≤1	2	≥4

2.3.2 CRE Related Infections: Mortality and Risk Factors

CRE may cause a variety of serious infections including bacteremia and urosepsis, and associated with a high mortality rate (Schwaber and Carmeli, 2008). Exposure to health care and antibiotics are among the most prominent risks for CRE infection. There are no specific symptoms of a CRE infection and infected patients may develop symptoms common to any bacterial infection. Infections caused by CRE have been associated with increased cost and length of hospitalization, frequent treatment failures and eventually death (CDC, 2009).

Patients suffering from CRE infections are treated under conditions of cohorting and contact isolation (Seigal *et al*. 2010), and the duration of CRE carriage following hospital discharge is unknown as yet. In South Africa, there is no standard procedure for treating and handling CRE cases, which causes uncontrollable transmission of CRE infections and worsening the status of CRE infections in South Africa. Currently, there are no standardized treatment options for CRE infections (Kanj and Kanafani, 2011), and clinicians have become increasingly dependent on combination therapy of polymyxins and tigecycline for treatment of these infections (Lee *et al.* 2009). In the review of case reports carried out by Lee and Burgess (2012), polymyxins monotherapy was reported to have higher treatment failure rates than combination therapy and a systematic review of 20 non randomized studies revealed a mortality rate of 67% among patients who were treated with colistin-carbapenem combination therapy, the mortality rate was up to 57% among patients treated with colistin monotherapy and up to 80% for tigecycline (Falagas *et al*. 2014). Resistance to these antibiotics has emerged, rendering infections with strains carrying these genotype and these infections are virtually untreatable (Kanj and Kanafani, 2011). Despite these inconsistencies, combination therapy is still recommended in patients with severe CRE infections (Qureshi *et al*. 2012).

2.4 The use of Imidazole and Triazoles

Imidazoles and triazoles are a group of antifungals that are also currently used in the treatment of nosocomial infections, and have compounds with potential antibacterial activity (Ghannoum and Rice, 1999). They interfere with biosynthesis of amino acids and proteins; altering cell membrane permeability by blocking production of essential enzymes (Ghannoum and Rice, 1999) and they have been extensively studied and successfully used in clinical practice, for instance metronidazole, fluconazole, posaconazole and voriconazole (Verma and Immam 1975). Since imidazoles and triazoles are primarily fungicides, comparison between mechanisms of resistance to antifungal and antibacterial agents is a significant way of developing a perspective on potential activity of these compounds against bacteria (Ghannoum and Rice, 1999).

Bacteria do not have sterols; however, fungicides may have indirect side effects on these organisms. The action of imidazole has been reported to inhibit *Escherichia coli*, correlated with the inhibition of the nitric oxide (Helmick *et al*. 2005) and this probably facilitates the task of host defense cells and might be the principal factor leading to clearance of infection (Ghannoum and Rice, 1999). Among many classes of antimicrobial agents, imidazoles have good antibacterial properties and some like micanozole have been reported to have antibacterial activity against multi-drug resistant bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Khabnadideh *et al.* 2012).

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2.4.1 Imidazole

Imidazoles enter the cell by passive diffusion and undergo reduction to yield nitro radical anion which oxidizes the DNA resulting in the destruction of DNA strand and causes cell death (Edwards, 1993). Imidazoles have been discovered to have antimicrobial properties and imidazole-based derivatives have many favorable properties including good tissue penetrability and permeability, good bioavailability and low incident of toxic and adverse effects (Verma and Immam 1975; Rani *et al*. 2013). They have a broad spectrum of pharmacological activities which includes anti-inflammatory, anti-cancer and antibacterial activity (Vijesh *et al.* 2011). Confirming the value of imidazole compounds in clinical practice, Vijesh *et al*. (2011) performed an in vitro antibacterial activity test of modified compounds from imidazoles. *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhimorium, Clostridium perfringens* and *Pseudomonas aeruginosa* were used to investigate the activity by well plate method and the antibacterial screening showed that some of the tested compounds have significant inhibition activity against various organisms.

2.4.2 Triazole

Triazoles are the most commonly used antifungal agents rendering activity against many fungal pathogens without causing serious nephrotoxic effects observed with amphotericin B (Gallagher *et al*. 2003). The pharmacological activity which includes antibacterial activity of triazoles and their derivatives has been established in various studies (Jantova, 1998: Agrwal and Pancholi, 2011). Due to the medicinal importance of triazoles and its derivatives, it is prompted to investigate the antibacterial activity these compounds against CRE.

2.4.4 Ketoconazole

Ketoconazole is a synthetic imidazole antifungal drug used to treat fungal infections such as cutaneous candidiasis. As with other imidazole antifungals, it also has some in vitro activity against Gram positive cocci (Heel *et al*. 1982).

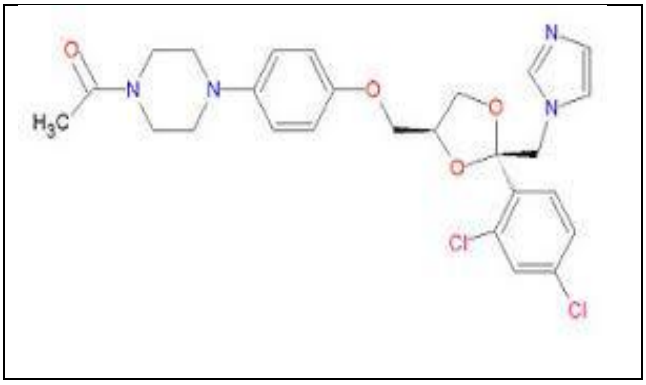

Figure 1.4 Chemical Structure of Ketoconazole (PubChem, 2005).

2.4.4 Fluconazole

Because of its efficacy, low toxicity and cost to patients, fluconazole is a commonly used triazole antifungal drug, to treat infections caused by *Candida spp*. Fluconazole has also proved to work synergistically with antibiotics like minocycline in resistant *Candida albicans* (Wenna *et al*. 2010).

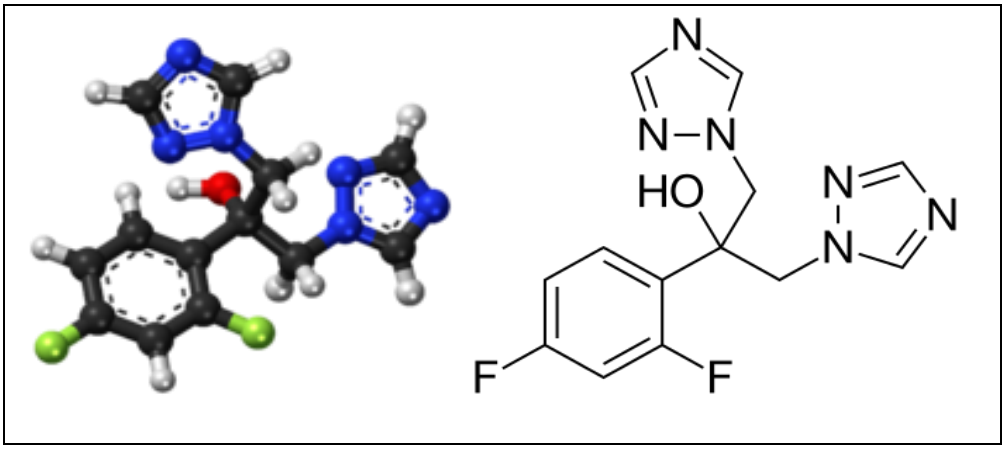

Figure 1.5 Chemical Structure of Fluconazole (PubChem, 2005).

