CLADE RELATED ANTIFUNGAL RESISTANCE AMONG SOUTH AFRICAN *CANDIDA ALBICANS* ISOLATES

By

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DECLARATION

I, **Julitha Molepo**, hereby declare that the work on which this thesis is based, is original (except where acknowledgements indicate otherwise) and that neither the whole work, nor any part of it has been, is being, or is to be submitted for another degree at this or any other university or tertiary education, institution or examination body.

_____J. Molepo_____

Signature of candidate

_____ day of ____ _____

DEDICATION

- I would like to thank God for making it possible for me to finally finish the study, despite all difficulties and obstacles.

- I dedicate this thesis to my husband, who has always been with me through thick and thin throughout the study. I would not have done it if it was not because of his support, encouragement, patience and advises.

- I also dedicate this to my daughters Mpho and Nthabi, and my son Thabo, who always encouraged me to push forward.

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SUMMARY

Background: Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Five genetic clades were described among *C. albicans* isolates using DNA fingerprinting methods (clades I, II, III, SA and NG). Although these clades have been described, little is known about their phenotypic characteristics, and not much is known about antifungal resistance with regard to each of these clades.

The widespread use of fluconazole has led to its increased resistance reported world-wide. Resistance to fluconazole can be caused by point mutations in the *ERG11* gene or over-expression of this gene, however, not much is known about the contribution of these mutations and over-expression to fluconazole resistance among different clades of *C. albicans*, and whether mutations or over-expression are clade-related.

There is evidence to suggest that phenotypic switching may play a significant role in the ability of *Candida* strains to survive under adverse conditions and perhaps cause more severe forms of disease in the immunocompromised host (Vargas et al., 2004). Only limited studies on the relationship between phenotypic switching and fluconazole resistance of *C. albicans* have been done, and not much is known about this relationship among different clades of *C. albicans*.

Objectives: This study undertook to investigate: (1) the induction of antifungal resistance among South African *C. albicans* isolates belonging to different clades, (2) the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, (3) the contributions of over-expression of *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, (4) and the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades.

Study populations and Methods: To investigate the induction of antifungal resistance among South African *C. albicans* isolates belonging to different clades, a total of 100 *C. albicans* isolates (20 from each of clades I, II, III, SA and NG) were used. These yeast isolates were obtained from surveillance cultures on patients attending HIV/AIDS clinics in the Pretoria region. Resistance to fluconazole, miconazole, amphotericin B and nystatin was induced in all 100 isolates according to the modified National Committee of Clinical

Laboratory Standards (NCCLS) broth microdilution method. Survival and retention of resistance among fluconazole resistant (n=100), miconazole resistant (n=100), amphotericin B resistant (n=100) and nystatin resistant (n==100) isolates after two years of storage at -80^{o}C was determined in the presence of highest concentrations of each antifungal.

To investigate the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, 30 isolates were used. These consisted of 3 isolates with induced fluconazole resistance and their 3 matching fluconazole susceptible isolates from each of clades I, II, III, SA, and NG. DNA was extracted, PCR performed with a high-fidelity *Pwo* DNA polymerase), and PCR products sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit on the GeneAmp® PCR System 9700. Obtained sequences were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989).

To investigate the contributions of over-expression of the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, 30 isolates were used. These consisted of 3 isolates with induced fluconazole resistance and their 3 matching fluconazole susceptible isolates from each of clades I, II, III, SA, and NG. RNA was extracted, cDNA synthesized and Real time PCR performed on a Rotor-Gene 6000 instrument. Relative gene expression of *ERG11* gene among resistant isolates, relative to susceptible isolates was quantified after normalization with the *18SrRNA* house-keeping gene.

To investigate the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades, 30 isolates were used. These consisted of 3 isolates with induced fluconazole resistance and their 3 matching fluconazole susceptible isolates from each of clades I, II, III, SA, and NG. Primary and secondary cultures were prepared on Lee's medium agar supplemented with arginine and zinc, and containing phloxine B. The switched colonies were counted and colony morphologies viewed and photographed. Phenotypic switching behavior and different colony morphologies obtained between the resistant and susceptible isolates from different clades were compared. Switch phenotypes among fluconazole resistant isolates in different clades were compared. Switch phenotypes and MIC levels among fluconazole resistant isolates from different clades were compared.

Results: Resistance to nystatin, AmB, fluconazole and miconazole was successfully induced in all of 20 (100%) *C. albicans* isolates from each of clades I, II, III, SA and NG. When survival and retention of resistance were determined, all 20 (100%) fluconazole resistant

isolates from clades I, II, SA, NG, and 19 (95%) from clade III survived and retained their resistance. Of miconazole resistant isolates, all 20 (100%) isolates from clade I, II, and SA, and 19 (95%) from clade III and NG survived and retained their resistance. Of AmB resistant isolates, 12 (60%) from Clade NG survived and retained their resistance; 9 (45%) from Clade I; 8 (40%) from Clade III; 7 (35%) from Clade II and 6 (30%) from Clade SA survived and retained their resistance. Of the isolates resistant to nystatin, 12 (60%) from clade I survived and retained their resistance, 8 (40%) from clade II, 10 (50%) from clade III, 11 (55%) from clade SA, and 15 (75%) from clade NG survived and retained their resistance.

No mutations associated with fluconazole resistance were observed in all isolates from clades I and II. Mutations associated with fluconazole resistance were observed in 33.3% of isolates from each of clades III, SA and NG , and some of the mutations observed in resistant isolates from clades III and NG were novel. A total of 50 novel mutations that have not been described previously were observed in both fluconazole resistant and susceptible isolates from this study. Previously described mutations, which were associated with fluconazole resistance, namely, D116E, K128T, V437I and E266D were also observed in this study.

When relative *ERG11* gene expression was quantified among fluconazole resistant and susceptible isolates from various clades, over-expression of *ERG11* gene was observed in 66.6% of isolates from each of clades I, II and SA, and in 33.3% of isolates from each of clades III and NG.

When the relationship between fluconazole resistance and phenotypic switching was investigated, phenotypic switching was related to resistance in 66.6% of the resistant isolates tested from each of clades I, II and III, and in 33.3% of the resistant isolates tested from each of clades SA and NG. When the switch phenotypes and MIC levels of resistant isolates from different clades were compared, stipple was the most common switch phenotype observed in all clades, and it was associated with the highest fluconazole MIC levels among isolates from all clades.

Conclusions: The results of this study showed that resistance to polyenes and azoles could readily be induced in *C. albicans* isolates from all clades, and that induction was not clade-related. The ease with which azole and polyene resistance could be induced in this study can hold serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles/polyenes are mostly used for *C. albicans* infections.

The study also showed that mutations contributed to fluconazole resistance in isolates from clades III, SA and NG, but not clades I and II, showing clade-relatedness. Novel mutations were observed, and their contribution to fluconazole resistance is at this stage not known. Genetic analysis of these mutations needs to be studied further to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa.

The results of the study showed that over-expression of *ERG11* gene contributed to fluconazole resistance in isolates from all clades. However, over-expression was observed in more isolates from clades I, II and SA, and in less isolates from clades III and NG, showing clade-relatedness of *ERG11* over-expression. The occurrence of over-expression of *ERG11* gene in these clades is a cause for concern, especially in HIV/AIDS patients with OPC, as the increased expression of *ERG11* allows for the cells to persist within the host, which in turn leads to the subsequent development of other more stable resistant isolates.

In this study, phenotypic switching was found to be related to fluconazole resistance in isolates from all clades, with a high number of switch phenotypes occurring more in isolates from clade II as compared to others. This suggests that isolates belonging to this clade may survive better under adverse conditions than isolates from other clades. These results suggest that further study of differences between different *C. albicans* clades may be warranted, and that isolates from this clade need to be studied further. The stipple phenotype was found to be the most dominant in isolates from all clades, and was found to be associated with the highest fluconazole MICs levels. These findings suggest that the evaluation of colony phenotypes and their antifungal susceptibilities in *C. albicans* isolates may be useful in therapy.

Recommendations: A continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is recommended. Pathogens that can potentially infect HIV-infected individuals need to be studied to subspecies level in order to improve treatment of these patients. Continued antifungal surveillance is needed to predict the evolution of resistance in a particular population and to take timely measures. Evaluation of colony phenotypes and their antifungal susceptibilities in *C. albicans* isolates is recommended as this may be useful in therapy. Genetic analysis of the novel mutations observed is recommended to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa.

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LIST OF ABBREVIATIONS

AMINO ACIDS

G	**-**	Glycine
A	**-**	Alanine
L	**-**	Leucine
M	**-**	Methionine
F	**-**	Phenylalanine
W	**-**	Tryptophan
K	**-**	Lysine
Q	**-**	Glutamine
E	**-**	Glutamic Acid
S	**-**	Serine
P	**-**	Proline
V	**-**	Valine
I	**-**	Isoleucine
C	**-**	Cysteine
Y	**-**	Tyrosine
H	**-**	Histidine
R	**-**	Arginine
N	**-**	Asparagine
D	**-**	Aspartic Acid
T	**-**	Threonine

NUCLEOTIDE CODES

G	-	Guanine
A	-	Adenine
C	-	Cytosine
T	-	Thymine
R	-	G or A
Y	-	T or C
M	-	A or C
K	-	G or T
S	-	G or C
W	-	A or T
H	-	A or C or T
B	-	G or T or C
V	-	G or C or A
D	-	G or A or T
N	-	G or A or T or C

ENZYMES

RNAse - Ribonuclease

DNAse - Deoxyribonuclease

***Pwo* polymerase**- Polymerase isolated from hyperthermophilic archaebacterium *Pyrococcus woesei*

MISCELLANEOUS

OPC - Orophanryngeal Candidiasis

HIV - Human immuno-deficiency virus

AIDS - Acquired immuno-deficiency syndrome

UNAIDS - United Nations Joint Programme for AIDS

WHO - World Health Organization

MF - Major Facilitators

ATP - Adenosine Triphosphate

ABCT - ATP Binding Cassettes

DEPC - Diethylene Pyrocarbonate

DNA - Deoxyribose Nucleic Acid

RNA - Ribonucleic acid

mRNA - Messenger RNA

cDNA - Complementary DNA

PCR - Polymerase Chain Reaction

RT-PCR - Reverse-Transcriptase PCR

AmB - Amphotericin B

DNTPs - Deoxyribose Nucleotide Triphosphates

CHAPTER ONE: EXPERIMENTAL PROPOSAL AND THESIS ORGANIZATION

EXPERIMENTAL PROPOSAL

Background

C. albicans is an opportunistic pathogen causing Oropharyngeal Candidiasis (OPC), the most common oral complication in HIV/AIDS patients (Greenspan and Greenspan, 1992; Glick et al., 1994). OPC was among the initial signs of HIV-induced immunodeficiency to be recognized (Gottlieb et al., 1981; Masur et al., 1981), and affects the majority of persons with advanced untreated HIV infection.

It is estimated that more than 5.7 million South Africans are currently infected with HIV (UNAIDS/WHO report, 2008), and that 80 to 95% of patients infected with HIV experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994). Even with the advent of highly active antiretroviral therapy (HAART) and the subsequent decline of opportunistic infections, oral-esophageal candidiasis still remains among the most frequent infections in HIV-infected individuals (Kaplan et al., 2000).

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been widely reported (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002). These antifungals are directed against ergosterol, the major sterol in the fungal plasma membrane. Ergosterol contributes to a number of cellular functions; it is important for the fluidity and integrity of the membrane and for the proper function of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division (Vanden Bossche et al., 1987; Joseph-Horne and Hollomon, 1997).

Azoles such as fluconazole, act by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase, an enzyme encoded by the *ERG11* gene of *C. albicans*. This enzyme is

important in the synthesis of ergosterol, and the inhibition of this enzyme leads to the inability of *C. albicans* to synthesize ergosterol (White et al., 1998).

Molecular mechanisms responsible for development of azole resistance in strains of *C. albicans* include changing the target enzyme, cytochrome P-450 lanosterol 14α-demethylase, either by over-expression of the *ERG11* gene (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Franz et al., 1998; Henry et al., 2000), or as a result of point mutations in the *ERG11* gene (Vanden Bossche et al., 1990; Vanden Bossche et al., 1994a; White et al., 1997; Sanglard et al., 1998; Franz et al., 1998). The point mutations in the *ERG11* gene lead to amino acid substitutions, causing a decreased affinity for azoles (Franz et al., 1998), and over-expression of *ERG11* gene results in increased production of lanosterol demethylase, making it difficult for the azoles to inhibit it (Henry et al., 2000).

Several mechanisms, including high-frequency phenotypic switching have been shown to be involved in the adaptability of *C. albicans* to different environments (Soll et al., 1991). Previous studies have demonstrated that most of *C. albicans* strains and related species have the capacity to switch spontaneously, at high frequencies and reversibly between different cell types that are distinguishable by the appearance of the resulting colonies on agar plates with low zinc concentrations (Slutsky et al., 1985; Slutsky et al., 1987; Gil et al., 1988; Soll, 1992; Hellstein et al., 1993; Soll, 2000).

In previous studies done, phenotypic switching was shown to play an important role in *C. albicans* pathogenesis, by regulating several phase-specific genes involved in pathogenesis (Soll, 1997; Soll, 2000). High-frequency switching, in addition to other mechanisms, is suggested to be another mechanism by which *C. albicans* achieves resistance against antifungal agents (Soll et al., 1992). In one study, switched *C. albicans* isolates from HIV positive individuals were found to be more resistant to a number of antifungal drugs including amphotericin B and fluconazole, than isolates from HIV-negative controls (Vargas et al., 2000).

Switching is usually distinguished by colony morphology and in some cases by the phenotype of cells in the budding phase (Anderson and Soll, 1987; Anderson et al., 1989;

Vargas et al., 2000). It has been suggested that switching in *C. albicans* involves differential gene expression (Kvaal et al., 1999; Sonneborn et al., 1999; Srikantha et al., 2000); therefore it is possible that different *C. albicans* phenotypes possess different physiologic and virulent traits.

Study problem

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Five genetic clades were described among *C. albicans* isolates using DNA fingerprinting methods (clades I, II, III, SA and NG). Although these clades have been described, little is known about their phenotypic characteristics, and not much is known about antifungal resistance with regard to each of these clades.

Fluconazole is the most used azole for treatment of *C. albicans* infections in AIDS patients due to its high oral bioavailability, good safety profile and wide tissue distribution (Lortholary and Dupont, 1997). The widespread use of this antifungal has led to its increased resistance reported world-wide. Resistance to fluconazole can be caused by point mutations in the *ERG11* gene or over-expression of this gene, however, not much is known about the contribution of these mutations and over-expression to fluconazole resistance among different clades of *C. albicans*, and whether mutations or over-expression are clade-related.

Several studies have demonstrated that the expression of specific virulence and pathogenesis factors of *C. albicans* are correlated with phenotypic switching (White et al., 1993; Miyasaki et al., 1994; White and Agabian, 1995; Vargas et al., 2000), therefore the importance of phenotypic switching in the *C. albicans* disease in humans is significant, and needs to be studied further. In addition, there is evidence to suggest that phenotypic switching may play a significant role in the ability of *Candida* strains to survive under adverse conditions and perhaps cause more severe forms of disease in the immunocompromised host (Vargas et al., 2004).

Only limited studies on the relationship between phenotypic switching and fluconazole resistance of *C. albicans* have been done, and not much is known about this relationship among different clades of *C. albicans*. It is not known whether a particular switch phenotype will be more common in one particular clade, especially the unique South African clade (SA), and whether switching is clade-related. Knowledge in this regard would contribute to a better understanding of fluconazole resistance and subsequently to improved management of South African patients infected with fluconazole resistant *C. albicans*.

Overall aim

The overall aim of the study was to investigate the antifungal resistance among South African *C. albicans* isolates belonging to different clades.

Broad objectives

- To investigate the induction of antifungal resistance among South African *C. albicans* isolates belonging to different clades.
- To investigate the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades
- To investigate the contributions of over-expression of *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades
- To investigate the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades.

To investigate the induction of antifungal resistance among South African C. albicans isolates belonging to different clades.

Rationale

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. When phenotypic characteristics of 5 *C. albicans* clades were studied, isolates from Clade SA showed a natural resistance mainly to amphotericin B (Blignaut et al., 2005) and those from Clade I almost

exclusively resistant to 5-flucytosine (Pujol et al., 2004). These results suggested that *C. albicans* clades differ phenotypically, and supported the need for a worldwide analysis of clade-specific phenotypic characteristics of *C. albicans* isolates. Apart from this information regarding clade SA and clade I, not much is known about resistance to other antifungals among *C. albicans* isolates belonging to different clades. Of specific concern is the antifungal resistance that could possibly be associated with particular clades, and more specifically the unique South African clade. Knowledge in this regard would contribute to a better understanding of drug resistance and subsequently to improved management of South African patients infected by *C. albicans*.

To investigate the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades

Rationale

Although various mutations in the *ERG11* gene have been reported to be responsible for fluconazole resistance among *C. albicans* isolates, not much is known about the contribution of these mutations to fluconazole resistance among different clades of *C. albicans*, and whether a particular mutation will occur more in one particular clade as compared to the others. Understanding mechanisms of fluconazole resistance in different clades of *C. albicans* would contribute in the designing and development of new antifungals, and also in the selection of the appropriate antifungal at the earliest possible time when treating South African patients infected with *C. albicans*.

To investigate the contributions of over-expression of *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades

Rationale

Although over-expression of *ERG11* gene has been reported to be responsible for fluconazole resistance among *C. albicans* isolates, not much is known about the contribution of over-expression of this gene to fluconazole resistance among different clades of *C. albicans*, and whether over-expression will occur more in one particular clade as compared to the others. Quantification of *ERG11* gene expression in different clades of *C. albicans* isolates resistant to fluconazole may produce a better understanding

of the structure of this enzyme, and may lead to the designing of new drugs, which will continue using *ERG11* gene product as the antifungal target.

To investigate the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades.

Rationale

Several studies have demonstrated that the expression of specific virulence and pathogenesis factors of *C. albicans* are correlated with phenotypic switching (White et al., 1993; Miyasaki et al., 1994; White and Agabian, 1995; Vargas et al., 2000), therefore the importance of phenotypic switching in the *C. albicans* disease in humans is significant, and needs to be studied further. In addition, there is evidence to suggest that phenotypic switching may play a significant role in the ability of *Candida* strains to survive under adverse conditions and perhaps cause more severe forms of disease in the immunocompromised host (Vargas et al., 2004).

Only limited studies on the relationship between phenotypic switching and fluconazole resistance of *C. albicans* have been done, and not much is known about this relationship among different clades of *C. albicans*. It is not known whether a particular switch phenotype will be more common in one particular clade, especially the unique South African clade (SA), and whether switching is clade-related. Knowledge in this regard would contribute to a better understanding of fluconazole resistance and subsequently to improved management of South African patients infected with fluconazole resistant *C. albicans*.

THESIS ORGANISATION

The thesis body is preceded by "**Experimental Proposal and Thesis organization".** The thesis consists of six Chapters, with chapters 2, 3, 4 and 5 each divided into Abstract, Introduction, Materials and Methods, Results, Discussions, Conclusions and References. **Chapter One** is: "Literature Review" and contains its own References; **Chapter two** is: "Induction of antifungal resistance in *Candida albicans* clades"; **Chapter three** is: "Contribution of mutations in *ERG11* gene to fluconazole resistance among *Candida albicans* clades"**; Chapter four** is: **"**Contribution of over-expression of *ERG11* gene to

fluconazole resistance in *Candida albicans* clades”**; Chapter five** is: “Relationship between phenotypic switching and fluconazole resistance among C*andida albicans* clades”**. Chapter six** is: “Combined discussions, conclusions and recommendations”, and contains its own References.

CHAPTER TWO: LITERATURE REVIEW

2.1 Historical background

The initial discovery of the thrush organism was made in 1839 by Langenbeck, who observed a fungus in scrapings of buccal thrush from a patient with typhus. Although he was the first to discover the organism, he mistook the fungus for the causative organism of typhus. In 1842, Gruby described the thrush fungus before the Academy of Sciences of Paris, and placed it in the genus *Sporotrichum.* Two years after Gruby's discovery, Bennett illustrated the microscopic characteristics of a fungus that appeared to be *Candida albicans* (*C. albicans*), in the sputum and lung of a patient who had tuberculous pneomothrax. In 1849, Wilkinson first described vaginal candidiasis and its mycotic origin. Robin recognized that the thrush fungus could cause systemic infections and named it *Oidium albicans* in 1853. In 1875, Haussmann recognized that the causative agents of oral and vaginal thrush were the same, and demonstrated transmission of the fungus to the mouth of infants from their mother's vaginal lesions (Kwon-chung and Bennet, 1992).

In 1877, Reess proposed another name for the thrush fungus, *Saccharomyces albicans,* and Grawitz noticed the dimorphic nature of the thrush fungi, describing the yeast form, mycelial form and chlamydospores, although he didn't use those terms. In 1890, Zopf named the thrush fungus *Monilia albicans*, from which moniliasis, the early name of candidiasis, originated. In 1923, Berkhout noted that the medical *Monilia* species differed physiologically and morphologically from the fruit-rotting *Monilia*. In 1912, Castellani suggested that the yeast species other than *Monilia albicans* might be involved in candidiasis and made descriptions of the species currently known as *C. krusei, C. guillierimondii, C. pseudotropicalis* and *C. tropicalis.* He persistently used the name *Monilia*, and the generic name Candida was finally accepted by the Eighth Botanical Congress at Paris in 1954 (Kwon-chung and Bennet, 1992).

In 1980, the genetic evidence that *C. albicans* is a diploid organism was demonstrated (Whelan et al., 1980).

2.2 Biology

2.2.1 Taxonomy

C. albicans belongs to the Class Fungi Imperfecti, the Order Monialiales and the Family Cryptococcaceae (Kwon-chung and Bennet, 1992).

2.2.2 Growth and nutrition

C. albicans grows on a defined medium consisting of a source of salts, carbon, nitrogen and phosphate, but it also requires biotin. The fungus grows over a temperature range of 20-40 ^{o}C and over a pH range of 2-8. The maximum growth rate in synthetic media is between 0.3-0.4h^{-1}, but in media supplemented with additional vitamins and amino acids the growth rate will be as high as 0.8^{-1} (Shepherd, 1990)**.** *C. albicans* readily grows in saliva supplemented with carbohydrates such as glucose and sucrose (Knight and Fletcher, 1971; Samaranayake et al., 1986)**.**

2.2.3 Cell wall and antigenic structure

The cell wall of *C. albicans* is composed of β-glucans, mannoproteins and a small quantity of chitin, which makes up 30% of the dry weight of the cell (Sullivan et al., 1983). The major component is carbohydrate (80-90% w/w), and proteins (3-6% w/w) and lipids (2% w/w) are also present. The same proportions of these substances are found in yeast, germ tubes and mycelial elements (Shepherd, 1987; Shepherd, 1990). The outer most layer of the cell wall is the mannoprotein, and these are distributed throughout the cell wall (Elorza et al., 1987). In addition to the mannoprotein layer is the fibrillar layer which can be seen when yeast cells are forming germ tubes (Hubbard et al., 1985)**.**

The β-glucans are responsible for the structural integrity of the cell wall (Gopal et al., 1984). Chitin is covalently bound to glucan and this leads to the development of the secondary wall structure (Surarit et al., 1988). The β-glucans and chitin are not present in the host and these compounds and the enzymes associated with their synthesis and degradation are potentially safe targets for new antifungal agents (Shepherd, 1990).

Ergosterol is the major sterol of the fungal plasma membrane, and is analogous to cholesterol in mammalian cells. Ergosterol contributes to a variety of cellular functions. It

is important for the fluidity and integrity of the membrane and for the proper functioning of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division (Vanden Bossche et al., 1987; Joseph-Horne and Hollomon, 1997).

2.2.4 Morphogenesis

C. albicans has a number of different morphological forms under different environmental conditions. These include budding yeast cells, which include blastospores and blastoconidia; pseudohyphae, which are elongated yeast cells appearing as filamentous cell chains; true hyphae and chlamydospores. At temperatures below 33^{o}C and low pH, yeast cell growth is favoured. The yeast cells are ovoid and are approximately 3x5µm in size. During yeast bud formation, the cell wall expansion is restricted to the bud, and the mother cell shows little growth (Staebell and Soll, 1985).

At elevated temperatures and near neutral pH, mycelial growth is favoured and the conversion of a yeast cells to a hyphal cell via a germ tube occurs (Odds, 1985; Shepherd, 1988). Germ tube formation is the initial stage in the yeast-hyphal transition. A blastospore gives rise to new cellular material in the shape of a cylinder, called a germ tube, which grows continuously by extension of the distal pole. Hyphae may arise as branches of already existing hyphae or by germination of spores. A mycelium includes hyphae with all their branches (Odds, 1988) **.**

Pseudohyphae are long, branching filaments of elongated yeast cells formed by polar budding, and are shortened by a septum. They are frequently encountered in both *in vivo* and *in vitro* growth. Apical growth in hyphal cells is continuous and the time at which secondary wall develops determines between yeast and hyphal growth. If the glucan-chitin complex is formed immediately behind the apical tip, this gives rise to a hyphal element. If secondary wall formation is delayed, a more plastic wall would result, forming spherical cells (Gow and Gooday, 1984).

2.2.5 Biochemical studies

Candida albicans metabolizes carbohydrates via glycolysis, pentose pathway and the citric acid cycle (Odds, 1988). Phosphofructokinase and pyruvate kinase are allosteric enzymes (Ram et al., 1983), responsible for utilization of glucose by growing yeast cells (Shepherd et al., 1985).

