

**PHENOTYPIC AND MOLECULAR ANTIFUNGAL SUSCEPTIBILITY TESTING
OF YEAST ISOLATES FROM PRETORIA**

By

MAREN HNAYA

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Department of Medical Microbiology

Faculty of Health Sciences, University of Pretoria
Pretoria, South Africa

Supervisor

Dr R Lekalakala

Co-Supervisor

Mr Y Dangor

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DECLARATION

I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signature: _____

Date: _____

DEDICATION

I owe a great many thanks to my parents for all of the sacrifices that they have made for me in the past, for the sacrifices that they continue to make for me till today and for all that they are doing in order to build a brighter tomorrow for me.

My deepest sense of gratitude to my husband for the confidence he has given me. He has always supported me. Because of him, I have never felt alone.

I wish to express my thanks to my sisters and my brothers for standing by my side constantly.

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SUMMARY

Antifungal drug resistance is a growing problem. Several mechanisms contribute to the development of resistance to antifungal agents. In South Africa, little is known about the antifungal susceptibility of local yeast isolates.

The aim of this study was therefore to determine the antifungal susceptibility profile of local *Candida* species and *Cryptococcus neoformans* isolates and the molecular mechanisms of resistance to different antifungal agents in Pretoria. A total of 250 yeast isolates were collected from the diagnostic laboratory in the Department of Medical Microbiology at the University of Pretoria-National Health Laboratory Services.

The isolates were subcultured on Sabouraud dextrose agar media for purity of yeast colonies. Identification to species level was performed using biochemical techniques. The antifungal susceptibility of 87 isolates was determined by the Etest for three azole antifungals (fluconazole, posaconazole and voriconazole), amphotericin B and caspofungin. Clinical breakpoint susceptibility was determined according to the CLSI clinical breakpoint reference methods. Polymerase chain reaction was performed on *C. albicans* isolates to amplify the *ERG11* gene and the *FKSI* gene. Sequencing was done for the amplification products and the sequence data were analysed by the CLC genome workbench software.

Among the 250 isolates collected, *Candida* species accounted for 82.8% and *C. neoformans* accounted for 17.2% of the isolates. *C. albicans* was the most commonly isolated (76.8% of *Candida* species), of which 30% were resistant to caspofungin. Fluconazole resistance was detected in 56.7% of *C. parapsilosis* isolates, the highest fluconazole resistance among *Candida* species. Cross-resistance was found between fluconazole and voriconazole. Resistance to posaconazole was detected in 66.7% of *C. glabrata* isolates, whilst all other *Candida* species and *C. neoformans* isolates were fully susceptible. Furthermore, *C. neoformans* var. *gattii* isolates were less susceptible to azole antifungal agents than *C. neoformans* var. *neoformans* isolates.

Molecular alterations are one of the mechanisms of resistance to azole and caspofungin antifungal agents. The amino acid substitutions D116E, K128T and V437I were detected in the *ERG11* gene of two azole susceptible isolates. The new amino acid substitution E517Q was detected in the *ERG11* gene of a resistant isolate. The S642L substitution was detected in the *FKS1* gene of all the isolates that were caspofungin resistant and caspofungin susceptible.

C. albicans was the most commonly isolated yeast species in Pretoria. Cross-resistance was detected between fluconazole and voriconazole. Therefore, these two agents are not a good alternative to use in the treatment of resistant isolates. With *Candida* species and *C. neoformans* isolates, there was less resistance to posaconazole than to fluconazole and voriconazole. The identification of the two varieties of *C. neoformans* is important in order to establish the differences in their antifungal susceptibility. Resistance to azole and caspofungin antifungal agents existed without the previously described molecular alterations in the *ERG11* and *FKS1* genes of resistant isolates. Further studies are required to explain the role of new amino acid substitutions, as well as the involvement of other mechanisms in resistance to antifungal drugs.

Key words: Antifungal, Yeast, Susceptibility, Resistance, Mechanism of resistance, *Candida*, *Cryptococcus neoformans*, Etest, Pretoria.

TABLE OF CONTENTS

	Page
DECLARATION	i
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
SUMMARY	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	x
CHAPTER 1: LITERATURE REVIEW	1
1.1 Introduction	1
1.2 Yeast taxonomy	3
1.3 Epidemiology of yeast infections	4
1.4 Clinical manifestations of yeast infections	8
1.5 Laboratory diagnosis of yeast infections	10
1.6 Antifungal agents	14
1.6.1 Azoles	15
1.6.2 Poleynes	16
1.6.3 Echinocandins	17
1.6.4 Pyrimidines	17
1.6.5 Allylamines	18
1.7 Mechanisms of resistance	18
1.7.1 Mechanism of resistance to azoles	18
1.7.2 Mechanism of resistance to poleynes	20
1.7.3 Mechanism of resistance to echinocandins	20
1.7.4 Mechanism of resistance to fluorinated pyrimidines	20
1.7.5 Mechanism of resistance to allylamines	21
1.8 Antifungal susceptibility testing	21

1.8.1 Broth dilution methods	22
1.8.2 Agar dilution methods	23
1.8.3 Non-growth based methods	24
1.9 Molecular aspects of resistance to antifungal agents	25
1.10 Aim	29
1.11 Objectives	29
CHAPTER 2: MATERIALS AND METHODS	30
2.1 Samples size and study population	30
2.2 Subculturing of yeast isolates	30
2.3 Identification of yeast isolates	30
2.3.1 Identification of <i>C. albicans</i> by germ-tube	30
2.3.2 Identification of <i>C. neoformans</i> by India ink staining	31
2.3.3 Identification of <i>C. neoformans</i> by urease test	31
2.3.4 Identification of <i>C. neoformans</i> varieties by using CGB culture medium	31
2.3.5 Identification of non-albicans <i>Candida</i> by the Vitek 2 system	32
2.4 Antifungal susceptibility testing by Etest	32
2.5 Extraction of genomic DNA	34
2.6 Detection of the <i>ERG11</i> gene mutations by PCR and gene sequencing	34
2.7 Detection of the <i>FKSI</i> gene mutations by PCR and gene sequencing	35
2.8 Data analysis	36
2.9 Ethical consideration	36
CHAPTER 3: RESULTS	37
3.1 Identification of yeast isolates	37
3.2 Antifungal susceptibility by Etest	39
3.3 Amplification of <i>ERG11</i> and <i>FKSI</i> genes of <i>C. albicans</i> isolates	42
3.4 Sequencing of the amplified genes	44
CHAPTER 4: DISCUSSION	47
CONCLUSION	54
LIMITATION OF THE STUDY	55
REFERENCE	56

LIST OF TABLES

	Page
Table 2.1 Clinical breakpoints for the antifungal agents against different yeast species	33
Table 2.2 Primer sequences for amplification of the <i>ERG11</i> gene by PCR	35
Table 2.3 Primer sequences for amplification of the <i>FKSI</i> gene by PCR	36
Table 3.1 Species distribution among yeast isolates	37
Table 3.2 Sources of the study isolates	38
Table 3.3 Antifungal susceptibility of yeast isolates by Etest method	40
Table 3.4 MIC and range values of yeast isolates by Etest	41

LIST OF FIGURES

	Page
Figure 1.1 Predominant drug resistance mechanisms of <i>Candida</i> species	19
Figure 3.1 A representative gel picture showing amplified products of the <i>ERG11</i> gene of <i>C. albicans</i> isolates	41
Figure 3.2 A representative gel picture showing amplified products of the <i>FKS1</i> gene of <i>C. albicans</i> isolates	42
Figure 3.3 Amino acid alignment of the <i>ERG11</i> gene of <i>C. albicans</i> isolates	43
Figure 3.4 Amino acid alignment of the <i>FKS1</i> gene of <i>C. albicans</i> isolates	45

LIST OF ABBREVIATIONS

ABC	Adenosine binding cassette
AIDS	Acquired immunodeficiency virus
ATP	Adenosine tri-phosphate
bp	Base pairs
BSIs	Blood stream infections
CGB	L-canavanine bromothymol blue
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
DNA	Deoxy ribonucleic acid
EUCAST	European Committee for Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FUMP	5-fluorodeoxyuridine monophosphate
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HS	Hot spot region
IFIs	Invasive fungal infections
KOH	Potassium hydroxide
MDR	Multidrug resistance
MFS	Major facilitators
MIC	Minimum inhibitory concentration
MOPS	3-N-morpholinopropanesulfonic acid
NCCLS	United States National Committee for Clinical and Laboratory Standards
NHDS	National Health Discharge Survey
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
TB	Tuberculosis
UNAIDS	United Nations program on HIV/AIDS
UPRTase	Urasil phosphoribosyl transferase
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The frequency of invasive fungal infections (IFIs) is increasing (Pfaller and Diekema, 2010); at the same time, the resistance of pathogenic fungi to antifungal therapy has become a serious problem in the management of fungal infections (Perea and Patterson, 2002). Hence, it is essential to undertake susceptibility testing and to understand mechanisms of resistance in order to ensure more efficient treatment of fungal infections and to improve outcomes when treating such infections.

Candida species can be part of the normal flora of the oral cavity, gastrointestinal tract and/or genitourinary tract of humans and 40% to 60% of adult population harbour *Candida albicans* (Jones, 1990). The main source of *Candida* infection (Candidiasis) is endogenous, but infection can also be transmitted to patients exogenously by healthcare workers and healthcare devices. Candidiasis can present as superficial skin or mucous membranes infections, or as invasive and systemic infections (Murray *et al.*, 2009). *C. albicans* is the most common cause of invasive fungal infections; but non-*albicans Candida* species have also been shown to be a major source of fungal infection (Pfaller *et al.*, 2004a).

Pathogenesis of *Candida* is facilitated by its adherence to host tissue or medical devices, cell surface hydrophobicity, proteinase production and phenotypic switching. Adherence is related to a combination of specific ligand receptor interaction and non-specific electrostatic mechanisms. Hydrophobicity of the cell surface can affect the degree of adhesion to epithelial cells. Hydrolytic enzymes such as aspartyl proteinases and phospholipases are able to hydrolyse proteins involved in host defense and can damage host cells. Phenotypic switching and transformation from yeast to hyphae allow the organism to grow along pores and grooves, to adapt to changes in the microenvironment and to elude host defenses (Murray *et al.*, 2009).

Infection with *Cryptococcus neoformans* (cryptococcosis) is mainly acquired when *C. neoformans* cells are inhaled from the environment. Although, cases of cryptococcosis have

been reported in immunocompetent individuals, they are more common in immunosuppressed patients such as patients with acquired immunodeficiency syndrome (AIDS), haematological malignancies or who have had organ transplantation. There are two varieties of *C. neoformans*, namely; *C. neoformans* var *neoformans* and *C. neoformans* var *gattii*. *C. neoformans* var *neoformans* predominates in immunocompromised patients, whereas *C. neoformans* var *gattii* has been identified as a cause of disease in immunocompetent individuals. Infection may be asymptomatic or may be associated with the respiratory system, prostate, eyes or skin (Chayakulkeeree and Perfect, 2006). Furthermore, infection may spread to the central nervous system (CNS) and it may present with meningitis or meningoencephalitis, which are more common in immunosuppressed patients (Kidd *et al.*, 2004).

The pathogenicity of *C. neoformans* is enhanced by its ability to grow at 37 °C, the presence of a capsule and the production of phenol oxidase (Kwon-Chung *et al.*, 1982). The capsule protects the organism from phagocytosis, suppresses both humoral and cellular immunity, interferes with antigen presentation and limits the production of toxic nitric oxide by the host cell. Furthermore, phenol oxidase utilizes hydroxybenzoic substrates such as catecholamines (such as dopamine and norepinephrine) and produces melanin, which then acts as an antioxidant and protects *C. neoformans* from host defenses; this phenomenon may explain the neurotropism of *C. neoformans* (Jacobson *et al.*, 1994; Murray *et al.*, 2009).

The antifungal agents used clinically are classified into four main groups, each of which relies on different mechanisms of action. Polyenes and azoles act on the cell membrane, echinocandins act by disrupting the fungal cell wall and pyrimidines interfere with the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Peman *et al.*, 2009).

Resistance to antifungal agents is increasing despite the introduction of new antifungal drugs (Pfaller, 2012). Several mechanisms of resistances for azoles have been described, namely; up-regulation of the efflux pump and over-expression of the target enzyme (Vanden-Bossche *et al.*, 1992). Another is point mutations in the *ERG11* gene (White, 1997a), which result in altered target or decreased affinity to bind with the antifungal agent. Echinocandin resistance is mediated by mutations in the *FKS1* gene, which encodes the expression of the enzyme that synthesizes 1, 3- β -D-glucan, a major component in the cell wall (Park *et al.*, 2005).

Different susceptibility testing methods have been introduced. These include; broth microdilution and macrodilution, disc diffusion, agar diffusion and the Etest (Ghannoum *et al.*, 1996; Rex *et al.*, 2001). Antifungal susceptibility testing is becoming essential in patient management. Studying the antifungal resistance profile and understanding of molecular mechanisms of resistance, will lead to the development of more efficient strategies for the prevention of fungal infections and the treatment of infected patients.

1.2 Yeast taxonomy

Fungi are eukaryotic organisms, characterized by a rigid cell wall composed of chitin and glucan and by a cell membrane with ergosterol as a major sterol component, compared to cholesterol in other eukaryotes. Medically important fungi have been classified into five classes according to their morphological appearance, spore production, ultrastructural and biochemical features. The five classes are: i. Hemiascomycetes: such as *Candida* species, ii. Basidiomycetes: such as *C. neoformans*, *Malassezia* and *Trichosporon*, iii. Euascomycetes: such as *Aspergillus* and *Histoplasma*, iv. Archiascomycetes: such as *Pneumocystis* and v. Zygomycetes: such as *Rhizopus* and *Mucor* (Murray *et al.*, 2009).

Other classifications are based on morphology and categorize fungi into yeasts and moulds. Moulds are multicellular fungi with threadlike structures known as hyphae that form mycelium. Yeast is defined as a fungus that reproduces by budding or fission, when a mother cell pinches off a portion of itself to produce a daughter cell (Murray *et al.*, 2009). The word “yeast” in English refers to an agent that causes something to foam and to raise, which indicates the fermentation processes induced by some yeasts, such as *Saccharomyces cerevisiae* which is used in the baking industry (Kurtzman and Fell, 1998).

Candida species are diploid, asexual and pleomorphic hemiascomycetous. They are oval yeast-like forms, 3 µm to 5 µm in diameter, can produce buds (blastospores) and form germ-tubes. In culture, *Candida* species form smooth creamy colonies. Some species, such as *C. albicans*, can undergo phenotypic switching in which they change to different morphotypes, ranging from the typical smooth creamy colonies to hairy colonies composed of pseudohyphal and hyphal structures (Murray *et al.*, 2009).

C. neoformans was discovered in 1894, when it was isolated by Busse and Buschke from a tibial lesion in Germany. In the same year Sanfelice isolated the organisms in nature from peach juice in Italy (Kwon-Chung and Bennett, 1992). *C. neoformans* is an encapsulated spherical to oval basidiomycetous, 2 µm to 20 µm in diameter, which replicates by budding. When they are stained with India ink, cells appear spherical to oval, surrounded by a halo that represents the polysaccharide capsule. There are two varieties; namely; *C. neoformans* var *neoformans* and *C. neoformans* var *gattii*, and four serotypes based on capsular epitopes; A, B, C and D. The serotypes of *C. neoformans* var *neoformans* are A, D and AD. They can be found worldwide in nature in soil enriched by pigeon droppings. The serotypes of *C. neoformans* var *gattii* serotypes are B and C. They can be found in eucalyptus trees in tropical and subtropical regions in Africa, Australia, Southern Asia and Southern California (Murray *et al.*, 2009).