2.4.5 Metronidazole

Metronidazole is one of the nitro-imidazole antifungals and it is primarily used to treat: bacterial vaginitis, oral infections and topical fungating wounds. It inhibits nucleic acid synthesis by disrupting the DNA of microbial cells (Rossi, 2013).

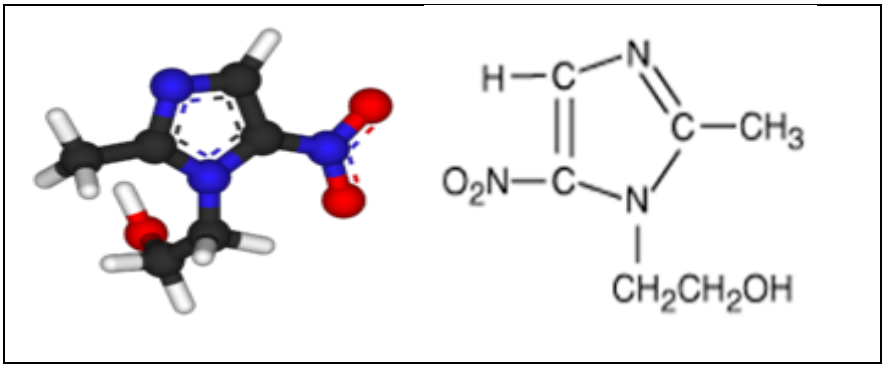

Figure 1.6 Chemical Structure of Metronidazole (PubChem, 2005).

2.5 Classes of Carbapenemases

2.5.1 Class A Carbapenemases

Class A beta-lactamases are characterized by their hydrolytic mechanisms that require an active-site serine at position 70 (Shibata *et al*. 2003). This group include penicillinases and cephalosporinases in the TEM, SHV, and CTX-M-type groups (which do not hydrolyse carbapenems), as well as additional groups that possess beta-lactamase (including carbapenemase) activity (Jacoby and Munoz-Price, 2005).

A variety of class A carbapenemases have been reported; some are chromosome encoded and others are plasmid encoded, but they all hydrolyze carbapenems effectively (Queenan and Bush, 2007). *Klebsiella pneumoniae* carbapenemases (KPC) are the most clinically common enzymes in this class. KPC producers are usually multidrug resistant (especially to all β-lactams), and therapeutic options for treating KPC- related infections are very limited (Nordmann, 2009). These infections are not specific to body sites and they are usually isolated in patients with multiple invasive devices, urinary tract infections and particularly in immuno-compromised patients (Bratu *et al.* 2005 and Marchaim *et al.* 2008). *Klebsiella pneumoniae* remains the most prevalent bacterial species carrying KPCs; however, the enzyme has been identified in several other bacterial species (Table 1.2) (Bamford *et al*. 2011).

Table 1.2 List of bacteria that produce the enzyme *Klebsiella pneumoniae* carbapenemases (KPC), (Bamford *et al*. 2011).

Enterobacteriaceae	**Non-Enterobacteriaceae**
Klebsiella pneumoniae	*Acinetobacter spp*
Klebsiella oxytoca	*Pseudomonas aeruginosa*
Escherichia coli	*Pseudomonas putida*
Proteus mirabilis	
Citrobacter freundii	
Serratia marcescens	
Salmonella enterica	
Enterobacter aerogenes	
Enterobacter cloacae	
Enterobacter gergoviae	

2.5.2 Class B Metallo-β-Lactamases

The common enzymes of this class are mostly of the Verona Integron–encoded Metallo-β-lactamase (VIM), Imipenemase (IMP) types and of the New Delhi Metallo- β-lactamase-1 (NDM-1) type (Queenan and Bush, 2007). New Delhi metallo-beta-lactamase-1 (NDM-1)-mediated carbapenemase is the most prevalent class B carbapenemase in *Enterobacteriaceae* and it has been increasingly detected in several countries (Rozales *et al*. 2014).

These enzymes hydrolyze all beta-lactam antibiotics except monobactams and infections from this class are mostly hospital acquired (Walsh *et al.* 2005). Unlike other carbapenem genes, the gene of this class ($bla_{\text{NDM-1}}$) is not implicated with a single clone but also with other non-clonally related isolates and species (Kumarasamy *et al.* 2010). Metallo-β-lactamases are able to hydrolyze carbapenems and are not inhibited by the serine-β-lactamase inhibitors (Crowder *et al.* 2006). These genes are encoded chromosomally mainly in environmental bacteria or opportunistic pathogens, such as BcII from *Bacillus cereus* (Lim *et al.* 1998). These isolate cause a major threat to public health globally, because of their apparent ease of transfer of the *bla*NDM gene (Pasteran *et al*. 2014). These carbapenemases constitute a versatile and heterogeneous group of enzymes hydrolyzing β-lactams and also exhibit resistance to β-lactamase inhibitors, making them almost impossible to treat (Gupta *et al*. 2011). Currently there is no clinical drug able to inhibit any of the Metallo-β-lactamases (King *et al.* 2013**).**

2.5.3 Class D OXA β-lactamases

OXA β-lactamases have an ability to hydrolyze oxacillin and includes several oxacillinases with an extended hydrolysis spectrum for cephalosporins caused by amino acid substitution (Naas and Nordmann, 1999: Bush, 1998). The enzymes of this class are distributed in various enterobacterial species (table 1.3) (Eser, 2017), however they are mostly identified in *Klebsiella pneumoniae* and *Escherichia coli*, and they are the most difficult carbapenemase producers to be identified, with unknown mortality rate (Cuzon *et al.* 2011 and Nordmann *et al.* 2011).

Enzymes, OXA-23, OXA-24/ 40, OXA-48, OXA-51, OXA-58, and OXA-143 are of major clinical significance due to their wide spread in pathogenic organism and the structure of OXA-48 reveals that the enzyme has a diverse mechanism for carbapenem hydrolysis compared to other oxacillinases (Docquier *et al.* 2009). These enzymes have been reported in South Africa (figure 1.7), (Perovic *et al*. 2016).