Candida albicans contains two respiratory systems: the classic cytochrome c oxidase and an alternative oxidase system which is insensitive to cyanide (Shepherd et al., 1978). It has been estimated that in growing cells the alternative pathway contributes less than 10% of the total respiration, but the role of this pathway in morphogenesis and pathogenicity is unclear (Aoki and Ito-Kuwa, 1984).

A number of metabolic end products have been detected in the culture media of *C. albicans* batch cultures. These include ethanol, acetoin and short chain carboxylic acids such as acetic, formic, lactic, propionic, pyruvic and succinic (Lategan et al., 1981; Samaranayake et al., 1986). The fungus ferments glucose, galactose and maltose with the formation of acid and carbon dioxide (Odds, 1988).

2.3 Epidemiology

C. albicans is distributed widely in nature, and is recovered commonly from both humans and animals. It has also been isolated from fresh and salt water, air and soil, but this is very uncommon and is usually related to contamination from human or animal sources (MacFarlane, 1990). In humans, *C. albicans* is commonly found as normal flora in the mouth (Arendorf and Walker, 1979), gastrointestinal tract (Saraswathamma and Naidu, 1979) and vagina (Peddie et al., 1984).

The prevalence of *C. albicans* reported from different anatomic sites varies greatly, depending on the subjects sampled and the isolation method. In a study by Soll (1991), the frequency of yeast carriage was highest in the mouth (56%), followed by vulvovaginal (40%), and anorectal (24%) regions. It is reported that the carriage rates of *C. albicans* in the mouths of healthy individuals determined by the swab method ranges from 2-69%. The carriage rate in adults is higher than in young children but lower than in

infants (Odds, 1988). Infants may acquire oral Candida during passage through the birth canal, during nursing or from contaminated bottles (Winner, 1975).

Wearing dentures increases the oral carriage of *C. albicans* in normal subjects (Tapper-Jones et al., 1981), as well as in diabetic patients (Fisher et al., 1987). A high-sugar diet may enhance the oral yeast carriage (Olsen and Birkeland, 1976). According to the imprint-culture study on the distribution of yeasts in the mouth, the tongue is the most prevalent site, followed by the palate and the buccal mucosa (Arendorf and Walker, 1980). T-cell abnormalities may be responsible for the extremely high rate of oropharyngeal candidiasis (OPC) in AIDS patients (Meunier and Meunier, 1989). *C. albicans* may be recovered from up to one third of the mouths of normal individuals and two thirds of those with advanced Human Immunodeficiency Virus (HIV) disease (Odds, 1988; Fichtenbaum et al., 2000). Oral colonization with drug-resistant organisms is more common in advanced HIV infection with CD4-lymphocyte counts of less than 50 cells/mm^3 (Fichtenbaum et al., 2000).

C. albicans is the most prevalent species in the gastrointestinal tract (51%), followed by *C. glabrata* and other species (Odds, 1988). The vaginal carriage rate in normal females is less than 30%, and the vaginal carriage is more prevalent in pregnant than in non-pregnant women (Odds et al., 1988). *C. albicans* is not a normal flora of the healthy human skin, but it has been isolated from toe clefts, fingers, or fecally contaminated diaper areas of babies. The highest carriage rate on the skin is seen from the toes of patients with athlete's foot (Odds, 1988).

2.4 Pathogenesis

The physiological status of the host determines development of candidiasis. *C. albicans* is a harmless commensal, but can become pathogenic due to its extensive variety of virulence determinants that are selectively expressed under suitable predisposing conditions (Sweet, 1997).

An interesting feature of *C. albicans* is its ability to grow in two different ways; reproduction by budding, and a hyphal form, which can periodically fragment and give rise to new mycelia, or yeast-like forms. Transitions between the two phenotypes can be induced *in vitro* in response to several environmental conditions such as pH or temperature, or different compounds such as N-acetylglucosamine or proline. The most important criterion for pathogenicity is the induction of mycelial form by serum or macrophages. In addition to this dimorphism, its ability to switch between the yeast and the hyphal mode of growth has been implicated in its pathogenicity (Ryley and Ryley, 1990; Cutler, 1991; Leberer et al., 1997; Lo et al., 1997).

2.4.1 Virulence factors

C. albicans is an opportunistic pathogen, and the major factor contributing to its virulence is its ability to persist on mucosal epithelia of healthy individuals (Shepherd et al., 1985). There is no single, major virulence factor of Candida which is predominantly responsible for candida infections, such as is found with certain toxins of bacterial pathogens.

C. albicans has developed several virulence factors and specific strategies to assist in its ability to colonize host tissues, cause disease, and overcome host defences. The virulence factors expressed by *C. albicans* to cause infections differ depending on the type of infection, the site and stage of infection, and the nature of the host response. Several virulence factors are involved in the infective process, but no single factor accounts for Candida virulence and not all expressed virulence factors may be necessary for a particular stage of infection (Cutler, 1991).

Although several virulence factors have been suggested to lead to the pathogenic nature of *C. albicans*, hyphal formation, surface recognition molecules, phenotypic switching, and extracellular hydrolytic enzyme production have been the most widely studied in recent years (Calderone et al., 2001).

2.4.1.1 Adherence

Adhesion of *C. albicans* to mucosa is an important initial step in its pathogenesis. This adhesion occurs by the interaction between yeast and epithelial cell receptors (King et al.,

1980; Enache et al., 1996; Sturtevant and Calderone, 1997), and is enhanced by several factors such as germ tube production, phospholipase, protease, other extracellular enzymatic activities, carbohydrates, pH and temperature (Barrett-Bee et al., 1985; Ghannoum and Abu Elteen, 1986; Macura et al., 1989; Hube, 1996; Cardaropoli et al., 1997; Vidotto et al., 1999).

Adhesion of Candida to host tissues allows the fungus to attach and colonize a specific niche environment. Under suitable predisposing conditions when the host is compromised, this colonized site provides the base for candidal multiplication, invasion, and spreading in some cases. Adherence of *Candida albicans* to host cells is a complex process involving several types of candidal adhesions (Calderone and Braun, 1991; Hostetter, 1994; Sundstrom, 2002).

2.4.1.2 Hydrolytic enzymes

Hydrolytic enzyme production is one of the factors known to play a central role in the pathogenicity of pathogenic yeasts (Ogrydziak, 1993). Although many microorganisms possess a variety of hydrolytic enzymes, proteinases are by far the most commonly associated with virulence. All proteinases catalyze the hydrolysis of peptide bonds (CO—NH) in proteins but can differ in specificity and mechanism of action (Barrett and Rawlings, 1991). Kvaal et al. (1999) reported that Sap1 proteinase might be involved in adherence, cavitation, and later penetration of *C. albicans* to host tissues. The precise mechanisms by which Sap proteins contribute to the adherence process are not clear, but two hypotheses are currently favoured. Firstly, *C. albicans* proteinases could act as attachments to surfaces on host cells. Secondly, *C. albicans* utilizes Sap proteins as active enzymes, changing the surface hydrophobicity or leading to conformational changes, allowing better adhesion of the fungus (Monod and Borg-von Zepelin, 2002).

One study showed that proteolytic strains of *C. albicans* adhered more strongly to human buccal epithelial cells *in vitro* than strains producing less proteinase (Ghannoum and Abu Elteen, 1986). Another report associated proteinase production with increased adherence to buccal epithelial cells and death of mice, with the higher-Sap-producing strains showing greater levels of tissue colonization in the liver, kidneys, and spleen (Abu-Elteen et al., 2001).

2.4.1.3 Hypha formation

C. albicans morphogenesis, as well as the identification and characterization of cell wall components that are associated with formation of hyphae and yeast forms, have been studied. *C. albicans* uses hyphae to adhere to and penetrate host tissues, therefore its ability to transform into hyphae is a pathogenic determinant in the invasion of host tissues. Morphogenesis is also considered important as a biological phenomenon (Brown and Gow, 1999; Ernst, 2000a; Ernst, 2000b; Liu, 2001; Gow et al., 2002; Brown, 2002a; Brown, 2002b). In culture medium, the main proteinases associated with hyphal formation are the *SAP4* to *SAP6* subfamily (Hube et al., 1994; White and Agabian, 1995).

2.4.1.4 Phenotypic switching

Most strains of *C. albicans* have been shown to switch at high frequencies between a number of general phenotypes, which are distinguishable by colony morphology. This switching is reversible and spontaneous (Gil et al., 1988; Soll, 1992; Soll, 2000).

Switching is suggested to play an important role in pathogenesis. It has been demonstrated to regulate several genes, leading to *C. albicans* being pathogenic (Soll, 1992; Soll, 1997). The genes that are responsible for this pathogenecity include the secreted aspartyl proteinase genes *SAP1* and *SAP3* (Morrow et al., 1993; White et al., 1993; Morrow et al., 1994; Hube et al., 1994; Kvaal et al., 1999), the drug resistance gene *CDR3* (Balan et al., 1997)), the white phase-specific gene *WH11* (Srikantha and Soll, 1993; Kvaal et al., 1997), the opaque phase-specific gene *OP4* (Morrow et al., 1993), the two-component histidine kinase regulator gene *CaNIK1* (Srikantha et al., 1998) and the transcription factor gene *EFG1* (Sonneborn et al., 1999; Srikantha et al., 2000).

Switching has been demonstrated to regulate several phenotypic characteristics involved in pathogenesis, namely antigenicity (Anderson et al., 1990), constraints on the budhypha transition (Anderson et al., 1993), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), adhesion (Kennedy et al., 1988; Vargas et al., 1994), secretion of aspartyl proteinase and virulence in a mouse systemic model and a mouse cutaneous model (Kvaal et al., 1997; Kvaal et al., 1999).

Switching has been shown to occur at sites of infection, and to occur between recurrent episodes of infection in certain cases (Soll et al., 1989; Bergen et al., 1990). It has been shown to be important in *C. albicans* antifungal drug resistance (Vargas et al., 1994). It may provide *C. albicans* with mechanisms for adapting to different environments, for evading host defense mechanisms and for adhering to different types of surfaces (Hellstein et al., 1993). In a study by Jones et al. (1994), it was demonstrated that infecting *C. albicans* isolates switch at significantly higher frequencies than commensal isolates. A study by Vargas et al. (2000) showed that isolates causing deep mycoses switch more frequently than isolates causing superficial mycoses (Vargas et al., 2000).

2.5 Oral Candidiasis

2.5.1 Clinical presentation

Candida colonizes the mouths of 64 to 84% of HIV/AIDS patients and causes symptomatic disease in up to 46% (Maenza et al., 1997). *C. albicans* is the causative agent of Oropharyngeal Candidiasis (OPC), the most common oral complication in HIV/AIDS patients (Greenspan and Greenspan, 1992; Glick et al., 1994). OPC was among the initial signs of HIV-induced immunodeficiency to be recognized (Gottlieb et al., 1981; Masur et al., 1981), and typically affects the majority of persons with advanced untreated HIV infection.

One of the earliest symptoms of OPC is loss of taste sensation, followed by a burning sensation, pain and later difficulty in eating and swallowing liquids (Greenspan and

Greenspan, 1992; Glick et al., 1994). Most persons with OPC present with pseudomembranous candidiasis or thrush, which are white plaques on the buccal mucosa, gums, or tongue; and less commonly with acute atrophic candidiasis, which leads to reddish mucosa; or chronic hyperplastic candidiasis involving the tongue; or angular cheilitis, which is inflammation and cracking at the corners of the mouth (Greenspan and Greenspan, 1992; Glick et al., 1994). Oral and oesophageal candidiasis are not life threatening, but can be severely debilitating. Many patients are asymptomatic (Sangeorzan et al., 1994; Maenza et al., 1997).

Severe OPC can interfere with the administration of medications and adequate nutritional intake, and may spread to the oesophagus (Tavitian et al., 1986). OPC is recurrent and becomes progressively severe as the immune system deteriorates (Greenspan and Greenspan, 1987; Glick et al., 1994; Greenspan and Greenspan, 1993; Arendorf et al., 1997; Arendorf et al., 1998). The infection is a useful marker for immune deterioration, and presenting months or years before more severe opportunistic disease, may be a signal indicating the presence or progression of HIV disease (Klein et al., 1984; Katz et al., 1992).

It is estimated that more than 5.7 million South Africans are currently infected with HIV (UNAIDS/WHO report, 2008), and that 80 to 95% of patients infected with HIV patients will experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994). The diagnosis of oral candidiasis is made on clinical appearance (Greenspan and Greenspan, 1992; Glick et al., 1994).

2.6 Laboratory diagnosis

2.6.1 Specimen collection

Samples are obtained by rubbing a sterile cotton-tipped swab over the lesional tissues. The swabs should be transported to the laboratory as quickly as possible to prevent drying up. *C. albicans* can survive at least 24hrs on a moist swab without loss of viability (Odds, 1982). For longer than 24hrs, Amies transport medium may be used to prevent drying up (Spijkervel et al., 1989).

2.6.2 Culture media

The most useful and popular medium for primary isolation of *C. albicans* is peptone-glucose or peptone-maltose agar described in 1896 by Sabouraud, and hence known as Sabouraud's agar. The medium has a pH of less than 6 and therefore suppresses many commensal oral bacteria. Bacterial growth can be further suppressed by addition of antibiotics such as chloramphenicol, penicillin, streptomycin and ciprofloxacin (Barnet et al., 1983). After inoculation, the media is incubated aerobically at 30-37 ^{o}C for 2-3 days. In this medium, *C. albicans* appears as cream-colored, smooth or rough, convex colonies (Samaranayake et al., 1983) .

The other medium used to differentiate Candida species from a single swab is known as CHROMagar. It is a selective and differential medium that allows selective isolation of yeasts and simultaneously identifies (by color reactions and colony morphology) colonies of *C. albicans*, *C. tropicalis*, and *C. krusei* with a high degree of accuracy (Odds et al., 1994). It facilitates the detection and identification of yeasts from mixed cultures and can provide results 24 to 48 h sooner than standard isolation and identification procedures. The medium contains chloramphenicol to suppress bacterial growth, and a chromogenic mixture to differentiate different species by colour. The swab is inoculated on the medium, and the medium incubated at 30-35 ^{o}C for 2 days. In this medium, *C. albicans* forms green colonies, *C. krusei* forms pale pink, rough, spreading colonies, *C. glabrata* forms lilac colonies, *C. tropicalis* blue and *C. parapsilopsis* off-white colonies (Odds et al., 1994).

Lee's medium is used to show different colony morphologies of *C. albicans* during phenotypic switching. The swabs are inoculated on Lee's medium and incubated at 35^{o}C for 48hrs, removed from the incubator and sealed with parafilm and incubated at room temperature in the dark for a further 10 days. To distinguish between different colony morphologies, the medium is supplemented with a low zinc concentration, a high amino acid concentration and a pink colorant, phloxin B (Lee et al., 1975).

2.6.3 Germ tube test

This is a rapid screening test to differentiate *C. albicans* from other Candida species. The yeast is lightly inoculated into approximately 1ml of sterile serum (rabbit or bovine) and incubated for 2-3hrs at 37 ^{o}C. After incubation, the suspension is placed on a glass slide, mounted with a coverslip and examined under the microscope for the presence of germ

tubes. Germ tubes appear as filamentous, cylindrical outgrowths from the yeast cell with no constriction present at base (Silverman et al., 1990).

Although several authors have recommended media other than serum for the germ-tube test, serum has been shown to be superior and the medium of choice.Two known inducers of hypha formation, *N*-acetylglucosamine (GlcNAc) and proline may contribute to the serum effect since they are generated by degradation of serum (glyco-) proteins (Feng et al., 1999).

2.7 Treatment: Antifungal drugs

Two groups of antifungal agents are used for treatment and/or prophylaxis of *C. albicans* infections. The first group, the azoles, include fluconazole (Diflucan), ketoconazole (Nizoral), itraconazole (Sporanox), clotrimazole (Canasten) and miconazole (Daktarin Oral Gel). The second group, the polyenes, include amphotericin B (AmB) and Nystatin.

2.7.1 Mechanisms of action of antifungal drugs

The antifungal drugs used are directed against ergosterol, the major sterol in the fungal plasma membrane, which is similar to cholesterol in mammalian cells. The fungal membrane ergosterol contributes to a number of cellular functions. It is important for the fluidity and integrity of the membrane and for the proper function of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division (Vanden Bossche et al., 1987; Joseph-Horne and Hollomon, 1997).

2.7.1.1 Polyenes

The polyenes antifungal agents include AmB and nystatin, which are amphipathic, having both hydrophobic and hydrophilic sides. The drugs are thought to intercalate into membranes, forming a channel through which potassium and magnesium ions leak, and thereby destroying the proton balance within the membrane (Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b).

AmB was isolated from *Streptomyces nodosus*, and acts by binding to ergosterol, and as it binds to ergosterol, it displaces the sterol from its normal phospholipid interaction, forming a sterol/AmB complex (Vanden Bossche et al., 1987). Several investigators have found that the aggregation of sterol/AmB complexes leads to formation of membrane pores (Bolard, 1986; Vanden Bossche et al., 1987; Brajtburg et al., 1990; Bolard et al., 1991; Warnock, 1991; Balakrishnan and Easwaran, 1993). The pores consist of eight alternating AmB molecules and eight sterol molecules (Kleinberg and Finkelstein, 1984).

The inside of the pore is hydrophilic due to the hydroxyl groups of AmB, while the outside of the pore is hydrophobic due to hydrocarbon portions of the AmB molecules and sterol molecules. The size of the pore has been estimated to be 4 Å in diameter by molecular modeling and permeability studies, and has higher affinity for cations rather than anions (Bolard et al., 1991; Khutorsky, 1992; Langlet et al., 1994). Some studies suggest that the formation of membrane pores leads to enhanced membrane permeabilities (Bolard et al., 1991; Khutorsky, 1992; Langlet et al., 1994) and ultimately to cell lysis and death (Vanden Bossche et al., 1987; Brajtburg et al., 1990).

The specificity of AmB for ergosterol-containing membranes may be associated with phospholipid fatty acids and the ratio of sterol to phospholipids (Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b). It has also been suggested that amphotericin B causes oxidative damage to the fungal plasma membrane (Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b; Vanden Bossche et al., 1987). There is evidence to suggest that amphotericin B has an antioxidant effect *in vivo*, protecting fungal cells against oxidative attack from the host (Osaka et al., 1997).

2.7.1.2 Azoles

Azoles are also known as ergosterol biosynthesis inhibitors, and act by interacting with enzymes involved in the synthesis of ergosterol from squalene. The azoles consist of

imidazoles (ketoconazole and miconazole) and triazoles (fluconazole, itraconazole), and are directed against lanosterol demethylase in the ergosterol pathway. This enzyme is a cytochrome P-450 enzyme containing a heme portion in its active site (Hitchcock, 1991; Vanden Bossche, 1991). Azoles bind to the heme iron through a nitrogen, inhibiting the enzymatic reaction (Joseph-Horne and Hollomon, 1997). A second nitrogen interacts directly with the apoprotein of lanosterol demethylase. It is thought that the affinity of different azoles for the enzyme is determined by the position of this second nitrogen (Hitchcock, 1991; Vanden Bossche, 1991). At high concentrations, the azoles may also interact directly with lipids in the membranes (Hitchcock et al., 1987; Joseph-Horne and Hollomon, 1997).

2.7.2 Clinical uses of antifungal drugs

2.7.2.1 Polyenes

AmB is considered to be the most effective of the systemically administered antifungal agents. It is fungicidal, and has *in vitro* and *in vivo* activity against Candida species. The use of this drug has been limited by its toxicity, and that it is frequently associated with renal dysfunction, and it is administered intravenously (Bennett, 1996).

Topical treatment is the route of choice for treating OPC. Since the drugs work topically, the target is to allow adequate contact time between the drug and the mucous surface, thus necessitating 5x daily doses. Treatment should be continued for at least 7-14 days. For treatment of OPC in HIV/AIDS patients in South Africa, nystatin is administered as 1ml of switch and spit solution 5x daily for 14 days. AmB is administered as lozenges 5x daily for 14 days (Rachanis, 2001). AmB has been used as a “gold standard” for the treatment of life-threatening, systemic fungal infections. For severe, persistent, resistant infections, 100mg of AmB is administered intravenously (Ellis, 2002).

2.7.2.2 Azoles

The azole antifungal agents are safe and easy to administer, and have therefore become an important component in treatment of fungal infections. The use of the first azoles was limited by their high toxicities and their levels that were inconsistent in the blood, until ketoconazole was developed and used to treat chronic mucocutaneous candidiasis in the early 1980s (Horsburgh and Kirkpatrick, 1983). The easy administration and extended activity of azoles against non-*Candida* fungal infections such as cryptococcosis, histoplasmosis, and coccidioidomycosis have made them a good choice as systemic drugs for the therapy and prophylaxis of AIDS-associated opportunistic infections (Como and Dismukes, 1994; Gallant et al., 1994).

Fluconazole has been found to be active against *C. albicans in vitro* and *in vivo* (Rogers and Galgiani, 1986), and has become the drug of choice for the treatment of OPC. It is the mostly used azole owing to its high oral bioavailability, good safety profile and wide tissue distribution (Lortholary and Dupont, 1997), although ketoconazole, which is less expensive, was still frequently used for antifungal treatment in other countries (De Wit et al., 1989). For treatment of OPC in HIV/AIDS patients in South Africa, miconazole gel (peasize) is applied orally 5 times daily. For severe/persistent infections, 100mg of fluconazole and 100mg of itraconazole are applied per day. Clotrimazole is applied as trouches (Rachanis, 2001).

2.7.3 Antifungal drug resistance

Dramatic increases in the incidence of fungal infections have been reported, which may be due to alterations in immune status associated with the AIDS epidemic (Nielsen et al., 1994). Antifungal drug resistance is a major problem in HIV/AIDS patients with OPC (Vanden Bossche et al., 1994b; Graybill, 1988). In one study, 33% of late-stage AIDS patients had drug-resistant strains of *C. albicans* in their oral cavities (Law et al., 1994).

2.7.3.1 Polyene resistance

Reports of amphotericin B resistance are limited, but severely immunocompromised patients, especially patients with cancer, are at the highest risk. Amphotericin B resistance in Candida species can develop in patients previously exposed to azole antifungal agents. This is thought to be due to an alteration of cellular membrane components (White et al., 1998). The incidence of resistance to amphotericin B has been

reported to be rare, but isolates resistant to this drug have been reported both *in vivo* (Dick et al., 1980; Powderly et al., 1988) and *in vitro* (Rex et al., 1995; Kelly et al., 1997; Barker et al., 2004).

Polyene-resistant isolates resistant to nystatin or amphotericin B have been reported and characterized.s, *Aspergillus nodulan*s, and *A. fennelliae* have been constructed *in vitro* (Borgers et al., 1986; Broughton et al., 1991; Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b). These strains were either exposed to chemical mutagenesis or serially passaged in media containing increasing amounts of polyene. Most polyene-resistant clinical isolates have a reduced ergosterol content in their membranes (Broughton et al., 1991). Based on an analysis of sterol composition, several clinical isolates of *C. albicans* may be defective in *ERG2* or *ERG3* genes (Kelly et al., 1994; Heald et al., 1996; Haynes et al., 1996; Nolte et al., 1997).

Resistant isolates that have been recovered during the treatment of patients with candida infections belonged to species other than *C. albicans*, in particular *C. lusitaniae* and *C. tropicalis*. However, amphotericin B resistance may be a greater problem in neutropenic patients than has been supposed. In one study, a higher mortality in neutropenic patients treated with amphotericin B was observed when the MIC for the infecting *C. albicans* was >0.8 µg/ml (Powderly et al., 1988).

In a recent study (Blignaut et al., 2002a), a natural resistance to amphotericin B was demonstrated in 8.4% of South African isolates. This prevalence was reported to be higher than the 0-0.4% reported by other overseas workers (Waltimo et al., 2000; Pizzo et al., 2002; Dar-Odeh and Shehabi, 2003; Hajjeh et al., 2004). Cross resistance between azoles and amphotericin B has also been reported in several studies (Kelly et al., 1996; Kelly et al., 1997; Nolte et al., 1997; Barker et al., 2004). Previous exposure to one polyene has also been hypothesised to lead to resistance to other polyenes (Conly et al., 1992)

Several strains of fluconazole and amphotericin B resistant *C. albicans* have been found in HIV-infected patients who have received prolonged courses of antifungal prophylaxis with azoles (Kelly et al., 1996).

2.7.3.2 Azole resistance

Azoles have little or no toxicity, but they are fungistatic. Therefore in AIDS patients, azoles must be administered for long periods of time. This extended use, combined with the increased use of azoles in recent years, has led to the increased isolation of azole-resistant strains of *C. albicans* (Ng and Denning, 1993; White, 1997a; Rex et al., 1998). A cross resistance between azoles has also been demonstrated (Barchiesi et al., 1994). Several investigators have noted that resistance can develop in *C. albicans* after only short exposures to fluconazole, both *in vitro* (Calvet et al., 1997) and *in vivo* (Marr et al., 1997; Nolte et al., 1997).

The prevalence of azole resistance has been estimated to be 21 to 32% in symptomatic patients and up to 14% in asymptomatic patients (Maenza et al., 1997). One study estimated that up to a third of all AIDS patients retain an azole resistant *C. albicans* strain orally (Law et al., 1994). It has also been established that antifungal drug-resistant Candida strains arise more often in HIV positive patients than in HIV negative patients (Johnson et al., 1995). Azole resistance has also been found in patients not infected with HIV and, in some cases, in patients not previously exposed to antifungal agents (White et al., 1998). Some studies have documented that infections due to azole resistant non-*albicans Candida* species have also increased (Price et al., 1994; Nguyen et al., 1996).

In a case-control study, advanced immunosuppression and previous exposure to oral azoles were found to be risk factors for the development of resistance (Maenza et al., 1996). In that study, patients with resistant infections had lower mean CD4 counts of 11 versus 71/mm^3, and a longer duration of antifungal therapy than the matched controls. These results are similar to those found by several other investigators (Sanguineti et al., 1993; Baily et al., 1994).