1.3 Epidemiology of yeast infections

Candida species are the fourth most commonly isolated organisms from bloodstream infections (BSIs), about 9% of all BSIs (Pfaller *et al.*, 2001a). The most commonly isolated *Candida* species from BSIs is *C. albicans*, which accounts for 50% to 70% of BSI isolates (Murray *et al.*, 2009). Between 1997 and 2003, Pfaller *et al.* (2005) collected more than 140000 yeast isolates from 39 countries in the world and reported that; in 2003, *Candida* species accounted for 95% of all isolates, *C. albicans* was the most common (60%), followed by *C. glabrata* (12 %), *C. tropicalis* (7%) and *C. parapsilosis* (7%). However, many geographical and institutional differences have been reported, for example; in Brazil, *C. tropicalis* is the second most common *Candida* species, followed by *C. parapsilosis* (Colombo *et al.*, 2006).

Many factors have contributed to the increase of serious systemic fungal infections. These factors include an increase in the number of immunocompromised cases due to human immunodeficiency virus (HIV) infection, cancer patients with neutropenia complicated by chemotherapy and transplantation patients on immunosuppressive therapy, as well as other factors such as the administration of broad spectrum antibiotics, glucocorticoids and parenteral nutrition (Lorthoraly and Dupont, 1997).

Candida species colonize the gastrointestinal tract, mucosal surfaces such as the vaginal tract and urethra, as well as skin. Candidiasis usually occurs by endogenous infection from mucosal or cutaneous sites with bloodstream dissemination, but exogenous infection may occur in patients when contaminated irrigation solutions are used, or via parenteral nutrition. Transmission from healthcare workers to patients or from patients to healthcare workers has also been documented (Murray *et al.*, 2009).

In the United States, the estimated annual incidence of invasive candidiasis, according to the National Health Discharge Survey (NHDS), was 228 per million in 1996 (Wilson *et al.*, 2002). The distribution of the pathogenic fungi varies, depending on the age and the underlying condition of the patient, for example; leukemic patients are more likely to be infected with *C. albicans* or *C. tropicalis*, whilst haematopoietic stem cell transplantation patients are more likely to be infected with species with primary resistance to azoles such as *C. krusei* and *C. glabrata* (Pfaller and Diekema, 2007). Children and infants are mostly infected with *C. albicans* and *C. parapsilosis* (Pappas *et al.*, 2003).

Approximately 3000 infants develop candidemia per year. Prematurity and low birth weight both are associated with the development of neonatal nosocomial infections (Saiman *et al.*, 2000). *Candida* species are the third most common nosocomial BSIs in premature neonates, with a mortality as high as 20%, despite adequate antifungal treatment (Stoll *et al.*, 1996).

In South Africa, *C. albicans* was the most commonly isolated *Candida* species among intensive care unit patients. *C. parapsilosis* and *C. tropicalis* were the main cause of candidaemia among children with haematological malignancies and HIV-infected children (Arendse and Orth, 2008).

According to the NHDS, the incidence of cryptococcosis in the United States was 29.6 per million of population in 1996 (Wilson *et al.*, 2002). *C. neoformans* has become an important cause of opportunistic fungal infections especially in immunocompromised patients, 7% to 10% of patients with AIDS are affected by cryptococcosis and 50% of all cryptococcal infections are associated with AIDS (Abadi *et al.*, 1999). Infection with *C. neoformans* is usually acquired by inhalation of *C. neoformans* cells from the environment and when the cells reach the alveoli, they start to reproduce and invade the tissues (Murray *et al.*, 2009).

The incidence of cryptococcosis has decreased since the introduction of highly active antiretroviral therapy (HAART) in the mid of 1990s (Manfredi *et al.*, 2003), but it is still causing up to 30% of mortality in AIDS patients in South Asia, Africa and Spain (Kumarasamy *et al.*, 2005; Perkins *et al.*, 2005). In South Africa, a study done between 2002 and 2004 demonstrated that the incidence of cryptococcosis was 15.6 per 100000 of the population and 95 per 100000 among HIV-infected persons; the mortality rate was 27 % (McCarthy *et al.*, 2006).

C. neoformans var *neoformans* is the major pathogen in AIDS patients and it is distributed worldwide. The natural habitat of the fungus is soil and areas enriched with avian excreta (Murray *et al.*, 2009). About 80% of cryptococcal infections in the general population are caused by *C. neoformans* var *gatti*. It was considered to be distributed only in tropical and subtropical regions, until the 1999 outbreak of *C. neoformans* var *gattii* in British Columbia, Canada, the Northwestern United States and Vancouver islands which suggested wide spread distribution of the fungus (Datta *et al.*, 2009). The pathogen is rarely isolated from immunocompromised patients (Murray *et al.*, 2009). *C. neoformans* var *gattii* was first isolated from the Eucalyptus trees (Ellis and Pfeiffer, 1990), but today more than 50 tree species harbour the organism (Springer and Chaturvedi, 2010).

Antifungal drug resistance can be classified into three groups namely; 1) primary antifungal drug resistance, which is present before exposure to the drug; 2) secondary resistance which develops after exposure to the drug due to genetic alterations and 3) clinical resistance, in which the organism is susceptible to the antifungal agent *in vitro*, but clinically, response is poor, as displayed by a relapse or a progression of the infection, which may occur in some cases due to an ineffective immune system (Kontoyiannis and Lewis, 2002).

Resistance of *Candida* species to azoles globally was less than 2.5% to fluconazole and less than 9% to itraconazole (Pfaller *et al.*, 2000). *C. albicans* and *C. dubliniensis* are the species most susceptible species to the antifungal azoles, whereas *C. glabrata* is less susceptible and *C. krusei* is intrinsically resistant to fluconazole *in vitro* (Pfaller *et al.*, 1999; Pfaller *et al.*, 2000). It has been reported that azoles resistance in *C. albicans* is 1.0% to 2.1%, in *C. parapsilosis* 0.4% to 4.2% and in *C. tropicalis* 1.4% to 6.6% (Pfaller and Diekema, 2004a; Pfaller *et al.*, 2005). In *C. glabrata*, resistance has increased from 7% in 2001 to 12% in 2004 (Pfaller *et al.*, 2006). A study done by Samie and Masheo in Limpopo, South Africa,

demonstrated fluconazole resistance in 80% of *C. albicans* isolates, 84.6% of *C. tropicalis*, 75% of *C. parapsilosis* and *C. glabrata* isolates (Samie and Masheo, 2012). About 10% of *Candida* species are primarily resistant to flucytosine. Secondary resistance develops in almost 30% of patients who receive flucytosine as a monotherapy (White *et al.*, 1998). Echinocandins such as caspofungin and micafungin have a good *in vitro* susceptibility with *Candida* species (Pfaller and Diekema, 2004b). Primary resistance to amphotericin B has been reported in some rare *Candida* species such as *C. guilliermondi* and *C. lusitaniae*. Secondary resistance has been described in other *Candida* species (Dick *et al.*, 1980; Blinkhorn *et al.*, 1989).

Drug resistance in cryptococcal infections remains a problem. This resistance can be due to many factors, such as development of primary or secondary resistance to the antifungal agent, pharmacokinetic properties, the underlying disease and the development of complications (Perea and Patterson, 2002). In South Africa, according to Samie and Masheo (2012), resistance of *C. neoformans* to fluconazole was as high as 69%. A study by Shadomy *et al.* (1987) reported that *C. neoformans* var. *gattii* was less susceptible to fluconazole than *C. neoformans* var. *neoformans*. Resistance of *Cryptococcus* to amphotericin B is rare (Vanden-Bossche *et al.*, 1998), but secondary resistance has been reported in severely immunocompromised patients (Powderly *et al.*, 1988). *Cryptococcus* species demonstrate primary resistance to echinocandin antifungal agents (Pfaller and Diekema, 2004b). Resistance of *C. neoformans* to flucytosine is a serious problem in the treatment of cryptococcal meningitis when flucytosine is used as a monotherapy (the failure rate of the treatment can be as high as 57%) (Hospenthal and Bennett, 1998) but combining amphotericin with flucytocine reduces flucytosine resistance (Bennett *et al.*, 1979).

Fungal infections are an important cause of morbidity and mortality in immunocompromised patients and have been reported in HIV patients co-infected with tuberculosis (TB) (Jarvis *et al.*, 2010). In South Africa; according to the United Nations programme on HIV/AIDS and the World Health Organization (UNAIDS/WHO report 2008), more than 5.7 million people are living with HIV and 75% of TB patients are co-infected with HIV (Heunis *et al.*, 2011). However, little is known about antifungal susceptibility profile (Blignaut *et al.*, 2002) and hence antifungal susceptibility tests are essential for proper patient management.

1.4. Clinical manifestations of yeast infections

Candida infections range from superficial candidiasis to disseminated disease. Infection of the mucous membranes such as oral candidiasis, presents as white creamy plaques known as oral thrush. Similar patches may develop in abdominal candidiasis with ulcers in the large and small intestine. Vaginal candidiasis produces a thick discharge associated with itching. Skin infections may occur in skin folds, or when the skin is wet and macerated, as in diaper dermatitis (Murray *et al.*, 2009).

Hematogenous candidiasis may be acute or chronic and may lead to deep tissues infection including infection of the heart, eyes, abdominal viscera, bones and joints. There are many predisposing factors for hematogenous candidiasis, including; solid organ transplantation, immunosuppression, prolonged antibiotic use and intensive care support (Murray *et al.*, 2009).

Urinary tract infection can range from bladder colonization to renal abscesses. Bladder colonization is mainly seen with patients who use indwelling catheters, diabetic patients, and patients who complain of urinary tract obstruction or who have had previous urinary surgery. Hematogenous spread can lead to renal abscesses, papillary necrosis or infection of the renal pelvis and ureters (Murray *et al.*, 2009).

Central nervous system infection can be due to haematogenous spread, or iatrogenic, for example, due to neurosurgery or ventriculoperitoneal shunt placement. Symptoms may resemble bacterial meningitis and may include fever, headaches and meningism, or may follow a chronic course (Murray *et al.*, 2009).

Pulmonary infection may occur as a result of aspiration of *candida* from an esophageal or mucous membranes infection (Haron *et al.*, 1993). Although, the fungus can be isolated from sputum, bronchoalveolar lavage or endotracheal tube secretions, definitive diagnosis of pulmonary infection can be made by a demonstration of *candida* in lung tissue (El-Ebiary *et al.*, 1997).

The heart may be involved and the symptoms may resemble that of infective endocarditis with fever and a new or changing heart murmur (Murray *et al.*, 2009). Endocarditis caused by *Candida* represents 48% of all cardiac fungal infections, in which 50% are caused by *C. albicans* (Ellis *et al.*, 2001).

Primary abdominal candidiasis can be seen in post-operative cases, in patients on dialysis or with a perforated bowel (Suarez *et al.*, 1994). Chronic hepatosplenic candidiasis may present with fever, elevated alkaline phosphatase and multiple lesions in the liver and spleen. *Candida* peritonitis may be seen in patients with chronic peritoneal dialysis, after abdominal surgery or intestinal perforation and can remain localized or can lead to hematogenous candidiasis (Murray *et al.*, 2009).

Eye infection usually presents as keratoconjunctivitis following trauma, corneal transplantation or topical steroid usage (Ainbinder *et al.*, 1998). Chorioretinitis and endophthalmitis may occur due to hematogenous spread (Parke *et al.*, 1982). Symptoms include redness, pain and blurred vision (Krishna *et al.*, 2000).

Bone and joint candidiasis is mainly caused by hematogenous spread and primary disease is extremely rare. Infection can be due to trauma, surgery or steroid injection. Furthermore, bone and joint candidiasis has been documented after several months of successful treatment of candidemia (Murray *et al.*, 2009). Symptoms are pain, swelling and abscess formation (Gathe *et al.*, 1987).

Symptoms of neonatal candidemia include lethargy, apnea, respiratory distress, hypotension, hypoglycemia, abdominal distension and feeding intolerance (Fanaroff *et al.*, 1998; Benjamin *et al.*, 2000). Meningitis has been reported in 15% of cases with disseminated disease (Benjamin *et al.*, 2003). The renal system may also be involved and infection varies in severity from candiduria to renal involvement (Karlłowicz, 2003). Candidemia in neonates has also been associated with endophthalmitis and the development of retinopathy of prematurity (Baley and Ellis, 2003). There are some other diseases which are unique neonatal presentations, such as congenital cutaneous candidiasis, which is acquired by ascending infection from the vagina into the uterus and presents as a diffuse erythematous papular rash developed at or during the first 24 hours of birth (Darmstadt *et al.*, 2000).

Cryptococcal infections can present as acute, subacute or chronic lung disease. Chest radiological imaging usually shows a pulmonary nodule that simulates pulmonary cancer (Singh *et al.*, 2008). In AIDS patients, chest cryptococcosis may present with miliary lesions resembling those of tuberculosis (Douketis and Kesten, 1993). Large mass lesions (cryptococcoma) are characteristic in *C. neoformans* var *gattii* chest infections (Pappalardo and Melhem, 2003).

Central nervous system involvement may occur due to haematogenous or lymphatic spread from pulmonary infection. In HIV negative patients, CNS cryptococcosis can present as subacute or chronic meningoencephalitis. Symptoms may be insidious, with headaches, behavioural changes and fatigue, or may be more serious, with visual problems and meningeal syndrome. In immunocompromised patients, meningoencephalitis is present in more than 90% of cases as an acute course. Clinical symptoms include fever, nausea, vomiting, visual disturbance, abnormal mental status and seizures. CNS infection due to *C. neoformans* var *gattii* is more severe, appears in 5% to 10% of cases with CNS involvement and usually present as a mass in the brain parenchyma (cryptococcoma) (Chayakulkeeree and Perfect, 2006).

Skin lesions are reported in 6% of AIDS patients with cryptococcosis. Lesions appear as tubercles, nodules or abscesses rarely associated with lymphangitis or lymphadenitis (Chayakulkeeree and Perfect, 2006). Other sites, such as the mucous membranes, bones, liver, spleen, adrenal glands, prostate, kidneys and lymphnodes may be involved (Murray *et al.*, 2009).

1.5 Laboratory diagnosis of yeast infections

Superficial candidiasis is diagnosed by taking a scraping of the mucosal or cutaneous lesion, which can be examined microscopically after treatment with 10% to 20% potassium hydroxide (KOH) with calcofluor white. Examination with a fluorescence microscope detects the budding yeast-like forms with pseudohyphae. Cultures on standard mycological media or selective media such as chromogenic media (such as CHROMagar Candida, France) are used to identify the species, based on morphological appearance. Skin lesions should also be

biopsied and stained with fungal stains for histologic examination; the presence of budding yeasts and pseudohyphae is specific to the diagnosis of candidiasis (Murray *et al.*, 2009).

Haematogenous spread is diagnosed by culturing blood, tissue, urine, sputum or other body fluid specimens. In contrast to bacterial pathogens, *Candida* infection may present as an invasive tissue disease, with the absence of the organism itself in the blood stream due to tissue tropism of *Candida* species (Brian Smith *et al.*, 2005).

C. albicans species form germ-tubes after incubation at 37°C for two to three hours, in the presence of albumin. The germ-tubes can be distinguished from pseudohyphae by the absence of separations in the tubes and the absence of constrictions at the mother cell neck. The germ-tube test is used to differentiate between *C. albicans* and non-*albicans Candida* species in diagnostic laboratories (Jones, 1990; Winn *et al.*, 2006).

Identification to species level of the infecting organism is important for treatment of *Candida* infection because *Candida* species differ in respect of their susceptibility to antifungal drugs (Pappas *et al.*, 2004). Furthermore, the severity of the clinical manifestations differs, depending on the infecting species. Therefore, knowledge of the infecting species can be used as a guide to patient therapy (Denning *et al.*, 2003).