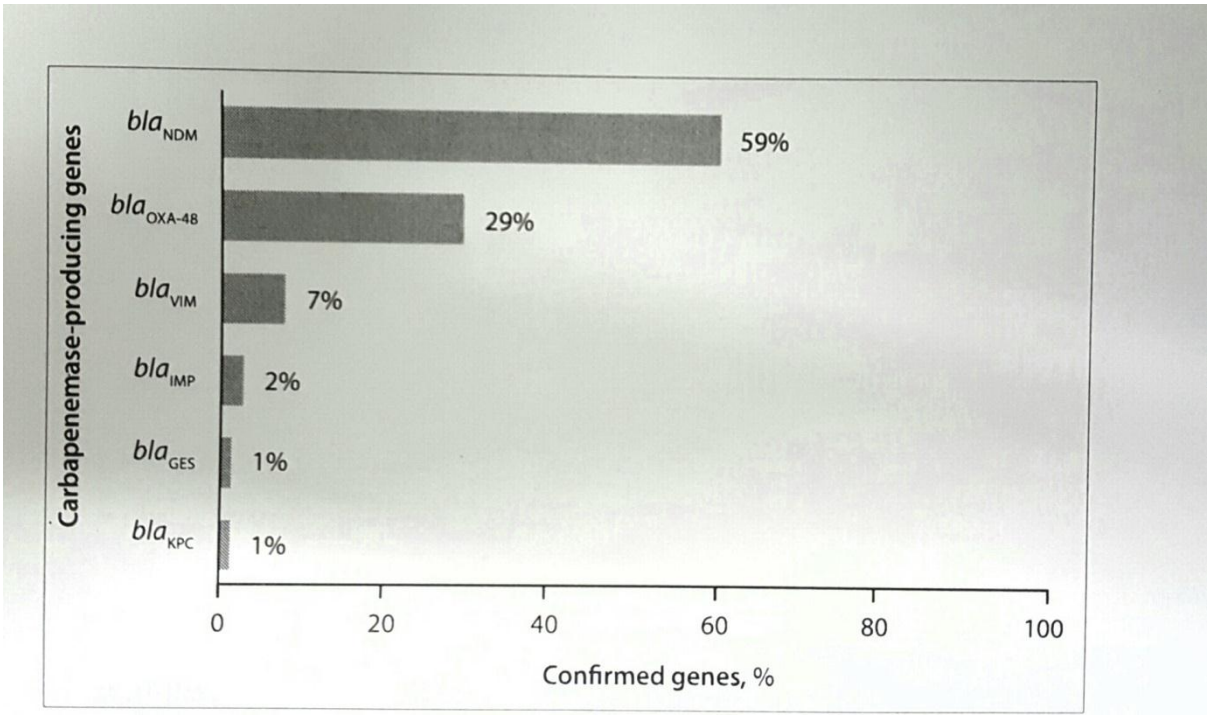

Figure 1.7 Genes detected in CRE isolates referred to the Antimicrobial Resistance Laboratory (AMRL) at the NICD South Africa, 2012 – 2015 (Perovic *et al*. 2016).

Table 1.3 Distribution of the different classes of carbapenemases in the Gram negative bacilli bacteria (Eser, 2017).

Microorganism	Class A (KPC,GES)	Class B (MBL)	Class D (OXA)
Pseudomonas aeruginosa	+	+	+
Acinetobacter baumannii		+	+
Klebsiella pneumoniae	+	+	+
Klebsiella oxytoca	+	+	
Esherichia coli	+	+	+
Proteus mirabilis	+	+	+
Serratia marscescens	+	+	
Entrobacter spp.	+	+	
Citrobacter freundii	+	+	
Morganella morganii		+	
Salmonella enterica	+		

2.6 Antimicrobial Screening: CRE infections

2.6.1 Selection of pathogens

The *Enterobacteriaceae* is a large family of Gram negative bacilli that are normal inhabitants of the gastrointestinal tract of humans and they are also most common cause of community acquired and health care acquired infections (Donnenberg, 2010). They are the most frequently isolated bacteria from clinical specimen and account for up to 80% of all clinically significant isolates from Gram-negative bacilli and up to 50% of all clinically significant bacteria (Murray *et al*. 2003). Multi-drug resistant Gram-negative organisms have been isolated more frequently compared to some other Gram positive multi-resistant organisms (O'Fallon *et al*. 2009).

For the past several decades the spread of *Enterobacteriaceae* with resistance to broad-spectrum antimicrobials have been noted and carbapenem antimicrobial class (imipenem, meropenem, doripenem, and ertapenem) have been reliable to treat infections caused by these resistant organisms (Nord, 1989). Carbapenem Resistant *Enterobacteriaceae* resistance is an additional resistance to penicillin and cephalosporin, therefore leaving a very limited number of treatment options for patients infected with these organisms which may cause serious infections, especially bacteremia and urosepsis associated with a very high mortality rate (Schwaber and Carmeli, 2008). *Enterobacteriaceae* producing KPC enzymes are increasingly reported in *K. pneumoniae*, *C. freundii*, *E. coli*, and *S. marcescens* (Cai *et al.* 2008). Carbapenemases from all important classes have recently been detected in South Africa (Brink *et al*. 2011).

2.6.1.1 *Klebsiella pneumoniae*

This Gram-negative bacterium is the fourth and fifth most common cause of pneumonia and bacteraemia respectively, among new born units, intensive care units (ICU) and in immunocompromised patients (Bratu *et al*. 2005).

Extended-spectrum beta-lactamases (ESBLs) producing *Klebsiella species* that are able to hydrolyse broad and extended-spectrum cephalosporins, monobactams and penicillins were reported in the early 1990s (Paterson, 2006). Therefore, carbapenems, one of the 'last resort antibiotics' were used to treat serious infections caused by ESBL carrying pathogens until *Klebsiella pneumoniae* carbapenemase (KPC), one of the epidemiologically significant carbapenemases first detected in United States of America, North Carolina in 1996 (Yigit *et al*. 2001).

KPC-associated infections are not specific to body sites, tissue or organs. Infections may be either systemic, occurring in patients with multiple invasive devices or urinary tract infections without an indwelling catheter, especially in patients with compromised immune system (Bratu *et al*. 2005). KPC producers are multidrug resistant (usually to all β-lactams), and treatment options of KPC related infections remain limited (Nordmann, 2009).

2.6.1.2 *Escherichia coli*

Escherichia coli is the most common cause of both nosocomial and community-acquired infections in humans (Oteo *et al*. 2005). Carbapenem resistance has been reported for *E. coli*, resulting from the production of chromosomal and plasmid-mediated cephalosporinases associated with decreased drug permeability through the outer membrane (Liu *et al*. 2008) and or from carbapenem-hydrolyzing enzymes (Bratu *et al*. 2007). The emergence and wide dissemination of *E. coli* strains showing resistance to broad-spectrum of antimicrobial agents has been reported from year 2000 and this has become a global concern, especially *on E. coli* sequence type (ST) 131, which is being increasingly reported in incidence of urinary tract infections and bacteraemia globally (de Kraker *et al*. 2012). Carbapenem resistance in *E. coli* isolates is usually attributed to the presence of an AmpC beta-lactamase in association with the loss of porins (Stapleton *et al*. 1999). New Delhi metallo-β-lactamase (NDM) and carbapenem-hydrolysing oxacillinase-48 (OXA-48) are the most common carbapenemases among *E. coli* worldwide (Nordmann and Poirel, 2014).

2.6.1.3 *Serratia marcescens*

Serratia marcescens is a significant nosocomial pathogen that may cause serious infections, including meningitis, pneumonia and bloodstream infection, with a significant impact on mortality and morbidity (Losifidis *et al*. 2012). Carbapenem-resistant *S. marcescens* has been reported in many countries (Yang *et al*. 1990) and this resistance could be due to decrease in outer membrane permeability (Fernandez and Hancock, 2012) coupled with AmpC or carbapenemase production, such as class A (KPC and SME types), B (metallo-β-lactamases: IMP, VIM, and NDM types) (Cuzon *et al*. 2010).

2.6.1.4 *Citrobacter freundii*

Citrobacter freundii is a species of facultative anaerobic Gram-negative bacteria, associated with opportunistic nosocomial infections of the respiratory tract, urinary tract, blood and many other normally sterile sites in immunocompromised patients (Whalen *et al*. 2007). Multidrug resistant *C. freundii* has been reported to have strains carry Amp-C b-lactamase (Amp-C), broad-spectrum b-lactamase, extended-spectrum b-lactamase (ESBL), plasmid-mediated quinolone resistance determinants, and carbapenemase (Samonis *et al*. 2009). *C. freundii* strains have also been associated with a higher rate of in-hospital mortality and poor prognostic factors compared to susceptible strains (Deal *et al*. 2007).