Azole resistance has been reported to occur in association with high doses of fluconazole (Millon et al., 1994; Vuffray et al., 1994), and with recent exposure to the drug (Revankar et al., 1996). Patients treated with intermittent therapy were more likely to develop

resistance than those treated continuously (Heald et al., 1996). Fluconazole resistance has also been found in patients treated with daily prophylaxis, weekly prophylaxis, and even episodic single doses (Sanguineti et al., 1993; Troillet et al., 1993; Baily et al., 1994; Laguna et al., 1997).

In one study, a patient with AIDS developed secondary resistance when he had several episodes of thrush caused by a single strain of *C. albicans* that acquired increasing resistance over time (Pfaller et al., 1994; Redding et al., 1994). Several investigators suggested that resistant strains develop with azole treatment (Barchiesi et al., 1995b), but nosocomial transmission (Bart-Delabesse et al., 1995) and transmission of resistant strains between partners (Barchiesi et al., 1995a) have also been reported. Infections due to azole-resistant Candida have also emerged in patients not infected with HIV. In another study, seventeen fluconazole-resistant isolates were found in a collection of 139 strains of *C. albicans* isolated from patients not infected with HIV at one institution (Goff et al., 1995).

2.7.4 Molecular mechanisms of antifungal drug resistance

2.7.4.1 Azoles

2.7.4.1a Molecular changes of the ERG11 Gene

The target enzyme of the azole drugs is cytochrome P-450 lanosterol 14-demethylase (*ERG11*p). The gene encoding this protein is *ERG11* in all fungal species, although it has previously been referred to as *ERG16* and *CYP51A1* in *C. albicans*. Several genetic changes associated with the *ERG11* gene of *C. albicans* have been identified. These include point mutations in the coding region, over-expression of the gene, gene amplification, and gene conversion or mitotic recombination (White et al., 1998).

One of the major molecular mechanisms responsible for development of azole resistance in strains of *C. albicans* involves changing the target enzyme, cytochrome P-450

lanosterol 14α-demethylase. This is achieved by over-expression of *ERG11*, the gene that encodes this enzyme (Sanglard et al., 1995; Albertson et al., 1996; White, 1997a; Franz et al., 1998; Henry et al., 2000) or by point mutations in the *ERG11* gene (Vanden Bossche et al., 1990; Vanden Bossche et al., 1994a; White, 1997b; Sanglard et al., 1998; Franz et al., 1998). The point mutations in the *ERG11* gene lead to amino acid substitutions, causing a decreased affinity for azoles (Franz et al., 1998).

In one study, a point mutation in *ERG11* was identified when an azole-resistant clinical isolate was compared with a susceptible isolate from a single strain of *C. albicans*. This point mutation (R467K) resulted in the replacement of arginine with lysine at amino acid 467 of the *ERG11* gene (White, 1997b). In another study, the point mutation T315A (the replacement of threonine [T] with alanine [A] at position 315) was constructed in the *C. albicans ERG11* gene (Lamb et al., 1997b). Several other point mutations that result in conformational changes that prevent effective binding between the azoles and cytochrome P-450 lanosterol 14α demethylase have been documented (Sanglard et al., 1998; Marichal et al., 1999; Perea et al., 2001).

ERG11 genes from a variety of fungal sources have been expressed and manipulated in *Saccharomyces cerevisiae* (Shyadehi et al., 1996; Lamb et al., 1997a; Lamb et al., 1997b). In one study, expression in *S. cerevisiae* was used to identify and characterize five *ERG11* point mutations from matched sets of susceptible and resistant isolates of *C. albicans* (Sanglard et al., 1998). Mutations identified in the *ERG11* gene of azole-resistant *C. albicans* isolates were found to be clustered in three diffuse hot-spot regions, including amino acid regions 105 to 165, 266 to 287, and 405 to 488 (Marichal et al., 1999). Five mutations located in these three hot spots, were observed in the three complete sequences, and these mutations included D116E, K128T, K143R, E266D and V437I (White et al., 2002).

Globally, the following amino acid substitutions were found to be associated with azole resistance; F126L, G129A, Y132H, K143E, K143R, F145L, A149V, T229A, S279F, K287R, G307S, S405F, G448E, G448R, F449L, V452A, G464S, G465S, R467K, I471T, G450E, D446N, F449S (Loffler et al., 1997; White, 1997b; Sanglard et al., 1998; Franz et al., 1998; Favre et al., 1999; Kelly et al., 1999a; Kelly et al., 1999b; Asai et al., 1999; Kakeya et al., 2000; Lamb et al., 2000; Perea et al., 2001; White et al., 2002).

Over-expression of *ERG11* has been described in several different clinical isolates (Sanglard et al., 1995; Albertson et al., 1996; White, 1997a; Franz et al., 1998; Henry et al., 2000). The over-expression of *ERG11* gene results in increased production of lanosterol demethylase, making it difficult for the azoles to inhibit it (Henry et al., 2000).

Previous work on the analysis of resistance mechanisms has routinely used matched sets of susceptible and resistant clinical isolates of the same strain (Sanglard et al., 1995; Lopez-Ribot et al., 1998; Perea et al., 2001). The use of matched sets is necessary, as *C. albicans* is mostly clonal in nature (Pujol et al., 1993), and isolates might differ considerably in their levels of expression of different genes, hence the use of matched sets in our study.

Franz et al. (1998) reported that even in the absence of fluconazole, some fluconazole-resistant isolates can express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug. In another study, mRNA levels of *ERG11* have been shown to be increased in several fluconazole-resistant isolates of *C. albicans* (Sanglard et al., 1995).

Over-expression of *ERG11* has been found in many other fluconazole-resistant *C. albicans* isolates compared with matched susceptible isolates (White, 1997a; White et al., 1997; Cowen et al., 2000; Perea et al., 2001; Morschhauser, 2002; Lee et al., 2004; Frade et al., 2004). In a study by Henry et al. (2000), azoles were described to show increased expression of *ERG11* within a few hours of exposure, contributing significantly to the survival of azole-treated cells. A recent study showed that up-regulation of *ERG11* gene was the predominant mechanism in experimentally induced fluconazole resistance (Ribeiro and Paula, 2007).

However, other studies have shown that multiple genes are involved in conferring fluconazole resistance. In one of those studies, different genes involved in drug resistance in *C. albicans* were concomitantly over-expressed, indicating that multiple mechanisms are operating to confer fluconazole resistance in these isolates (Albertson et al., 1996; Goldman et al., 2004).

Other studies showed that changes in expression levels of *ERG11* gene were not correlated with fluconazole resistance. One of those studies used quantitative real-time PCR with molecular beacons to determine gene expression in fluconazole-resistant isolates. Changes in expression levels for *ERG11* were not statistically correlated with fluconazole resistance (Park and Perlin, 2005). In other studies, which did not use matched sets, resistance was not found to correlate with over-expression of *ERG11* gene (White et al., 2002; Martinez et al., 2002).

2.7.4.1b Drug efflux Pumps

C. albicans contains two types of efflux pumps that are known to contribute to drug resistance, namely, ATP binding cassette (ABC) transporters (ABCT) and major facilitators (MF) (Marger and Saier, 1993; Michaelis and Berkower, 1995).

2.7.4.1b(i) ABC transporters

The ABCT are associated with the active efflux of molecules that are toxic, hydrophobic or lipophilic, such as most azole drugs. They are composed of four protein domains: two membrane-spanning domains (MSD), and two nucleotide binding domains (NBD). The NBD of ABCT binds ATP through an ABC. The ATP that is bound to the ABC is used as a source of energy for the ABCT, although the mechanism by which the ATP energy causes transport of the substrate molecule is unknown (Michaelis and Berkower, 1995).

The genes coding for several ABC transporters in *C. albicans* include *CDR1* and *CDR2 (Candida drug resistance*), which were the first two members of this family to be identified in *C. albicans*, and both have been described as playing a role in fluconazole resistance (Sanglard et al., 1995; Sanglard et al., 1996; Sanglard et al., 1997; Lopez-Ribot et al., 1998). These genes are highly homologous, and encode ATP-binding cassette (ABC) transporters, using adenosine triphosphate (ATP) as the energy source (Prasad et

al., 1995; Sanglard et al., 1997). The *CDR1* (*Candida* drug resistance) gene is homologous to the *S. cerevisiae* pleiotropic drug resistance gene *PDR5*, and was cloned by complementation of the cycloheximide hypersensitivity of an *S. cerevisiae* pdr5 mutant (Prasad et al., 1995).

When *CDR1* mRNA levels were monitored in azole-resistant clinical isolates of *C. albicans*, matched sets of susceptible and resistant isolates showed an increase in *CDR1* expression (Sanglard et al., 1995; Albertson et al., 1996). In an unrelated study, five azole-resistant strains of *C. albicans* showed an increased expression of *CDR1* compared to azole- susceptible controls (White, 1997a). In another study, *CDR1* over-expression was correlated with increasing fluconazole MICs in a series of clinical isolates (Sanglard et al., 1996). Genetic deletion of both alleles of *CDR1* in *C. albicans* resulted in a strain that is hyper susceptible to azole drugs (Niimi et al., 1997). The gene disruption did not affect susceptibility to amphotericin B or flucytosine. In one study, over-expression of *CDR1* in a *CDR1* null mutant resulted in an increased resistance to fluconazole and itraconazole (Sanglard et al., 1997).

CDR2 is closely related to *CDR1* and confers resistance to azoles (Sanglard et al., 1997). Disruption of *CDR2* gene in isolates did not change their susceptibility to azoles, but isolates with *CDR1* and *CDR2* disruption were more susceptible than the strains with disruption in only one gene. *CDR2* over-expression was observed in the resistant isolates from two matched sets of susceptible and resistant clinical isolates. *CDR1* over-expression was also observed in the resistant isolates in these pairs, but at a lower level (Alarco et al., 1997). In *C. albicans* strains containing a *CDR1* disruption, expression of *CDR2* was also observed (Fling et al., 1991).

CDR1 and *CDR2* have been described as playing a role in fluconazole resistance (Sanglard et al., 1995; Sanglard et al., 1996; Sanglard et al., 1997; Lopez-Ribot et al., 1998). ABC transporters also lead to resistance to other azole drugs, and therefore over-

expression of *CDR* genes results in cross-resistance to other azoles (Sanglard et al., 1995; Sanglard et al., 1996; Lopez-Ribot et al., 1998). Over-expression of these efflux pumps prevents the accumulation of sufficient amounts of the azole antifungal agents in the fungal cell. Over-expression of the *CDR* genes leads to resistance to several azoles, whereas over-expression of *MDR1* is specific to fluconazole (White et al., 1998).

2.7.4.1b(ii) Major facilitators

The MF have not been studied as extensively as the ABCT, but are also associated with hydrophobic molecules such as tetracycline (Marger and Saier, 1993). The MF do not contain NBD. They are composed primarily of 12 to 14 transmembrane segments. The MF use the proton motive force of the membrane as a source of energy. The MF work by antiport meaning that protons are pumped into the cell and substrate molecules are pumped out of the cell (Marger and Saier, 1993).

MF are encoded by *MDR1* (multi drug resistance) gene, which is also referred to as *Ben*-R (Fling et al., 1991) and *FLU1* (fluconazole resistance) gene, which has recently been found to be homologous to *MDR1* in *C. albicans* (Calabrese et al., 2000). The *MDR1* gene was originally cloned for its ability to confer both benomyl and methotrexate resistance when transformed into *S. cerevisiae* (Ben-Yaacov et al., 1994). This transformant also demonstrated resistance to cycloheximide (Goldway et al., 1995). In one study, both alleles of the *MDR1* gene were deleted by genetic manipulation, and the cells remained viable and resistant to benomyl (Sanglard et al., 1995).

The *C. albicans MDR1* gene was shown to be overexpressed in the fluconazole-resistant isolate in one of five matched sets of clinical isolates (Albertson et al., 1996) and in one of five azole-resistant isolates when compared to azole- susceptible isolates including one matched set (White, 1997a). In a third study of 17 sequential isolates, over-expression of *MDR1* occurred early in this series, correlating with a major increase in resistance (Sanglard et al., 1995). The over-expression of *MDR1* then continued throughout the series. These experiments suggest a correlation between *MDR1* over-expression and azole resistance in clinical isolates.

Genetic manipulations have also demonstrated that major facilitators can be involved in azole resistance. When the *MDR1* gene was overexpressed in *S. cerevisia*e, the cells became resistant to fluconazole but not to ketoconazole or itraconazole (Sanglard et al., 1996). When both allelic copies of the *MDR1* gene were deleted in a laboratory strain of *C. albicans*, the cells were unaltered in their susceptibility to azoles (Sanglard et al., 1996)**.** If the *MDR1* deletion was combined with the *CDR1* deletion, the cells became more susceptible to cycloheximide than did cells with either single gene deletion (Hitchcock et al., 1987).

The *MDR1* gene encodes a major facilitator that is proton motive force-dependent, and leads to azole drug resistance (Fling et al., 1991; Sanglard et al., 1995; Albertson et al., 1996; White, 1997a; Walsh et al., 1997; Lopez-Ribot et al., 1998), and its over-expression leads exclusively to fluconazole resistance (Sanglard et al., 1995; Sanglard et al., 1996; Lopez-Ribot et al., 1998).

2.7.4.2 Polyenes

Polyene resistance has been reported to be rare, but polyene-resistant isolates resistant to nystatin or amphotericin B have been reported and characterized, and resistant strains of *C. albicans*, *C. neo-formans*, *Aspergillus nidulans*, and *A. fennelliae* have been constructed *in vitro* (Borgers et al., 1986; Broughton et al., 1991; Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b). These strains were either exposed to chemical mutagenesis or serially passaged in media containing increasing amounts of polyene. Most polyene-resistant clinical isolates have a reduced ergosterol content in their membranes (Broughton et al., 1991). Based on an analysis of sterol composition, several clinical isolates of *C. albicans* may be defective in *ERG2* or *ERG3* (Kelly et al., 1994; Heald et al., 1996; Haynes et al., 1996; Nolte et al., 1997).

One of these strains with an apparent *ERG2* defect was unable to form amphotericin B-generated pores in the membrane (Broughton et al., 1991). There is evidence to suggest that alterations in the membrane structure or in the sterol-to-phospholipid ratio in the

membrane may be associated with resistance (Hitchcock et al., 1993). Resistance to polyenes is caused by a decrease in either the amount of ergosterol in the fungal cell membrane, or a mutation in the ergosterol biosynthesis pathway, leading to production of ergosterol-like compounds with less binding affinity for AmB (Ellis, 2002).

Another mechanism by which antifungal resistance can occur in polyenes is through changes in other components of the ergosterol biosynthesis pathway resulting in the accumulation of sterol intermediates (Lupetti et al., 2002). In clinical isolates of *C. albicans*, combined resistance to both amphotericin B and azoles has been associated with accumulation of ergosta-7,22-dienol. This leads to reduced activity of C-5 desaturase, encoded by *ERG3* gene (Kelly et al., 1996; Kelly et al., 1997; Nolte et al., 1997). Such changes in *ERG3* are also thought to reduce the conversion of episterol to potentially toxic metabolites that accumulate in the cell membrane during azole exposure (Lupetti et al., 2002).

2.8 Molecular techniques

2.8.1 DNA sequencing

DNA sequencing is a biochemical method for determining the order of the nucleotide bases, adenine(A), guanine(G), cytosine(C), and thymine(T), in a DNA oligonucleotide. The sequence of DNA contains the genetic information for the development of all living organisms. Determining the DNA sequence is therefore useful in basic research studying fundamental biological processes, as well as in applied fields such as diagnostic or forensic research (Sambrook and Russel, 2001).

In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases (Maxam and Gilbert, 1977). This method rapidly became popular, since purified DNA could be used directly. However, this sequencing method is no longer used due to its technical complexity and extensive use of hazardous chemicals. In addition, chemicals used in this method could not be easily used in a standard molecular biology kit (Maxam and Gilbert, 1977).

The chain-termination sequencing method was developed by Frederick Sanger and coworkers in 1975 (Sanger et al., 1977). This method requires a single-stranded DNA

template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. In the labelled-primer chain termination method, the DNA sample is divided into four separate sequencing reactions, containing the four standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP). These dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Incorporation of a dideoxynucleotide into the elongating DNA strand therefore terminates DNA strand extension, resulting in various DNA fragments of varying lengths (Sanger et al., 1977).

The newly synthesized and labeled DNA fragments are heat denatured, and separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel. Each of the four DNA synthesis reactions is run in one of four individual lanes (lanes A, T, G, C); the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. A dark band in a lane indicates a DNA fragment that is the result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The terminal nucleotide base can be identified according to which dideoxynucleotide was added in the reaction giving that band. The relative positions of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence (Sanger et al., 1977).

The dye-terminator sequencing is a method in which sequencing can be performed in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with a different fluorescent dye, each fluorescing at a different wavelength. The DNA is then separated on a gel, but they all run on the same lane as opposed to four different

ones. Since the four dyes fluoresce at different wavelengths, a laser then reads the gel to determine the identity of each band according to the wavelength at which it fluoresces (Russell, 2002).

The results are then depicted in the form of a chromatogram, which is a diagram of colored peaks that corresponds to the nucleotide in that location in the sequence. This method is now mainly used for the vast majority of sequencing projects because of its greater speed, easy performance and lower cost. It is now the mainstay in automated sequencing with computer-controlled sequence analyzers (Russell, 2002).

2.8.2 Real-time Polymerase Chain Reaction (Real-time PCR)

Real-time PCR is used to measure the quantity of a PCR product in real-time. It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. It is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample (Higuchi et al., 1993).

This method uses fluorescent dyes, such as Sybr Green, or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. The method uses PCR together with fluorescence detection of amplification products (Higuchi et al., 1993; Wittwer et al., 1997). The amplification is monitored through each cycle, which leads to more accurate quantification, and eliminates the need for post-PCR sample processing to visualize and analyze products. Quantitative real-time RT-PCR has been successfully used for the measurement of gene expression in a variety of fields, including microbiology (Kreuzer et al., 1999; Okeke et al., 2001; Smith et al., 2000).

Currently, four different chemistries, TaqMan® (Applied Biosystems, Foster City, CA, USA), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes), are available for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA (Higuchi et al., 1993; Wittwer et al., 1997).

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. It binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed (Higuchi et al., 1993; Wittwer et al., 1997).

The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. Therefore, detection by SYBR Green requires extensive optimization, and follow up assays are needed to validate results. To check that the correct fragments are being amplified, a melting curve has to be done (Higuchi et al., 1993; Wittwer et al., 1997).

The real-time machine used for Real-time PCR monitors DNA synthesis during the PCR, and also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a DNA double helix depends on its base composition. All PCR products for a particular primer pair should have the same melting temperature, unless there is contamination, mispriming, primer-dimer artifacts, or some other problem. Since SYBR green does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature, and this is done by doing a melt curve (Higuchi et al., 1993; Wittwer et al., 1997).

After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) [-d (RFU)/dT] on the Y-axis versus the temperature on the X-axis, and this will peak at the melting temperature. In this curve, a primer-dimer artefact would give a peak with a lower melting temperature because it is a

short DNA. If the peaks of the melt curve are not similar, this might suggest contamination, mispriming or primer-dimer artefact (Higuchi et al., 1993; Wittwer et al., 1997). Several instruments are used in performing Real-time PCR, including Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany) and Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia).

2.9 Candida albicans clades (Groups)

DNA fingerprinting methods, using the complex Ca3 probe, were used to reveal 5 distinct clades among clinical isolates of *Candida albicans.* Using these methods, groups (clades) I, II, III were distinguished from studies done in the United States. Group II was found to be absent in the Southwest USA and South America (Pujol et al., 1997; Lott et al., 1999). In another study, a new clade among South African isolates that differed from the three US clades, which accounted for 53% of isolates in HIV positive South Africans was demonstrated, and was named clade SA (Blignaut et al., 2002b). A fifth clade has also been established among isolates from Europe, clade E or NG (Pujol et al., 2002; Soll and Pujol, 2003). The Ca3 fingerprinting patterns of *Candida albicans* clades are shown in Figure 2.1, as demonstrated by Blignaut et al. (2002b).

When phenotypic characteristics of these groups were studied, isolates from Clade SA were found to be mainly resistant to amphotericin B (Blignaut et al., 2005) and those from Clade I exclusively resistant to 5-flucytosine (Pujol et al., 2004). These results demonstrated that clades differ phenotypically, and showed that one strain of *C. albicans* does not necessarily represent all strains. This also supports the need for a worldwide analysis of population structure and clade-specific phenotypic characteristics of *C. albicans* isolates. It also demonstrates that in future, pathogenic characteristics must be analyzed in representatives from all five clades.

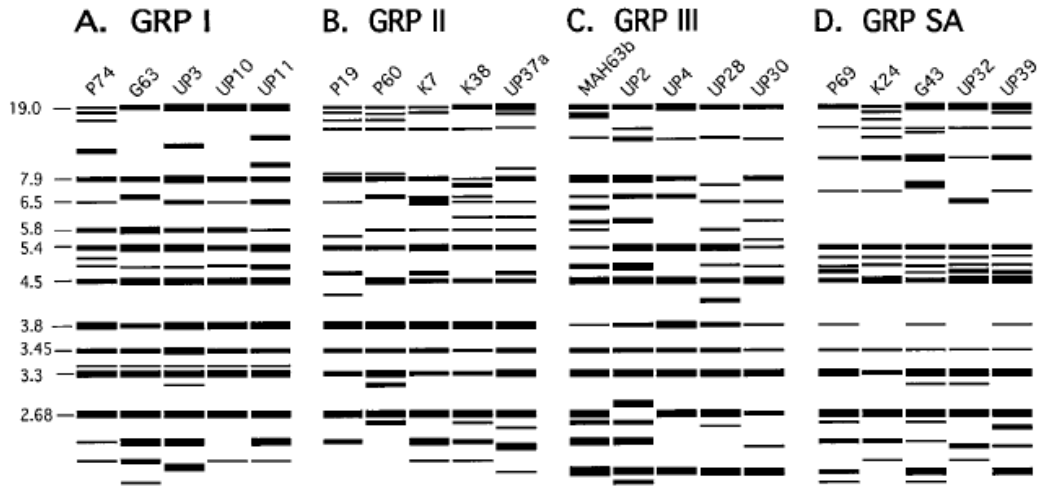

Figure 2.1: Ca3 fingerprinting patterns of *Candida albicans* clades (groups). Adapted from Blignaut et al. (2002b).

2.10 Phenotypic switching

2.10.1 Historical Review

C. albicans isolates showing varying colony morphology were reported in 1932. A variant rough colony morphology, the R-variant, was described in *Mycotorula albicans*, an early name for *C. albicans*. In 1968, it was demonstrated that various colonies occurred in a number of strains of *C. albicans* and that it affected the basic physiology of the cell. By streaking strains on malt agar, the colonial phenotype and the genesis of sectors were demonstrated. It was also found that morphological differences in streaks were the result of differences in the proportions of buds, hyphae, and pseudohyphae, as well as the result of variations in pseudohyphal phenotype. More than 300 strains of *C. albicans* were examined, and 15 different morphological forms were identified (Brown-Thomsen, 1968).

2.10.2 High frequency switching

In 1985, two reports of reversible high-frequency switching in *C. albicans* for phenotypic variability other than the bud-hypha transition were made (Pomes et al., 1985) demonstrated that, in strain 1001, ultraviolet (UV) irradiation gave rise to rough colonies. They found that a low dose of UV which killed 13% of the cell population increased the frequency of rough colonies. In another report, (Slutsky et al., 1985) reported that the common laboratory strain 3153A was capable of high frequency reversible switching.

They demonstrated several characteristics of switching in strain 3153A which have since been demonstrated for switching systems in other strains (Slutsky et al., 1987).

2.10.2.1 Switch phenotypes

When cells of the standard laboratory strain 3153A were plated on an amino acid-rich agar with low zinc concentrations (Slutsky et al., 1987), and then incubated at 25°C, the predominant colony phenotype observed was "smooth"(Slutsky et al., 1985). Various colonies appeared spontaneously at a high frequency and included several variant phenotypes, namely, "star", "ring", and "irregular wrinkle". When cells of strain 3153A were irradiated with low doses of UV light, which killed 8% of the population, variant colonies appeared at a combined frequency, and more switch phenotypes including "stipple", "hat", "fuzzy"and reverant smooth were observed. These various phenotypes are shown in 2.2 as indicated by Soll et al. (1992).

When cells from a single colony of any phenotype were plated, majority of the cells maintained their original phenotype, showing that the cells inherited the parent phenotype, but some showed other switch phenotypes, including the original smooth morphology. Different switch phenotypes observed included: a switch from ring to star, star to ring, star to irregular wrinkle, ring to irregular wrinkle, and ring to smooth. Sectoring was also observed (Slutsky et al., 1985).

Another switching system was demonstrated in *C. albicans* strain WO-1. This strain was isolated from a bone marrow transplant patient at the University of Iowa Hospitals and Clinics in Iowa City in 1985 (Slutsky et al., 1987). When the original cultures from the blood and lungs of this patient were replated on defined amino acid-rich nutrient agar and incubated at 25°C for 7 days, two distinct morphologies were observed; smooth white colony and a flat grey colony, which was originally labeled "opaque" because of the way in which it refracted light under the lighting conditions used in the original study. The

other new switch phenotypes observed in this study were “medusa” and “fried egg” (Slutsky et al., 1987).

The studies by (Pomes et al., 1985) and (Slutsky et al., 1985) demonstrated that UV stimulates switching and that switching is reversible at relatively high frequency. These studies demonstrated that there can be more than two phenotypes in a switching isolate, that switching can occur from one variant phenotype to another, and that one phenotype can revert back, and that there are different frequencies of switching, which in some cases are characteristic of particular colony phenotypes.