Different methods and formats have been developed for *Candida* species identification. These methods include: the RapID Yeast Plus (Innovate Diagnostic System, USA), API AUX kits (20C and 32C) (BioMerieux, France), the Vitek yeast cards, using the Vitek 2 system (BioMerieux, France), the MicroScan yeast identification panel (Dade Behring Inc., USA), the Yeast star system (CLARRC Laboratories, Netherlands), the Auxacolor identification system (Sanofi Diagnostic Pasteur, France), the API Candida (BioMerieux, France) and the CHROMagar medium (Microbiology, France). These systems are discussed in more details below.

The RapID Yeast Plus System (Innovate Diagnostic Systems, USA) contains conventional and chromogenic substrates and is based on the utilization of carbohydrate substrates, the hydrolysis of fatty acids and the enzymatic hydrolysis of glycoside and aryl amide. Performance studies showed that its accuracy ranges from 84% to 99% (Espinel-Ingroff *et al.*, 1998; Wadlin *et al.*, 1999).

The API 20C and 32C AUX kits (BioMerieux, France) contain 20 and 32 microtubes, respectively. The API AUX kits depend on an increase in turbidity and identification performance has been reported to reach 99.8% (Bernal *et al.*, 1998; Campbell *et al.*, 1999).

The Vitek system (BioMerieux Vitek, USA) is also an automated biochemical system used for phenotypic *Candida* species identification. It consists of 30 wells which contain 26 biochemical broths and four negative control broths. After incubation at 30°C for 24 to 48 hours with the identification cards, colour changes can be read spectrophotometrically. The database includes 36 yeast species. Performance ranges from 84.9% to 98%, in comparison with conventional methods (Verweij *et al.*, 1999).

The Vitek 2 coloremtric system (BioMerieux, France) consists of 64 wells and 46 coloremtric biochemical tests. Final results can be obtained at 18 hours. The database of this system contains 53 species and 14 genera (Aubertine *et al.*, 2006).

The MicroScan Yeast Identification Panel (Dade Behring Inc., USA) is based on the detection of preformed enzymes rather than growth. After incubation at 37°C for four hours, the panels can be read visually or automatically. Accuracy is comparable with that of the API 20C system for the identification of common yeasts (95.8% to 96.6%) (St Germain and Beauchesne, 1991).

The Yeast Star system (CLARRC Laboratories, Netherlands) is based on the inhibitory effects of specific dyes on fungal growth. Six dyes are applied on top of a pre-inoculated growth medium and then incubated at 37°C for 24 to 48 hours. Results can be read in the form of six numerical codes. The data base contains 16 yeast species (Verweij *et al.*, 1999).

The Auxacolor yeast identification system (Sanofi Diagnostic Pasteur, France) is based on carbohydrate assimilation. Growth can be seen by the colour change of a pH indicator. The plates can be read after incubation at 30°C for 24 to 72 hours. Several other characteristics are used to complete the identification, such as ability to grow at 37°C, the formation of mycelium and the presence of a capsule. The database contains 26 yeast species. Performance ranges between 63.8% and 95.2%, in comparison with conventional methods (Buchaille *et al.*, 1998; Verweij *et al.*, 1999).

The API Candida system (BioMerieux, France) is using sugar acidification or enzymatic reactions for yeast identification. Results are read after 24 and 48 hours of incubation at 30°C and a four digit code is obtained. The database contains 26 yeast species (Verweij *et al.*, 1999).

The CHROMagar medium (Microbiology, France) was developed for the identification of *Candida* species. This medium contains chromogenic substrates linked to chemical dyes and can react with enzymes secreted by *Candida* species, producing colonies with different colours and allowing identification of the organisms to species level by colour and growth characteristics (Odds and Bernaerts, 1994; San-Millan *et al.*, 1996).

Molecular identification of *Candida* species is also available. Polymerase chain reaction (PCR) techniques are used by amplifying the highly conserved regions of the DNA of each species (Sandhu *et al.*, 1995). Furthermore, identification can be performed by real time PCR assays, such as TaqMan (Guiver *et al.*, 2001) or fluorescent energy transfer methods (Loeffler *et al.*, 2000).

Histopathological examination of a tissue section is an important method to diagnose systemic fungal infection. The presence of blastospores and pseudohyphae of *Candida* species in a stained histopathological tissue section is one of the parameters for the diagnosis of invasive *Candida* infection (Ellepola and Morrison, 2005).

Serological diagnosis of invasive candidiasis is complicated, because *Candida* could be a normal commensal of mucosal surfaces and the circulating antibodies to *Candida* species may be found in a normal person. In addition, immunocompromised patients produce low or undetectable levels of antibodies, which may lead to false negative results. Several circulating antigens have been used to diagnose systemic candidiasis, such as the extracellularly secreted aspartyl proteinase and mannan, which is the major cell wall mannoprotein of *C. albicans*. *Candida* antigens are often rapidly cleared from the circulation, with the result that antigen detection tests often lack the exact level of sensitivity needed (Ellepola and Morrison, 2005).

The structural component of the cell walls of *Candida* species, 1,3-β-D-glucan has been used as an indicator of invasive fungal infection, as it is not found in bacteria, viruses or mammals (Ellepola and Morrison, 2005). Candidiasis may be diagnosed by the metabolite D-arabinitol

in serum or urine, as it is produced by most medically important *Candida* species, except *C. krusei* and *C. glabrata* (Christensson *et al.*, 1999).

C. neoformans can be identified by microscopic examination of blood samples, cerebrospinal fluids or other body fluids. Staining with India ink reveals the encapsulated budding cells of *C. neoformans*. Incubation on most mycological media at 25°C to 37°C for 48 hours produces moist white mucoid colonies (Casadevall and Perfect, 1998). *C. neoformans* can be identified using a urease test, which is based on urea hydrolysis by the enzyme urease, which is produced by *C. neoformans*, producing ammonia and a change in the colour of the indicator from yellow to red (Roberts *et al.*, 1978). *C. neoformans* can also be identified by a production of brown colonies in niger seed agar with production of melanin by phenoloxidase activity (Casadevall and Perfect, 1998; Klein *et al.*, 2009).

Serological detection of the capsular polysaccharide antigen in cerebrospinal fluid or serum can be performed by latex agglutination with specific rabbit anti *C. neoformans* antisera (Mitchell and Perfect, 1995; Casadevall and Perfect, 1998). Histopathological analysis of a tissue specimen (Gazzoni *et al.*, 2008) can be performed to diagnose cryptococcosis.

Differentiation between *C. gattii* and *C. neoformans* can be performed by L-canavanine glycine bromothymol blue (CGB) agar, which is a simple and efficient method that can be incorporated in routine laboratory screening tests to identify *C. neoformans* varieties. *C. gattii* can grow in the presence of L-canavanine and utilizes glycine as a source of carbon and nitrogen, which cause the pH to rise and the agar to turn blue after incubation at 35°C for 72 hours. Other *C. neoformans* var *neoformans* cannot grow in the presence of canavanine and cannot utilize glycine, so the colour will not change (Klein *et al.*, 2009).

1.6 Antifungal agents

It is essential to understand the mechanism of action of antifungal drugs to understand the mechanism of resistance. Three of the main antifungal drugs used clinically, namely: azoles, polyenes and allylamines; act by inhibiting the synthesis of or the interaction with ergosterol, which is the main component of the cell membrane (Parks and Casey, 1996). Another group of the antifungal drugs is pyrimidines; which inhibit nucleic acids synthesis (Diasio *et al.*,

1978). The echinocandin antifungal drugs are active against the fungal cell wall (Denning, 2003).

1.6.1 Azoles

Azoles are divided, depending on their structure, into: imidazole (which has two nitrogen atoms in theazole ring) such as ketoconazole; and triazole (which has three nitrogen atoms in theazole ring) such as fluconazole, voriconazole and posaconazole (Murray *et al.*, 2009). These agents act by inhibition of cytochrome P450 dependent 14 α -lanosterol demethylase, leading to the depletion of ergosterol and the accumulation of sterol precursors and result in an alteration of the structure and the function of fungal cell membrane (Hitchcock *et al.*, 1990).

Fluconazole is a first generation triazole and an active agent against most *Candida* species and *C. neoformans*. Clinically, it is effective in the treatment of candidaemia, mucosal candidiasis and maintenance treatment of cryptococcal meningitis in AIDS patients. Fluconazole is a water soluble agent and can be administered by oral or intravenous route. It is well distributed in organs and tissues and side effects, such as liver failure and exfoliative dermatitis, are uncommon (Murray *et al.*, 2009).

Voriconazole, a new broad spectrum antifungal, is active against *Candida* species and *C. neoformans*. It is available in oral and intravenous preparations and is widely distributed in the body. It is fungistatic against yeast-like fungi and fungicidal against *Aspergillus* species. Voriconazole is generally a well tolerated drug; side effects include liver enzyme abnormalities, skin reactions, confusion and about one third of the patients experience transient visual disturbances (Murray *et al.*, 2009).

Posaconazole is a water insoluble lipophilic agent. It is available only in oral route preparations that are not affected by changes in gastric acidity and must be administered with food or food supplements (Courtney *et al.*, 2004). Posaconazole is active against *Candida* species; it displays fungicidal activity against *C. krusei* and *C. lusitaniae* and fungistatic activity against other *Candida* species (Schiller and Fung, 2009). It is also active against *C. neoformans*, *Rhodotorula* species and zygomycetes (Zaas *et al.*, 2003; Sabatelli *et al.*, 2006).

Clinically, it is used for the treatment of oropharyngeal and esophageal candidiasis, as well as invasive aspergillosis and zygomycoses (Skiest *et al.*, 2007; Walsh *et al.*, 2007). Side effects are mainly gastrointestinal symptoms, headaches and elevated transaminase levels (Ullmann *et al.*, 2007).

1.6.2 Polyenes

The basic structure of polyenes consists of a lactose ring, with a lipophilic portion bearing several double bonds and a hydrophilic portion bearing hydroxyl groups (Murray *et al.*, 2009). They act by interaction with membrane sterol, resulting in altered permeability of the cell membrane, leakage of cytoplasmic components and associated cell death (Kerridge, 1985).

Amphotericin B is a polyene macrolide antifungal agent. It is poorly absorbed in water and not absorbed by an oral or intramuscular route of administration. Its mechanism of action is binding to the ergosterol component of the fungal cell membrane, leading to leakage of the intracellular contents and cell death. Furthermore, it can pass through oxidative reactions and produce toxic free radicals, leading to direct cell membrane damage and cell death. Amphotericin B has a wide spectrum activity, including *Candida* species, *C. neoformans*, *Aspergillus* species, zygomycetes and dimorphic pathogens. It is widely distributed in the body, including the liver, kidneys, spleen, bone marrow and lungs and effective in treating CNS fungal infections. The main side effects are nephrotoxicity and infusion-related side effects such as: fever, rigor and hypotension. Lipid formulation of amphotericin B has less nephrotoxicity than the conventional deoxycholate amphotericin B (Murray *et al.*, 2009).

Nystatin, a topical preparation, acts by the same mechanism as amphotericin B. A lipid formulation has been developed for systemic uses but it is still under investigation (Murray *et al.*, 2009).

1.6.3 Echinocandins

Echinocandins were the first lipopeptide antifungal agents introduced. They inhibit synthesis of 1,3- β -glucan, an important component in fungal cells. Caspofungin, micafungin and anidulafungin were the first members of the antifungal group that targets the cell wall (Denning, 2003; Murray *et al.*, 2009).

Caspofungin displays broad spectrum action against *Candida* and *Aspergillus* species, but is less effective against *C. neoformans*, *Fusarium* species and zygomycetes (Denning, 2003). Caspofungin and anidulafungin are used for the treatment of invasive candidiasis such as esophageal candidiasis and candidaemia. Caspofungin is also recommended for empirical treatment of presumed fungal infections in neutropenic febrile patients, as well for the treatment of patients with invasive aspergillosis that is resistant to other approved antifungal therapies. Caspofungin is a well tolerated drug and has few side effects (Bachmann *et al.*, 2002; Murray *et al.*, 2009).

1.6.4 Pyrimidine

Flucytosine, a fluorinated pyrimidine analogue, acts by interfering with the DNA, RNA and protein synthesis in the fungal cell. It enters the cell by permease enzyme and is first converted to 5-fluorouracil, then to 5-fluorouridylic acid, which becomes phosphorylated and incorporated into the RNA, disrupting protein synthesis (White *et al.*, 1998). It can be converted to 5-fluorodeoxyuridine monophosphate (FUMP), which inhibits DNA synthesis and nuclear division (Diasio *et al.*, 1978).

Flucytosine is active against *Candida* species, *C. neoformans*, *Rhodotorula* species, *S. cerevisiae* and other molds. Primary resistance to flucytosine is rare, but resistance can develop during monotherapy. Flucytosine is water soluble and can reach high concentrations in the CSF, serum and other body fluids. Side effects include bone marrow suppression, liver toxicity and gastrointestinal disturbances. Monotherapy with flucytosine is not recommended, but combinations with amphotericin B or fluconazole are effective for treatment of candidiasis and cryptococcosis (Murray *et al.*, 2009).

1.6.5 Allylamines

Allylamines inhibit the early stages of sterol biosynthesis, which leads to an accumulation of the sterol precursor squalene (Kerridge, 1980). An accumulation of squalene leads to increased cell wall permeability and the disruption of cell contents, which is the main cause of cell death, more than ergosterol deficiency (Ryder and Favre, 1997).

Terbinafine is a lipophilic allylamine antifungal agent with a broad spectrum activity including *Candida* species and *C. neoformans*. It is highly distributed in fatty tissues, skin, hair and nails. It has been shown to be effective in the treatment of fluconazole resistant fungal infections if it is combined with fluconazole (Murray *et al.*, 2009).

1.7 Mechanisms of resistance

Candida species became important pathogens not only because of the severity of infections, but also because of their ability to develop resistance against antifungal agents. Several mechanisms have contributed to the development of resistance in *Candida* species and other fungal pathogens, including: (a) Reduced drug uptake, (b) Rapid efflux of drug, (c) Over-expression of the genes encoding the drug target, (d) Mutation in drug target and (e) activation of alternate pathways (Prasad and Kapoor, 2005) (Figure 1.1).

1.7.1 Mechanism of resistance to azoles

Resistance to azole antifungal agents can be enhanced by a modification of the quality or quantity of the target enzyme, reduced access of the drug to the target, or a combination of both mechanisms (Ghannoum and Rice, 1999).

Modification of the target enzyme of azoles, which is the cytochrome P450 lanosterol 14 α -demethylase (Erg11p), occurs by over-expression (Vanden-Bossche *et al.*, 1992) or point mutation (White, 1997a) of the *ERG11* gene that encodes this enzyme. Over-expression produces a higher amount of the enzyme which then requires a higher concentration of the

drug to be effective. Point mutations lead to amino acid substitutions and decreased affinity of the antifungal agent toward the target enzyme (Sanglard *et al.*, 1998; Asai *et al.*, 1999).

Failure of the antifungal agent to accumulate inside the yeast cell, as a result of active efflux, is another mechanism of resistance to azoles. This mechanism is mediated by two types of transporters; the major facilitators (MFS), which are encoded by multidrug resistance genes (*MDR*) and the adenosine tri-phosphate (ATP) binding cassette super family (ABC transporters), which are encoded by *CDR* genes (White, 1997b).