Chapter three: Research methodology

3.1 Introduction

Imidazole and triazole-based antimicrobial agents have been studied and used in clinical practice (Verma and Immam, 1975), they have been discovered to have antimicrobial properties and imidazole-based derivatives have been identified to have many favourable properties including good tissue penetrability and permeability, good bioavailability and low incident of toxic and adverse effects (Verma and Immam, 1975; Rani *et al*. 2013). A number of imidazole and triazole-based drugs have been modified to optimise the efficacy of the compounds. Recently, the development of high bacterial resistance to virtually all available antibiotics has led researchers to investigate alternative drugs derived from imidazole and triazoles.

This section describes the preparation of the stock solutions for each antimicrobial agent used in this study (ketoconazole, fluconazole and metronidazole). For optimum activity and results, disc and agar diffusion methods were both used to determine inhibitory effects of these antimicrobials against CRE. The antibacterial activity was investigated using four pathogenic bacterial strains of CRE (*K.pneumoniae, E.coli, S.marcescens* and *C.freundii*) and compared to control organism. Serial dilutions were also performed for evaluation of Minimum Inhibitory Concentration. To determine the biochemical effects of the test antimicrobial agents, a series of biochemical tests were performed on each test organism before and after exposure to imidazole and triazoles.

3.2 Materials and Methods

3.2.1 Organisms

The bacterial isolates utilized in this study were isolated from clinical specimen processed at Lancet Laboratory (Durban) and each isolate was confirmed for the production of enzymes carbapenemases using the Vitek 2 automated ID/AST instrument, bioMerieux Clinical Diagnostics.

3.2.2 Imidazole and Triazole drugs

The imidazole and triazoles selected for this study were purchased in tablet form, from Oval pharmacy Ladysmith, KZN (Ketoconazole 100mg, Fluconazole 200mg and Metronidazole 100mg).

3.2.3 Quality control material

There are no known or standardized antibiotics for infections caused by CRE (Kanj and Kanafani, 2011). ATCC strain of *Staphylococcus epidermidis* (ATCC 12228) and *Escherichia coli* (ATCC 25922) was used as control organism for antibacterial activity testing and as a negative control; distilled water purchased from Diagnostic Media Products SA was used.

3.3 Experiment: Antimicrobial Susceptibility Testing

3.3.1 Isolation of Pathogenic Bacteria

The bacterial cultures of the test organisms (*K. pneumoniae, E. coli, S. marcescens and C. freundii*) obtained from Lancet Laboratory were sub-cultured on blood agar to obtained fresh and pure cultures for testing. MacConkey agar made up of agar, bile salts, lactose and neutral red was used for the validation of colonial morphology of each organism. MacConkey culture media is selective and differential for Gram negative bacilli, recommended for cultivation and identification of enteric organisms (MacConkey, 1905). 10ml suspensions of each organism were prepared using sterile water and each suspension was standardized to 0.5 McFarland standard using a turbidity reader, bioMerieux DensiCheck plus instrument.

3.3.2 Preparation of Imidazole and Triazole Stock Solutions

The selected test imidazole and triazole antimicrobials were purchased in tablet form and for optimum dissolving; stock solutions were prepared using dimethyl sulfoxide (DMSO) solvent, as described by Kelava *et al.* (2011). Ketoconazole 200mg tablet was reconstituted in a beaker filled with 200ml of DMSO, 200mg Fluconazole in 200ml DMSO and 100mg Metronidazole in 100ml DMSO. The solutions were then placed on a magnetic stirrer until completely dissolved and the final concentration of each antimicrobial stock solution was therefore 1mg/mL and smaller concentrations were prepared from those stock solutions.

3.3.3 Preparation of Antimicrobial Discs

Whatman filter paper No. 3 was used to prepare blank discs. The concentration and name of each imidazole and triazole antimicrobial was written on the filter paper for the identification. An office punching machine was disinfected and used to make discs of approximately 6mm diameter. The discs were then autoclaved for 30 minutes. Using an automated pipette, a fixed volume of 20ul of each antifungal solution was impregnated on the sterile blank discs, respectively. The discs were then dried in a 37°C incubator for 4 hours, and then stored at the refrigerator. This method was validated and accepted by the NCCLS Standards.

3.3.4 Preparation of Mueller Hinton Agar

Mueller Hinton Agar was prepared according to the manufacturer's instruction. 36 g of powder was weighed into 1 L glass beaker. Sterile distilled water was added to the 1 L mark using a measuring cylinder. The glass beaker was placed on a magnetic stirrer until completely dissolved. The medium was sterilized by autoclaving for 15 minutes at 121 °C at 15 psi pressure. Sterile molten cool (50 °C) agar was then poured aseptically into sterile Petri plates (approximately 15 ml) to solidify at room temperature under a sterile biosafety cabinet. This agar was chosen based on the method by Bauer, (Bauer *et al*. 1966).

3.3.5 Preparation of Nutrient Broth

Nutrient broth is a basal medium suitable for cultivation of a wide range of non-fastidious micro-organisms. According the manufacturer's instructions 13 g of Nutrient broth powder was weighed into a 1 L glass beaker. Sterile distilled water was added to the 1 L mark with the aid of a measuring cylinder. The media was completely dissolved using a magnetic stirrer and sterilized by autoclaving for 15 minutes at 121 °C. After cooling, 9 ml was dispensed into sterile test tubes.

3.3.6 Preparation of Optimized MacConkey Agar

MacConkey agar was prepared following the manufactures instructions. 52 g of powder was suspended in 1 L of sterile distilled water and sterilized by autoclaving at 121 °C for 15 minutes. Cool sterile agar was then poured into petri plates containing 1 ml of each antimicrobial solution, respectively. This was allowed to solidify at room temperature under a sterile biosafety cabinet.

3.4 Antimicrobial susceptibility testing

For optimum in-vitro susceptibility testing, the culture media must be nutritionally adequate and free of antagonistic agents. Disc and agar diffusion methods for testing antimicrobial activity were developed in the 1940's and to minimize and eliminate variability in this testing;

Bauer *et al*. developed a standardized procedure in which Mueller Hinton Agar was used as the test medium (Bauer *et al*. 1966).

Antibacterial activity of imidazole and triazoles was tested using the Kirby-Bauer disc diffusion method recommended by the CLSI (2012). The purpose of this test is to determine the sensitivity or resistance of the selected test organisms against selected test antimicrobials. Mueller-Hinton agar is non-selective, non-differential medium, almost all organisms cultured on it will grow and it has been used in standardized antimicrobial disk susceptibility testing, as described by Bauer, (Bauer *et al*. 1966).

For Disc diffusion method, Mueller-Hinton agar (MHA) plates were allowed to warm to room temperature so that any excess moisture will be absorbed into the medium and the organism suspensions were mixed using a vortex. Using a sterile cotton swab, each inoculum was evenly streaked on the surface of MHA. Using a sterile forceps, the discs impregnated with each test imidazole and triazole antimicrobial were placed one by one the surface of MHA streaked with each test organism respectively.

For agar diffusion method, sterile bore was used to cut wells out from the agar plates as described by Sivaranjani and Meenakshisundaram 2013, and after each organism was inoculated, these wells were filled with 30ul of each imidazole and triazole solution, respectively. The plates were then incubated for 24 hours at 37 °C.