In many cases, the phenotype of a colony changed with prolonged incubation of more than 7 days or when incubated at another temperature. Media like yeast-peptone dextrose agar (Odds and Merson-Davies, 1989), used in many yeast studies, are rich in zinc and suppress expression of particular colony phenotypes, but they may not affect the switching mechanism (Soll et al., 1991).

In addition to UV and temperature, colony age also appears to affect switching. When white colonies are incubated for >10 days on defined nutrient agar containing phloxine B to visualize sectoring, there is a definite increase in the number of opaque sectors at a colony periphery (Soll et al., 1991). However, it has not been determined in this case whether the increase in sectors at the colony periphery is due to an increase in switching frequency or to the differential rate of growth of opaque cells at the colony periphery. Wrapping a dish with Parafilm was observed to stimulate sectoring at the white colony periphery (Anderson et al., 1990; Kolotila and Diamond, 1990).

In another study, the third phenotypic system was described; the unmyceliated-heavy myceliated system, which includes strains that switch between colonies with and without dense myceliation (Soll et al., 1987). Switching usually is discriminated by colony morphology and in some cases by the phenotype of cells in the budding phase (Anderson and Soll, 1987; Anderson et al., 1989; Vargas et al., 2000).

There is evidence to suggest that switching plays a significant role in *C. albicans* pathogenesis. In these studies, switching was demonstrated to regulate a number of phase-specific genes, some of which were suggested to be involved in pathogenesis (Soll,

1997; Soll, 2000). These genes included the secreted aspartyl proteinase genes *SAP1* and *SAP3* (Kolotila and Diamond, 1990; Morrow et al., 1992; Morrow et al., 1993; Hube et al., 1994; Morrow et al., 1994), the drug resistance gene *CDR3* (Balan et al., 1997), the white phase-specific gene *WH11* (Srikantha and Soll, 1993; Kvaal et al., 1997), the opaque phase-specific gene *OP4* (Morrow et al., 1993), the two-component histidine kinase regulator gene *CaNIK1* ((Srikantha et al., 1998) and the transcription factor gene *EFG1* (Sonneborn et al., 1999; Srikantha et al., 2000).

Several studies have demonstrated that switching can regulate a number of phenotypic characteristics involved in pathogenesis (Soll et al., 1992). These include antigenicity (Anderson et al., 1990), constraints on the budhypha transition (Anderson et al., 1993), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), adhesion (Vargas et al., 1994), secretion of aspartyl proteinase (Morrow et al., 1992), and virulence in a mouse systemic model and a mouse cutaneous model (Kvaal et al., 1999).

In addition, switching has been demonstrated at sites of infection. In a detailed study of a single recurrent vaginitis patient, it was demonstrated by DNA fingerprinting that the same strain was responsible for three sequential infections and that phenotypic switching occurred between episodes (Soll et al., 1989). The results of that study suggested that each switching phenotype of the infecting strain may exhibit a specific drug susceptibility profile (Soll et al., 1989). This conclusion was supported by studies of drug susceptibility for switch phenotypes in the 3153A system and the white-opaque transition (Soll et al., 1991).

High-frequency switching is another mechanism by which *C. albicans* achieves resistance to antifungal agents (Soll et al., 1992). In one study, switched *C. albicans* isolates from HIV positive individuals were found to be more resistant to a number of antifungal drugs including amphotericin B and fluconazole, than isolates from HIV-

negative controls (Vargas et al., 2000). Results from several studies have demonstrated that infecting isolates switch at significantly higher frequencies, on average, than commensal isolates (Gallagher et al., 1992; Hellstein et al., 1993), and that isolates causing deep mycoses switch more frequently than isolates causing superficial mycoses (Jones et al., 1994).

Switching may provide *C. albicans* with mechanisms for adapting to different environments, for evading host defense mechanisms and for adhering to different types of surfaces (Vargas et al., 1994). Phenotypic switching play a role in virulence of pathogenic fungi, facilitating invasion and escape from the host's defense mechanism (Soll, 2002). Switching usually is discriminated by colony morphology and in some cases by the phenotype of cells in the budding phase (Anderson and Soll, 1987; Anderson et al., 1989; Vargas et al., 2000).

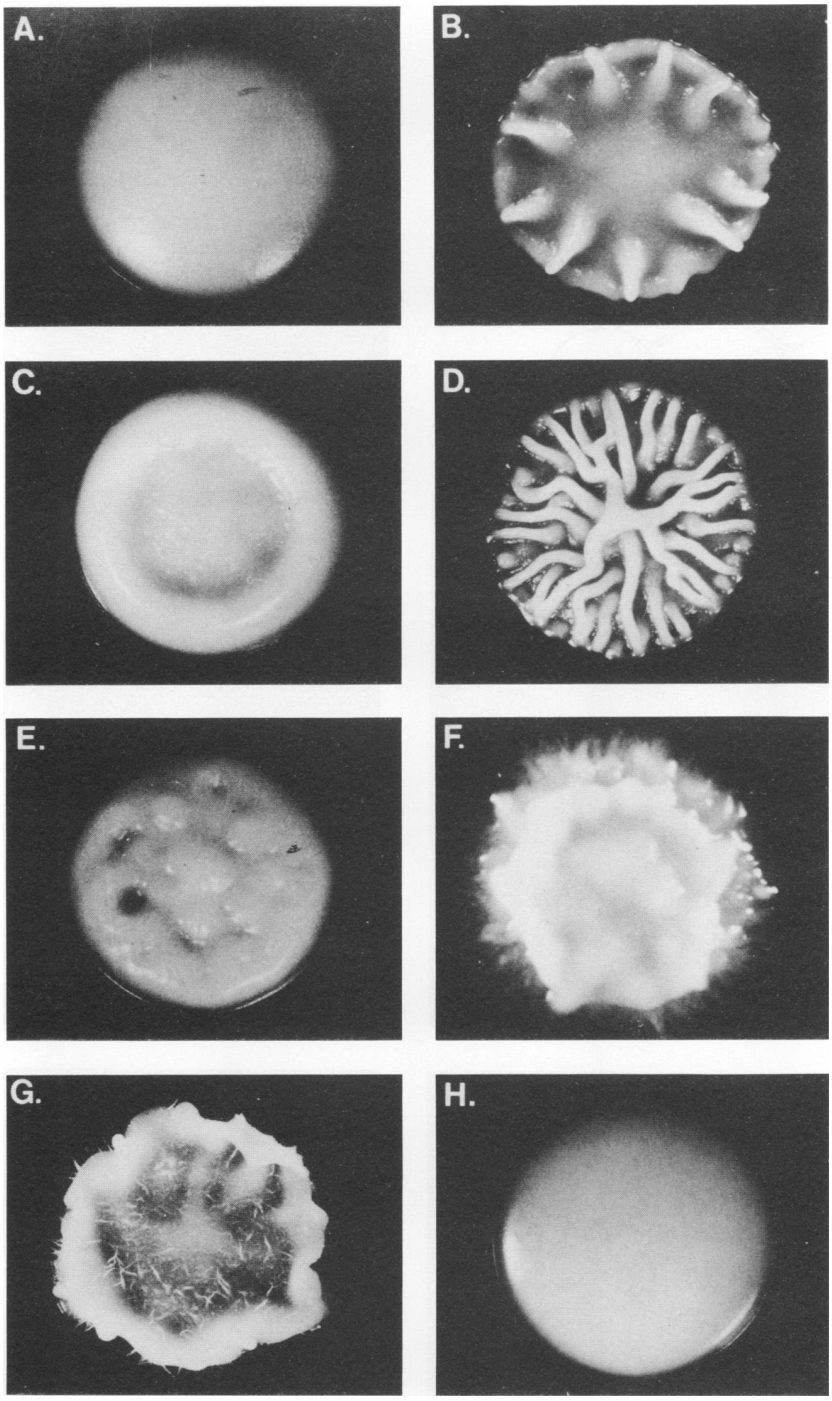

Figure 2.2: Various colony phenotypes in the switching of *C. albicans* strain 3153A. (A) Original smooth; (B) star; (C) ring; (D) irregular wrinkle; (E) stippled; (F) hat; (G) fuzzy; (H) revertant smooth (Slutsky et al., 1985). Picture adapted from Soll et al. (1992).

2.11 References

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CHAPTER THREE: INDUCTION OF ANTIFUNGAL RESISTANCE IN *CANDIDA ALBICANS* CLADES

3.1. Abstract

Background

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *Candida albicans* (*C. albicans*) infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Five genetic clades were described among *C. albicans* isolates using DNA fingerprinting methods (clades I, II, III, SA and NG). Although these clades have been described, little is known about their phenotypic characteristics. Apart from the resistance of Clade SA to amphotericin B and Clade I to 5-flucytosine, not much is known about antifungal resistance with regard to each of the *C. albicans* clades. Of specific concern is the antifungal resistance that could possibly be associated with particular clades, and more specifically the unique SA clade. Knowledge in this regard would contribute to a better understanding of drug resistance and subsequently to improved management of South African patients infected by *C. albicans*.

Objectives

To induce resistance against azoles (fluconazole and miconazole) and polyenes (amphotericin B and nystatin) in *C. albicans* isolates belonging to different clades, and to determine whether induction was clade-related, and also to determine the survival and retention of resistance among antifungal resistant isolates after long-term storage at -80^{o}C, and to determine whether survival and resistance retention were clade-related.

Materials and Methods

Twenty *C. albicans* isolates selected from each of the previously typed clades, I, II, III, SA and NG were used in the study (n=100). Resistance to fluconazole, miconazole, amphotericin B and nystatin was induced in all 100 isolates according to the modified National Committee of Clinical Laboratory Standards (NCCLS) broth microdilution method. Isolates were exposed to increasing concentrations of each antifungal until they

became resistant, and induction rates between different antifungals and clades were determined and compared.

Survival and retention of resistance among fluconazole resistant (n=100), miconazole resistant (n=100), amphotericin B resistant (n=100) and nystatin resistant (n==100) isolates after two years of storage at -80^{o}C was determined by culturing the stored isolates on RPMI agar containing highest concentrations of each antifungal and in RPMI agar without antifungals. Survival and resistance retention rates between different antifungals and clades were determined and compared.

Results

Resistance to nystatin, AmB, fluconazole and miconazole was successfully induced in all of 20 (100%) *C. albicans* isolates from each of clades I, II, III, SA and NG. When survival and retention of resistance were determined, all 20 (100%) fluconazole resistant isolates from clades I, II, SA, NG, and 19 (95%) from clade III survived and retained their resistance. Of miconazole resistant isolates, all 20 (100%) isolates from clade I, II, and SA, and 19 (95%) from clade III and NG survived and retained their resistance. Of AmB resistant isolates, 12 (60%) from Clade NG survived and retained their resistance; 9 (45%) from Clade I; 8 (40%) from Clade III; 7 (35%) from Clade II and 6 (30%) from Clade SA survived and retained their resistance. Of the isolates resistant to nystatin, 12 (60%) from clade I survived and retained their resistance, 8 (40%) from clade II, 10 (50%) from clade III, 11 (55%) from clade SA, and 15 (75%) from clade NG survived and retained their resistance.

Conclusion

To our knowledge, this study is the first to be performed on the *in vitro* induction of antifungal resistance in isolates representing the various clades of *C. albicans*. The results of this study show that resistance to polyenes and azoles could readily be induced in *C. albicans* isolates from all clades, and that induction was not clade-related. The results also show that azole resistant isolates survived and retained their resistance better than polyene resistant isolates after long-term storage at -80^{o}C. Survival and retention of resistance among azole resistant isolates was not clade-related, while those of polyene resistant isolates were clade-related.

The ease with which polyene resistance could be induced in this study can hold serious implications, especially for patients with systemic *C. albicans* infections, in whom AmB is mostly used, as it has remained a "gold standard" for the treatment of life-threatening systemic fungal infections. The ease with which azole resistance could be induced in this study can hold serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles are mostly used for *C. albicans* infections.

The high survival and resistance retention rates among azole resistant isolates after freezing could explain the widespread reported increase in azole resistance among clinical isolates due to increased and extended use of azoles. The reduced survival and resistance retention rates among AmB resistant isolates from SA clade is in support of the results of the study by Blignaut et al. (2005), where a high natural resistance to AmB was observed in isolates from clade SA. The high survival and resistance retention rates among AmB and nystatin resistant isolates from clade NG, and the low survival and resistance retention rates among nystatin resistant isolates from clade II suggest that further study of differences between different *C. albicans* clades may be warranted, and that isolates from these clades need to be studied further.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000). Continued antifungal surveillance is equally important to predict the evolution of resistance in a particular population and to take timely measures.

3.2 Introduction

C. albicans is an opportunistic pathogen causing Oropharyngeal Candidiasis (OPC), the most common oral complication in HIV/AIDS patients (Greenspan and Greenspan, 1992; Glick et al., 1994). OPC was among the initial signs of HIV-induced immunodeficiency to be recognized (Gottlieb et al., 1981; Masur et al., 1981), and affects the majority of persons with advanced untreated HIV infection.

It is estimated that more than 5.7 million South Africans are currently infected with HIV (UNAIDS/WHO report, 2008), and that 80 to 95% of patients infected with HIV patients will experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994). Even with the advent of highly active antiretroviral therapy (HAART) and the subsequent decline of opportunistic infections, oral-esophageal candidiasis still remains among the most frequent infections in HIV-infected individuals (Kaplan et al., 2000).

The azoles, including fluconazole and miconazole, are used in the treatment and/or prophylaxis of *C. albicans* infections (White et al, 1998). Due to their convenient administration and extended activity against non-*Candida* fungal infections such as cryptococcosis, histoplasmosis, and coccidioidomycosis, azoles have become a good choice as systemically administered drugs for the therapy and prophylaxis of AIDS-associated opportunistic infections (Como and Dismukes, 1994; Gallant et al., 1994). Fluconazole is the most widely used azole for systemic candidiasis due to its high solubility, low toxicity, and wide tissue distribution (Lortholary and Dupont, 1997).

Azoles act by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase, an enzyme encoded by the *ERG11* gene of *C. albicans*. This enzyme is important in the synthesis of ergosterol, a primary sterol in the fungal cell membrane. Ergosterol is important for the fluidity and integrity of the membrane and for the proper functioning of many membrane-bound enzymes. The inhibition of this enzyme leads to the inability of *C. albicans* to synthesize ergosterol (White et al., 1998).

Two major molecular mechanisms are responsible for development of azole resistance in strains of *C. albicans*. The first mechanism involves changing the target enzyme, cytochrome P-450 lanosterol 14α-demethylase, either by over-expression of the *ERG11*

gene (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Franz et al., 1998; Henry et al., 2000), or as a result of point mutations in the *ERG11* gene (Vanden Bossche et al., 1990; Vanden Bossche et al., 1994a; White et al., 1997; Sanglard et al., 1998; Franz et al., 1998). The point mutations in the *ERG11* gene lead to amino acid substitutions, causing a decreased affinity for azoles (Franz et al., 1998). The over-expression of *ERG11* gene results in increased production of lanosterol demethylase, making it difficult for the azoles to inhibit it (Henry et al., 2000).

A second major mechanism is failure of azole antifungal agents to accumulate inside the yeast cells due to increased drug efflux. This is mediated by two types of multidrug efflux pumps; the proton motive force-dependent major facilitators (MF) and the energy-dependent ATP Binding Casettes (ABC) transporters (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Walsh et al., 1997; Lopez-Ribot et al., 1998).

Azoles have little or no toxicity, but they are fungistatic, therefore in AIDS patients, azoles must be administered for long periods of time. This extended use, combined with the increased use of azoles in recent years, has led to the increased isolation of azole-resistant strains of *C. albicans* (Ng and Denning, 1993; White, 1997; Rex et al., 1998). A cross resistance between azoles has also been demonstrated (Barchiesi et al., 1994). Several investigators have noted that resistance can develop in *C. albicans* after only short exposures to fluconazole, both *in vitro* (Calvet et al., 1997) and *in vivo* (Marr et al., 1997; Nolte et al., 1997).

Azole resistance in clinical isolates of *C. albicans* is well documented (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002). One study estimated that up to a third of all AIDS patients retain an azole resistant *C. albicans* strain orally (Law et al., 1994). It has also been established that antifungal drug-resistant Candida strains arise more often in HIV positive patients than in HIV negative patients (Johnson et al., 1995). In other studies, a patient with AIDS developed secondary resistance when he had several episodes of thrush caused by a single strain of *C. albicans* that acquired increasing resistance over time (Pfaller et al., 1994; Redding et al., 1994). Fluconazole resistant isolates have also been constructed *in vitro* (Calvet et al., 1997; Cowen et al., 2000; Marr et al., 2001; Barker et al., 2004).

The polyenes antifungal agents used for treatment of *C. albicans* infections include nystatin and amphotericin B (AmB). Polyenes act by binding to ergosterol, a major sterol in the fungal cell membrane. This leads to disruption of the osmotic integrity of the membrane, causing leakage of intracellular K^+ and Mg^{2+} from the cell, and also disrupting oxidative enzymes in the cells (Vanden Bossche et al., 1994a), thus killing the cell.

Amphotericin B (AmB) is a polyene antifungal drug derived from *Streptomyces nodosus*. It has remained a "gold standard" for the treatment of life-threatening systemic fungal infections, but its toxic nature has limited doses used for treatment (Ellis, 2002). Despite its toxic nature, AmB is still used because it has several positive features required for an effective antifungal drug, namely, its high antifungal activity, broad antifungal spectrum and fungicidal activity (Baginski et al., 2005). Resistance to AmB is caused by a decrease in either the amount of ergosterol in the fungal cell membrane, or a mutation in the ergosterol biosynthesis pathway, leading to production of ergosterol-like compounds with less binding affinity for AmB (Ellis, 2002).

Another mechanism by which antifungal resistance can occur in polyenes is through changes in other components of the ergosterol biosynthesis pathway resulting in the accumulation of sterol intermediates (Lupetti et al., 2002). In clinical isolates of *C. albicans*, combined resistance to both AmB and azoles has been associated with accumulation of ergosta-7, 22-dienol. This leads to reduced activity of C-5 desaturase, an enzyme encoded by *ERG3* gene (Kelly et al., 1996; Kelly et al., 1997; Nolte et al., 1997). These changes in *ERG3* are thought to reduce the conversion of episterol to potentially toxic metabolites that accumulate in the cell membrane during azole exposure (Lupetti et al., 2002).

The incidence of resistance to amphotericin B has been reported to be rare, but isolates resistant to this drug have been reported both *in vivo* (Dick et al., 1980; Powderly et al., 1988) and *in vitro* (Rex et al., 1995; Kelly et al., 1997; Barker et al., 2004). Polyene-resistant isolates have been reported and characterized, and resistant strains of *C. albicans* have been constructed *in vitro* (Broughton et al., 1991; Vanden Bossche et al., 1994a; Vanden Bossche et al., 1994b). These strains were either exposed to chemicals or serially passaged in media containing increasing amounts of polyene. Most polyene-resistant

clinical isolates were observed to have a greatly reduced ergosterol content in their membranes (Broughton et al., 1991). In studies done to analyse sterol composition, several clinical isolates of *C. albicans* were found to be defective in *ERG2* or *ERG3* genes (Kelly et al., 1994; Heald et al., 1996; Haynes et al., 1996; Nolte et al., 1997).

In previous studies done in the United States, three groups (clades) of *C. albicans* isolates (groups I, II, III) were distinguished by using various DNA fingerprinting methods (Pujol et al., 1997; Lott et al., 1999). In another study, a new clade among South African isolates that differed from the three US clades, which accounted for 53% of isolates in HIV positive South Africans was demonstrated, and was named clade SA (Blignaut et al., 2002b). A fifth clade has also been established among isolates from Europe (Pujol et al., 2002; Soll and Pujol, 2003).

In a recent study, a natural resistance to AmB was demonstrated in 8.4% of South African isolates (Blignaut et al., 2002a). This prevalence was found to be higher than the 0-0.4% reported by other overseas workers (Waltimo et al., 2000; Pizzo et al., 2002; Dar-Odeh and Shehabi, 2003; Hajjeh et al., 2004). In a follow-up study, this high incidence of natural AmB resistance was found to be dominant among *C. albicans* isolates belonging to clade SA (Blignaut et al., 2005). Cross-resistance between azoles and AmB has also been reported in several studies (Kelly et al., 1996; Kelly et al., 1997; Nolte et al., 1997).

3.3 Study Problem

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. When phenotypic characteristics of 5 *C. albicans* clades were studied, isolates from Clade SA showed a natural resistance mainly to amphotericin B (Blignaut et al., 2005) and those from Clade I almost exclusively resistant to 5-flucytosine (Pujol et al., 2004). These results demonstrated that *C. albicans* clades differ phenotypically, and supported the need for a worldwide analysis of clade-specific phenotypic characteristics of *C. albicans* isolates. Apart from this information regarding clade SA and clade I, not much is known about resistance to other antifungals among *C. albicans* isolates belonging to different clades. Of specific concern is the antifungal resistance that could possibly be associated with particular clades, and more specifically the unique South African clade. Knowledge in this regard would contribute to a better understanding of drug resistance and subsequently to improved management of South African patients infected by *C. albicans*.

3.4 Aim

To investigate the induction of antifungal resistance among South African *C. albicans* isolates belonging to different clades.

3.5 Objectives

a) To culture *C. albicans* isolates belonging to clades I, II, III, SA, and NG.
b) To induce resistance in these isolates by growing them in increasing concentrations of fluconazole, miconazole, AmB and nystatin.
c) To confirm antifungal resistance by growing isolates in medium containing highest concentration of each antifungal.
d) To determine the minimum inhibitory concentrations of fluconazole resistant and susceptible isolates by using E test.
e) To determine survival of resistant isolates and retention of resistance after long-term storage at -80 ^{o}C by growing them in medium containing highest concentrations of each antifungal.
f) To compare induction rates between different antifungals and clades.

g) To compare survival and resistance retention rates of resistant isolates between different antifungals and clades.

3.6 Materials and Methods

3.6.1 Yeast isolates

Twenty isolates were selected from each of the previously typed clades of *C. albicans* (Blignaut et al., 2002b), clades I, II, III, SA and NG. These yeast isolates were obtained from surveillance cultures on patients attending HIV/AIDS clinics in the Pretoria region, and were previously tested and found to be susceptible to fluconazole (MIC≤8µg/ml), miconazole (MIC <0.5µg/ml), AmB (MIC<1µg/ml) and nystatin (MIC <8µg/ml) (Blignaut et al., 2002a). These isolates were selected from a bank stored in the MEDUNSA Oral Health Centre, Stomatological Studies Laboratory library. The isolates were grown on Sabouraud's Dextrose (SD) agar for 24 hours at 35^{o}C. The study was approved by the Research, Ethics, and Publications Committee (REPC), Faculty of Dentistry, University of Limpopo (Medunsa Campus), and REPC of Senate.

3.6.2 Antifungal stock solutions and working dilutions

Stock solutions of AmB (0.012g), nystatin (0.01g), miconazole (0.01g) (Sigma Chemical Co., St Louis, Mo) and fluconazole (0.1g) (Pfizer, N.J.) were prepared in 10ml dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany). Dilutions of 1:10, 1:100 and 1:1000 were prepared from stock solutions, filter sterilized, covered with aluminium foil and then stored at -80^{o}C till needed. Antifungals working dilutions (0.25µg/ml to 4µg/ml of AmB, 0.5µg/ml to 8µg/ml of nystatin**,** 0.5µg/ml to 32µg/ml of miconazole and 0.125µg/ml to 64µg/ml of fluconazole) were prepared from these stored original dilutions, in RPMI liquid medium (10.4g/liter RPMI powder, 34.53g/liter MOPS, 20g/litre glucose, pH 7.0). Hundred microlitres of these working dilutions were transferred to wells of 96 U-bottomed microtitre plates, covered with parafilm and stored at -80^{o}C till needed.

3.6.3 Preparation of inoculum

An isolated, single colony from each culture on SD agar was picked off and dissolved in 5ml of sterile saline to make up a 0.5 MacFarland standard (Scientific Device Laboratory Inc., Illinois, USA). From that suspension, 50µl was taken and transferred to 4.95ml of sterile saline to make up a 1:1000 dilution. After mixing, 600µl was transferred to 5.4ml

RPMI liquid medium(10.4g/liter RPMI powder, 34.53g/liter MOPS, 20g/litre glucose, pH 7.0) to make up a 1:20 working dilution, giving approximately 1x 10^3 to 5x10^3 cells/ml.

3.6.4 Induction of resistance

Induction of antifungal resistance was conducted according to the modified microdilution method of the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards [NCCLS] (NCCLS, 2002). Isolates were exposed to increasing concentrations of antifungals: from 0.25µg/ml to 4µg/ml of AmB, 0.5µg/ml to 8µg/ml of nystatin**,** 0.5µg/ml to 32µg/ml of miconazole and 0.125µg/ml to 64µg/ml of fluconazole. One hundred microlitres of antifungal dilutions in wells of 96 U-bottomed well microtitre plates were diluted to the final working concentration with the addition of 100µl of the prepared yeast inoculum. Isolates were run in duplicate, and controls containing only RPMI and RPMI with antifungal were run with the samples to check for contamination.

The isolates were incubated at first in the lowest concentration of each antifungal agent for 48 hours at 35^{o}C. After measuring growth on an automated microtitre plate reader at 405nm, another standard inoculum was prepared as described above, from this growth and inoculated into the next higher concentration of each antifungal agent and incubated. The procedure was repeated for all antifungal agents, up to 4µg/ml for amphotericin B, 64µg/ml for fluconazole, 32µg/ml for miconazole and 8µg/ml for nystatin.