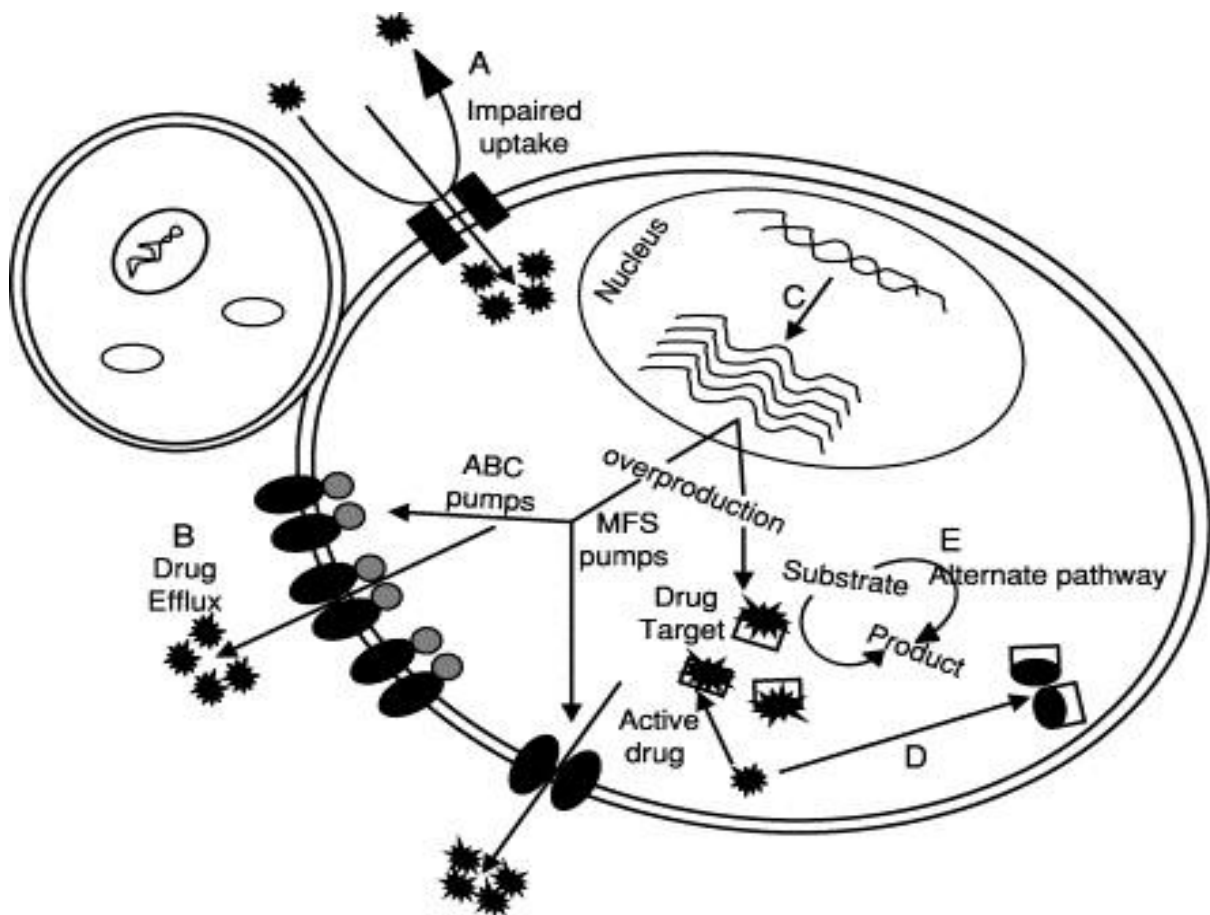


Figure 1.1 Predominant drug resistance mechanisms of *Candida* species (adopted from: Prasad and Kapoor, 2005)

1.7.2 Mechanism of resistance to polyenes

Amphotericin B is a rapidly acting, potent broad spectrum antifungal agent. Resistance to this agent is uncommon and has been reported mainly in *C. glabrata*, *C. guilliermondii*, *C. krusei* and *C. lusitaniae* (Collin *et al.*, 1999; Rex *et al.*, 2000). In *Candida* species, resistance occurs due to an alteration in the membrane ergosterol due to mutations in ergosterol biosynthesis. Defects in the *ERG3* gene can lead to defects in ergosterol biosynthesis and sterols accumulation in the fungal membranes (Dick *et al.*, 1980). Resistance in *Cryptococcus* species is rare, it is also due to mutations in ergosterol biosynthesis pathway, such as the 5,6-desaturase enzyme (Perfect and Cox, 1999).

1.7.3 Mechanism of resistance to echinocandins

In *Candida* species, resistance to echinocandins is associated with point mutations in two hot spot (HS) regions (HS1 and HS2) in the *FKS1* gene encoding the 1,3- β -D-glucan synthase complex (Perlin, 2007). A range of mutations have been reported in the HS1 and HS2 of the *FKS1* gene from resistant isolates (Park *et al.*, 2005).

1.7.4 Mechanism of resistance in fluorinated pyrimidines

Resistance to flucytosine arises from decreasing uptake of the drug associated with defects in permease. This mechanism has been reported in some species such as *C. glabrata*, but it is not an important mechanism in *C. albicans* and *C. neoformans*. The other mechanism of resistance is loss of enzymatic activity for the conversion of flucytosine to FUMP, which may occur due to a loss of cytosine deaminase or a loss of uracil phosphoribosyl transferase activity (Whelan, 1987).

1.7.5 Mechanism of resistance to allylamines

Resistance to allylamines has not been reported in human pathogenic fungi (Ghannoum and Rice, 1999). However, some cross-resistance between fluconazole and terbinafinate in *C. glabrata* has been reported (Vanden-Bossche *et al.*, 1992).

1.8 Antifungal susceptibility testing

Different antifungal susceptibility testing methods have been proposed, such as broth microdilution and macrodilution, disc diffusion and Etest. Susceptibility testing by the broth microdilution and macrodilution and the Etest is estimated by the measurement of the minimum inhibitory concentration (MIC) of the antimicrobial agent.

Intensive efforts are being made to develop standardized, reproducible and clinically relevant drug susceptibility testing for yeasts. The first optimized and standardized method was developed by the United States National Committee for Clinical and Laboratory Standards (NCCLS) (now the Clinical and Laboratory Standards Institute, CLSI) (Johnson, 2008), which proposed a reference method (M27-P) for antifungal testing in yeast by broth macrodilution and microdilution in 1992. The final approved method (M27-A) was published in 1997 (CLSI, 1997) and is used as a reference for resistance breakpoints for several antifungal agents in *Candida* and *Cryptococcus* species. Further modifications were adopted by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2008). Both the CLSI and EUCAST use broth microdilution, but there are some differences in the inoculum size and the MIC determination. They both provide clinically useful results and can discriminate between the susceptible wild type strains and resistant strains with intrinsic or acquired resistance (Pfaller *et al.*, 2010; Pfaller *et al.*, 2011). The CLSI has also developed an agar based disc diffusion method to test for antifungal susceptibility (CLSI, 2004).

A number of commercial methods that conform to CLSI method standards have been developed. Three products have been cleared by the United States Food and Drug Administration (FDA) for testing antifungal drugs: the Sensititre YeastOne colorimetric plate

(TREK Diagnostics, USA), which is based on broth microdilution with a chromogenic substrate to facilitate end point interpretation (Davey *et al.*, 1998), the Vitek 2 yeast susceptibility test (BioMerieux, France) and the Etest (Pfaller, 2012). Commercial products represent modifications of the agar test or broth microdilution test with advantages such as flexibility, rapidity and ease of use and they allow for testing of amphotericin B, flucytosine, itraconazole, voriconazole and echinocandin susceptibilities (Pfaller, 2012).

Breakpoints for antifungal agents can be established based on the MIC values for wild type organisms, the pharmacokinetics and pharmacodynamics characteristics of the agent (Johnson, 2008). However, MIC values are not always associated with response to antifungal therapy. The relation between *in vivo* and *in vitro* data can be summarized by the “90-60 rule”, which means that infections due to susceptible strains respond to the treatment in about 90% of cases, whereas infections due to resistant strains respond in about 60% of cases (Rex and Pfaller, 2002).

1.8.1 Broth dilution based methods

Broth microdilution and macrodilution methods for antifungal susceptibility testing were described by the CLSI. The first optimized and standardized method was a broth macrodilution, which was later adapted to allow a microdilution method and was reported in the CLSI M27-A2 document. Detailed procedures for performing antifungal susceptibility testing by microdilution and macrodilution methods are described in the CLSI guidelines. The Roswell Park Memorial Institute (RPMI) 1640 broth with 3-N-morpholino propanesulfonic acid (MOPS) and buffered to pH 7.0 was indicated as a standard medium, with a spectrophotometric reading at 0.5 McFarland standard and incubation at 35°C for 48 hours for *Candida* species and 72 hours for *C. neoformans* (CLSI, 2002; Espinel-Ingroff *et al.*, 2005).

The MIC for flucytosine and azoles by microdilution is defined as the lowest drug concentration at which growth is reduced to 50%. In the broth macrodilution method, the MIC is the lowest drug concentration that causes 80% growth inhibition. For amphotericin B, the MIC is the lowest drug concentration that causes 100% growth inhibition by both the microdilution and macrodilution methods (CLSI, 2002).

Other methods based on microbroth dilution have been developed, such as the YeastOne colorimetric antifungal panel (TREK Diagnostics, USA). It consists of a microtitre plate with the antifungal drugs and incorporates an oxidation reduction indicator, alamar blue, which changes to pink in the presence of fungal growth (Davey *et al.*, 1998; Pfaller *et al.*, 2006). Agreement with the CLSI was 98% for *Candida* species, with five different antifungals, for *C. neoformans*, it was 94% to 100% with azoles and flucytosine and 74% with amphotericin B (Pfaller *et al.*, 2004b).

Boimerieux introduced the Vitek 2 cards (Biomerieux, France) for antifungal susceptibility testing. Agreement with the CLSI was more than 95% for fluconazole, flucytosine and amphotericin B tested against many *Candida* species (Pfaller *et al.*, 2007a; Pfaller *et al.*, 2007b).

Other broth microdilution based methods include the Fungitest (Sanofi Diagnostic Pasteur, France). It is performed by using two different concentrations of antifungals in a modified RPMI 1640 medium in the presence of a redox indicator. Agreement with the CLSI was 56% to 100%, with poor agreement with azoles (Davey *et al.*, 1998).

1.8.2 Agar based methods

The disc diffusion method for antifungal susceptibility testing was published by the CLSI M44-A2 (CLSI, 2004). It is suitable for water soluble agents such as fluconazole and flucytosine. It has also been shown to be suitable for echinocandin testing producing a sharp and easy to read area of inhibition (Pfaller *et al.*, 2001a).

This method is applied by subculturing the organism in under study in Sabouraud dextrose media, preparation followed by preparation of cell suspension in normal saline on 0.5 McFarland standard, then inoculation in Muller Hinton agar plates with 2% glucose and 0.5 µg/ml methylene blue, application of antifungal disc and incubation for 24 hours for *Candida* species (72 hours for *C. neoformans*). The MIC is determined by the resulting circular area of inhibition (CLSI, 2004). Agreement with the CLSI broth microdilution was 70 % to 80% for posaconazole and 50% to 70% for amphotericin B (Vanden-Bossche *et al.*, 2002; Espinel-Ingroff, 2006).

Etest is a commercially available agar based test that was introduced in 1988 (Bolmstrom *et al.*, 1988). It is comprised of a predefined concentration gradient of antifungal agent on the surface of a plastic strip. When this strip is applied to the surface of an inoculated agar plate, the antifungal agent will be transferred to the agar in a continuous gradient as on the strip (Johnson, 2008).

The recommended medium is RPMI 1640 supplemented with 2% glucose and buffered with MOPS at pH 7.0. A yeast suspension is standardized to a 0.5 McFarland standard and inoculated on the surface of the plate, followed by incubation for 48 hours (Pfaller *et al.*, 2001b). After incubation, an ellipse appears indicating the MIC value in $\mu\text{g/ml}$. For amphotericin B and caspofungin, MIC is defined as the lowest concentration at which the zone of complete inhibition intersects the strip (100% inhibition). For azoles, MIC is defined as the lowest concentration at which the border of the elliptical inhibition zone intercepts the scale on the strip (80% inhibition) (Baixench *et al.*, 2007). Agreement with the CLSI was more than 90% for *Candida* species with fluconazole, voriconazole, posaconazole, amphotericin B, flucytosine and caspofungin (Pfaller *et al.*, 2001b; Sims *et al.*, 2006). For *C. neoformans*, agreement was 94% for voriconazole and 99% for amphotericin B (Maxwell *et al.*, 2003).

1.8.3 Non-growth based methods

Several methods based on principles other than growth of the fungus have been developed for antifungal susceptibility testing. The rapid susceptibility assay is an example of a method based on colorimetric reaction. It provides an MIC reading within eight hours, compared to 48 hours required for broth microdilution. It is performed by introducing an exogenous substance such as glucose, which is suppressed in the presence of drug susceptible fungi. The residual glucose concentration can be represented by optical density. Susceptibility can be determined by the relationship between the drug resistance and the optical density (Espinel-Ingroff *et al.*, 2005).

The ergosterol assay is based on a quantification of ergosterol in the cell wall. This method is only applicable with azoles, as they inhibit ergosterol synthesis. The assay measures the amount of the intracellular ergosterol content after the introduction of different concentrations

of antifungal agent. This assay eliminates subjectivity in reading and can be read in 18 hours (Arthington-Skaggs *et al.*, 1999).

Flow cytometry is another non-growth based method. It measures the change of fungal cell membrane potential and metabolic activity in the presence of an antifungal agent. It also measures the uptake of a DNA binding dye such as acridine orange. This method requires a specialized instrument and a highly trained technician (Ramani and Chaturvedi, 2000; Balajee and Marr, 2002).

Several molecular assays have been developed to provide more rapid and specific results than the other methods. Azole resistance has been investigated by determining point mutations in *ERG11* gene or over-expression of the drug efflux transporter genes, *CDR1* and *CDR2* (Ribeiro and Paula, 2007). Mutations in the *FKS1* gene which is associated with caspofungin resistance, have been screened by molecular methods as well (Balashov *et al.*, 2006).

1.9 Molecular aspects of resistance to antifungal agents

Multiple molecular mechanisms contribute to the resistance of *Candida* species to azole antifungal agents. These mechanisms include: genetic mutations in the *ERG11* gene encoding lanosterol 14 α -demethylase enzyme, over-expression of the *ERG11* gene, over-expression of genes encoding efflux transporters (*CDR1*, *CDR2* and *MDR*) or an alteration in the ergosterol biosynthesis pathway (Sanglard and Odds, 2002).

The *ERG11* gene contains 1851bp. The transcription starting codon is located at 148 to 150bp and the stop codon is located at 1732 to 1734bp (published *ERG11* sequence in GenBank, accession number X13296). Mutations in the *ERG11* gene, which represent the main mechanism related to azole resistance in *Candida* species, can result in changes in the enzyme 14- α demethylase configuration and cause a decrease in the affinity between azole and the enzyme (White *et al.*, 2002). This mechanism has also been reported in *C. neoformans* resistance to azole antifungal drugs (Rodero *et al.*, 2003).

To date, more than 140 amino acid substitutions have been reported, but only 20 of them have been detected in azole resistant strains, including F126L, G129A, Y132H, K143R, K143E,

F145L, A149V, T229A, S279F, K287R, G307S, S405F, G448E, G448R, F449L, V452A, G464S, G465S, R467K and I471T. These mutations except T229A are clustered in three hot spot regions ranging from amino acids 105 to 165, 266 to 287 and 405 to 488 (White *et al.*, 1997a; Sanglard *et al.*, 1998; Marichal *et al.*, 1999; White *et al.*, 2002). The mutations Y132H, S405F, K143R, I471T, R467K and G464S have been recovered exclusively from azole resistant strains and have been confirmed to be involved in azole resistance (Sanglard *et al.*, 1998; Lamb *et al.*, 2000).