Each organism was then cultured on MacConkey agar containing 1mg of each imidazole and triazole solution. These plates were also incubated overnight at 37 °C.

3.5 Minimum Inhibitory Concentration (MIC) Testing

For imidazole and triazole, three tubes containing 9 ml of nutrient broth was used to perform serial dilution. 1 ml (1mg/ml) of each solution was pipetted into the first tube, mixed and 1ml was transferred from the first to the second tube and finally 1ml from second tube was transferred to the third and from the third tube 1ml was discarded. Distilled water was used as control material. The final dilutions were therefore: tube 1 (1/10); tube 2 (1/100) and tube 3 (1/1000). Each tube was then inoculated with 100 µl of each bacterial suspension of the test organisms. The tubes were then incubated for 24 hours at 37 °C. The results were compared with 0.5 McFarland standard and growth was interpreted by visible turbidity. The MIC was regarded as the lowest concentration of imidazole and triazole showing no visible growth.

3.6 Biochemical reaction testing

Analytical Profile Index (API 20E) for *Enterobacteriaceae* (lot no. 1005214990) was purchased from bioMerieux SA and used to test for biochemical tests and sugar reactions. Using the procedure recommended by the manufacturer on the package insert, the suspensions of the test organisms were standardized to 0.5 McFarland standard and each bacterial suspension was pipetted into the tubes of the API strip. To evaluate the biochemical effects of imidazole and triazole against the test organisms, the test organisms were then exposed to the test antmicrobial agents by emulsifying each organism on distilled water containing each antimicrobial solution, respectively and API test was also performed on these suspensions. For reactions that requires anaerobic environment, the tubes on the strip were sealed with mineral oil. Purity plates were culture on MacConkey agar for all bacterial suspensions. Purity plates and API 20E strips were then incubated at 37^0C for 24 hours.

Chapter four: Results

4.1 *In vitro* antibacterial activity

Imidazole and triazole-based antimicrobial agents have been previously studied as drugs candidates, and most of them have been and currently used in clinical practice, for instance metronidazole, fluconazole, posaconazole and voriconazole (Verma and Immam, 1975). The antibacterial activity of imidazole and triazoles were tested against various pathogenic CRE bacterial strains of health significance using Kirby-Bauer disc diffusion method and modified agar diffusion method. Haloes Caliper was used to measure the diameters of zones of inhibition. Disc and agar diffusion method for selected imidazole and triazoles (metronidazole, fluconazole and ketoconazole) at 1mg/ml, 500ug/ml and 250ug/ml showed no activity against all CRE test organisms and *Escherichia coli* (ATCC 25922) control, (Figure 4.1 – 4.4). However, metronidazole showed a significant inhibition activity against a control organism, *Staphylococcus epidermidis* (ATCC 12228), which unlike the CRE, belongs to family of Gram positive bacteria.

Following 24 hours of incubation at 37^0C, the tubes from the serial dilution technique were also examined for MIC, which is determined by no visible turbidity and all tubes showed visible turbidity, revealing that the imidazole and triazole used have no activity against the organisms tested.

API 20E was used to test for the biochemical reactions of the test organisms before and after exposure to test organisms. The biochemical reactions of the organisms were all the same before and after exposure to 1mg/ml, 500ug/ml and 250 ug/ml of each antimicrobial agent, respectively. Therefore, the imidazole and triazoles tested have no biochemical activity on the test organisms.

Table 1.4: Disc Diffusion zones of inhibition report

Organism	Disc Diffusion zone diameter (mm)								
	Metronidazole			Ketoconazole			Fluconazole		
	1mg/ml	500ug/ml	250ug/ml	1mg/ml	500ug/ml	250ug/ml	1mg/ml	500ug/ml	250ug/ml
E.coli	0	0	0	0	0	0	0	0	0
K.pneumoniae	0	0	0	0	0	0	0	0	0
S.marcescens	0	0	0	0	0	0	0	0	0
C.freundii	0	0	0	0	0	0	0	0	0
S.epidermidis (ATCC 12228)	34	32	28	0	0	0	0	0	0
E.coli (ATCC	0	0	0	0	0	0	0	0	0
Sterile water (negative control)	0	0	0	0	0	0	0	0	0

Table 1.5: Agar Diffusion zones of inhibition report

Organism	Disc Diffusion zone diameter (mm)								
	Metronidazole			Ketoconazole			Fluconazole		
	1mg/ml	500ug/ml	250ug/ml	1mg/ml	500ug/ml	250ug/ml	1mg/ml	500ug/ml	250ug/ml
E. coli	0	0	0	0	0	0	0	0	0
K.pneumoniae	0	0	0	0	0	0	0	0	0
S.marcescens	0	0	0	0	0	0	0	0	0
C.freundii	0	0	0	0	0	0	0	0	0
S.epidermidis (ATCC 12228)	34	32	28	0	0	0	0	0	0
E. coli (ATCC	0	0	0	0	0	0	0	0	0
Sterile water (negative control)	0	0	0	0	0	0	0	0	0

The findings on both disc and agar diffusion test showed that the imidazole and triazoles used have no inhibitory activity against the CRE tested. All tested antimicrobials have 0 mm diameter on all CRE organisms and this is interpreted as resistant. Unlike the CRE, the control organism (*S.epidermidis*) belongs to the family of Gram positive bacteria and metronidazole showed significant inhibitory against this organism, with a zone of 34 mm.

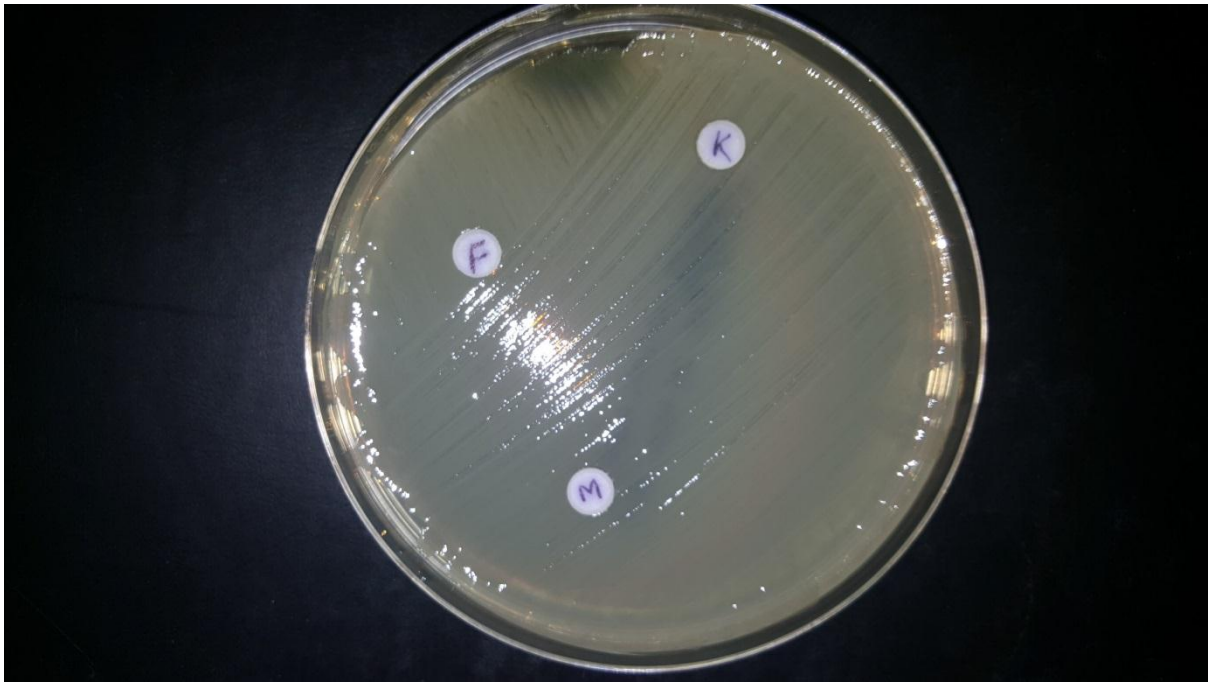

Figure 1.8 Disc Diffusion: *K.pneumoniae* showing resistance to all imidazole and triazoles, image taken by Radebe SP (2017).