3.6.5 Confirmation of antifungal Resistance

Induced resistance of all twenty isolates from each of clades I, II, III, SA and NG was confirmed by using a method described by Patterson et al. (1996). RPMI agar (10.4g/liter RPMI powder, 34.53g/liter MOPS, 20g/litre glucose, 15g/litre Bacto agar, pH 7.0) containing different antifungal agents to final highest concentrations of 8µg/ml for nystatin, 4µg/ml for AmB, 64µg/ml for fluconazole and 32µg/ml for miconazole was prepared. Antifungal agents were added to molten RPMI agar at 45 ^{o}C with thorough stirring, and agar was poured into plates and allowed to solidify. A sterile, 10µl loop of isolates was used to inoculate RPMI agar plates with different antifungals. Plates were incubated for 48 hours at 35^{o}C. The isolates were considered resistant to fluconazole, miconazole, nystatin and AmB if they showed large, visible colonies on RPMI containing

concentrations of 64µg/ml, 32µg/ml, 8µg/ml and 4µg/ml respectively. The resistant isolates that grew in the medium with antifungals, together with their matching susceptible isolates (parent strains) were aliquoted and stored in 50% glycerol at -80°C until required (Calvet et al., 1997).

3.6.6 Determination of Minimum Inhibitory Concentrations (MICs) of fluconazole resistant and susceptible isolates

To determine the MICs of fluconazole resistant and susceptible isolates which were to be used later for determination of mutations, gene expression and phenotypic switching, the E-test was performed on randomly selected six matching (3 fluconazole susceptible and 3 resistant) isolates from each of clades I, II, III, SA and NG, according to manufacturer's directions (AB Biodisk, Sweden). The isolates were grown on Sabouraud's Dextrose agar for 24 hours at 35°C, and an inoculum of a 0.5 McFarland standard was prepared. The inoculum was plated on RPMI MOPS glucose agar (8.4g/liter RPMI powder, 34.53g/liter MOPS, 20g/litre glucose, 15g/litre Bacto agar, pH 7.0). The E-test strips containing fluconazole ranging in concentration from 0.016 to 256 µg/ml were used. The plates were incubated at 35°C for 72 hours. The MIC was read visually where the inhibition ellipse intersects the scale on the strips, and the MIC was taken as the point of 80% inhibition. The isolates were considered to be fluconazole resistant if they had MIC of ≥64µg/ml, and fluconazole susceptible if they had MIC of ≤8µg/ml.

3.6.7 Determination of survival and retention of resistance among polyene- and azole-resistant isolates after storage at -80°C

To check for survival and retention of resistance of resistant isolates after long-term storage at -80°C, aliquots of all twenty stored isolates from each of clades I, II, III, SA and NG, with induced resistance to AmB, nystatin, miconazole and fluconazole, were thawed. RPMI agar (10.4g/liter RPMI powder, 34.53g/liter MOPS, 20g/litre glucose, 15g/litre Bacto agar, pH 7.0) containing different antifungal agents to final highest concentrations of 8µg/ml for nystatin, 4µg/ml for AmB, 64µg/ml for fluconazole and 32µg/ml for miconazole was used. Antifungal agents were added to molten RPMI agar at 45°C with thorough stirring. A sterile, 10µl loop of isolates was used to inoculate RPMI agar plates with different antifungals. All isolates were also cultured on RPMI agar without antifungals. Plates were incubated for 48 hours at 35° and viewed for growth (Patterson et al., 1996). Resistant isolates were considered to have survived and retained

their resistance if they showed visible growth in both RPMI medium with and RPMI without antifungals. Isolates were considered not to have survived and to have lost their resistance if they did not grow in RPMI with antifungals and in RPMI without antifungals.

3.6.8 Statistical analyses

The statistical analyses were performed using Statistical Software Package of Social Sciences (SPSS) 12.0 for windows. One-way Analysis of Variance (ANOVA) was performed to analyze the data. Statistical differences between survived isolates among the clades were determined. A *p* value less than 0.05 was considered significant.

3.7 Results

3.7.1 Induction of antifungal resistance

To induce antifungal resistance, twenty previously typed *C. albicans* isolates belonging to each of clade I, II, III, SA and NG were exposed to increasing concentrations of nystatin, AmB, fluconazole and miconazole according to the modified National Committee of Clinical Laboratory Standards (NCCLS) broth microdilution method (NCCLS, 2002). Resistance to AmB, nystatin, fluconazole and miconazole was successfully induced in all of 20 (100%) isolates from clade I, II, III, SA and NG without any re-incubation.

3.7.2 Confirmation of antifungal Resistance

All twenty isolates from each of clades I, II, III, SA and NG with induced resistance to AmB, fluconazole, miconazole and nystatin showed large, visible colonies on RPMI agar containing 4µg/ml, 64µg/ml, 32µg/ml and 8µg/ml respectively, confirming that they were resistant.

3.7.3 Determination of isolates MIC

The E-test method was performed on six matching (fluconazole susceptible and resistant) isolates from each of clades I, II, III, SA and NG, according to manufacturer's directions (AB Biodisk, Sweden). All isolates with induced resistance to fluconazole were resistant, with MIC ranging from 1:128µg/ml to ≥1:256µg/ml. These results are summarized in

Table 3.1, and representative MIC patterns of fluconazole resistant and susceptible are shown in Figure 3.1.

Table 3.1: MICs of *C. albicans* fluconazole-resistant and susceptible isolates from different clades

ISOLATE	CLADE	MIC PRIOR TO INDUCTION	MIC AFTER INDUCTION
G63	**I**	0.25	1:128
K239	**I**	0.38	1:256
K21	**I**	0.19	≥1:256
K162	**II**	0.19	1:128
G19	**II**	0.125	1:128
K153	**II**	0.75	≥1:256
OKP25	**III**	0.125	1:128
UP30	**III**	0.19	≥1:256
G58	**III**	0.25	≥1:256
G116	**SA**	0.5	≥1:256
G118	**SA**	1.0	≥1:256
K306	**SA**	0.75	≥1:256
G22	**NG**	0.25	≥1:256
G6	**NG**	0.38	1:128
K86	**NG**	0.125	≥1:256

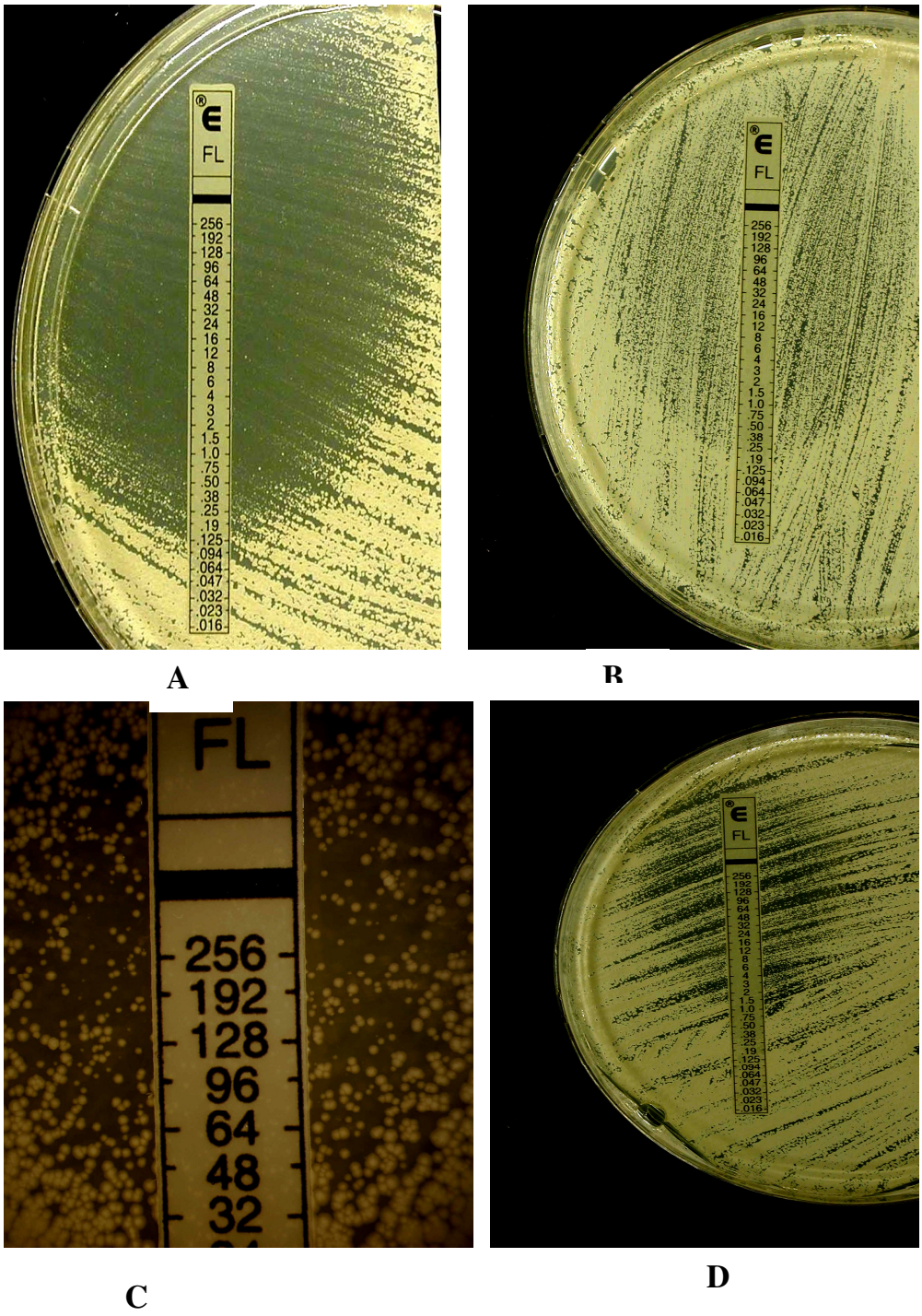

Figure 3.1: MIC patterns of some *C. albicans* fluconazole resistant and susceptible isolates. **(A):** MIC 0.19**; (B):** MIC 0.25**; (C):** MIC ≥1:256; **(D)**: MIC 1.0

3.7.4 Survival and retention of resistance among resistant isolates after long-term storage at -80^{o}C

To check for survival and retention of resistance of resistant isolates after long-term storage at -80^{o}C, the stored isolates were subcultured on RPMI agar containing highest concentration of nystatin (8µg/ml), AmB (4µg/ml), fluconazole (64µg/ml) and miconazole (32µg/ml), and on RPMI agar without antifungal, according to a method described by (Patterson et al., 1996).

3.7.4.1 Survival and retention of resistance among polyene-resistant isolates

Of the isolates resistant to nystatin, the following numbers of isolates survived and retained their resistance: 12 (60%) from clade I, 8 (40%) from clade II, 10 (50%) from clade III, 11 (55%) from clade SA, and 15 (75%) from clade NG. When ANOVA was done, there was no statistically significant difference between resistant isolates that survived and retained their resistance among various clades (ANOVA, F=2.14; p=0.18). However, these results show that isolates from clade NG survived and retained their resistance better (75%), than isolates from other clades and those from clade II were the least in surviving and retaining their resistance (40%). These results are shown in Figure 3.2.

Of AmB resistant isolates, the following numbers of isolates survived and retained their resistance: 12 (60%) from Clade NG; 9 (45%) from Clade I; 8 (40%) from Clade III; 7 (35%) from Clade II and 6 (30%) from Clade SA. When ANOVA was done, there was no statistically significant difference between resistant isolates that survived among various clades (ANOVA, F=4.8; p=0.06). However, these results show that isolates from clade NG survived and retained their resistance better than isolates from other clades (60%), and those from clade SA survived and retained their resistance the least (30%). These results are shown in Figure 3.3.

3.7.4.2 Survival and retention of resistance among azole-resistant isolates

All 20 (100%) of fluconazole resistant isolates from clades I, II, SA, NG, and 19 (95%) from clade III survived and retained their resistance. These results show that survival and retention of resistance among these isolates was not clade-related. These results are shown in Figure 3.4.

Of miconazole resistant isolates, all 20 (100%) isolates from clade I, II, and SA, and 19 (95%) from clade III and NG survived and retained their resistance. These results show that survival and retention of resistance among miconazole resistant isolates was not clade-related. These results are shown in Figure 3.5.

When survival and resistance retention rates between polyene resistant isolates and azole resistant isolates were compared, the azole resistant isolates from all clades survived and retained their resistance better than the polyene resistant isolates.

The results of comparison of survival and resistance retention rates between azole and polyene resistant isolates are shown in Figure 3.6.

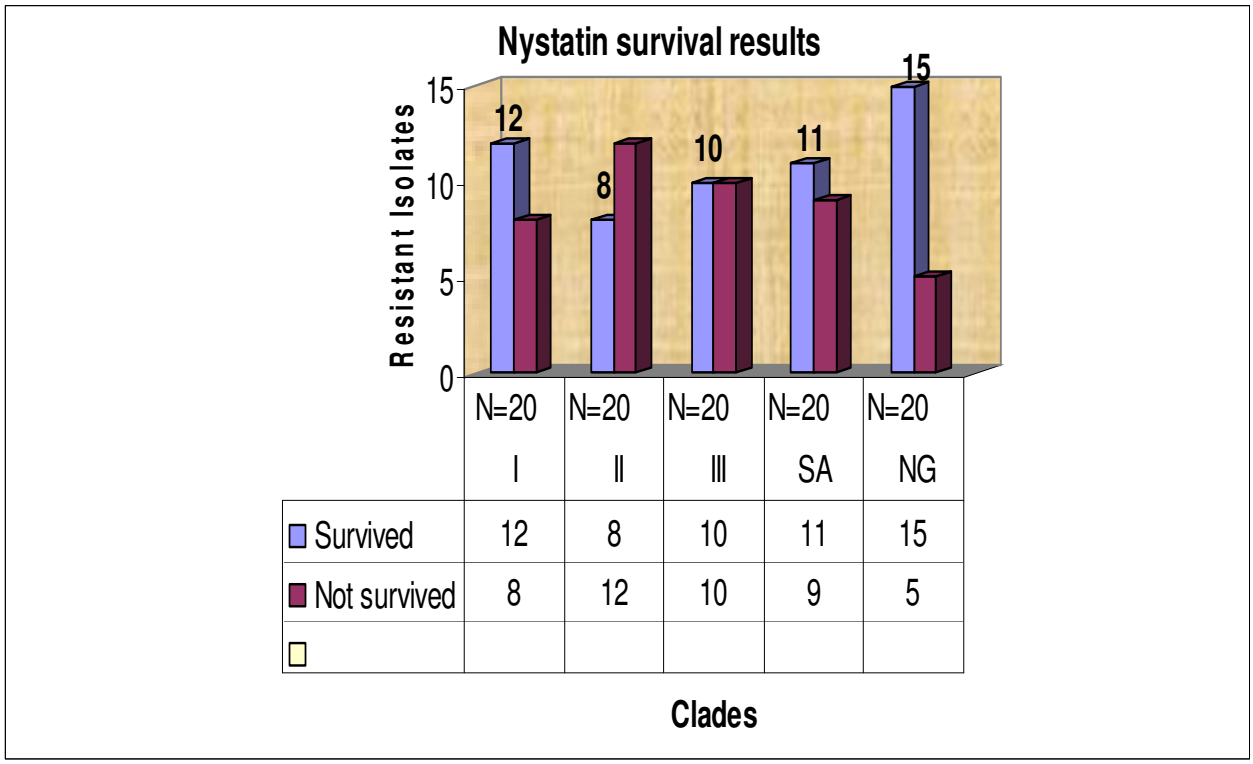

Figure 3.2: Survival and resistance retention rates among *C. albicans* isolates with induced nystatin resistance

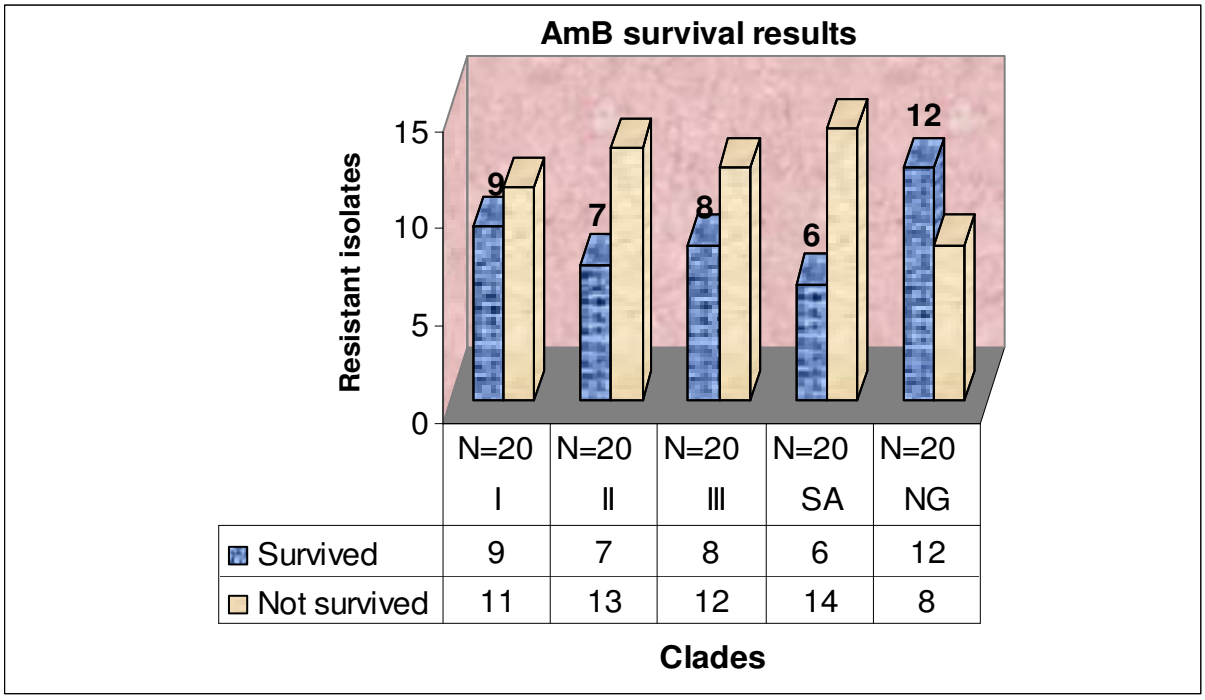

Figure 3.3: Survival and resistance retention rates among *C. albicans* isolates with induced AmB resistance

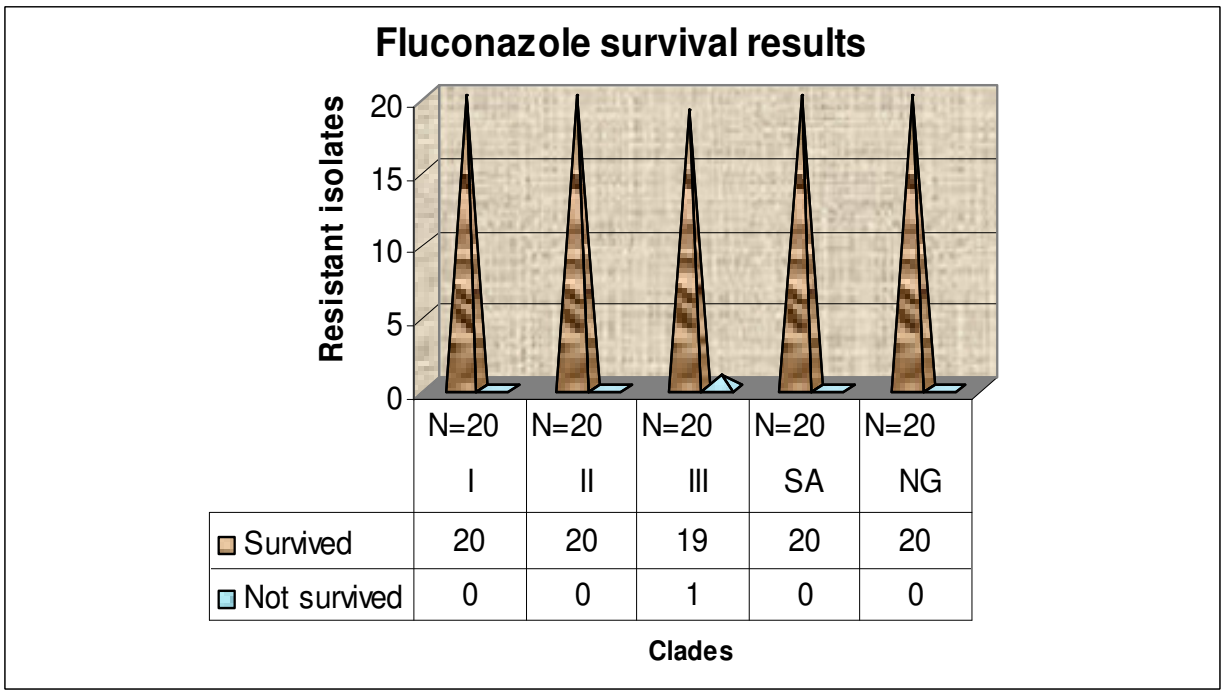

Figure 3.4: Survival and resistance retention rates among *C. albicans* isolates with induced fluconazole resistance

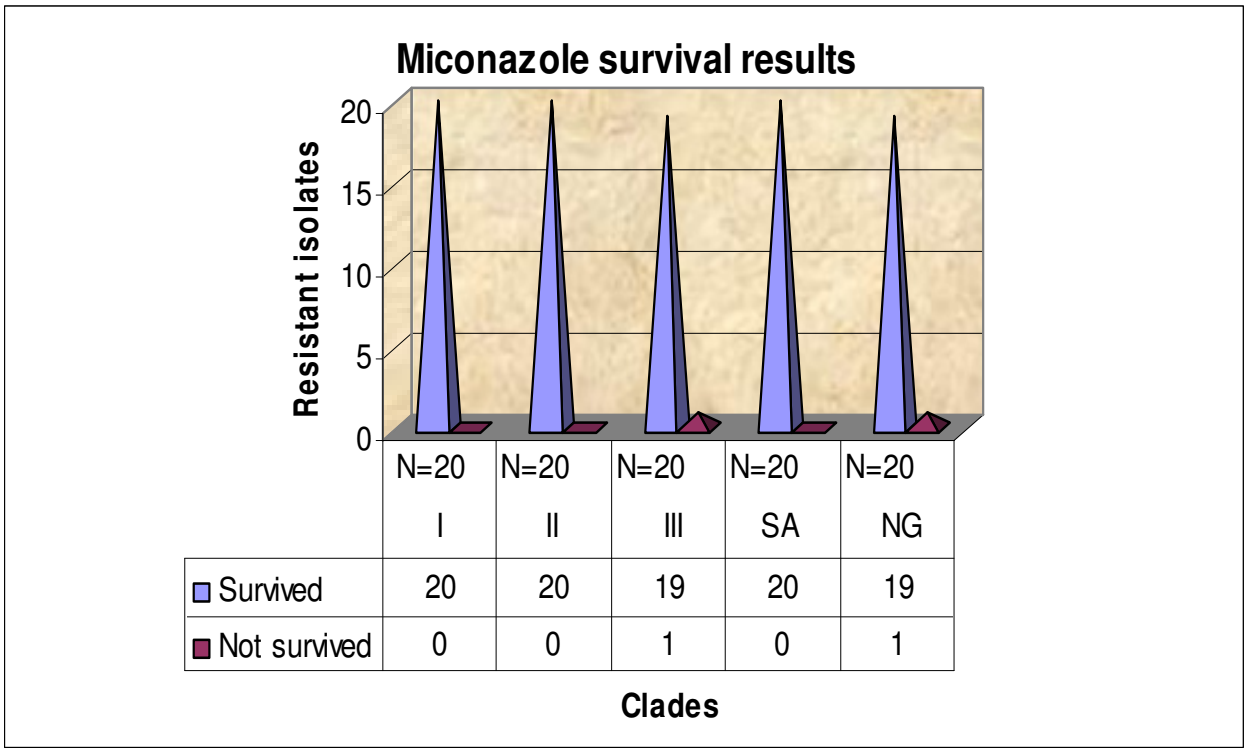

Figure 3.5: Survival and resistance retention rates among *C. albicans* isolates with induced miconazole resistance

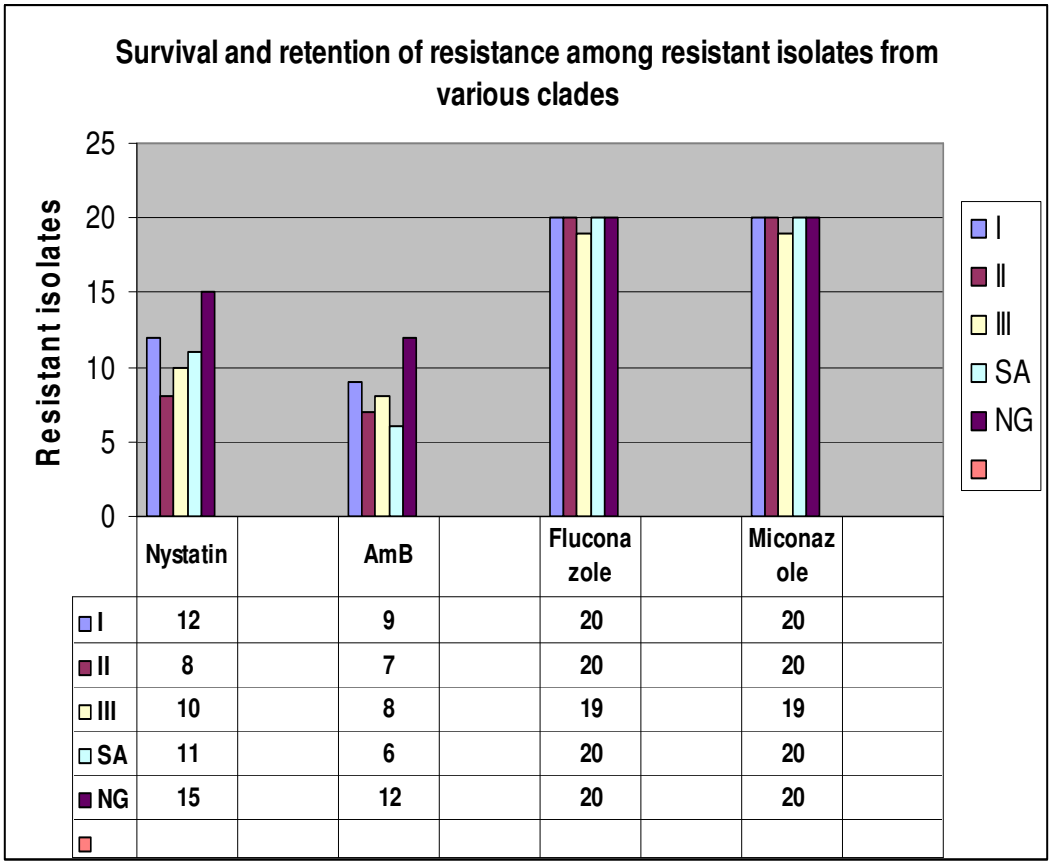

Figure 3.6: Comparison of survival and resistance retention rates between azole and polyene resistant isolates

3.8 Discussion

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been widely reported (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002).