Some mutations can cause resistance only if combined with other mutations, such as G129A, which is insufficient to cause azole resistance, but when this mutation is combined with G464S mutation it has shown to be responsible for a 16 fold increase in fluconazole MIC. Furthermore, other mutations such as F72L, K99T, K147R and D153E may exist in azole resistant and azole susceptible strains, but they are not thought to be involved in azole resistance (Sanglard *et al.*, 1998; Marichal *et al.*, 1999; White *et al.*, 2002).

Resistance to azoles can be due to over-production of the enzyme lanosterol 14- α demethylase, which creates a need for a higher concentration of the antifungal agent to combine with all the enzyme molecules in the fungal cell. Over-production can result from up-regulation of the *ERG11* gene, which encodes an enzyme involved in the demethylation of 14- α lanosterol, increased transcription or decreased degradation of the product. However, this mechanism contributes little to any other resistance mechanisms, because only a modest increase in the enzyme level has been reported in resistant strains (White *et al.*, 1997b; Sanglard and Odds, 2002; Kanafani and Perfect, 2008).

Failure of the antifungal agent to accumulate inside the fungal cell is one of the main mechanisms involved in azole resistance. This mechanism has been reported in many post treatment clinical isolates of *Candida* species and *C. neoformans* (Venkateswarlu *et al.*, 1997; Barchiesi *et al.*, 2000). Decreased drug concentration at the site of action occurs due to enhanced drug efflux pumps by over-expression of the ABC genes, such as *CDR1* and *CDR2* in *C. albicans* and *Cg CDR1* in *C. glabrata*, or the *MDR* of the major facilitator class (Sanglard and Odds, 2002; Prasad and Kapoor, 2005). The *CDR* genes confer resistance to almost all azole antifungal drugs, while the *MDR* is only associated with fluconazole resistance (Podust *et al.*, 2001).

Modification of the ergosterol biosynthetic pathway is another mechanism of azole resistance, which gives resistance not only to the drug to which the organisms are exposed, but also to other drugs with the same mechanism of action. A defect in lanosterol demethylase results in an accumulation of 14- α sterols, which can modify the function of plasma membrane and increase sensitivity to an oxygen-dependent microbicidal host defence mechanism (Shimokawa and Nakayama, 1992; Kelly *et al.*, 1995). Sterol analysis in *C. albicans* strains suggests that an alteration in the *ERG3* gene may be a major cause of azole resistance (Kelly *et al.*, 1997). In *C. neoformans*, analysis of fluconazole resistant strains suggests that these strains may have defects with *ERG2* and *ERG3* genes (Venkataswarlu *et al.*, 1997).

Depending on the sterol composition analysis in the cell membrane of polyene resistant isolates, polyene resistance is due to defects in the *ERG2* and *ERG3* genes (Vanden-Bossche *et al.*, 1994; Haynes *et al.*, 1996). Defects in the *ERG3* gene have been implicated in ergosterol biosynthesis, which causes the accumulation of other sterols in the cell membrane. Studies have shown that *Candida* and *C. neoformans* isolates that are resistant to polyenes have low ergosterol levels compared to polyene susceptible isolates (Dick *et al.*, 1980). However, the molecular mechanisms that cause polyene resistance are not yet completely understood (Joseph-Horne *et al.*, 1996) and many other mechanisms may be involved, such as increased catalase activity and decreased susceptibility to oxidative damage (Sokol-Anderson *et al.*, 1986).

Resistance to echinocandins is mainly associated with mutations in the target site, because echinocandins are poor substrates for drug efflux transporters (Bachmann *et al.*, 2002). Point mutations in the *FKS1* gene encoding 1,3- β -D-glucan synthase have been reported in resistant clinical isolates of *C. albicans*. The identified mutations tended to cluster around the two hot spot regions, HS1 and HS2, of the *FKS1* gene; mutations in HS1 are located at positions 641 to 649 and mutations in HS2 are located at positions 1345 to 1365. Substitutions of the serine at position 645 to phenylalanine, proline or tyrosine (S645P, S645F and S645Y) within the HS1 region are the most common mutation observed (Park *et al.*, 2005; Perlin, 2007). This mechanism is extended to other non-*albicans Candida* species as well. In *C. glabrata* and *C. guilliermondii*, hot spot mutations have been observed in the *FKS2* gene too (Katiyar *et al.*, 2006). The mechanism of *C. neoformans* resistance to echinocandin is not yet completely understood. The *FKS1* gene encoding a 1,3- β -D-glucan synthase is reported to be the primary site of action of echinocandin and glucan synthase was strongly inhibited by caspofungin in *in*

vitro studies, but *C. neoformans* appears to be intrinsically resistant to echinocandin (Thompson *et al.*, 1999; Maligie and Selitrennikoff, 2005). Other mechanisms such as efflux pumps and degradation pathway alterations may be involved (Maligie and Selitrennikoff, 2005).

Resistance to flucytosine is either related to decreased uptake or to a loss of enzymatic activity responsible for conversion to FUMP (Whelan, 1987). The formation of FUMP can be blocked by a loss of cytosine deaminase activity or a loss of uracil phosphoribosyl transferase activity (UPRTase). A decrease of this enzyme activity is associated with the manner of resistance. In the diploid *C. albicans*, the homozygous resistance gene (FCY/FCY) possesses high UPRTase activity, while in heterozygous resistance gene (FCY/fcy) possesses less activity (Whelan and Kerridge, 1984). In the haploid *C. neoformans*, resistance is caused by a mutation in the *FCY1* and *FCY2* genes (Alexander and Perfect, 1997).

Allylamine resistance has not been reported in human pathogenic fungi. However, some resistant strains to fluconazole expressed cross-resistance to terbinafinate (Vanden-Bossche *et al.*, 1992). Terbinafinate can be a substrate to *CDR1* (Sanglard *et al.*, 1996), which means that the mechanism to develop resistance to allylamines is already present.

Antifungal drug resistance is a broad issue and collaboration between laboratory studies and clinical trials is essential to prevent and control this problem. Furthermore, testing antifungal resistance in yeast isolates and understanding the molecular mechanisms of resistance will improve the infection treatment and will assist in the development of more effective strategies to overcome antifungal resistance in future.

1.10 Aim

The aim of this study was to determine the antifungal susceptibility profile and the molecular mechanism of resistance among yeast isolates in Pretoria.

1.11 Objectives

- To collect 250 yeast isolates from the diagnostic laboratory in the Diagnostic Medical Microbiology, University of Pretoria-National Health Laboratory Services (NHLS)
- To subculture the collected yeast isolates on Sabouraud dextrose agar medium for purification
- To identify the isolates using biochemical methods
- To determine the antifungal susceptibility profile of the yeast isolates with three azole antifungal agents (fluconazole, posaconazole and voriconazole), amphotericin B and caspofungin using the Etest
- To perform polymerase chain reaction (PCR) on *C. albicans* isolates for amplification of the *ERG11* gene and the *FKS1* gene
- To sequence the amplified *ERG11* and *FKS1* genes and analyze sequencing data using CLC Genomic Workbench software

CHAPTER 2

MATERIALS AND METHODS

2.1 Sample size and study population

A total of 250 yeast isolates were collected from stored isolates in the Diagnostic Medical Microbiology Laboratory/NHLS. The isolates were detected from clinical samples of patients who visited Steve Biko Academic Hospital Complex and referring hospitals in Pretoria between January 2010 and December 2012. The samples were submitted to the Diagnostic Medical Microbiology Laboratories/NHLS for routine investigation. A descriptive study was performed to determine the proportion of resistant isolates to antifungal agents and describe the genetic mutations that might cause resistance.

2.2 Subculturing of yeast isolates

The collected yeast isolates were subcultured on Sabouraud dextrose agar with 0.005% chloramphenicol (prepared in-house in the Department of Medical Microbiology Laboratory-NHLS, South Africa) to purify the isolates. Cultures were incubated aerobically at 30°C for 24 to 48 hours, identified by biochemical procedures and then stored in sterile distilled water at room temperature until needed for further testing.

2.3 Identification of yeast isolates

The yeast isolates were identified by biochemical methods. *Candida* isolates were identified to species level and *C. neoformans* were further identified to a subspecies level.

2.3.1 Identification of *C. albicans* by germ-tube

Germ-tube testing was performed on the isolates to distinguish *C. albicans* from other species. A colony from each isolate was suspended in a 0.5 ml horse serum (Medical

Microbiology Laboratory-NHLS, South Africa) by a wooden stick and incubated for three hours at 35°C. Then a drop from the suspension was placed on a glass slide and examined under a light microscope at a 40x magnification for the presence or absence of germ-tubes. Germ-tube positive isolates were reported as *C. albicans*. *C. albicans* ATCC 90028 was included as a positive control and *C. parapsilosis* ATCC 22019 as a negative control.

2.3.2 Identification of *C. neoformans* by India ink staining

C. neoformans isolates were identified by India ink staining for all germ-tube negative isolates. A colony of the isolate to be tested was mixed with a drop of saline on a glass slide, a drop of India ink was added, then the slide was examined under a light microscope at 40x magnification. *C. neoformans* was identified by the presence of a bright halo around the yeast cell, which indicated the presence of encapsulated cells.

2.3.3 Identification of *C. neoformans* by urease test

Urease test was performed on India ink positive isolates by inoculating a colony of the isolate to be tested on a urea slant (Medical Microbiology Laboratory-NHLS, South Africa) and incubating it at 37°C for 24 hours. A change in the slant colour from yellow to pink was interpreted as positive for *C. neoformans*. *C. neoformans* ATCC 66031 was included as a positive control.

2.3.4 Identification of *C. neoformans* varieties by using CGB culture medium

Inoculation of *C. neoformans* isolates on L- canavanine bromothymol glycine blue medium (CGB) (Diagnostic Media Preparations-NHLS, South Africa) was used to identify *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii*. The inoculated plates were incubated at 30°C for 72 hours. *C. neoformans* var. *gattii* was considered to be positive when the medium changed from yellow to blue after 72 hours. Plates with no colour change were considered as *C. neoformans* var. *neoformans*.

2.3.5 Identification of non albicans *Candida* by the Vitek 2 system

Isolates were processed using the Vitek 2 system (BioMerieux, Germany) according to the manufacturer's protocol. A sterile cotton swab was used to pick five yeast colonies and these were then added to a tube containing 3 ml of sterile saline. The inoculum turbidity was adjusted to (1.0 to 2.2) McFarland standard by means of Densicheck instrument (BioMerieux, France). The tubes were loaded into the slots of the cassette along with an identification card for each isolate. The carrier was then loaded into the Vitek 2 machine where it was incubated at 35.5°C. The isolates were identified and results were generated after 18 to 24 hours.

2.4 Antifungal susceptibility testing by Etest

Due to financial constraints, of the 250 isolates collected, only 87 isolates were tested for antifungal susceptibility, including 10 *C. albicans*, 30 *C. parapsilosis*, 9 *C. glabrata*, 6 *C. tropicalis*, 3 *C. lipolytica*, 21 *C. neoformans* var. *neoformans* and 8 *C. neoformans* var. *gattii*. Antifungal susceptibility testing was performed using Etest strips (Biomérieux, UK) according to the manufacturer's protocol. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were included as control strains as recommended by the Clinical and Laboratory Standard Institute (CLSI). Isolates were subcultured in Sabouraud dextrose media with chloramphenicol. Five colonies were picked up and suspended in saline water, then adjusted to a 0.5 McFarland standard using the Densicheck instrument (Biomérieux, France). Five Roswell Park Memorial Institute agar media (RPMI) with 2% glucose (Diagnostic Media Preparations-NHLS, South Africa) were used for each isolate. The plates were inoculated using a cotton swab dipped in the standardized suspension. When the surface was completely dry, the Etest strip of each antifungal was applied on a separate inoculated RPMI medium by using a sterile forceps and the plates were incubated aerobically at 35°C for 48 hours. For amphotericin B and caspofungin, minimal inhibitory concentration (MIC) was defined as the lowest concentration at which the zone of complete inhibition (100% growth inhibition) intersected with the strip. For fluconazole, posaconazole and voriconazole, MIC was defined as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip (80% growth inhibition) and any microcolonies were ignored (Baixench *et al.*, 2007). Clinical breakpoints susceptibility for the antifungal agents tested against various yeast isolates are shown in Table 2.1 (according to the CLSI clinical breakpoints reference method) (Pfaller *et al.*, 2012).

Table 2.1 Clinical breakpoints for the antifungal agents against different yeast species

Yeast	Antifungal	S (µg/ml)	SDD (µg/ml)	I (µg/ml)	R (µg/ml)
<i>C. albicans</i>	Fluconazole	≤2	4	0.25-0.5	≥8
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤0.25		0.5	≥1
<i>C. parapsilosis</i>	Fluconazole	≤2	4	0.25-0.5	≥8
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤2		4	≥8
<i>C. glabrata</i>	Fluconazole	≤2	≤32	0.25-0.5	>32
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤0.12		0.25	≥0.5
<i>C. tropicalis</i>	Fluconazole	≤2	4	0.25-0.5	≥8
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤0.25		0.5	≥1
<i>C. lipolytica</i>	Fluconazole	≤2	4	0.25-0.5	≥8
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤0.25		0.5	≥1
<i>Cryptococcus neoformans</i>	Fluconazole	≤2	4	0.25-0.5	≥8
	Posaconazole	<1	2		≥4
	Voriconazole	≤0.12			≥1
	Amphotericin B	≤2		>2	
	Caspofungin	≤0.25		0.5	≥1

S=Susceptible, SDD=Susceptible Dose Dependent, I=Intermediate, R=Resistant.

2.5 Extraction of genomic DNA

The extraction of genomic DNA from colonies of *C. albicans* isolates was performed by using the ZR Fungal/Bacterial DNA MicroPrep Kit (Zymo Research Corp, USA) according to the manufacturer's instructions. The isolates were subcultured in Sabouraud dextrose medium with chloramphenicol. Colonies were resuspended in 200 µl of water. Thereafter, 100 mg of the fungal cells were put in a lysis tube with 750 µl of the lysis solution was and then centrifuged for 1 minute, followed by centrifusion of 400 µl of the supernatant for 1 minute. Thereafter, 1 200 µl of the Fungal/Bacterial DNA Binding Buffer was added and 800 µl of the mixture was centrifuged for 1 minute, 200 µl of DNA Pre-Wash Buffer was added and the mixture was centrifuged again. 500 µl of Fungal/Bacterial DNA Wash Buffer was added and the mixture was centrifuged. 100 µl of DNA Elution Buffer wad added and the mixture was centrifuged to elute DNA. The extracted DNA was stored at -20 °C until needed.

2.6 Detection of the *ERG11* gene mutations by PCR and gene sequencing

The complete *ERG11* open reading frame was amplified using primers described by Lee *et al.* (2004) (Table 2.2). The reaction mixture contained 5 µl of genomic DNA, 25 µl of FastStart PCR master (Roche, Germany), 5 µl of each primer and sterile nuclease-free water up to a final volume of 50 µl. Amplification was carried out in a Master Cycler (Eppendorf, Germany), with a first cycle of denaturation at 95°C for ten minutes, 35 cycles of denaturation at 95°C for one minute, annealing at 56°C for one minute, elongation at 72°C for two minutes and a final extension step at 72°C for seven minutes. A negative control was included containing 5µl of sterile nuclease-free water with the FastStart master, primers and water without the genomic DNA. *C. albicans* ATCC 90028 was used as a positive control. Amplification products were detected by gelelectrophoresis on a 1% agarose gel, which was stained with ethidium bromide. A 100 bp molecular weight marker was used (Fermentas, ThermoScientific, USA). Gel electrophoresis was performed at 100 V for 75 minutes. Products were visualized under a UV (ultraviolet) light with an ultraviolet transilluminator instrument (Ultraviolet products, USA) .