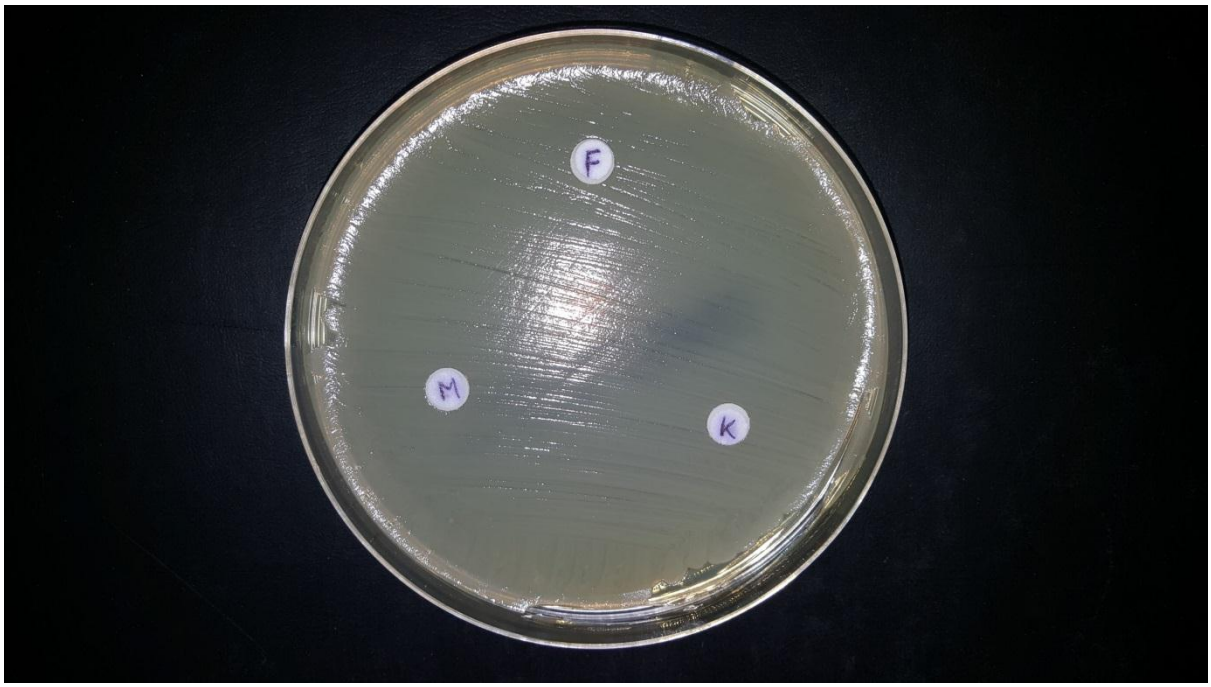

Figure 1.9 Disc Diffusion: *E. coli* showing resistance to all imidazole and triazoles, image taken by Radebe SP (2017).

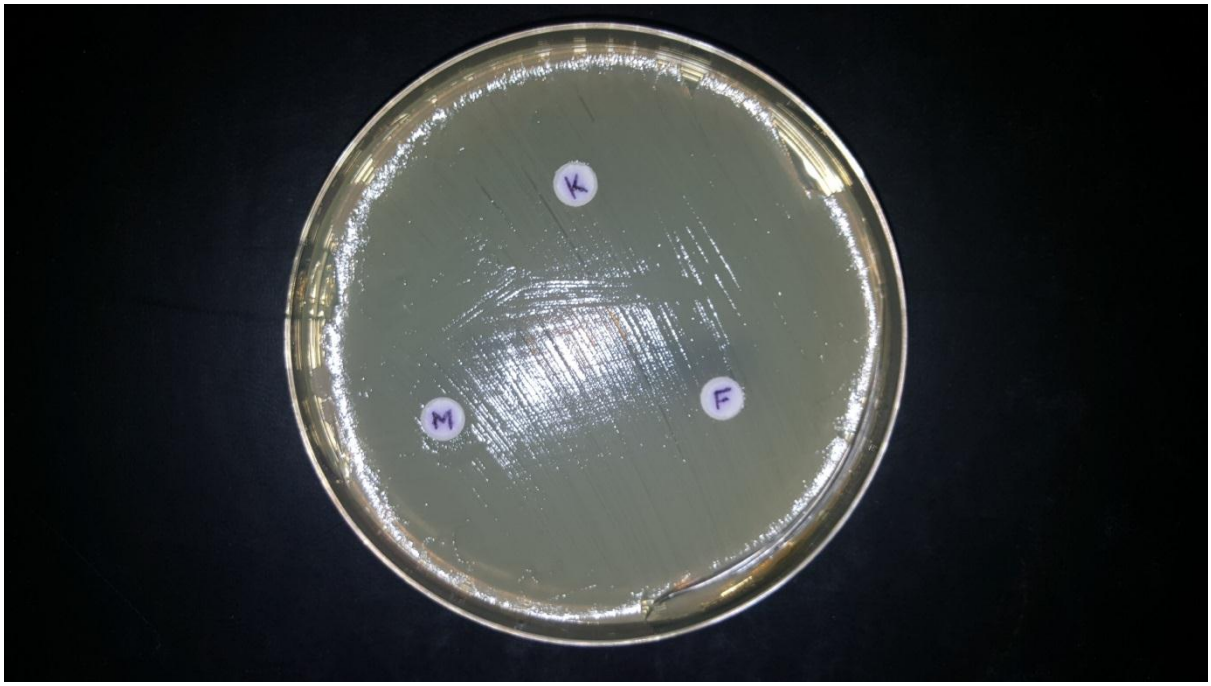

Figure 2.0 Disc Diffusion: *S. marcescens* showing resistance to all imidazole and triazoles, image taken by Radebe SP (2017).