Although 5 unique genetic clades of *C. albicans* have been described, little is known about their phenotypic characteristics. Apart from the resistance of Clade SA to amphotericin B (Blignaut et al., 2005) and Clade I to 5-flucytosine (Pujol et al., 2004), not much is known about antifungal resistance with regard to each of the *C. albicans* clades. Of specific concern is the antifungal resistance that could possibly be associated with particular clades, and more specifically the unique South African clade (Clade SA). Knowledge in this regard would contribute to a better understanding of drug resistance and subsequently to improved management of South African patients infected by *C. albicans*.

This study was done to induce antifungal resistance among *C. albicans* isolates, and to determine whether induction was clade-related, and to determine survival of resistant isolates after long-term storage in freezing conditions, and whether survival was clade-related. To our knowledge, this study is the first to be performed on the *in vitro* induction of antifungal resistance in *C. albicans* isolates representing the various clades of *C. albicans*. In this study, resistance to azoles, namely fluconazole and miconazole, and to polyenes, namely AmB and nystatin was experimentally induced by exposing 20 *C. albicans* isolates from each of clades I, II, III, SA and NG in increasing concentrations of these antifungals.

The results of this study show that resistance against AmB and nystatin was successfully and readily induced in all 20 *C. albicans* isolates from each of clades I, II, III, SA and NG after exposure to increasing concentrations of these antifungals. These results are in agreement with those of limited previous studies done, where resistance to polyenes was experimentally induced. In a study by Athar and Winner (1971), a high-level resistance of greater than 100-fold increase in the MIC to AmB was successfully induced in *C. albicans* strains after 30 to 40 passages in drug-containing solid medium, although our

studies differed because we used liquid medium instead of a solid medium. In a study by Broughton et al. (1991), nystatin and AmB resistant isolates were constructed *in vitro*, and these isolates were observed to have a greatly reduced ergosterol content in their membranes. These results are in agreement with those of our study, since polyene resistance could be easily induced after several passages in medium with AmB or nystatin, although ergosterol content was not measured in our study.

In their study, Haynes et al. (1996) induced AmB resistance by exposing an AmB susceptible isolate to AmB, and this isolate was found to have decreased ergosterol content. In their study, Kelly et al. (1997) induced AmB resistance after experimental exposure of susceptible isolates to increasing concentrations of fluconazole. A study by Barker et al. (2004) exposed a susceptible isolate to increasing concentrations of AmB, and the isolate developed resistance to AmB (MIC>32) and cross-resistance to fluconazole (MIC>256). This AmB resistant isolate was found to have sterol intermediates eburicol and lanosterol, instead of ergosterol.

The ease with which polyene resistance could be induced in our study can have serious implications, especially for patients with systemic *C. albicans* infections, in whom AmB is mostly used. AmB has remained a "gold standard" for the treatment of life-threatening systemic fungal infections (Ellis, 2002).

The results of this study also show that induction of resistance against nystatin and AmB was not clade-related, as resistance could be induced equally among isolates from all clades. However, our results cannot be compared with results from other studies regarding clade-relatedness, as this is the first study where induction of AmB and nystatin resistance was done in isolates belonging to different clades.

The results of our study show that resistance against fluconazole and miconazole was successfully and readily induced in all 20 *C. albicans* isolates from each of clades I, II,

III, SA and NG after exposure to increasing concentrations of these antifungals. The results of this study show that resistance to azoles in *C. albicans* isolates from different clades can be easily induced, and that resistance is induced equally among all clades. These results are in contrast to those observed by Iwata (1992), where azole resistance could not be induced after several attempts of exposure to azoles.

However, these results are in agreement with those of previous studies, where fluconazole resistance was successfully induced. In a study by Hernandez et al. (1995), fluconazole resistance was induced by serially passing *C. albicans* in liquid medium containing fluconazole. However, in their study, an increase in the MIC was found in only 2 of 4 *C. albicans* strains assayed, whereas in our study we successfully managed to induce fluconazole resistance in all isolates. In a study by Albertson et al. (1996), fluconazole resistance was induced by growing a fluconazole susceptible *C. albicans* isolate in RPMI medium containing increasing concentrations of fluconazole. The final MIC of the isolate was found to be 32 μg/ml.

In a study by Calvet et al. (1997), rapid development of fluconazole resistance was reported in *C. albicans* after serial growth in the presence of drug. In their study, high-level fluconazole resistance of >256μg/ml developed in the isolates after serial passage in medium containing 8, 16, or 128μg/ml of fluconazole, but not in isolates passed in 4μg/ml of fluconazole. Their results are in agreement with those of our study, although they exposed isolates to individual concentrations of fluconazole instead of serial passaging.

In their study, Cowen et al. (2000) induced fluconazole resistance by serially exposing six azole-susceptible *C. albicans* isolates for 100 days to RPMI 1640 medium containing fluconazole at twice their most recently measured MIC. However, in their study, fluconazole resistance could only be induced in 3 of 6 isolates, while in our study resistance was induced in all isolates. In a study by Marr et al. (2001), 2 fluconazole-susceptible isolates were serially transferred in YAD medium containing fluconazole at a concentration equivalent to two times the original MIC for the isolate. The increase in fluconazole MIC was observed in both clinical isolates, although these authors used YAD medium instead of RPMI used in our study.

In a study by Ribeiro and Paula (2007), fluconazole resistance was induced by serially culturing fluconazole susceptible vaginal *C. albicans* isolates from HIV-infected patients in RPMI-1640 broth containing increasing concentrations of fluconazole up to a concentration of 64µg/ml. Although these investigators used vaginal isolates in their study, while we used oral isolates, they successfully induced fluconazole resistance in these isolates. In a recent study by Angiolella et al. (2008), a fluconazole susceptible isolate was made resistant by 10 growth passages in stepwise-increasing concentrations of fluconazole in agar-solidified yeast nitrogen base (YNB) medium, up to a concentration of 128µg/ml. These investigators successfully induced resistance in the isolate although they used a solid medium, while we used a liquid medium in our study.

The ease with which azole resistance could be induced in our study can have serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles are mostly used for *C. albicans* infections. This ease with which resistance against azoles could be induced may also explain the widespread increase in azole resistance among clinical isolates, as described by several investigators (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002). The results of our study confirm that continuous exposure of isolates to azoles will finally enable isolates to become resistant to these antifungals.

The results of our study also show that induction of azole resistance was not clade-related, as resistance was induced equally among isolates from all clades. However, we cannot compare these results to other studies concerning clade-relatedness, as this is the first study where induction of azole resistance was done in different clades of *C. albicans.*

To check for survival and retention of resistance among antifungal resistant isolates after long-term storage at -80^{o}C, the stored isolates were grown on RPMI agar containing

highest concentration of each antifungal, and on RPMI agar without antifungals, according to a method described by Patterson et al. (1996).

To our knowledge, this study is the first in which survival and retention of resistance among azole resistant isolates from different *C. albicans* clades was determined after long-term storage in freezing conditions. The results of this study show that 95-100% of fluconazole and miconazole resistant isolates survived and retained their resistance, as compared to AmB and nystatin resistant isolates. The high survival and resistance retention rates of azole resistant isolates could be attributed to the fact that these isolates have intact ergosterol due to possible over-expression of *ERG11* gene, allowing these isolates to grow and survive. This is in agreement with results observed in a study by Henry et al. (2000), where isolates grown in presence of fluconazole were observed to have intact ergosterol and over-expression of *ERG11* gene.

Ergosterol is important for the fluidity and integrity of *C. albicans* membrane, and for the proper functioning of many membrane-bound enzymes (White et al., 1998). Over-expression of *ERG11* gene results in increased production of lanosterol demethylase, an enzyme responsible for ergosterol synthesis, leading to increased synthesis of ergosterol and contributing significantly to the survival of azole-treated cells (Henry et al., 2000). The high survival and resistance retention rates of of azole resistant isolates could explain the widespread reported increase in azole resistance among clinical isolates due to increased and extended use of azoles (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002).

The results of our study also show that the survival and retention of resistance among azole resistant isolates was not clade-related, as isolates from all clades survived and retained their resistance equally. These results cannot be compared with other studies, as this is the first study where survival and retention of resistance among *C. albicans* resistant isolates after storage at -80^{o}C has been done.

The results of the study also show that the survival and resistance retention rates among polyene (AmB and nystatin) resistant isolates was low as compared to that of azoles, with survival and resistance retention rates ranging from 35-75%. The reduced survival and

resistance retention rates of polyene resistant isolates could be attributed to the fact that these isolates have decreased ergosterol content in their membranes, or have sterol-like compounds (Ellis, 2002; Lupetti et al., 2002), which is one of the mechanisms they use to develop resistance. Therefore their membranes are not as intact as those of azoles, and they therefore die easily and survive less.

The decreased ergosterol content in AmB resistant isolates was demonstrated in several previous studies. In a study by Haynes et al. (1996), an isolate with induced AmB resistance was found to have decreased ergosterol content. In their study, Kelly et al (1997) induced AmB resistance after experimental exposure of susceptible isolates to increasing concentrations of fluconazole. These isolates were found to have sterol-like compounds instead of ergosterol. A study by Barker et al. (2004) exposed a susceptible isolate to increasing concentrations of AmB, and the isolate developed resistance to AmB (MIC>32). This AmB resistant isolate was found to have sterol intermediates eburicol and lanosterol, instead of ergosterol. The decreased ergosterol and the replacement of ergosterol by sterol intermediates lead to weak cell wall of *C. albicans*, which then lead to fungal low survival rate.

The results of our study also show that the survival and resistance retention rates of polyene resistant isolates was clade-related. Although when ANOVA was done there was no general statistical significant difference between AmB resistant isolates that survived and retained their resistance among various clades (ANOVA, F= 4.8; p=0.06), these results show that AmB resistant isolates from clade NG survived and retained their resistance better (60%) than isolates from other clades, and those from clade SA survived and retained their resistance the least (30%).

Although when ANOVA was done there was no general statistical significant difference between nystatin resistant isolates that survived and retained their resistance among

various clades (ANOVA, F= 2.14; p=0.18), these results show that nystatin resistant isolates from clade NG survived and retained their resistance better (75%), than isolates from other clades, and those from clade II survived and retained their resistance the least (40%).

The reduced survival and resistance retention rates of AmB resistant isolates from clade SA is in support of the results of the study by Blignaut et al. (2005), where a high natural resistance to AmB was observed in isolates from clade SA. The high survival and resistance retention rates of AmB and nystatin resistant isolates from clade NG, and the low survival and resistance retention rates of nystatin resistant isolates from clade II suggests that further study of differences between different *C. albicans* clades may be warranted, and that isolates from these clades need to be studied further.

The findings of this study stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000). Continued antifungal surveillance is equally important to predict the evolution of resistance in a particular population and to take timely measures.

3.9 Conclusion

To our knowledge, this study is the first to be performed on the *in vitro* induction of antifungal resistance in isolates representing the various clades of *C. albicans*. The results of this study show that resistance to polyenes and azoles could readily be induced in *C. albicans* isolates from all clades, and that induction was not clade-related. The results also show that azole resistant isolates survived and retained their resistance better than polyene resistant isolates after long-term storage at -80^{o}C. Survival and retention of resistance among azole resistant isolates was not clade-related, while that among polyene resistant isolates was clade-related.

The ease with which polyene resistance could be induced in this study can hold serious implications, especially for patients with systemic *C. albicans* infections, in whom AmB is mostly used, as it has remained a "gold standard" for the treatment of life-threatening systemic fungal infections. The ease with which azole resistance could be induced in this study can hold serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles are mostly used for *C. albicans* infections.

The high survival and resistance retention rates among azole resistant isolates after freezing could explain the widespread reported increase in azole resistance among clinical isolates due to increased and extended use of azoles. The reduced survival and resistance retention rates among the AmB resistant isolates from SA clade is in support of the results of the study by Blignaut et al. (2005), where a high natural resistance to AmB was observed in isolates from clade SA. The high survival and resistance retention rates among AmB and nystatin resistant isolates from clade NG, and the low survival and resistance retention rates among nystatin resistant isolates from clade II suggest that further study of differences between different *C. albicans* clades may be warranted, and that isolates from these clades need to be studied further.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000).

Continued antifungal surveillance is equally important to predict the evolution of resistance in a particular population and to take timely measures.

3.10 References

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CHAPTER FOUR: CONTRIBUTION OF MUTATIONS IN *ERG11* GENE TO FLUCONAZOLE RESISTANCE AMONG *CANDIDA ALBICANS* CLADES

4.1 Abstract

Background

A high increase in antifungal resistance among clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Fluconazole, an antifungal agent used for treatment of *C. albicans* infections, acts by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase of this fungus. This enzyme is encoded by the *ERG11* gene, and is important in the synthesis of ergosterol, a primary sterol important in the integrity of the fungal cell membrane. Resistance to fluconazole can be caused by point mutations in the *ERG11* gene, which leads to amino acid substitutions, causing a decreased affinity for fluconazole. Although various mutations in the *ERG11* gene have been reported to be responsible for fluconazole resistance among *C. albicans* isolates, nothing is known about the contribution of these mutations to fluconazole resistance among different clades of *C. albicans*, and whether these mutations are clade-related.

Objectives

To determine whether induced fluconazole resistance among *C. albicans* isolates from different clades was due to mutations in the *ERG11* gene, and whether mutations were clade-related.

Materials and Methods

Three isolates from each of previously typed *C. albicans* clades I, II, III, SA, and NG (Blignaut et al., 2002), in which fluconazole resistance (MIC≥64µg/ml) was induced in Chapter 2, were selected. Their three matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were also included in the study. The susceptible isolates were grown on Saboraud's Dextrose agar, and resistant isolates were grown in Saboraud's Dextrose agar containing 64µg/ml of fluconazole. DNA was extracted from the isolates. Polymerase chain reaction (PCR) was carried out with a high-

fidelity *Pwo* DNA polymerase, using PCR reactions and cycling conditions suggested by the manufacturer (Roche Diagnostics, Mannheim, Germany). The PCR products were sequenced by using the dye-terminator sequencing method. The obtained sequences of resistant and susceptible isolates were compared with the published *ERG11* gene sequence, and sequences of resistant isolates were also compared with those of their matching susceptible isolates.

Results

No mutations associated with fluconazole resistance were observed in all isolates from clades I and II. Mutations associated with fluconazole resistance were observed in 33.3% of isolates from each of clades III, SA and NG , and some of the mutations observed in resistant isolates from clades III and NG were novel. A total of 50 novel mutations that have not been described previously were observed in both fluconazole resistant and susceptible isolates from this study. Previously described mutations, which were associated with fluconazole resistance, namely, D116E, K128T, V437I and E266D were also observed in this study.

Conclusions

To our knowledge, this study is the first to be performed where contribution of mutations to fluconazole resistance was determined among different clades of *C. albicans*. The results of the study show that mutations were not responsible for fluconazole resistance among isolates from clades I and II, but mutations were associated with fluconazole resistance in isolates from clades III, SA and NG. The occurrence of mutations in fluconazole resistant isolates from clades III, SA and NG, but not in clades I and II shows clade-relatedness of mutations in the *ERG11* gene. This suggests that further study of differences between different clades may be warranted.

Some of mutations observed in resistant isolates from clades III and NG were novel, and have never been described previously. Other novel mutations were also observed in both resistant and susceptible isolates from all clades. Further genetic analysis of these new mutations is required to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa. Some of the novel mutations observed in this study were found in the previously described hotspots, while others were outside the described hotspots. These novel mutations observed outside the previously described hotspots

may warrant further study and probably a review of the hotspots, especially among South African *C. albicans* isolates.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000).

4.2 Introduction

C. albicans is the causative agent of oropharyngeal candidiasis (OPC), the most common oral complication causing morbidity in individuals infected with Human Immunodeficiency Virus (HIV) (Arendorf et al., 1998). It is estimated that more than 5.7 million South Africans are currently infected with HIV (UNAIDS/WHO report, 2008), and that 80 to 95% of patients infected with HIV will experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994).

In previous studies done in the United States, three clades of *C. albicans* isolates (clades I, II, III) were distinguished by using various DNA fingerprinting methods (Pujol et al., 1997; Lott et al., 1999). A recent study revealed a new clade among South African isolates that differed from the three US clades, and which accounted for 53% of isolates in HIV positive South Africans (Blignaut et al., 2002). Later, a fifth clade was established among isolates from Europe (Pujol et al., 2002; Soll and Pujol, 2003).

Azoles antifungal agents, such as fluconazole, have become an important component in treatment of fungal infections because of their relative safety and ease of delivery. Due to their convenient administration and extended activity against non-*Candida* fungal infections such as cryptococcosis, histoplasmosis, and coccidioidomycosis, azoles have become a good choice as systemically administered drugs for the therapy and prophylaxis of AIDS-associated opportunistic infections (Como and Dismukes, 1994; Gallant et al., 1994).

Fluconazole has been found to be active against *C. albicans in vitro* and *in vivo* (Rogers and Galgiani, 1986), and has become the drug of choice for the treatment of OPC. It is the most widely used azole for systemic candidiasis due to its high solubility, low toxicity, and wide tissue distribution (Lortholary and Dupont, 1997). Azoles have little or no toxicity, but they generally lack fungicidal activity. This has led to azoles being administered for long periods of time in AIDS patients. This extended use, together with the increased use of azoles in recent years, has resulted in high increase of azole-

resistance among strains of *C. albicans* (Pfaller et al., 1994; Redding et al., 1994; White et al., 1998; Lopez-Ribot et al., 1998).

Azoles act by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase (P45014DM), which is encoded by the *ERG11* gene of *C. albicans* (Kelly et al., 1993). *ERG11* gene is also known as *ERG16* or *CYP51A1*. Cytochrome P-450 lanosterol 14α demethylase is important in the synthesis of ergosterol, a primary sterol in the fungal cell membrane. Ergosterol is important for the fluidity and integrity of the membrane and for the proper functioning of many membrane-bound enzymes. The inhibition of this enzyme leads to the inability of *C. albicans* to synthesize ergosterol (White et al., 1998).

One of the major molecular mechanisms responsible for development of azole resistance in strains of *C. albicans* involves changing the target enzyme, cytochrome P-450 lanosterol 14α-demethylase. This is achieved by overexpression of *ERG11*, the gene that encodes this enzyme (Sanglard et al., 1995; Albertson et al., 1996; White, 1997a; Franz et al., 1998; Henry et al., 2000) or by point mutations in the gene (Vanden Bossche et al., 1990; Vanden Bossche et al., 1994; White, 1997b; Sanglard et al., 1998; Franz et al., 1998). The point mutations in the *ERG11* gene lead to amino acid substitutions, causing a decreased affinity for azoles (Franz et al., 1998).

Azole resistance in clinical isolates of *C. albicans* is well documented (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002). One study estimated that up to a third of all AIDS patients retain an azole resistant *C. albicans* strain orally (Law et al., 1994). It has also been established that antifungal drug-resistant Candida strains arise more often in HIV positive patients than in HIV negative patients (Johnson et al., 1995).

Point mutations in the *ERG11* gene have been documented to lead to azole resistance in *C. albicans* strains (Vanden Bossche et al., 1990; Vanden Bossche et al., 1994; White, 1997b; Sanglard et al., 1998; Franz et al., 1998). To identify alterations in the *ERG11* gene that might cause fluconazole resistance, several investigators compared the sequence of the *ERG11* gene of fluconazole resistant *C. albicans* strains with the published *ERG11* sequence and that of fluconazole-susceptible strains. Using matched sets of susceptible and resistant clinical isolates of the same strain is recommended, as *C.*

albicans is mostly clonal in nature (Pujol et al., 1993), and isolates might differ considerably in their levels of expression of different genes.

In one study, a point mutation in *ERG11* was identified when an azole-resistant clinical isolate was compared with a susceptible isolate from a single strain of *C. albicans*. This point mutation (R467K) resulted in the replacement of arginine with lysine at amino acid 467 of the *ERG11* gene (White, 1997b). In another study, the point mutation T315A (the replacement of threonine [T] with alanine [A] at position 315 was constructed in the *C. albicans ERG11* gene (Lamb et al., 1997).

Mutations identified in the *ERG11* gene of azole-resistant *C. albicans* isolates were found to be clustered in three diffuse hot-spot regions, including amino acid regions 105 to 165, 266 to 287, and 405 to 488 (Marichal et al., 1999). Five mutations, located in these three hot spots, were observed in the three complete sequences, and these mutations included D116E, K128T, K143R, E266D and V437I (White et al., 2002). Several other point mutations that result in conformational changes that prevent effective binding between the azoles and cytochrome P-450 lanosterol 14α demethylase have been documented (Sanglard et al., 1998; Marichal et al., 1999; Perea et al., 2001).

Globally, the following amino acid substitutions (mutations) were found to be associated with azole resistance: F126L, G129A, Y132H, K143E, K143R, F145L, A149V, T229A, S279F, K287R, G307S, S405F, G448E, G448R, F449L, V452A, G464S, G465S, R467K, I471T, G450E, D446N, F449S (Loffler et al., 1997; White, 1997b; Sanglard et al., 1998; Franz et al., 1998; Favre et al., 1999; Kelly et al., 1999a; Kelly et al., 1999b; Asai et al., 1999; Kakeya et al., 2000; Lamb et al., 2000; Perea et al., 2001; White et al., 2002).

4.3 Study problem

Although various mutations in the *ERG11* gene have been reported to be responsible for fluconazole resistance among *C. albicans* isolates, nothing is known about the contribution of these mutations to fluconazole resistance among different clades of *C. albicans*, and whether a particular mutation will occur more in one particular clade as compared to the others. Understanding mechanisms of fluconazole resistance in different clades of *C. albicans* would contribute in the designing and development of new antifungals, and also in the selection of the appropriate antifungal at the earliest possible time when treating South African patients infected with *C. albicans*.

4.4 Aim

To investigate the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades

4.5 Objectives

h) To culture matching fluconazole resistant and susceptible isolates belonging to *C. albicans* clades I, II, III, SA, and NG
i) To extract DNA from the isolates
j) To perform polymerase chain reaction (PCR) on DNA samples
k) To sequence PCR products using a dye-terminator method
l) To compare mutations obtained in fluconazole susceptible and resistant isolates with the published sequence of the wild-type susceptible isolate
m) To compare mutations obtained between fluconazole susceptible and resistant isolates in different clades.

4.6 Materials and Methods

4.6.1 Isolates and culture conditions

Glycerol stocks of three isolates from each of previously typed *C. albicans* clades, namely, clade I, II, III, SA, and NG (Blignaut et al., 2002) with induced fluconazole resistance (MIC≥64µg/ml) were subcultured on Saboraud's Dextrose agar containing

64µg/ml of fluconazole. Their three matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were subcultured on Saboraud's Dextrose agar without fluconazole. All isolates were incubated at 35^{o}C for 48 hours. Two clinical isolates with fluconazole MIC of 1:128µg/ml (resistant) and MIC of 2µg/ml (susceptible) were included in the study as controls, and were treated the same as resistant and susceptible isolates respectively.

4.6.2 Genomic DNA Extraction

Genomic DNA was extracted from all fluconazole resistant and susceptible isolates according to a method described by Scherer and Stevens (1987). Cells were lysed by using 20µl of Zymolyase 20T (Kirin Brewery, Tokyo, Japan) followed by incubation at 37 ^{o}C for 2 hours. Proteins were removed with 50µl of 10% sodium dodecyl sulphate (SDS) and 100µg/ml proteinase K (Sigma, Germany) and incubation at 55ºC overnight. DNA was extracted with 1:1 phenol-chloroform, followed by washing with 500µl of 95% ethanol. Traces of RNA were removed with 10mg/ml RNAase (Sigma Aldrich, Germany) and incubation at 37ºC for 1 hour. After second extraction with phenol-chloroform, excess phenol was removed with 600µl chloroform. Extracted DNA was precipitated with 600µl of 100% isopropanol, washed with 95% ethanol, air-dried, resuspended in 50µl TE buffer and stored at -20^{o}C until required.

4.6.3 Polymerase Chain Reaction (PCR)

Before PCR, the absorbances of all DNA samples were measured on a Genequant spectrophotometer (Pharmacia, CA, USA) at 260nm, and DNA concentrations were determined using standard calculations (Absorbance x dilution factor x 50). The DNAs were used as templates for amplification of *ERG11* gene.