The PCR products were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing, using the same primers as those used for PCR. The sequencing results were analysed by creating consensus sequences and nucleotide alignment with the *C. albicans* *ERG11* reference sequence (Genbank accession number X13296) (Lai and Kirsch, 1989). The translation of the consensus sequences into protein and alignment with the protein sequence of the reference *ERG11* gene was done using CLC Genomic Workbench software.

Table 2.2 Primer sequences for the amplification of the *ERG11* gene by PCR

Primer	Sequence (5'-3')
<i>ERG11</i> -forward	GAAAGGGAATTCAATCG
<i>ERG11</i> -reverse	TGTTAATCCAATAAGTAAC

2.7 Detection of the *FKSI* gene mutations by PCR and gene sequencing

The previously described primers by Park *et al.* (2005) were used for amplification of a fragment of 450 base pairs (bp) of the *FKSI* gene (Table 2.3). This fragment contains the HS1, of which the mutations are known to confer reduced susceptibility to echinocandins. The reaction solution consisted of 5µl of genomic DNA, 25µl of FastStart PCR master (Roche, Germany), 5µl of each primer and sterile nuclease-free water up to a final volume of 50µl. PCR was carried out in a Master Cycler (Eppendorf, Germany) set-up with a first cycle of denaturation at 95°C for ten minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 30 seconds and final extension at 72°C for ten minutes. A negative control was included containing the reaction mixture with 5µl of sterile nuclease-free water instead of the genomic DNA. *C. albicans* ATCC 90028 was used as a positive control. After PCR, the amplicons were detected by electrophoresis on a 2% agarose gel. A 100 bp molecular weight marker was used (Fermentas, ThermoScientific, USA). Gel electrophoresis was performed at 100 V for 75 minutes. The gel was stained with ethidium bromide and photographed under a UV light with an ultraviolet transilluminator system (Ultraviolet products, USA).

The PCR products were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing using the same primers used for PCR. The sequence data were compared to the wild type *C. albicans FKS1* reference sequence (Genbank accession number D88815) (Mio *et al.*, 1997), using CLC Genomic Workbench software. The consensus sequences were created and nucleotide alignment was done, followed by the translation of the consensus sequences to protein and the alignment with the protein sequence of the reference.

Table 2.3 Primer sequences for amplification of the *FKS1* gene by PCR

Primer	Sequence (5'-3')
<i>FKS1</i> -forward	GAAATCGGCATATGCTGTGTC
<i>FKS1</i> -reverse	AATGAACGACCAATGGAGAAG

2.8 Data analysis

The distribution of yeast organisms was tabulated, giving percentage of each species present. The results of the antifungal susceptibility testing were entered in a Microsoft Excel data sheet and the proportion of resistant isolates was determined. The MIC₅₀ and MIC₉₀ of the isolates were calculated as the concentrations of the antifungal agent that were able to inhibit 50% and 90% of the isolates, respectively.

2.9 Ethical consideration

This study was approved by The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (195/2012). All data were confidentially used. Unique numbers for the isolates were used without any link to the names of the patients or the laboratory numbers.

CHAPTER 3

RESULTS

3.1 Identification of yeast isolates

Among the 250 yeast isolates collected, 207 (82.8%) were *Candida* species and 43 (17.2%) were *Cryptococcus neoformans*. Of the *Candida* species, 159 isolates (76.8%) were identified as *C. albicans*, in which, 67% (106/159) were from genitourinary tract specimens.

Of the non-*albicans Candida*, *C. parapsilosis* accounted for 14.5% (30/207) and 46% (14/30) of these were from blood cultures. *C. glabrata* accounted for 4.3% (9/207), the majority of specimens were from blood (33.3%) and genitourinary tract (33.3%). *C. tropicalis* accounted for 2.9% (6/207) and *C. lipolytica* for 1.4% (3/207) .

C. neoformans was identified in 43/250 (17.2%) of the yeast isolates. Of these, 33 (76.7%) were *C. neoformans* var. *neoformans* and 10 (23.3%) were *C. neoformans* var. *gattii*. All *C. neoformans* isolates were from cerebrospinal fluid (Table 3.1 and Table 3.2).

Table 3.1 Species distribution among yeast isolates

Yeast	Number of isolates (% of isolates)
<i>Candida</i> species	207/250 (82.8)
<i>C. albicans</i>	159/207 (76.8)
<i>C. parapsilosis</i>	30/207 (14.5)
<i>C. glabrata</i>	9/207 (4.3)
<i>C. tropicalis</i>	6/207 (2.9)
<i>C. lipolytica</i>	3/207 (1.4)
<i>Cryptococcus neoformans</i>	43/250 (17.2)
<i>C. neoformans</i> var. <i>neoformans</i>	33/43 (76.7)
<i>C. neoformans</i> var. <i>gattii</i>	10/43 (23.3)

Table 3.2 Sources of the study isolates

Source of specimen	Species (Number of Isolates)						
	<i>C. albicans</i> (159)	<i>C. parapsilosis</i> (30)	<i>C. glabrata</i> (9)	<i>C. tropicalis</i> (6)	<i>C. lipolytica</i> (3)	<i>C. neoformans</i> var. <i>neoformans</i> (33)	<i>C. neoformans</i> var. <i>gattii</i> (10)
Blood	16 (10%)	14 (46%)	3 (33.3%)	2 (33.3%)	2 (66.6%)	-	-
Genitourinary	106 (67%)	-	3 (33.3%)	3 (50%)	-	-	-
Sputum	6 (3.8%)	-	1 (11.1%)	-	-	-	-
Cerebrospinal fluid	1 (0.6%)	-	-	-	-	33 (100%)	10 (100%)
Tracheal aspirate	11 (6.9%)	2 (6.6%)	1 (11.1%)	-	-	-	-
Nasopharyngeal aspirate	-	-	-	1 (17%)	-	-	-
Pus	7 (4.4%)	-	1 (11.1%)	-	-	-	-
Peritoneal fluid	2 (1.2%)	-	-	-	-	-	-
Central venous catheter tip	5 (3.1%)	4 (13.4%)	-	-	-	-	-
Tissue	1 (0.6)	-	-	-	-	-	-
Unknown	4 (2.5%)	10 (34%)	-	-	1 (33.4%)	-	-

Var=Variety

3.2 Antifungal susceptibility of yeast isolates by Etest

The antifungal susceptibilities and the MIC values of the yeast isolates are shown in Tables 3.3 and 3.4. Resistance of *C. albicans* to fluconazole (4/10, MIC $\geq 8\mu\text{g/ml}$) and voriconazole (3/10, MIC $>32\mu\text{g/ml}$) was detected. Three isolates were resistant to caspofungin (MIC ≥ 1). All *C. albicans* isolates were fully susceptible to posaconazole and amphotericin B.

C. parapsilosis was resistant to fluconazole (17/30, MIC $\geq 16\mu\text{g/ml}$) and voriconazole (15/30, MIC $\geq 1\mu\text{g/ml}$). Only three isolates were resistant to amphotericin B (MIC $>2\mu\text{g/ml}$). Interestingly, 15 isolates showed intermediate resistance (MIC $4\mu\text{g/ml}$) to caspofungin.

Among *C. glabrata* isolates, resistance to fluconazole was (5/9, MIC $>256\mu\text{g/ml}$), that to voriconazole was (5/9, MIC ≥ 1.5) and that to posaconazole was (6/9, MIC $\geq 4\mu\text{g/ml}$). Only one isolate was resistant to amphotericin B (MIC = $3\mu\text{g/ml}$). Intermediate resistance to voriconazole (MIC = $0.25\mu\text{g/ml}$) was found in four of the isolates and to caspofungin (MIC = $0.25\mu\text{g/ml}$) was found in five of the isolates.

No resistance was detected in *C. tropicalis* to fluconazole, voriconazole, posaconazole and caspofungin. However, only one isolate was resistant to amphotericin B (MIC = $3\mu\text{g/ml}$).

Of the three *C. lipolytica* isolates tested, all were susceptible to voriconazole, posaconazole and amphotericin B. However, resistance to fluconazole was detected in one isolate (MIC $>256\mu\text{g/ml}$) and resistance to caspofungin (MIC $\geq 1.5\mu\text{g/ml}$) was detected in two isolates.

Resistance of *C. neoformans* var. *neoformans* to fluconazole (MIC $\geq 8\mu\text{g/ml}$) was detected in 9/21 (43%) of the isolates tested. Nine isolates were susceptible dose dependent to fluconazole (MIC = $4\mu\text{g/ml}$). Intermediate resistance to voriconazole was seen in four isolates (MIC = $0.25-0.5\mu\text{g/ml}$). All the isolates were susceptible to posaconazole and amphotericin B, whereas, all the isolates were resistant to caspofungin (MIC $\geq 1\mu\text{g/ml}$). All *C. neoformans* var. *gattii* isolates (8) were resistant to fluconazole (MIC $\geq 12\mu\text{g/ml}$) and to caspofungin (MIC $>32\mu\text{g/ml}$). Five isolates showed intermediate resistance to voriconazole (MIC = $0.25-0.5\mu\text{g/ml}$). All the isolates were susceptible to posaconazole and amphotericin B.

Table 3.3 Antifungal susceptibility of yeast isolates by Etest method (N=87)

Yeast (Number of isolates)	Antifungal agents														
	Fluconazole			Posaconazole			Voriconazole			Amphotericin B			Caspofungin		
	S (%)	SDD (%)	R (%)	S (%)	SDD (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
<i>C. albicans</i> (10)	6(60)	-	4(40)	10(100)	-	-	6(60)	1(10)	3(30)	10(100)	-	-	7(70)	-	3(30)
<i>C. pareapsilosis</i> (30)	9(30)	4(13.3)	17(56.7)	30(100)	-	-	13(43.3)	2(6.7)	15(50)	27(90)	-	3(10)	15(50)	15(50)	-
<i>C. glabrata</i> (9)	4(44.4)	-	5(55.6)	-	3(33.3)	6(66.7)	-	4(44.4)	5(55.6)	8(88.9)	-	1(11.1)	-	5(55.6)	4(44.4)
<i>C. tropicalis</i> (6)	6(100)	-	-	6(100)	-	-	5(83.3)	1(16.7)	-	5(83.3)	-	1(16.7)	3(50)	3(50)	-
<i>C. lipolytica</i> (3)	2(66.7)	-	1(33.3)	2(66.7)	1(33.3)	-	2(66.7)	1(33.3)	-	3(100)	-	-	-	1(33.3)	2(66.7)
<i>C. neoformans</i> var. <i>neoformans</i> (21)	3(14.3)	9(42.9)	9(42.9)	21(100)	-	-	17(81)	4(19)	-	21(100)	-	-	-	-	21(100)
<i>C. neoformans</i> var. <i>gattii</i> (8)	-	-	8(100)	8(100)	-	-	3(37.5)	5(62.5)	-	8(100)	-	-	-	-	8(100)

N= Number, var= variety, S=Susceptible, SDD=Susceptible Dose Dependent, I=Intermediate, R=Resistance

Table 3.4 MIC values of yeast isolates by Etest

Yeast (Number of isolates)	Antifungal agents														
	Fluconazole			Posaconazole			Voriconazole			Amphotercin B			Caspofungin		
	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)	Range (µg/ml)
<i>C. albicans</i> (10)	0.75	>256	0.19 - >256	0.094	0.125	0.047 - 0.25	0.023	>32	0.004 - >32	1	1.5	0.5 - 1.5	0.19	1	0.094 - 1
<i>C. parapsilosis</i> (30)	0.75	>256	0.75 - >256	0.19	0.125	0.064 - 0.25	0.38	>32	0.016 - >32	1.5	2	0.75 - 3	2	6	0.5 - 6
<i>C. glabrata</i> (9)	>256	>256	8 - >256	>32	>32	1.5 - >32	1.5	2	0.38 - 6	1.5	2	1 - 3	0.38	0.5	0.25 - 0.75
<i>C. tropicalis</i> (6)	0.5	0.75	0.38 - 2	0.125	0.5	0.064 - 0.5	0.064	0.125	0.016 - 0.25	1.5	2	1.5 - 3	0.25	0.5	0.19-0.5
<i>C. lipolytica</i> (3)	-	-	1.5 - >256	-	-	0.38 - 2	-	-	0.047 - 0.38	-	-	1.5	-	-	0.5 - 2
<i>C. neoformans</i> var. <i>neoformans</i> (21)	6	24	0.5 - 96	0.38	0.5	0.094 - 0.75	0.094	0.25	0.016 - 0.38	0.75	1.5	0.38 - 1.5	>32	>32	1 - >32
<i>C. neoformans</i> var. <i>gattii</i> (8)	1	48	12 - >256	0.5	1	0.25 - 1	0.19	0.38	0.047 - 0.5	0.75	1	0.5 - 1	>32	>32	>32

MIC=Minimum Inhibitory Concentration, var=variety

3.3 Amplification of *ERG11* and *FKS1* genes of *C. albicans* isolates

Amplification of the complete *ERG11* gene of 10 *C. albicans* isolates (in which, 3 were both fluconazole and voriconazole resistant, 1 was only fluconazole resistant and 6 were azole susceptible) was performed by polymerase chain reaction (PCR). Each PCR product resulted in a clear band at 1851 base pair (bp) on ethidium bromide stained, ultraviolet (UV) transilluminated agarose gel (Figure 3.1).

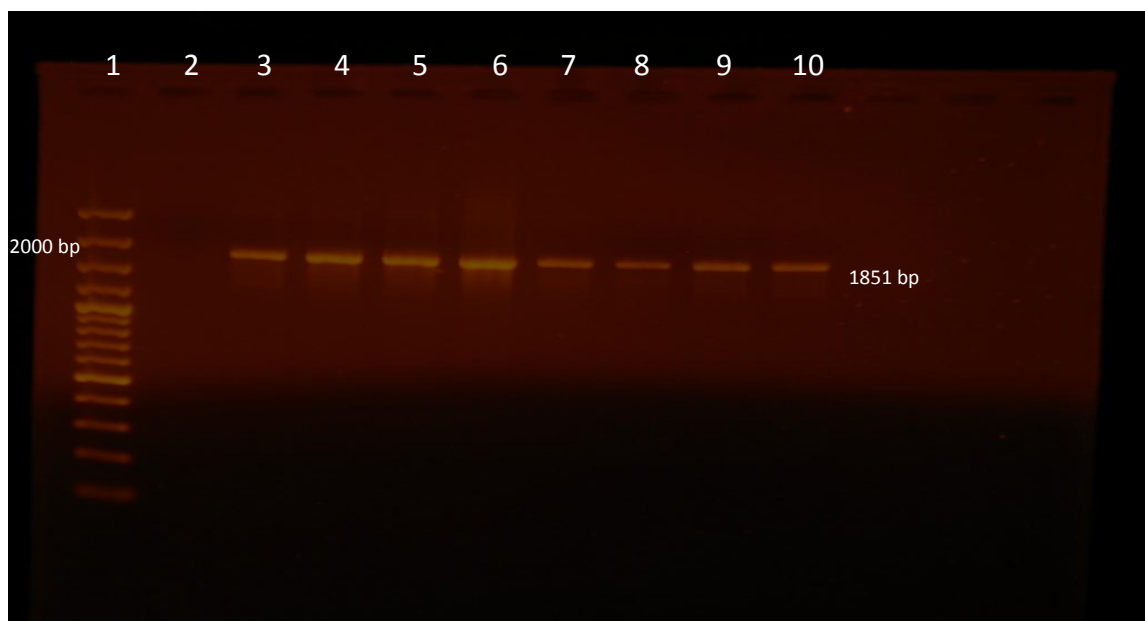


Figure 3.1 A representative gel picture showing amplified products of the *ERG11* gene of *C. albicans* isolates

Lane 1: size ladder; Lane 2: negative control; Lane 3: positive control; Lane 4 to 6: fluconazole and voriconazole resistant *C. albicans* specimens; Lane 7: fluconazole resistant *C. albicans* specimen; Lane 8 to 10: azole susceptible *C. albicans* specimens

A fragment of 450 bp of the *FKS1* gene of 10 *C. albicans* isolates (3 caspofungin resistant and 7 caspofungin susceptible) was amplified by PCR. All the amplification products resulted in clear bands visualised by ethidium bromide stained agarose gel under the UV light (Figure 3.2). This fragment contains the hot spot region (HS1) which mutations are known to confer reduced susceptibility to echinocandin.