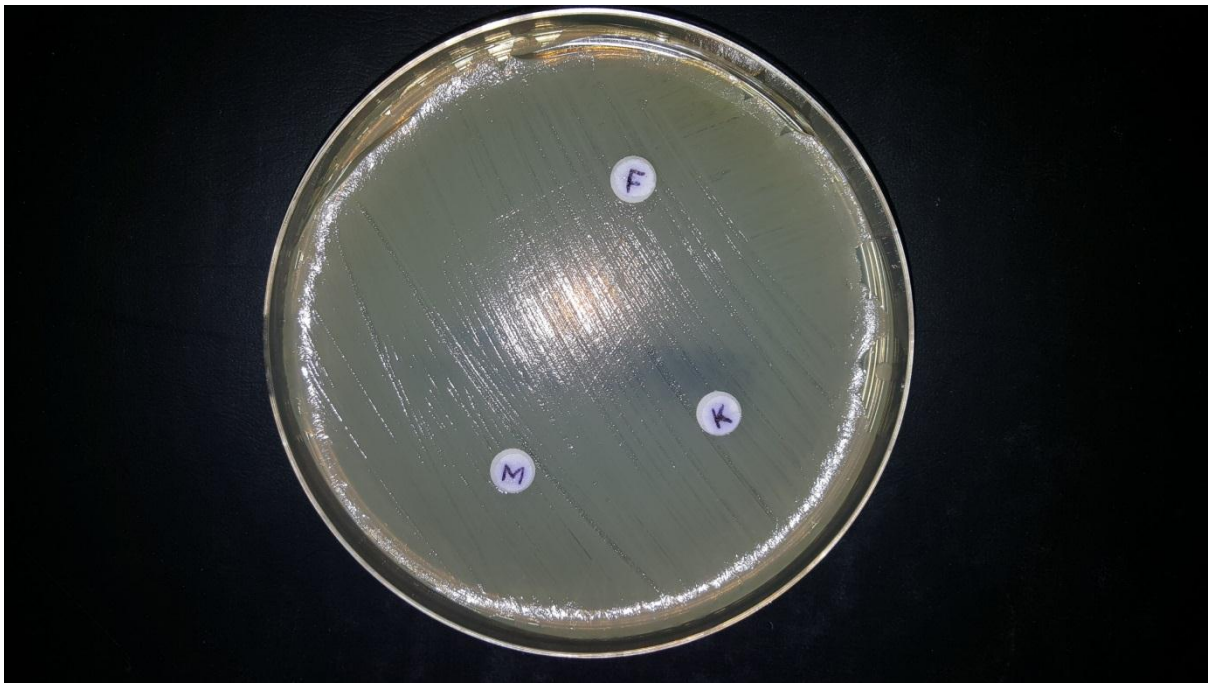

Figure 2.1 Disc Diffusion: *C. freundii* showing resistance to all imidazole and triazoles, image taken by Radebe SP (2017).

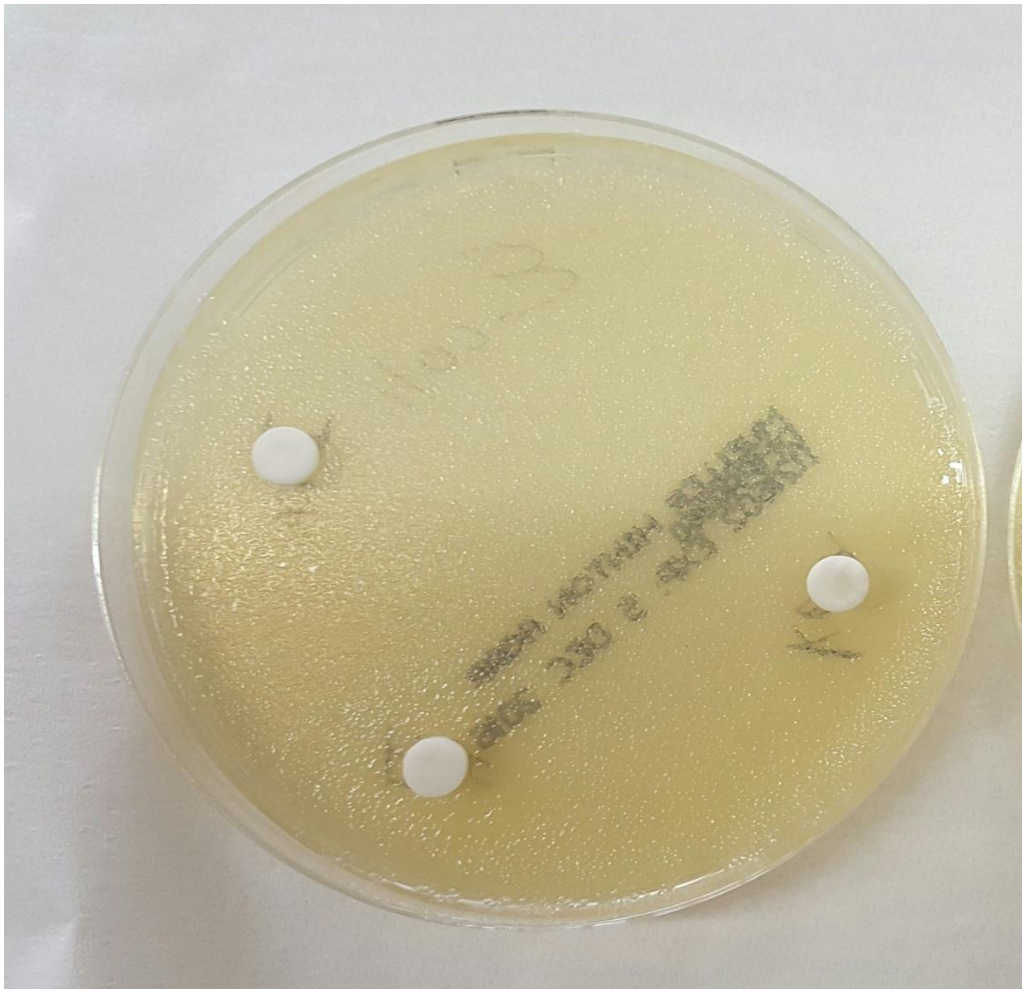

Figure 2.2 Disc Diffusion: *E.coli* control (ATCC 25922) showing resistance to all imidazole and triazoles, image taken by Radebe SP (2017).