PCR was carried out with a high-fidelity *Pwo* DNA polymerase (Roche Diagnostics, Mannheim, Germany), and PCR reactions and cycling conditions suggested by the manufacturer (Roche Diagnostics, Germany) were used. PCR was performed in a total reaction volume of 100µl consisting of 0.75µg of extracted DNA, 2µl deoxynucleoside triphosphates mix (10mM each of dTTP, dCTP, dATP and dGTP), 0.3µM of each primer, 10µl of 10x PCR buffer with $MgSO_4$ (100mM Tris-HCl, 250mM KCl, 50mM $(NH_4)_2SO_4$, 20mM $MgSO_4$), 0.5µl (2.5U) of *Pwo* DNA polymerase (Roche Diagnostics,

Mannheim, Germany) and PCR grade water to make up 100µl. The primers used were 5'-GTT GAA ACT GTC ATT GAT GG-3' (Forward) and 5'-TCA GAA CAC TGA ATC GAA AG-3' (Reverse), which were amplifying a fragment of 1.6kb (Perea et al., 2001). Primers were obtained from Inqaba Biotech, Pretoria, South Africa.

PCR was performed on a PTC-100 thermocycler (MJ Research Inc, Watertown, MA, USA). PCR amplification consisted of an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 51°C for 30 seconds, and extension at 72°C for 2 minutes. Final extension consisted of one cycle at 72°C for 7 minutes. The PCR products were resolved by gel electrophoresis on a 1% gel for 1h 30 minutes at 100V, together with O'GeneRuler 100bp DNA ladder (Fermentas UAB, Lithuania, EU). To determine if the PCR products were enough for sequencing, 2µl of the PCR products were resolved by gel electrophoresis on a 1% gel for 1h 30 minutes at 100V, together with O'GeneRuler 100bp DNA ladder (Fermentas UAB, Lithuania, EU). The gel was then photographed using a gel documentation system.

Stringent laboratory procedures were implemented to avoid contamination during the procedures: A dedicated room with a laminar flow hood was used for DNA extraction. Aerosol-free, filter pipette tips and separate pipettes were used for DNA extraction and PCR set-up. For PCR assays, a non-template control was included in each run.

4.6.4 DNA Sequencing

4.6.4.1 PCR products purification

All PCR products were purified using Nucleofast PCR clean-up kit according to manufacturer's directions (Macherey-Nagel Inc., PA, USA). Briefly, PCR products were transferred directly onto the membrane on the NucleoFast 96 PCR Plate. To remove contaminants (primers, dNTPs, salts), vacuum was applied on the sample for 15 minutes. The membrane was washed by releasing the vacuum and adding 100µl nuclease-free water into each well of the NucleoFast® 96 PCR Plate, and applying vacuum until water has passed the membrane. The purified PCR samples were recovered by dispensing 25µl of nuclease-free water directly onto the membrane of the NucleoFast 96 PCR Plate, and then mixing up and down. A multichannel pipette was used to recover the purified PCR products completely from the wells.

4.6.4.2 Sequencing reactions

Cycle sequencing was performed by using BigDye® Terminator v3.1 Cycle Sequencing Kit according to manufacturer's directions (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed in a total reaction volume of 20µl consisting of 0.04µg purified PCR product, 8µl Terminator Ready Reaction Mix {**A**-Dye Terminator labeled with dichloro[R6G]; **C**-Dye Terminator labeled with dichloro[ROX]; **G**-Dye Terminator labeled with dichloro[R110]; **T**-Dye Terminator labeled with dichloro[TAMRA]; Deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP); AmpliTaq DNA Polymerase, FS, with thermally stable pyrophosphatase; $MgCl_2$; Tris-HCl buffer, pH 9.0}, 3.2 pmol of each primer and deionised water to make up 20µl volume. The following primer pairs were used: Forward: 5'-GTT GAA ACT GTC ATT GAT GG-3' and Reverse: 5'-TCA GAA CAC TGA ATC GAA AG-3'; Forward: 5' CAT TTA ACT ACT CCA GTT TTC G 3' and Reverse: 5'CCC ATA ATC AAC TTC ATC AG 3'.

Cycle sequencing was performed on the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA), and consisted of initial denaturation step at 96°C for 10 seconds, followed by 25 cycles of 96 °C for 10 sec, 50ºC for 10 seconds, 60^{o}C for 4 minutes, then holding at 4^{o}C. The sequencing products were purified by using Centri-Sep™ spin columns according to manufacturer's directions (Applied Biosystems, Foster City, CA, USA). After purification of cycle sequencing products, sequence analysis was performed on an ABI3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were then compared with the published *ERG11* sequence from a wild type, fluconazole-susceptible, *C. albicans* strain (Lai and Kirsch, 1989) by using Variant Reporter software (Applied Biosystems, Foster City, CA, USA). The obtained sequences of susceptible and resistant isolates were also compared.

4.7 Results

4.7.1 PCR

After PCR, the PCR products were resolved by gel electrophoresis on a 1% gel for 1h 30 minutes at 100V, and the expected fragment of size 1.6kb was observed. O'GeneRuler 100bp DNA ladder (Fermentas UAB, Lithuania, EU) was used as a molecular weight marker. These results are shown in Figure 4.1.

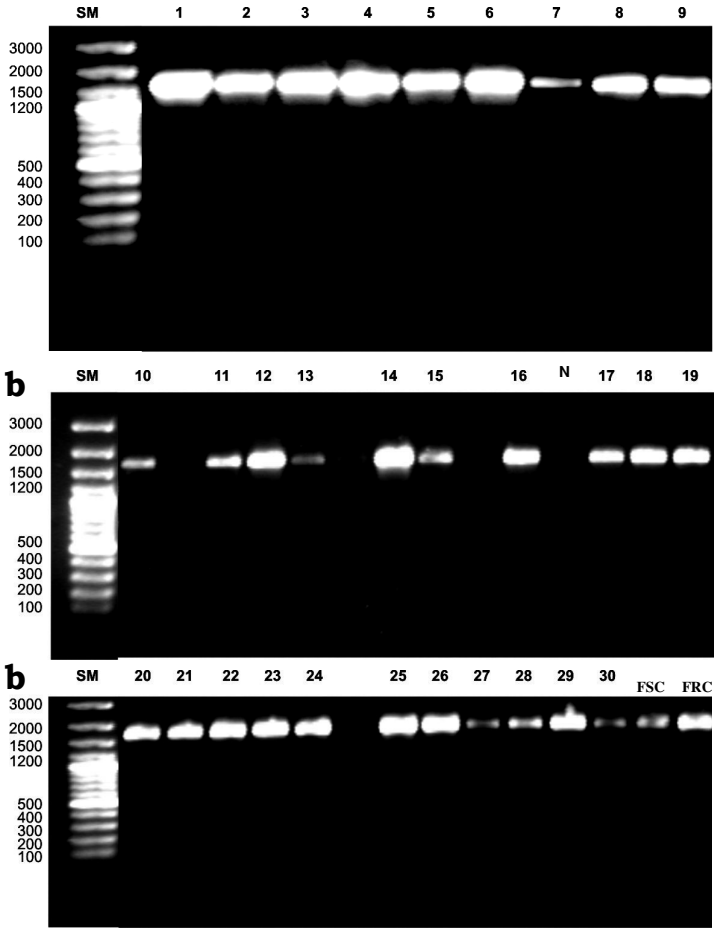

Figure 4.1: PCR results for fluconazole resistant and susceptible *C. albicans* isolates. **Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29=**Fluconazole susceptible isolates; **Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30=** Fluconazole resistant isolates as they appear in Table 4.1**; SM=** O'GeneRuler 100bp DNA ladder**; N=** Non-template control**; FSC=** Fluconazole susceptible control**; FRC=** Fluconazole resistant control

4.7.2 Sequencing results

Sequencing of *ERG11* gene was done on all PCR products. The sequences were compared to the published sequence (Lai and Kirsch, 1989), and sequences of resistant isolates were also compared with those of their matching susceptible isolates. The sequencing results are shown in Table 4.1. Published sequence is shown in Figure 4.2.

4.7.2.1 Mutations observed among fluconazole susceptible isolates compared to the published sequence

These results are summarized in Table 4.1.

4.7.2.1a Mutations among susceptible isolates from clade I

When 3 fluconazole susceptible isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G63 fluconazole susceptible (G63FS) had these homozygous mutations (point mutations in both alleles): D116E, K128T, L340C, L480W and N490T, and these silent nucleotide substitutions (Nucleotide substitutions with no change in a protein sequence) T462Y, A504G, C558T, C805T, C1257T. Isolate K239FS had no amino acid or nucleotide substitutions. Isolate K21FS had no amino acid substitutions, but had a C deletion at nucleotide base position 256.

4.7.2.1b Mutations among susceptible isolates from clade II

When 3 fluconazole susceptible isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate K162FS had these homozygous mutations: V332S, F380S, Y401T, V437F, V437I, L480W and these silent nucleotide substitutions: C558T, T696C, C805T, C1257T, T1404C. Isolate G19FS had these homozygous mutations: E266D, E266T, V332S, K342R, Y401T, A432L, A434P, V488F, and these silent nucleotide substitutions: T462Y, C558Y, T696Y, C805T, C1257T. Isolate K153FS had these homozygous mutations: I333S, R371L, R381S, V402T, E420P, V437L, V437I, and G481W, and a deletion of T at base position 851 and 877 and a deletion of A at position 861.

4.7.2.1c Mutations among susceptible isolates from clade III

When 3 fluconazole susceptible isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate OKP25FS had these heterozygous mutations (point mutations in one allele): D116 (E,D), E266 (E,D), E266 (N,T), V332 (S,L), L340 (Y,C), F380 (S,L), Y401 (T,M), V437 (I, V), N490 (T,I), D443 (M,N), and these homozygous mutations: V437F and L480W, and these silent nucleotide substitutions: T462Y, A504R, C558T, T696Y, C805T and C1257T. Isolate UP30FS had these heterozygous mutations: M189 (I, M), V332 (S, L), L370 (S, L), Y401 (T, M), P419 (P, L) and nonsense mutation (stop codon mutation) L480 (*, W), and these homozygous mutations: V437I and V437F, P419R, F380Q, and silent nucleotide substitution C558T. Isolate G58FS had these heterozygous mutations: K128 (K, T), M189 (I, M), and homozygous mutation L340N, and silent nucleotide substitutions T462Y, A504R, C558Y, C805Y and C1257Y.

4.7.2.1d Mutations among susceptible isolates from clade SA

When 3 fluconazole susceptible isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G116FS had these homozygous mutations: L220I, L220Y, V332S, S361R, Y401T, A432L, A434P, H183T, and these silent nucleotide substitutions: C1257T, T1350C. Isolate G118FS had these homozygous mutations: L340I, L340C, L480I, L480W, N490T and nonsense mutation N490*, and no silent nucleotide substitutions. Isolate K306FS had these homozygous mutations: V437I, H183T, V332S, F380S, S137L, L220Y, Y401T, and these silent nucleotide substitutions: T1404C, C1257T, A1587R.

4.7.2.1e Mutations among susceptible isolates from clade NG

When 3 fluconazole susceptible isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G22FS had these homozygous mutations V332S, F380S, Y401T, V437F, V437I, L480W and the silent nucleotide substitutions: C363T, T462Y, C805T, C1257T. Isolate G6FS had these homozygous mutations: E266T, V332S, K342R, Y401T, A432L, A434P, V488F, S137L, H183T, L220Y, E266D, and the following silent nucleotide substitutions: T462Y and C1257T. Isolate K86FS had these homozygous mutations: L220Y, V332H, Y401R, V437I, nonsense mutation L370*, and the following silent nucleotide substitutions: C363T, T1287C and A1587G.

4.7.2.1f Mutations in a fluconazole susceptible control isolate

No mutations were observed in a fluconazole susceptible control (FSC) isolate used in the study.

4.7.2.2 Mutations observed among fluconazole resistant isolates compared to the published sequence

These results are summarized in Table 4.1

4.7.2.2a Mutations among resistant isolates from clade I

When 3 fluconazole resistant isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G63 fluconazole resistant (G63FR) had no nucleotide and no amino acid substitutions. Isolate K239FR had no amino acid or nucleotide substitutions. Isolate K21FR had no nucleotide or amino acid substitutions.

4.7.2.2b Mutations among resistant isolates from clade II

When 3 fluconazole resistant isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate K162FR had these homozygous mutations: V332S, F380S, Y401T, V437F, V437I, L480W and these silent nucleotide substitutions: C558T, T696C, C805T, C1257T, T1404C. Isolate G19FR had these homozygous mutations: E266D, E266T, V332S, K342R, Y401T, A432L, A434P, V488F, and these silent nucleotide substitutions: T462Y, C558Y, T696Y, C805T, C1257T. Isolate K153FR had these homozygous mutations: I333S, R371L, R381S, V402T, E420P, V437L, V437I, G481W, and these silent nucleotide substitutions: C558T and T696C.

4.7.2.2c Mutations among resistant isolates from clade III

When 3 fluconazole resistant isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolates OKP25FR had these heterozygous mutations: D116 (E,D), E266 (E,D), E266 (N,T), V332 (S,L), L340 (Y,C), F380 (S,L), Y401 (T,M), V437 (I, V), N490 (T,I), D443 (M,N), these homozygous mutations:V437F and L480W, and these silent nucleotide substitutions: T462Y, A504R, C558T, T696Y, C805Tand C1257T. Isolate UP30FR had these heterozygous mutations: L340 (Y, C), K342 (K, R), M189 (I, M), V332 (S, L), L370 (S, L), Y401 (T, M), P419 (P, L) and L480 (*, W), and these homozygous mutations: V437I, V437F, D116E, K128T, E266D, and silent nucleotide substitutions T462Y and C558T. Isolate G58FR had these heterozygous mutations: K128 (K,T), M189 (I,M), and homozygous mutation L340N, and silent nucleotide substitutions T462Y, A504R, C558Y, C805Y, C1257Y, A1587R and T1617Y.

4.7.2.2d Mutations among resistant isolates from clade SA

When 3 fluconazole resistant isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G116FR had these homozygous mutations: L220I, L220Y, V332S, S361R, Y401T, A432L, A434P, H183T, and these silent nucleotide substitutions: C1257T, T1350C. Isolate G118FR had these mutations: L340I, L340C, L480I, L480W, N490T and nonsense mutation N490*, and no silent nucleotide substitutions. Isolate K306FR had these homozygous mutations: D116E and K128T, and homozygous mutations: S137L and L220Y, and these silent nucleotide substitutions T462Y, A504R, A1167R, T1617Y, C1257T and A1587R.

4.7.2.2e Mutations among resistant isolates from clade NG

When 3 fluconazole resistant isolates from this clade were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989), the following results were observed:

Isolate G22FR had these homozygous mutations: V332S, F380S, Y401T, V437F, V437I, L480W and the nucleotide substitutions: C363T, T462Y, C558T, C805T, C1257T. Isolate G6FR had these homozygous mutations: E266T, V332S, K342R, Y401T, A432L, A434P, V488F, S137L, H183T, L220Y, E266D, and the following silent nucleotide substitutions: T462Y and C1257T. Isolate K86FR had these homozygous mutations: L220Y, V332H, Y401R, E266P, K342G, A434Q and V488L, and nonsense mutations L370* and A432*.

4.7.2.2f Mutations in a fluconazole resistant control isolate

Mutations observed in a fluconazole resistant control (FRC) isolate used in the study were: D116E, K128T, K342R and A434P.

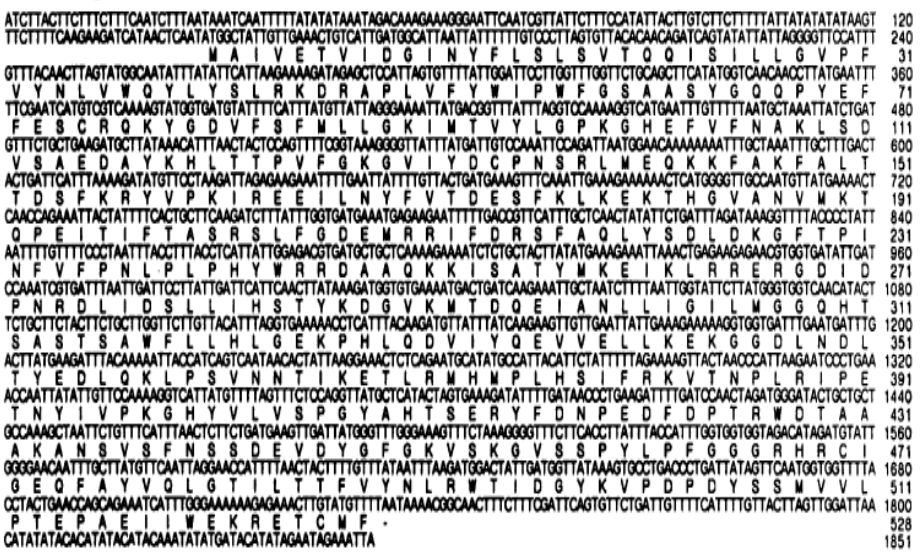

Figure 4.2: *ERG11* published sequence. Adapted from Lai and Kirsch (1989)

Table 4.1: Amino acid (Point mutations) and nucleotide substitutions in *C. albicans ERG11* gene of fluconazole-resistant and susceptible isolates in comparison to the published sequence

ISOLATE	CLADE	MIC	AMINO ACID SUBSTITUTIONS (POINT MUTATIONS)	NUCLEOTIDE SUBSTITUTIONS WITH NO CHANGE IN PROTEIN SEQUENCE(SILENT MUTATIONS)
1. G63FS	**I**	0.25	D116E, K128T, L340C, L480W, N490T	T462Y, A504G, C558T, C805T, C1257T
2. G63FR	**I**	1:128	NONE	NONE
3. K239FS	**I**	0.38	NONE	NONE
4. K239FR	**I**	1:256	NONE	NONE
5. K21FS	**I**	0.19	NONE, C del at 259	NONE
6. K21FR	**I**	≥1:256	NONE	NONE
7. K162FS	**II**	0.19	V332S, F380S, Y401T, V437F, V437I, L480W	C558T, T696C, C805T, C1257T, T1404C
8. K162FR	**II**	1:128	V332S, F380S, Y401T, V437F, V437I, L480W	C558T, T696C, C805T, C1257T, T1404C
9. G19FS	**II**	0.125	E266T, E266D, V332S, K342R, Y401T, A432L, A434P, V488F	T462Y, C558Y, T696Y, C805T, C1257T
10. G19FR	**II**	1:128	E266T, E266D, V332S, K342R, Y401T, A432L, A434P, V488F	T462Y, C558Y, T696Y, C805T, C1257T
11. K153FS	**II**	0.75	I333S, R371L, R381S, V402T, E420P, V437L, V437I, G481W. Deletion of T base position 851 and 877, a deletion of A at position 861	NONE
12. K153FR	**II**	≥1:256	I333S, R371L, R381S, V402T, E420P, V437L, V437I, G481W	C558T, T696C
13. OKP25FS	**III**	0.125	D116 (E,D), E266 (E,D), E266 (N,T), V332 (S,L), L340 (Y,C), F380 (S,L), Y401 (T,M), V437 (I,V), N490 (T,I), D443 (M,N), V437F, L480W	T462Y, A504R, C558T, T696Y, C805T, C1257T
14. OKP25FR	**III**	1:128	D116 (E,D), E266 (E,D), E266 (N,T), V332 (S,L), L340 (Y,C), F380 (S,L), Y401 (T,M), V437 (I,V), N490 (T,I), D443 (M,N), V437F, L480W	T462Y, A504R, C558T, T696Y, C805T, C1257T
15. UP30FS	**III**	0.19	M189 (I,M), V332 (S,L), L370 (S,L), Y401 (T,M), P419 (P,L), L480 (*,W), V437I, V437F, P419R, F380Q	C558T
16. UP30FR	**III**	≥1:256	**L340 (Y,C), K342 (K,R)**, M189 (I,M), V332 (S,L), L370 (S,L), Y401 (T,M), P419 (P,L), L480 (*,W), V437I,	T462Y, C558T

			V437F, **D116E, K128T, E266D**	
17. G58FS	**III**	0.25	K128 (K,T), M189 (I,M), L340N	T462Y, A504R, C558Y, C805Y, C1257Y
18. G58FR	**III**	≥1:256	K128 (K,T), M189 (I,M), L340N	T462Y, A504R, C558Y, C805Y, C1257Y, A1587R, T1617Y.
19. G116FS	**SA**	0.5	L220I, L220Y, V332S, S361R, Y401T, A432L, A434P, H183T	C1257T , T1350C
20. G116FR	**SA**	≥1:256	L220I, L220Y, V332S, S361R, Y401T, A432L, A434P, H183T	C1257T , T1350C
21. G118FS	**SA**	1	L340I, L340C, L480I, L480W, N490T, N490*.	NONE
22. G118FR	**SA**	≥1:256	L340I, L340C, L480I, L480W, N490T, N490*.	NONE
23. K306FS	**SA**	0.75	V437I, H183T, V332S, F380S, Y401T, S137L, L220Y	T1404C, C1257T, A1587R
24. K306FR	**SA**	≥1:256	**D116E, K128T**, S137L, L220Y	T462Y, A504R, A1167R, T1617Y, C1257T, A1587R
25. G22FS	**NG**	0.25	V332S, F380S, Y401T, V437F, V437I, L480W	C363T, T462Y, C558T, C805T, C1257T
26. G22FR	**NG**	≥1:256	V332S, F380S, Y401T, V437F, V437I, L480W	C363T, T462Y, C558T, C805T, C1257T
27. G6FS	**NG**	0.38	E266T, V332S, K342R, Y401T, A432L, A434P, V488F, S137L, H183T, L220Y, E266D	T462Y, C1257T
28. G6FR	**NG**	1:128	E266T, V332S, K342R, Y401T, A432L, A434P, V488F, S137L, H183T, L220Y, E266D	T462Y, C1257T
29. K86FS	**NG**	0.125	L220Y, V332H, L370*, Y401R, V437I	C363T, T1287C, A1587G
30. K86FR	**NG**	≥1:256	L220Y, V332H, L370*, Y401R, **E266P, K342G, A432*, A434Q, V488L**	T462Y, C558Y, T696Y
FRC		1:128	D116E, K128T, Y401T, A432L	T462Y, A504R, C1257T
FSC		2	NONE	NONE

FS=Fluconazole susceptible isolate; **FR**=Fluconazole resistant isolate; **FRC**=Fluconazole resistant control; **FSC=** Fluconazole susceptible control. **Bold mutations** are those observed only in fluconazole resistant isolates, and associated with resistance in thisstudy. "Y" indicates a C or a T; R = A or G; W = A or T; M = A or C; S = G or C; K = T or G. *indicates a stop codon (Nonsense mutation).

4.7.2.3 Comparison between mutations observed among fluconazole susceptible and resistant isolates from different *C. albicans* clades.

These results are shown in Table 4.1

4.7.2.3a Mutations observed in isolates from Clade I

When 6 matching *C. albicans* isolates from clade I were compared with each other and the published sequence (Lai and Kirsch, 1989), isolate G63 fluconazole susceptible (G63FS) had these homozygous mutations (point mutations in both alleles): D116E, K128T, L340C, L480W and N490T, and these silent nucleotide substitutions: T462Y, A504G, C558T, C805T, C1257T, while its matching resistant isolate (G63FR) had no nucleotide and no amino acid substitutions.

Matching isolates K239FS and K239FR had no amino acid or nucleotide substitutions when compared to the published sequence. Isolate K21FS had no amino acid substitution, but had a C deletion at nucleotide base position 256, while isolate K21FR had no nucleotide or amino acid substitutions.

In isolates from clade I, no mutations associated with fluconazole resistance were observed among all 3 fluconazole resistant isolates.

4.7.2.3b Mutations observed in isolates from Clade II

When 6 matching isolates from clade II were compared with each other and the published sequence, both matching isolates K162FS and K162FR shared these homozygous mutations: V332S, F380S, Y401T, V437F, V437I, L480W and these nucleotide substitutions: C558T, T696C, C805T, C1257T, T1404C.

Both matching isolates G19FS and G19FR shared these homozygous mutations: E266D, E266T, V332S, K342R, Y401T, A432L, A434P, V488F, and these silent nucleotide substitutions: T462Y, C558Y, T696Y, C805T, C1257T. Both matching isolates K153FS and K153FR shared these homozygous mutations: I333S, R371L, R381S, V402T, E420P, V437L, V437I, and G481W. Isolate K153FS had the following nucleotide changes when compared to K153FR and the published sequence: a deletion of T at base position 851 and 877 and a deletion of A at position 861. Isolate K153FR had these silent

nucleotide substitutions when compared to the matching susceptible isolate and the published sequence: C558T and T696C.

In isolates from clade II, no mutations associated with fluconazole resistance were observed among all 3 fluconazole resistant isolates, as the same mutations occurred in matching resistant and susceptible isolates.

4.7.2.3c Mutations observed in isolates from Clade III

When 6 matching isolates from clade III were compared with each other and the published sequence, both matching isolates OKP25FS and OKP25FR shared the following heterozygous mutations: D116 (E,D), E266 (E,D), E266 (N,T), V332 (S,L), L340 (Y,C), F380 (S,L), Y401 (T,M), V437 (I, V), N490 (T,I), D443 (M,N), these homozygous mutations: V437F and L480W, and these silent nucleotide substitutions: T462Y, A504R, C558T, T696Y, C805Tand C1257T.

Isolate UP30FR had these heterozygous mutations when compared to UP30FS and the published sequence: L340 (Y, C), K342 (K, R), and silent nucleotide substitution T462Y, and homozygous mutations: D116E, K128T and E266D. Isolates UP30FS and UP30FR shared these heterozygous mutations: M189 (I, M), V332 (S, L), L370 (S, L), Y401 (T, M), P419 (P, L), a nonsense mutation L480 (*, W), homozygous mutations: V437I and V437F, and nucleotide substitution C558T. Isolate UP30FS had these silent mutations when compared with UP30FR and the published sequence: P419R, F380Q.