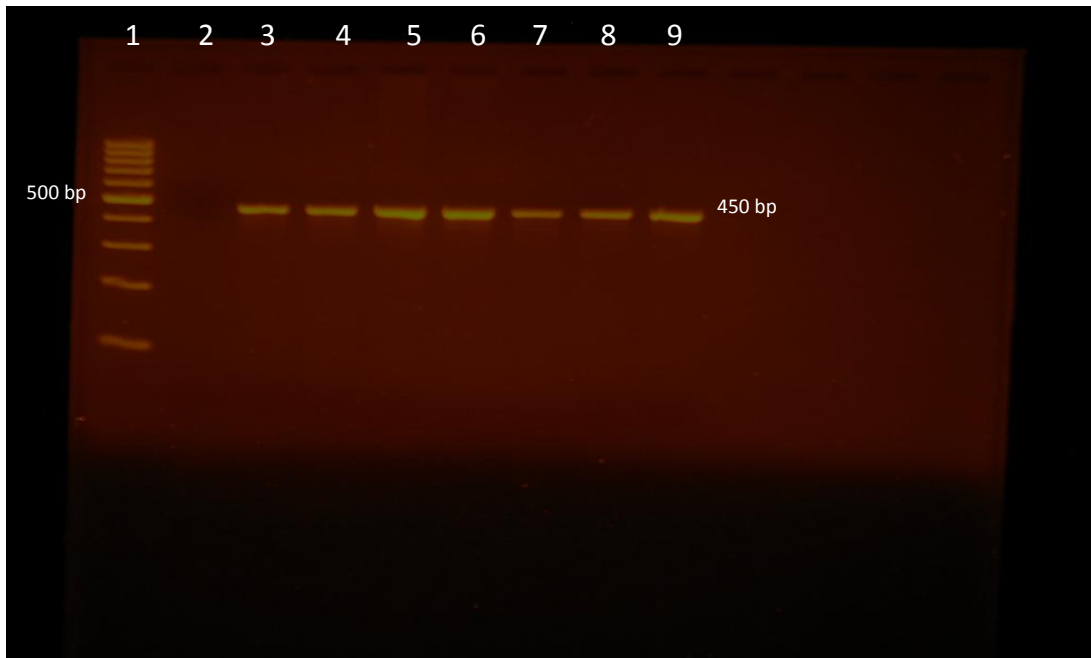


Figure 3.2 A representative gel picture showing amplified products of the *FKS1* gene of *C. albicans* isolates

Lane 1: size ladder; Lane 2: negative control; Lane 3: positive control; Lane 4 to 6: caspofungin resistant *C. albicans* specimens; Lane 7 to 9: caspofungin susceptible *C. albicans* specimens

3.4 Sequencing of the amplified genes

The amplified *ERG11* genes of the ten *C. albicans* isolates tested for antifungal susceptibility (3 were both fluconazole and voriconazole resistant, 1 was only fluconazole resistant and 6 were azole susceptible) were sequenced and then the nucleotide alignment with the reference and the screening of amino acid substitution were performed on the sequencing data. A large number of silent single nucleotide polymorphisms were identified, but missense mutations that led to amino acid substitutions were detected in three isolates. The amino acid substitutions D116E and K128T were found simultaneously in one azole susceptible isolate. The amino acid substitution V437I was found in another azole susceptible isolate. E517Q was detected in fluconazole and voriconazole resistant isolate (Figure 3.3).

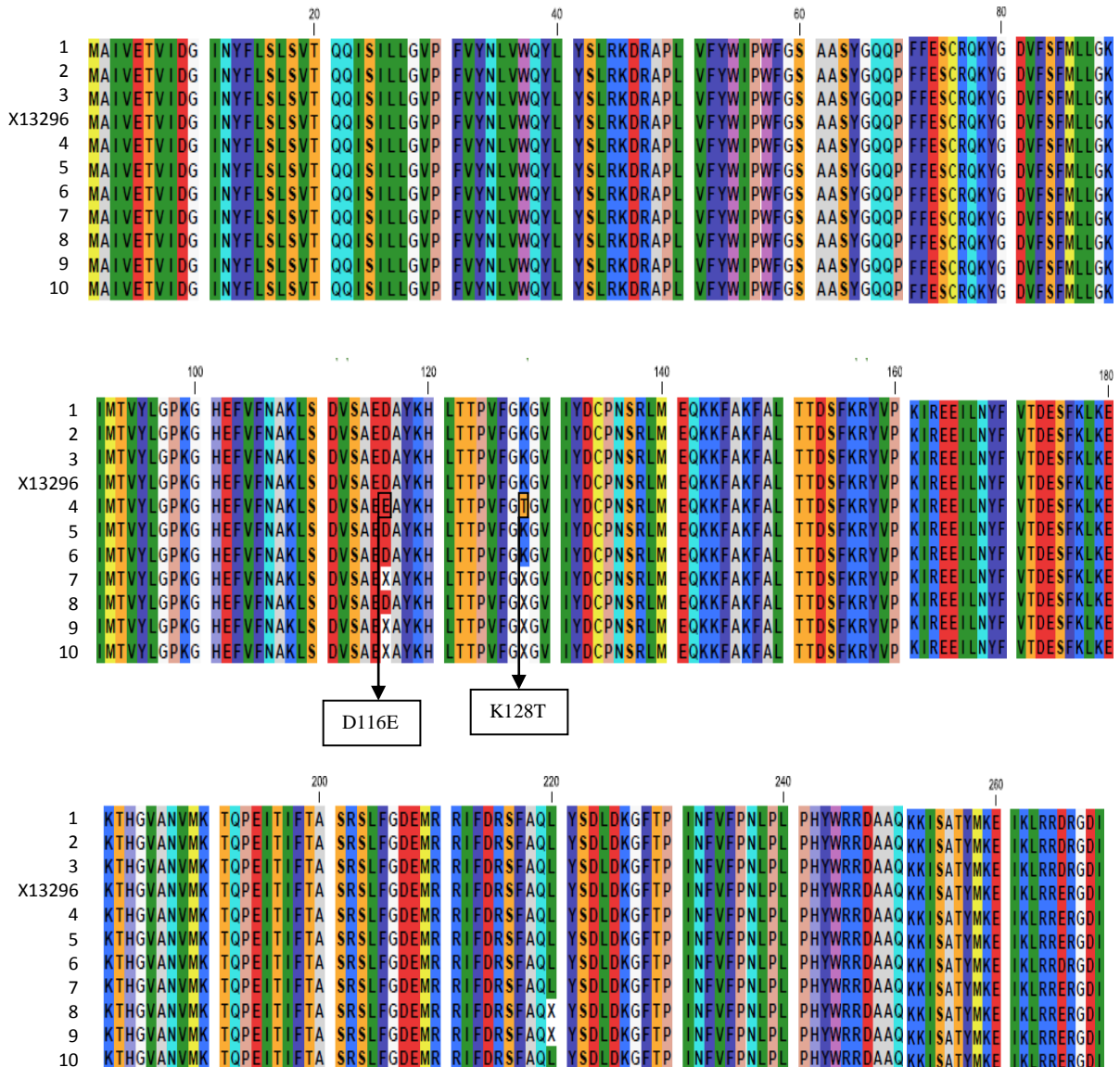


Figure 3.3 Amino acid alignment of the *ERG11* gene of *C. albicans* isolates

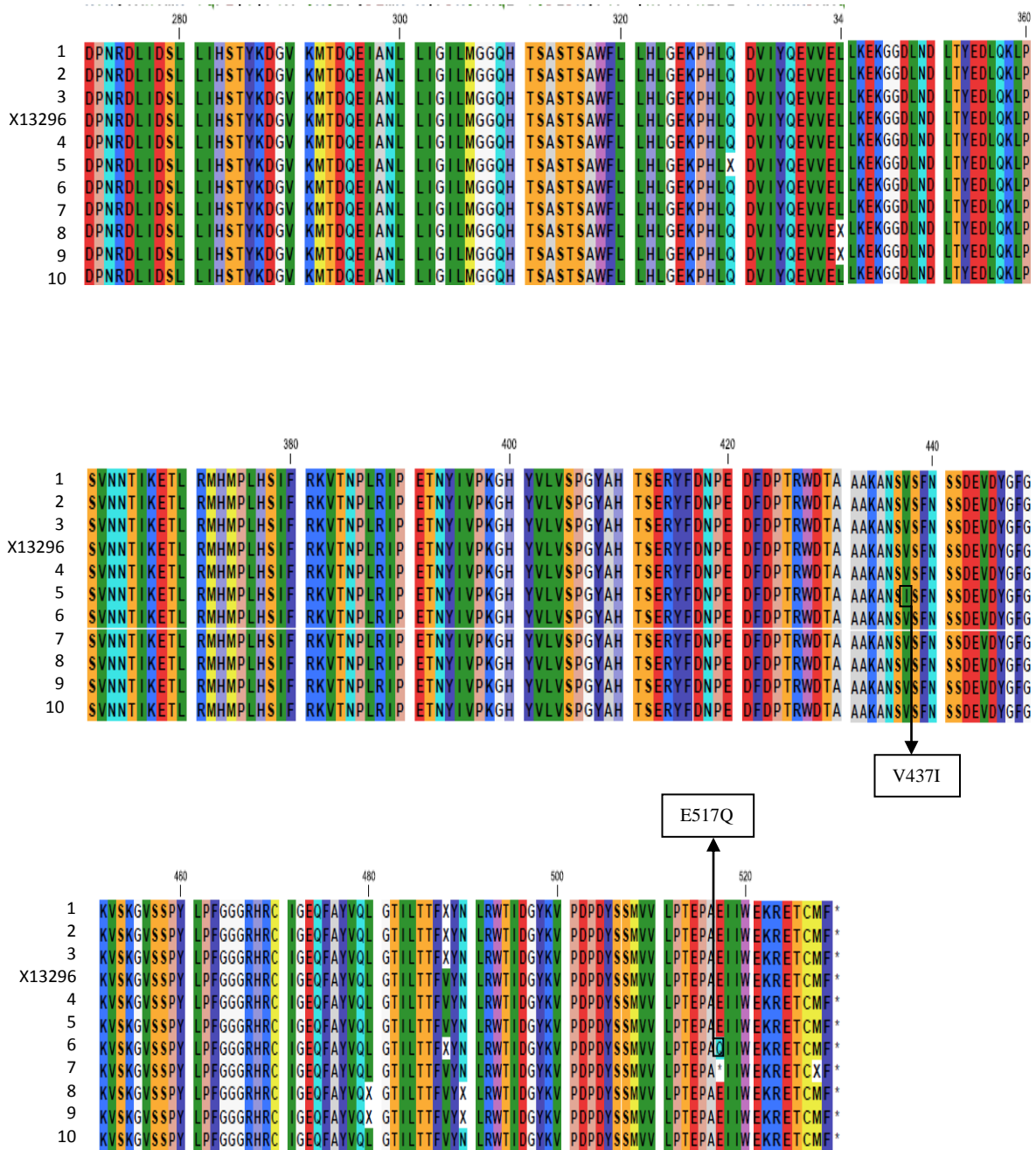


Figure 3.3 (continued) Amino acid alignment of the *ERG11* gene of *C. albicans* isolates

The sequencing of the amplified fragments of the *FKS1* genes was performed, followed by nucleotide alignment and protein alignment for the detection of amino acid substitution. Among the ten *C. albicans* isolates (3 caspofungin resistant and 7 caspofungin susceptible), one missense mutation was detected in all the isolates (resistant and susceptible). This mutation causes the substitution of the polar amino acid serine (S) in the reference *FKS1* gene

(GenBank accession number D88851) by the non-polar amino acid leucine (L) in the isolates at position 642 (S642L) (Figure 3.4).

2

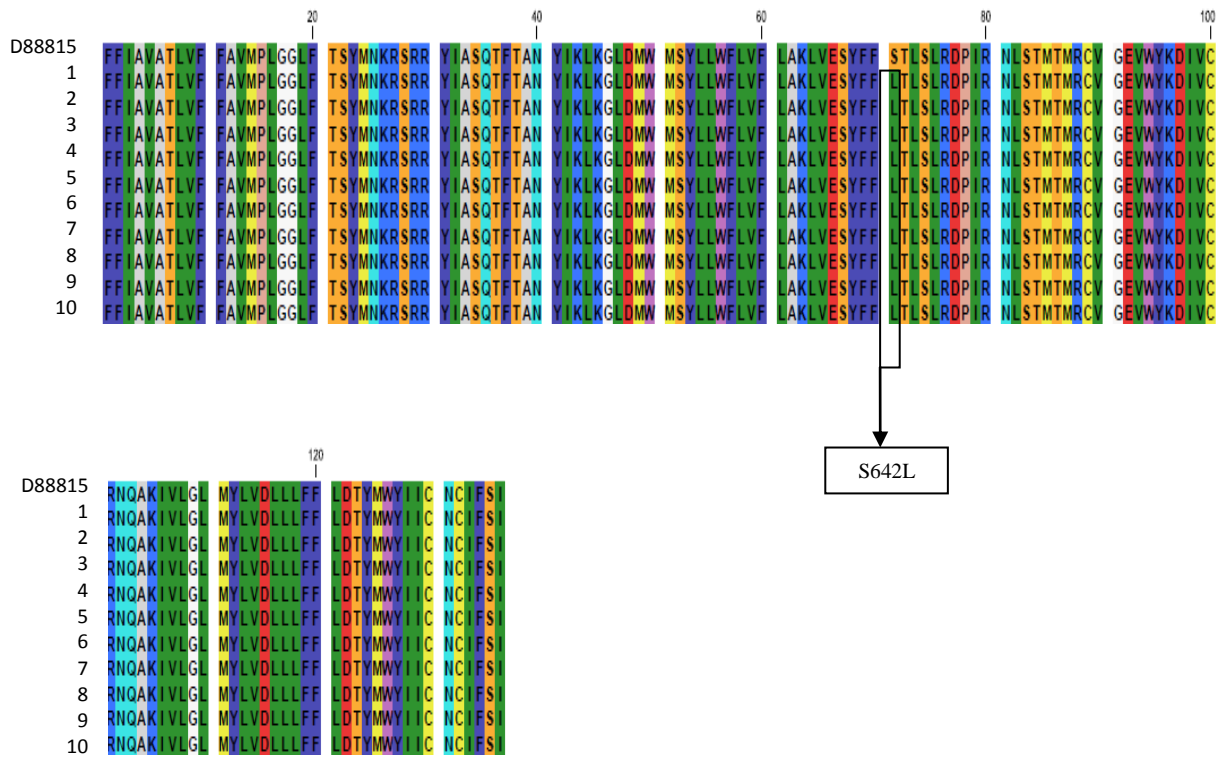


Figure 3.4 Amino acid alignment of the *FKS1* gene of *C. albicans* isolates

CHAPTER 4

DISCUSSION

The frequency of superficial candidiasis, as well as, invasive fungal infections (IFIs) is increasing, particularly in patients with HIV/AIDS (Pfaller and Diekema, 2010), whilst the resistance of pathogenic fungi to antifungal therapy is becoming a serious problem in the management of fungal infections (Perea and Patterson, 2002). Therefore, susceptibility testing and understanding of the mechanisms of resistance are essential for efficient treatment of fungal infections and improvement of the outcomes of these infections.