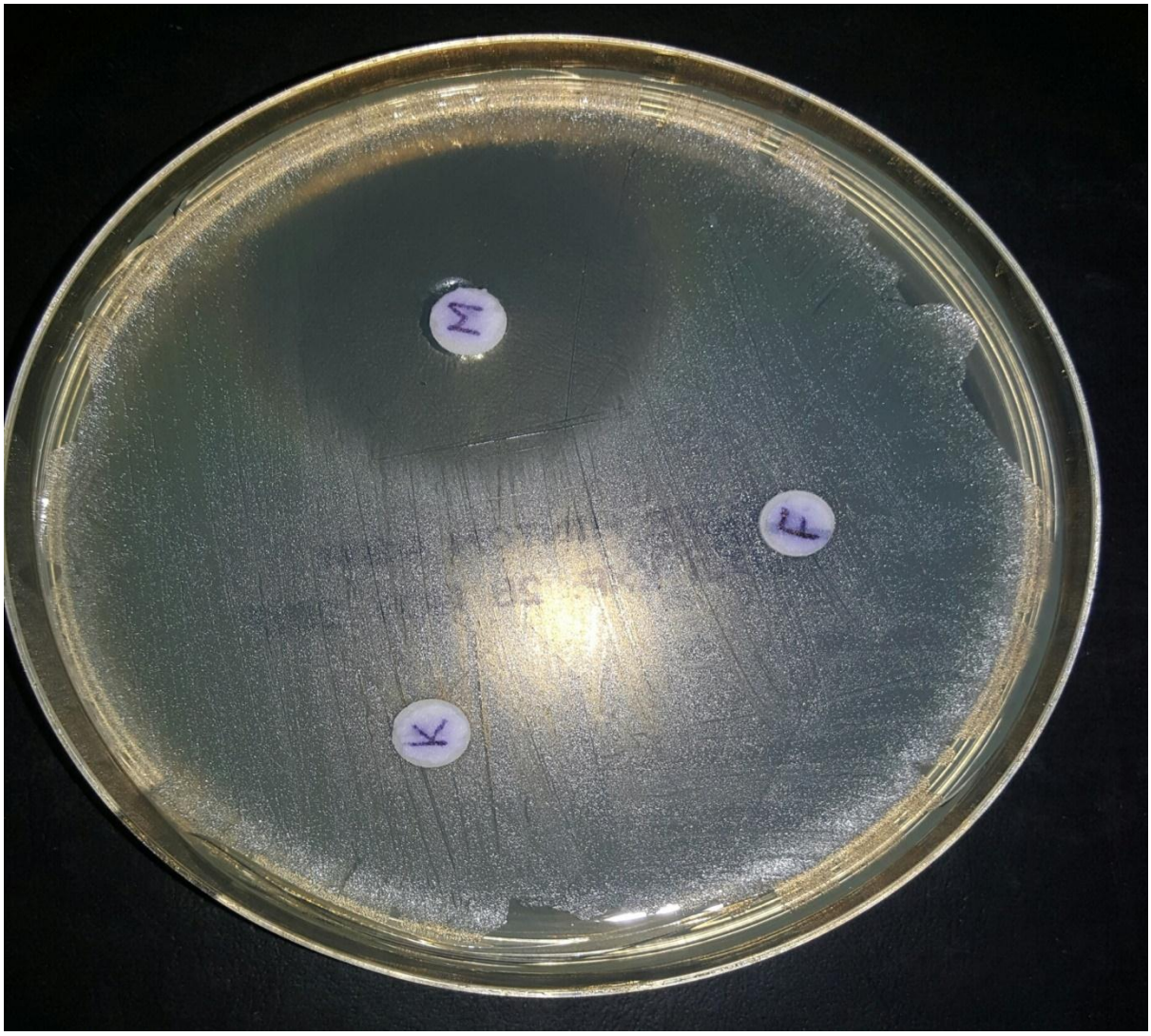

Figure 2.3 Disc Diffusion: metronidazole showing activity against control organism, *S. epidermidis* (ATCC 12228), image taken by Radebe SP (2017).

Figure 2.4 Agar Diffusion: metronidazole showing activity against control organism, *S. epidermidis* (ATCC 12228), image taken by Radebe SP (2017).

Chapter Five: Discussion and Conclusion

5.1 Discussion

This study investigated the *in vitro* antibacterial activity of imidazole and triazooles against CRE. The results showed that the selected antimicrobials have no activity against CRE. Our findings are in keeping with recent reports from (Yamomoto & Pop-Vicas, 2014), who found that none of the newly developed antibiotic classes and antimicrobials demonstrate activity against the entire spectrum of carbapenemase-producing Gram-negative bacteria. Imidazoles and triazoles have a broad spectrum of pharmacological activities which includes anti-inflammatory, anti-cancer and antibacterial activity (Vijesh and Isloor, 2011). Vijesh *et al*. (2011) carried out an in vitro antibacterial activity test of synthesized compounds from imidazoles. *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhimorium, Clostridium perfringens* and *Pseudomonas aeruginosa* were used to investigate the activity by well plate method and the antibacterial screening revealed that some of the tested compounds showed good inhibition against various bacterial strains. These results are different to the ones obtained in our study and these discrepancies may be due to the fact that, the imidazole and triazoles used in our study were not synthesized or modified as in the study by Vijesh *et al* (2011).In this present study, an in vitro antibacterial activity of imidazole and triazoles (metronidazole, fluconazole and ketoconazole) was tested against four pathogenic strains of Carbapenem Resistant Enterobacteriaceae (*Escherichia coli, Klebsiella pneumonia, Citrobacter freundii and Serratia marcescens).* A panel of biochemical and sugar reaction tests was also performed on the four bacterial strains of CRE before and after exposure to these antimicrobials. The reaction results showed no biochemical effects against the test organisms.

Antimicrobial compounds from imidazole and triazole class have been proven to possess antibacterial properties. Although the selected antimicrobials for this study showed no activity against the selected strains of Carbapenem Resistant *Enterobacteriaceae*, metronidazole showed a significant inhibition activity against a control organism, *Staphylococcus epidermidis*, which is a Gram positive bacterium. Our findings are in contrast to Vijesh *et al*. (2011) who found that imidazoles have good activity against certain Gram negative bacteria. This may be due to the fact that CRE contains evolved enzymes on genetic elements that can transfer resistance among *Enterobacteriaceae* and other Gram negative organisms, thus conferring resistance to virtually all available antimicrobial agents.

5.2 Strengths and Weaknesses

There is little documented data on the use of unmodified imidazole and triazoles against bacteria and since there are no recommended standards of imidazole and triazole concentrations against bacteria, the results were based on researcher's choice of imidazole and triazole concentrations and this introduce bias into the study. The sample size of this study was limited due to the number of CRE that have been isolate in Durban and the surrounding areas at the time of the study. Recommended standards for all tests were used in this study and the qualitative data obtained have provided essential elements of the imidazole and triazole efficacy against bacterial infections.

5.3 Conclusion

Since the discovery of penicillin in 1928, antimicrobial resistance has been a ceaseless evolving phenomenon and this is a global public health concern which requires immediate strategic interventions. Currently, infections caused by CRE have become a significant clinical threat compromising the selection of optimum treatment options due to the fact these organisms are resistant to all existing antibiotic classes. Clinicians have become reliant on combination therapy of polymyxin and tigecycline for treatment of infections caused by CRE (Lee *et al.* 2009). Resistance to these antibiotics has emerged, rendering infections with strains carrying these genotype and these infections are virtually untreatable (Kanj and Kanafani, 2011). Reports of colistin-resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* rendering the bacteria pan-drug resistant are already documented (Denton *et al*. 2002 and Lee *et al.* 2012). Currently there are no known or standardized treatment options for infections caused by these CRE (Kanj and Kanafani, 2011). These infections are slowly causing a global massacre associated with increased cost and length of stay as well as habitual treatment failures and eventually, death.

As a result, the indiscriminate use of available drugs has increased, thus planting seeds for more antimicrobial resistance complications to emerge in the future. This calls for an indispensable need for the investigation and development of more effective and diverse spectrum antimicrobials with fewer side effects. Since a number antimicrobial classes have been identified as risk factors for CRE acquisition, antibiotic stewardship is crucial in preventing the spread and further evolving of these organisms. Local and international health care facilities should develop and implement clinical policies that will limit the irrational use of antibiotics and strategies that will improve the practice of infection prevention and control. As a precaution strategy, patients with CRE infection should be placed on contact precautions and health care staff should be specially trained in attending these patients. Furthermore,

CRE are still evolving and thus becoming more complex and diverse, eventually the detection will become a serious challenge for clinical microbiology laboratories. Therefore, improved laboratory based surveillance is required for an early detection of resistance and monitoring the spread of these bacterial strains

Although, results obtained in this study indicated no activity of imidazole and triazoles against CRE, laboratory studies are still necessary in modification of the imidazole and triazoles to synthesize derived substituents that can optimize the antibacterial potency of these compounds.

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