The distribution of yeast isolates and antifungal susceptibility patterns show marked variation between different geographic areas. Several mechanisms have been involved in the development of antifungal resistance in pathogenic fungi. Thus, it is necessary to monitor the pathogen distribution at a species level and the local antifungal susceptibility profiles with understanding of the mechanism of resistance of each species in each country. In South Africa, little is known about species distribution and the antifungal susceptibility of local isolates (Blignaut *et al.*, 2002).

Candida species are the most common cause of IFIs and the fourth most frequently isolated microorganisms from blood stream infections (BSIs) (Pfaller *et al.*, 2001a). *C. albicans* is the most commonly isolated *Candida* species from nosocomial BSIs worldwide (50 % to 70%) (Pfaller *et al.*, 2004a; Murray *et al.*, 2009). In North America, *C. albicans* accounted for 50% during the 1990s (Poikonen *et al.*, 2003).

In South Africa, Arendse and Orth (2008) reported *C. albicans* to be the most commonly isolated species (69%) at Tygerberg Hospital in Cape Town. Samie and Masheo (2012) reported that *C. albicans* accounted for 15% of yeast isolates from sputum samples of HIV patients co-infected with tuberculosis (TB). In this study, *C. albicans* was the most commonly isolated *Candida* species at Steve Biko Hospital and referring hospitals in Pretoria and accounted for 76.8% of yeast isolates. The majority (more than 67%) were from genitourinary tract. Only 10% were from blood cultures.

Several studies have reported an increase in the rate of non-albicans *Candida* such as *C. krusei*, *C. parapsilosis* and *C. glabrata* (Pfaller *et al.*, 2004a; Murray *et al.*, 2009). Pfaller *et al.* (2005) collected a total of 140767 yeast isolates from different body site specimens from 127 different medical centres worldwide. They reported an increase in non-albicans *Candida* isolates with a steady decrease in the percentage of *C. albicans*.

In our study, *C. parapsilosis* was the most commonly isolated (14.5%) non-albicans *Candida* species (46% were from blood cultures). In South Africa, *C. parapsilosis* was the most commonly isolated species (19%) from respiratory samples of HIV-TB patients (Samie and Masheo, 2012) and the second most commonly isolated (10%) in Cape Town (Arendse and Orth, 2008). According to Pfaller *et al.* (2005), *C. parapsilosis* was the third most commonly isolated species from different body site samples worldwide.

C. glabrata is the most common non-albicans *Candida* species worldwide (21.5%) (Pfaller *et al.*, 2005). According to Samie and Masheo (2012) *C. glabrata* was the third most commonly isolated (4%) from the respiratory tracts of HIV infected patients with TB in Limpopo province, South Africa. Arendse and Orth (2008) reported that 10% of the yeast isolates from Tygerberg Hospital in Cape Town were *C. glabrata*. In our study, *C. glabrata* was the second most commonly isolated (4.3%) and more than 50% were from blood and genitourinary samples.

C. tropicalis, the most common cause of invasive candidiasis in patients with haematological malignancies, was the second most commonly isolated non-albicans *Candida* species worldwide and the third most isolated in North America (Laupland *et al.*, 2005; Pfaller *et al.*, 2005). In South Africa, it was the third most commonly isolated (4%) in Tygerberg Hospital in Cape Town (Arendse and Orth, 2008). Our study showed rates of 2.9%, mostly from urine and genital swab specimens.

Other unusual non-albicans *Candida* species such as *C. lipolytica* accounted for only a small percentage worldwide. According to Pfaller *et al.* (2005), *C. lipolytica* constituted less than 0.1% during 2003. In South Africa, *C. lipolytica* accounted for similar rates (Arendse and Orth, 2008). In our study, *C. lipolytica* accounted for 1.4%.

C. neoformans has a variable geographic distribution and it mainly infects AIDS patients and other immunosuppressed patients. Globally, about one million persons with HIV infection are developing cryptococcal meningitis each year (Park *et al.*, 2009). In this study, all *C. neoformans* isolates were detected from cerebrospinal fluid samples. These accounted for 17% (43/250) of the yeast isolates of which (33/43) were *C. neoformans* var. *neoformans* and (10/43) *C. neoformans* var. *gattii*. Almost all cases of cryptococcosis in patients with AIDS globally are due to *C. neoformans* var. *neoformans*, even in countries where *C. neoformans* var. *gattii* is endemic (Dromer *et al.*, 1994). In South Africa, where more than 5.7 million people are infected with HIV (UNAIDS/WHO report, 2008), this may explain the predominance of *C. neoformans* var. *neoformans* among the *C. neoformans* isolates in our study.

In the current study, the Etest was used to determine the susceptibility of three azole antifungal agents (fluconazole, posaconazole and voriconazole), amphotericin B and caspofungin against *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. lipolytica* and *C. neoformans* isolates in Pretoria, South Africa.

Azoles has been widely used for treatment of fungal infections. However, resistance to this group of antifungal agents has become a major problem since the 1990s (White *et al.*, 1998). Samie and Masheo (2012) reported that 80% of *C. albicans*, 75% of *C. parapsilosis* and 69% of *C. neoformans* were resistant to fluconazole (MIC $\geq 8\mu\text{g/ml}$) in Limpopo province, South Africa. In this study, fluconazole resistance was detected among 56.7% of *C. parapsilosis* isolates, the most fluconazole resistant of *Candida* species.

Differences in the antifungal susceptibility profiles among *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* have been reported. *C. neoformans* var. *gattii* isolates have been documented to be less susceptible than *C. neoformans* var. *neoformans* to azole antifungal agents (Chong *et al.*, 2010). In the present study, all *C. neoformans* var. *gattii* isolates were resistant to fluconazole, whereas only 42.9% (9/21) of *C. neoformans* var. *neoformans* isolates were resistant to fluconazole, a finding consistent with that of previous studies (De Bedout *et al.*, 1999; Chong *et al.*, 2010). This study documents the importance of differentiating between these two varieties of *C. neoformans* in order to establish the differences in their antifungal susceptibility.

Resistance to voriconazole was seen in 50% (15/30) of *C. parapsilosis* isolates, 30% (3/10) of *C. albicans* isolates and 55.6% (5/9) of *C. glabrata* isolates. These results are high, in comparison with the worldwide results (2% for *C. parapsilosis*, 1% for *C. albicans* and 10% for *C. glabrata*) reported by Pfaller *et al.* (2005).

Cross-resistance between fluconazole and voriconazole among *Candida* isolates has been documented in previous studies (Swinne *et al.*, 2004; Sabatelli *et al.*, 2006). Mulu and colleagues (2013) collected 221 samples from HIV patients complaining of oropharyngeal candidemia in Ethiopia, they reported concomitant resistance between fluconazole and other azole antifungals. In our study, the three *C. albicans* isolates that were resistant to voriconazole were also resistant to fluconazole. Furthermore, 15 *C. parapsilosis* isolates that were resistant to voriconazole were also resistant to fluconazole. The five isolates of *C. glabrata* that were resistant to fluconazole were also resistant to voriconazole. Therefore, these two agents are not a good alternative to use in the treatment of resistant isolates.

Carillo-Munoz (2005) reported that posaconazole displays a superior activity in comparison to fluconazole against *Candida* species, however, higher MIC₉₀ values were obtained with *C. glabrata* (16 µg/ml) compared to other species (0.125 µg/ml). According to Espenil-Ingroff (1998), posaconazole was highly effective against *C. neoformans*, compared to caspofungin, which displayed very poor *in vitro* activity. In our study, resistance to posaconazole was detected in 66.7% of *C. glabrata* isolates (≥ 32 µg/ml). All other *Candida* species, as well as *C. neoformans* isolates, were fully susceptible to the antifungal agent.

Amphotericin B was the first systemic antifungal agent used to treat IFIs. However, because of its nephrotoxicity in 80% of the patients, use of this agent has been limited (Chen and Sorrell, 2007). Amran *et al.* (2011) tested amphotericin B against 159 different *Candida* species from patients with IFIs and reported that only one isolate of *C. lusitaniae* was resistant to this agent. However, in Limpopo, South Africa, 33.7% of yeast isolates from respiratory system of HIV-TB infected patients were reported to be resistant to amphotericin B (Samie and Masheo, 2012). In the present study, *C. parapsilosis* (3/30), *C. glabrata* (1/9) and *C. tropicalis* (1/6) were resistant to this antifungal agent.

Caspofungin is a broad spectrum antifungal agent against *Candida* species and was the first of the three echinocandins that became available (Deresinski and Stevens, 2003). Pfaller *et al.*

(2006), collected 8197 *Candida* isolates from 91 medical centres in the world and determined the *in vitro* susceptibilities of caspofungin against these isolates. Over the four years of their study, the susceptibility profile of caspofungin against these isolates remained unchanged and 99% were inhibited by <1 µg/ml in each year. In our study, *C. albicans* (3/10) and *C. glabrata* (4/9) isolates were resistant to caspofungin, but all *C. parapsilosis* and *C. tropicalis* isolates were susceptible to caspofungin.

Mutations in the *ERG11* gene is one of the mechanisms that lead to azole resistance in *C. albicans* isolates (Morio *et al.*, 2010). More than 140 amino acid substitutions have been documented to date, most of these mutations are located in three hot spot regions (HS); namely, the 105 to 165, 266 to 287 and 405 to 488 amino acid regions (Marichal *et al.*, 1999). Some of these mutations contributed exclusively to azole resistance, but others are not associated with azole resistance (Sanglard *et al.*, 1998; Lamb *et al.*, 2000).

A study done by Morio *et al.* (2010) reported 23 amino acid substitutions among 60 of 73 *C. albicans* isolates, 19 substitutions have been reported previously and four were newly described by their study (N136Y, Y221H, L276S and Y447H). Furthermore, their study documented that 11 of the 23 amino acid substitutions were found in both azole resistant and azole susceptible isolates including D116E, K128T, V437I, G129A, D153E, V159I, E266D, G464S, V488I, Y221H and L276S. Lee *et al.* (2004) studied the *ERG11* gene mutations in 12 *C. albicans* isolates and documented nine different amino acid substitutions in 10 isolates. Five of these (K143R, R467K, V404I, V509M, and F449V) were found exclusively in isolates with reduced fluconazole susceptibility, while three were found in both susceptible and resistant isolates (D116E, E226D and V437I) and K128T was found only in susceptible isolates. Three novel amino acid substitutions (F449V, V404I and V509M) were reported by their study, two of them were located just outside the third HS region.

In our study, the complete *ERG11* sequence of *C. albicans* isolates was amplified to provide a full picture of the genetic alterations occurring in this gene. Point mutations were frequently observed among the *ERG11* gene of both azole resistant and azole susceptible isolates, but amino acid substitutions were detected in only one azole resistant and in two azole susceptible isolates. The amino acid substitutions D116E and K128T were found simultaneously in a susceptible isolate and V437I was found in another susceptible isolate. All mutations were located within the three HS regions reported by Marichal *et al.* (1999) and all of them have been described previously in both susceptible and resistant isolates (Sanglard *et al.*, 1998;

Marichal *et al.*, 1999). E517Q substitution was found in a resistant isolate just outside the third HS region. This mutation has not been described before and further study is needed to provide information regarding its contribution to azole resistance.

The *ERG11* gene is the primary target of azole antifungal agents, but resistance observed in our isolates could not be explained by studying the *ERG11* gene mutations. Other mechanisms such as over-expression of the *ERG11* gene, over-expression of genes encoding efflux transporters and alteration in the ergosterol biosynthetic pathway may be involved (Prasad and Kapoor, 2005).

Point mutations within the *FKSI* gene encoding the 1,3- β -glucan synthase were identified in *C. albicans* isolates with reduced susceptibility and resistance to echinocandins (Park *et al.*, 2005). These mutations were located at two HS regions, namely, at the amino acid regions 641 to 649 (HS1) including the F641, L642, T643, S645, R647, D648 and P649 mutations, and at the amino acid regions 1345 to 1365 (HS2), including the R1352 mutation (Park *et al.*, 2005; Perlin, 2007). Balashov and colleagues (2006) studied the resistance to caspofungin in *C. albicans* isolates by profiling the *FKSI* mutations. Among the 85 resistant isolates detected, all were identified with mutations in *FKSI*.

Other mechanisms of resistance to echinocandin, such as the stimulation of the synthesis of the cell wall components other than 1,3- β -D glucan have been described as an alternative for the *FKSI* gene mutation (Stevens *et al.*, 2006; Drakulovski *et al.*, 2011). The chitin synthesis pathway in *C. albicans* is a complex mechanism that involves up to four genes (*CSH1*, *CSH2*, *CSH3* and *CSH8*) and alterations in these genes can lead to caspofungin resistance without any mutations in the *FKSI* gene (Munro and Gow, 2001). A study was conducted by Drakulovski *et al.* (2011) on *C. albicans* isolates exhibiting high MIC for caspofungin without any *FKSI* gene mutations. This study reported that these isolates had a low level of 1,3- β -D glucan with a high level of chitin in their cell wall. Their study also reported the presence of mutations in the *CTH2* and *CTH3* genes, which may be the cause of the high chitin level and the survival of these strains with a low amount of 1,3- β -D glucan in the presence of caspofungin.

In our study, among the three caspofungin resistant and seven caspofungin susceptible *C. albicans* isolates, no mutations were found except the substitution of the polar amino acid serine by the non-polar amino acid leucine (S642L) in the *FKSI* gene of all susceptible and

resistant isolates. Although, the substitution of serine by leucine (and *vice versa*) in CTG-clade fungi such as *C. albicans* has been reported before (Santos *et al.*, 1993; Miranda *et al.*, 2007), which was due to a mutation in the anticodon loop that allows the recognition of the CUG codon by both the seryl and the leucyl-tRNA synthetases (Santos *et al.*, 1993; Miranda *et al.*, 2007), the substitution found in our study was due to TCG to TTG missense mutation. Further research is required to study the nature of this mutation; functional or structural, as well as its involvement in strain evolution and virulence.

In this study, we found that *C. albicans* isolates can be resistant to caspofungin antifungal agent without mutations in the HS1 region of the *FKS1* gene. Other mechanisms may be involved such as mutations in the HS2 region or increase in the chitin level of the cell wall and mutations in the chitin synthase genes.

CONCLUSION

The distribution of yeast isolates and the antifungal susceptibility profile found in Pretoria differed from those found in and other geographical regions. Therefore, it is necessary to identify the isolates at species level and perform susceptibility testing for the efficient treatment of fungal infections. *C. albicans* was the most commonly isolated yeast species at Steve Biko Academic Hospital and referring hospitals in Pretoria. Cross-resistance between fluconazole and voriconazole was found. Therefore, these two agents are not a good alternative to use in the treatment of resistant isolates. *Candida* species and *C. neoformans* isolates were less resistant to posaconazole than to fluconazole and voriconazole. Furthermore, it is important to identify the two varieties of *C. neoformans* in order to establish the differences in their antifungal susceptibility.

Resistance to azole and caspofungin antifungal agents existed without the previously described mutations in the *ERG11* and *FKSI* genes of resistant isolates. Although, resistance could not be explained by studying the alterations in these two genes, further studies are required to explain the role of new amino acid substitutions, as well as, the involvement of other mechanisms in resistance to the antifungal agents.

LIMITATION OF THE STUDY

Germ tube testing was used as an identification method for *C. albicans* isolates. Ells *et al.* (2011) reported that *C. dubliniensis* shares many phenotypic characteristics with *C. albicans* including germ tube formation. Therefore, the use of this method alone may lead to misidentification. In our study, because of financial constraints, other methods could not be used to differentiate *C. dubliniensis* from *C. albicans* and all germ tube positive isolates were considered as *C. albicans*.

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