Matching isolates G58FS and G58FR shared these heterozygous mutations: K128 (K,T), M189 (I,M), and homozygous mutation L340N, and nucleotide substitutions T462Y, A504R, C558Y, C805Y and C1257Y. Isolate G58FR had these nucleotide substitutions when compared to G58FS and published sequence: A1587R and T1617Y.

In isolates from clade III, mutations were associated with fluconazole resistance in 1 (33%) isolate of the resistant isolates tested, which is isolate UP30FR, with mutations L340 (Y, C), K342 (K, R), D116E, K128T and E266D.

4.7.2.3d Mutations observed in isolates from Clade SA

When 6 matching isolates from clade SA were compared with each other and the published sequence, both matching isolates G116FS and G116FR shared the following homozygous mutations: L220I, L220Y, V332S, S361R, Y401T, A432L, A434P, H183T, and these silent nucleotide substitutions: C1257T, T1350C. Both matching isolates G118FR and G118FS shared the following homozygous mutations: L340I, L340C, L480I, L480W, N490T, and a nonsense mutation N490*.

Isolate K306FR had these mutations: D116E and K128T, and nucleotide substitutions T462Y, A504R, A1167R, T1617Y when compared to K306FS and the published sequence. Isolate K306FS had these homozygous mutations when compared to K306FR and the published sequence: V437I, H183T, V332S, F380S and Y401T, and these nucleotide substitutions: T1404C. Isolates K306FS and K306FR shared the following homozygous mutations: S137L and L220Y, and nucleotide substitutions C1257T and A1587R when compared with the published sequence.

In isolates from clade SA, mutations were associated with fluconazole resistance in 1 (33%) isolate of the resistant isolates tested, which is isolate K306FR with mutations D116E and K128T.

4.7.2.3e Mutations observed in isolates from Clade NG

When 6 matching isolates from clade NG were compared with each other and the published sequence, both matching isolates G22FS and G22FR shared the following homozygous mutations V332S, F380S, Y401T, V437F, V437I, L480W and the silent nucleotide substitutions: C363T, T462Y, C805T, C1257T.

Matching isolates G6FS and G6FR shared these homozygous mutations: E266T, V332S, K342R, Y401T, A432L, A434P, V488F, S137L, H183T, L220Y, E266D, and the following silent nucleotide substitutions: T462Y and C1257T.

Matching isolates K86FS and K86FR shared the following homozygous mutations: L220Y, V332H, Y401R, and a nonsense mutation L370*. Isolate K86FS had the following mutations when compared to K86FR and published sequence: V437I, and the following silent nucleotide substitutions: C363T, T1287C and A1587G. Isolate K86 FR had the following mutations when compared to K86FS and published sequence: E266P, K342G, A434Q, V488L and a nonsense mutation A432*.

In isolates from clade NG, mutations were associated with fluconazole resistance in 1 (33%) isolate of the resistant isolates tested, which is isolate K86 FR with mutations E266P, K342G, A434Q, V488L, and a nonsense mutation A432*. The results of fluconazole resistant isolates with mutations among various clades are shown in Figure 4.3.

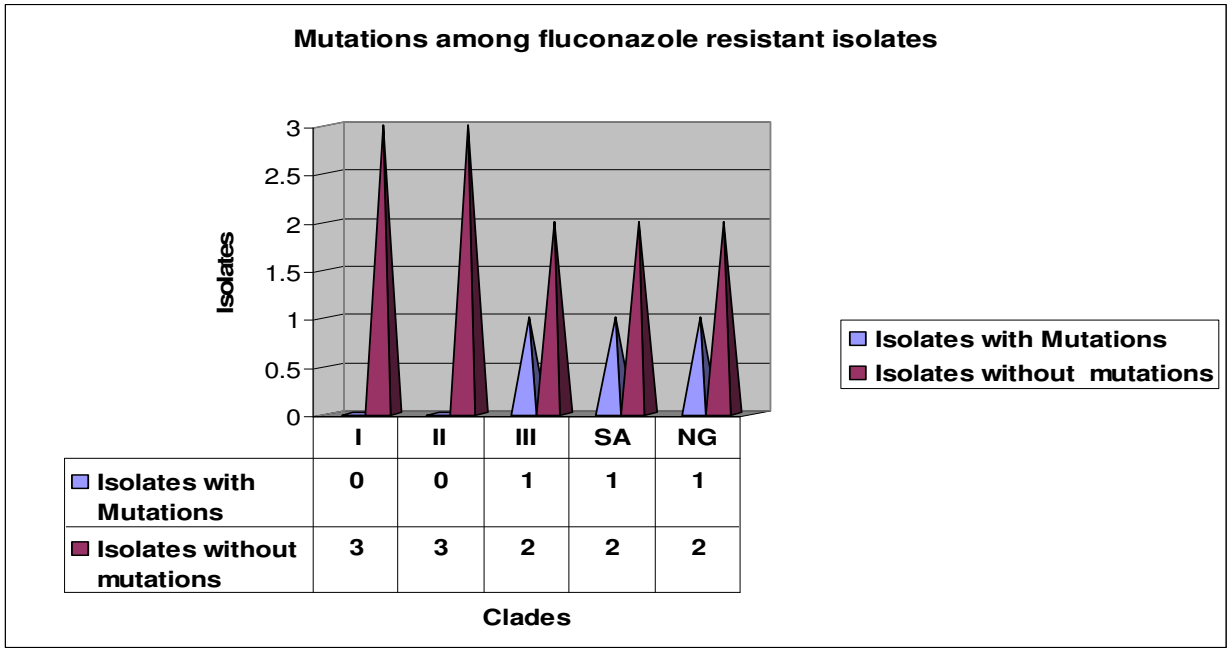

Figure 4.3: Fluconazole resistant isolates with mutations among various *C. albicans* clades

4.7.2.3f Summary of all mutations observed among *C. albicans* fluconazole resistant and susceptible isolates from all clades

When total mutations among all the clades in this study were reviewed, the following results were observed:

In matching isolates from clade I, 5 mutations were observed, of which 2 were previously described (D116E, K128T), and 3 were novel. In matching isolates from clade II, 18 muations were identified, of which 3 were previously described (V488F, V437I, E266D), and 15 were novel. In matching isolates from clade III, 21 mutations were identified, of which 4 were previously described (D116E, E266D, V437I, K128T), and 16 mutations and 1 nonsense mutation were novel. In matching isolates from clade SA, 18 amino acid substitutions were identified, of which 3 were previously described (V437I, D116 (E,D, K128(K,T), and 14 mutations and 1 nonsense mutation were novel. In matching isolates from clade NG, 25 amino acid substitutions were identified, of which 3 were previously described (E266D, V488F, V437I), and 21 mutations and 1 nonsense mutation were novel. However, most of the novel mutations occurred in more than 1 clade, therefore in total, 50 novel mutations were observed in this study. Heterozygous mutations occurred mostly in isolates from clade III, and nonsense mutations in isolates from clades III, SA and NG. These results are shown in Figure 4.4.

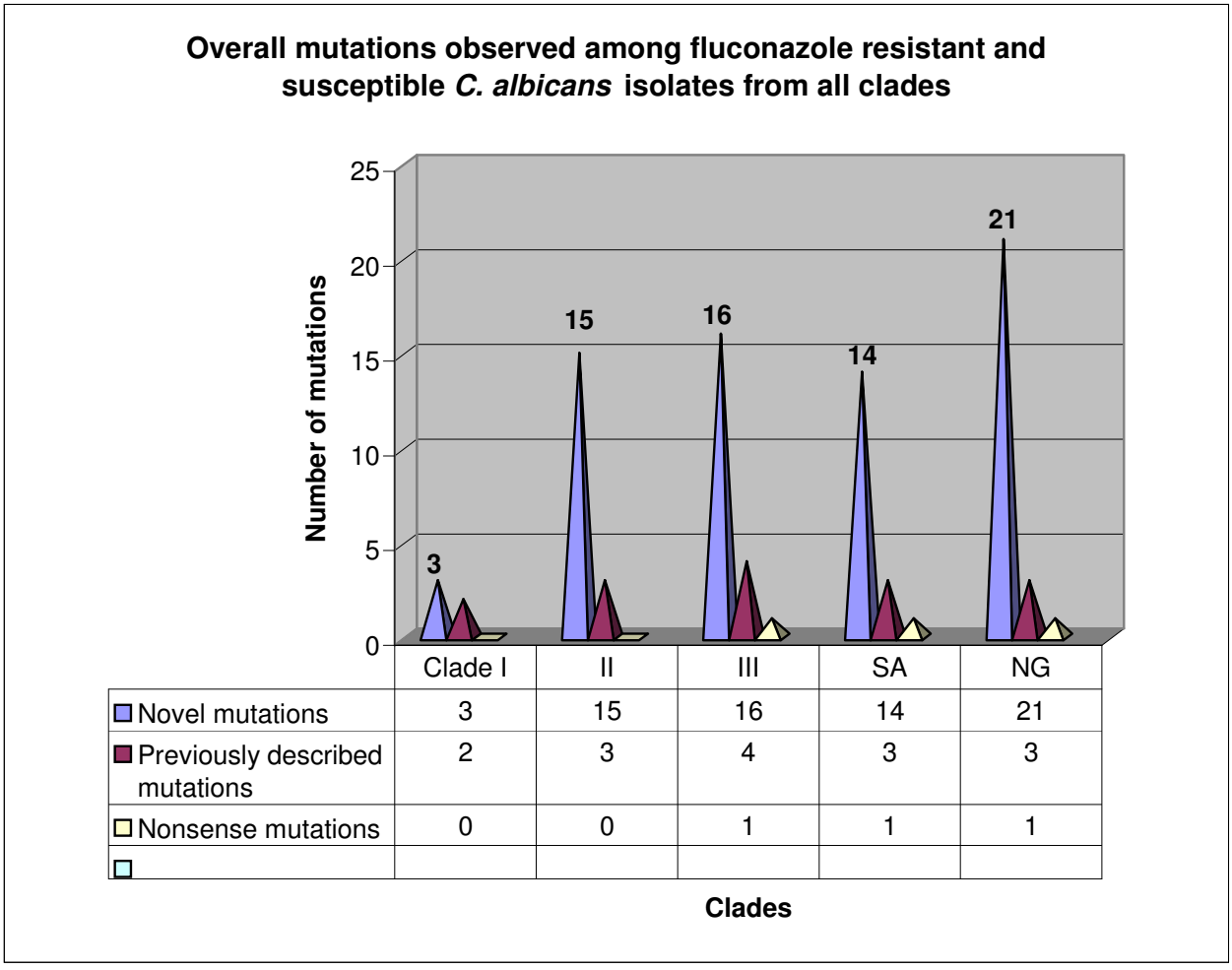

Figure 4.4: Summary of mutations observed among *C. albicans* fluconazole resistant and susceptible isolates from all clades

4.8 Discussion

Fluconazole has been found to be active against *C. albicans in vitro* and *in vivo* (Rogers and Galgiani, 1986), and has become the drug of choice for the treatment of OPC. It is the most widely used azole for systemic candidiasis due to its high solubility, low toxicity, and wide tissue distribution (Lortholary and Dupont, 1997). The increased use of azoles in recent years in HIV positive patients has resulted in high increase of azole-resistance among strains of *C. albicans* (Lopez-Ribot et al., 1998; Pfaller et al., 1994; Redding et al., 1994; White et al., 1998).

Although various mutations in the *ERG11* gene have been reported to be responsible for fluconazole resistance among *C. albicans* isolates, nothing is known about the contribution of these mutations to fluconazole resistance among different clades of *C. albicans*, and whether a particular mutation will occur more in one particular clade as compared to the others.

Understanding this mechanism of fluconazole resistance in different clades of *C. albicans* would contribute in the designing and development of new antifungals, and also in the selection of the appropriate antifungal agent in the earliest possible time when treating South African patients infected with *C. albicans*. This study was done to determine whether the induced resistance to fluconazole in *C. albicans* clades was due to point mutations in the *ERG11* gene, and whether these mutations were clade-related.

To our knowledge, this study is the first to be performed where contribution of mutations to fluconazole resistance was determined among different clades of *C. albicans*, therefore the results of this study can only be compared with studies done on *C. albicans* strains which were not genotyped into different clades.

In this study, 3 isolates from each of previously typed *C. albicans* clades I, II, III, SA and NG with induced resistance to fluconazole, together with their 3 matching susceptible

isolates, were tested for point mutations in the *ERG11* gene. DNA was extracted from the isolates, PCR was performed and PCR products were sequenced. Obtained sequences were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989).

The results of the study show that in isolates from clade I, mutations were not associated with fluconazole resistance in all 3 resistant isolates tested. Mutations D116E, K128T, L340C, L480W and N490T were observed in one fluconazole-susceptible isolate (G63FS), but not in its matching resistant isolate.

The occurrence of mutation D116E in a susceptible isolate G63FS from clade I in our study is in agreement with the results obtained by previous several investigators, where this mutation was observed in fluconazole susceptible isolates. In a study by Marr et al. (1998), mutation D116E was observed in a fluconazole susceptible clinical isolate after sequencing and comparison with the published sequence. Sanglard et al. (1998) sequenced fluconazole susceptible and resistant isolates, and this mutation was observed in 4 of the fluconazole susceptible isolates with MICs varying from 0.25 to 2µg/ml. In a study of matched resistant and susceptible isolates from HIV-infected patients, Perea et al. (2001) observed this mutation in matching susceptible and resistant isolates. These investigators concluded that the mutation was not responsible for resistance in these isolates. Park and Perlin (2005) sequenced clinical isolates displaying a range of fluconazole susceptibilities. In their study, mutation D116E was observed in a fluconazole susceptible isolate from a patient with OPC.

The occurrence of mutation K128T in a fluconazole susceptible isolate in our study is in agreement with the results obtained in previous studies. In a study by Sanglard et al. (1998), this mutation was found in a fluconazole isolate with MIC 1µg/ml. Kallakuri et al. (1996) observed mutation K128T in a fluconazole susceptible isolate investigated in their study. In a study by Loeffler et al. (1997), when *C. albicans* strains were sequenced, mutation K128T was observed in a fluconazole susceptible isolate. Perea et al. (2001) sequenced isolates from HIV-infected patients, and this mutation was observed in a

fluconazole susceptible isolate. Park and Perlin (2005) investigated a collection of 59 clinical isolates, where this mutation was observed in 2 fluconazole susceptible isolates from the oropharynx.

In our study, mutations D116E and K128T were observed only in a susceptible isolate from clade I, but not in its matching resistant isolate, therefore these mutations do not account for fluconazole resistance in this isolate. These mutations are in the first hotspot 105 to 165 as previously described (White, 1997b; Sanglard et al., 1998; Marichal et al., 1999; Manavathu et al., 1999).

In addition to the 2 discussed mutations, novel mutations L340C, L480W and N490T were also observed in this susceptible isolate. Mutation L480W is within the third hotspot as previously described (Marichal et al., 1999), while mutations L340C and N490T are outside the previously described hotspots. The presence of these novel mutations may suggest that the clinical environment in South Africa can select novel mutations in *C. albicans ERG11* gene, and these mutations need to be further characterized to determine whether they may mediate fluconazole resistance in other *C. albicans* isolates.

The results of this study show that no mutations associated with fluconazole resistance were observed in all 3 fluconazole resistant isolates from clade I. The absence of mutations in these resistant isolates is in agreement with the results obtained in several previous studies. In a study by Perea et al. (1998), no mutations were observed in 3 fluconazole resistant isolates in that study. In a study by Favre et al. (1999), mutations contributed to fluconazole resistance in only 20% of resistant isolates, and these investigators concluded that mutations were not the sole mechanism of fluconazole resistance in their study. Goldman et al. (2004) found no mutations in 2 fluconazole resistant clinical isolates obtained from AIDS patients. In a study by Pancholi et al. (2004), no mutations were observed in a clinical isolate which developed fluconazole resistance after exposure to fluconazole.

The absence of mutations in these resistant isolates belonging to clade I could be due to the fact that fluconazole resistance in these isolates could have been caused by over-expression of *ERG11* gene or drug efflux genes such as *MDR1, CDR1* or *CDR2.* Resistance could have also been caused by uncharacterized mechanisms as suggested by Sanglard et al. (1998). The absence of mutations in these resistant isolates could not have been caused by growing these isolates in the absence of fluconazole, as these isolates were grown in the presence of fluconazole all the time to ensure that they did not lose their resistance.

The results of the study show that in isolates from clade II, mutations were not associated with resistance in all 3 resistant isolates tested, as similar mutations were observed in matching isolates, therefore the observed mutations were not responsible for resistance in isolates from this clade.

Mutations observed in these isolates included E266D, E266T, V332S, K342R, Y401T, A432L, A434P, L480W, V488F, I333S, R371L, R381S, F380S, V402T, E420P, V437L, V437I, V437F and G481W. Mutations E266D and V437I have previously been observed in both fluconazole resistant and susceptible isolates, while mutations E266T, I333S, R371L, R381S, V332S, Y401T, V402T, E420P, A432L, A434P, V437L, V437F, L480W, G481W and V488F were novel.

The occurrence of mutation E266D in both fluconazole resistant and susceptible isolates in our study is in agreement with the results observed in previous studies, although most of these studies did not use matched isolates. In a study by Kallakuri et al. (1996), a fluconazole-resistant clinical isolate of *C. albicans* was characterized, and mutation 266D was observed in this isolate. Loeffler et al. (1997) observed this mutation in 4 fluconazole resistant isolates when they were characterized. In a study by Ryder and Favre (1997), mutation E266D was observed in a fluconazole resistant isolate characterized. In a study by Franz et al. (1998), fluconazole susceptible isolates from AIDS patients with OPC were characterized, and this mutation was observed in these isolates. In their study, Marichal et al. (1999) observed mutation E266D in 2 fluconazole susceptible *C. Albicans*

strains from AIDS patients. Manavathu et al. (1999) sequenced clinical isolates from AIDS patients, and E266D was observed in 2 resistant isolates in that study. In a study by Favre et al. (1999), 4 selected fluconazole-resistant clinical isolates were sequenced and compared with the sequence from a sensitive strain SC5314. In that study, mutation E266D was observed in 1 of these resistant isolates.

When a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans* were analyzed in a study by White et al. (2002), mutation E266D was observed in 1 of the resistant isolates in that study. Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates. Mutation E266D was observed in 1 of the fluconazole resistant isolates in that study. When Lee et al. (2004) sequenced clinical isolates from patients infected with HIV, mutation E266D mutation was observed in 2 resistant isolates in that study.

The finding of mutation V437I in both resistant and susceptible isolates in our study is in agreement with the results observed in previous studies. In a study by Ryder and Favre (1997), mutation V437I was observed in a fluconazole susceptible isolate characterized. Sanglard et al. (1998) observed this mutation in a fluconazole susceptible clinical isolate. When fluconazole resistant *C. albicans* isolates obtained from five HIV-infected patients with recurrent OPC were analyzed, Lopez-Ribot et al. (1998) observed mutation V437I in 2 of the resistant isolates. These investigators suggested that this mutation was not responsible for fluconazole resistance in these isolates, as they also detected this mutation in fluconazole susceptible isolates.

In a study of matched resistant and susceptible isolates from HIV-infected patients, Perea et al. (2001) observed mutation V437I in 2 matching susceptible and resistant isolates. These investigators concluded that this mutation was not responsible for resistance in these isolates. In their study, White et al. (2002) analyzed a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans,* and mutation V437I was observed in 2 of the resistant isolates in that study. Lee et al. (2004) analyzed 12

resistant and susceptible clinical isolates for *ERG11* mutations by DNA sequencing. In that study, mutation V437I was observed in 2 of the resistant isolates.

When Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates, mutation V437I was observed in 1 resistant isolate from that study. Park and Perlin (2005) evaluated a diverse collection of 59 clinical isolates of *C. albicans* consisting of fluconazole susceptible, dose-dependent, and resistant phenotypes by DNA sequence analysis of *ERG11* gene. In that study, mutation V437I was observed in 3 resistant and 2 susceptible isolates. These investigators concluded that this mutation was not responsible for resistance as it was also found in susceptible isolates.

Mutations E266T, I333S, R371L, R381S, V332S, Y401T, V402T, E420P, A432L, A434P, V437L, V437F, L480W, G481W and V488F observed in isolates from clade II were novel, and have never been described previously. The presence of these new mutations may suggest that the clinical environment in South Africa can select novel mutations in *C. albicans ERG11* gene, and these mutations need to be further characterized to determine whether they may mediate fluconazole resistance in other *C. albicans* isolates. Mutations E266D and E266T are within the second hotspot 266 to 287, while mutations E420P, A432R, A434P, V437I, V437L, V437F, L480W, G481W and V488F are in the third hot spot, 405 to 488, which was previously described (Marichal et al., 1999). However, mutations I333S, R371L, R381S, V332S, K342R, Y401T, V402T are outside the previously described hotspots, and the observation of these novel mutations outside the previously described hotspots may warrant further study and probably a review of the hotspots, especially among South African isolates from clade II.

However, mutation V488I, which is similar to V488F, has previously been identified in fluconazole resistant isolates in a study by Loeffler et al. (1997). Goldman et al. (2004) also detected mutation V488I in a susceptible isolate in their study. Mutation E266D, which is similar to E266T has been previously observed in fluconazole resistant isolates

from studies as has been discussed earlier (Kallakuri et al., 1996; Ryder and Favre, 1997; Manavathu et al., 1999; Favre et al., 1999; Goldman et al., 2004; Lee et al., 2004). This mutation was also observed in fluconazole susceptible isolates in a study by Franz et al. (1998).

The occurrence of mutations in both matching susceptible and resistant isolates from this clade shows that these mutations were not responsible for fluconazole resistance in these isolates. Resistance in isolates from this clade could have been due to over-expression of *ERG11* gene or drug efflux pumps genes *CDR1, CDR2* or *MDR1,* or some uncharacterized mechanisms as suggested by Sanglard et al. (1998).

The results of the study show that in isolates from clade III, mutations were associated with fluconazole resistance in 1(33.3%) of the 3 resistant isolates tested. Mutations were not associated with resistance in 2 remaining isolates, as similar mutations were observed in these matching isolates. Mutations associated with resistance were D116E, K128T, L340 (Y, C), K342 (K, R) and E266D, which were observed in one resistant isolate (UP30FR), but not in its matching susceptible isolate (UP30FS). The occurrence of mutations D116E and K128T in a fluconazole-resistant isolate (UP30FR) from clade III is in agreement with the results observed in other studies, where these mutations were found simultaneously in fluconazole resistant isolates. In a study by Kallakuri et al. (1996), mutations D116E and K128T were observed in the same resistant isolate. In their study, Marichal et al. (1999) observed mutations K128T and D116E in the same 3 fluconazole resitant *C. albicans* strains from AIDS patients. In a study by Asai et al. (1999), when 2 clinical strains were characterized, these 2 mutations were observed simultaneously in these isolates**.** Manavathu et al. (1999) studied six clinical isolates of *C. albicans* with high level of resistance to fluconazole. Mutations D116E and E266D were detected in the same resistant isolate in that study.

The occurrence of mutation E266D in resistant isolate UP30FR is in agreement with previous studies, where this mutation was observed in fluconazole resistant isolates. In a study by Kallakuri et al. (1996), mutation E266D was observed in a resistant isolate when

it was characterized. Loeffler et al. (1997) characterized fluconazole resistant isolates, and mutation E266D was observed in 4 of the resistant isolates. In a study by Ryder and Favre (1997), mutation E266D was observed in a fluconazole resistant isolate characterized. In their study, Manavathu et al. (1999) investigated six clinical isolates of *C. albicans* with high level of resistance to fluconazole, and mutation E266D was observed in 2 of the isolates. In a study by Favre et al. (1999), 4 selected fluconazole-resistant clinical isolates were sequenced and compared with the sequence from a sensitive strain SC5314. In that study, mutation E266D was observed in 1 of these resistant isolates. When a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans* were analyzed in a study by White et al. (2002), mutation E266D was observed in 1 of the resistant isolates in that study. In their study, Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates. Mutation E266D was observed in 1 resistant isolate from that study. Lee et al. (2004) analyzed 12 resistant and susceptible clinical isolates for *ERG11* mutations by DNA sequencing. In that study, mutation E266D was observed in 2 of the resistant isolates.

The occurrence of two or more amino acid substitutions appears to be common in *ERG11* gene from fluconazole-resistant strains as has been observed in this study and previous studies discussed above. It is unclear as yet whether all identified mutations are important for conferring reduced affinity for azoles. In their study, Sanglard et al. (1998) found by introducing some amino acid substitutions in *ERG11* gene from a sensitive strain that double point mutations usually have a synergistic effect over single amino acid substitution, explaining the apparent natural occurrence of more than one mutation in azole-resistant *ERG11* gene.

Mutations L340 (Y, C), K342 (K, R) observed in this resistant isolate were novel, and have never been described previously. The presence of these novel mutations may suggest that the clinical environment in South Africa allow the appearance of novel mutations in *C. albicans ERG11* gene. Further characterization of these new mutations is needed to determine their contribution to fluconazole resistance in *C. albicans* isolates.

The other two resistant isolates and their matching susceptible isolates shared heterozygous mutations K128 (K,T), M189 (I, M), D116 (E,D), E266 (E,D), E266 (N,T), V332 (S, L), L340 (Y,C), F380 (S,L), L370 (S, L), Y401 (T, M), P419 (P, L), V437 (I, V), N490 (T,I), D443 (M,N), homozygous mutations V437I, L340N, V437F, L480W, and nonsense mutation L480 (*, W). A susceptible isolate had mutations P419R and F380Q, and therefore these mutations were not responsible for resistance in these isolates.

The occurrence of mutations E266D and V437I in both fluconazole resistant and susceptible isolates in clade III is in agreement with previous studies, as has already been discussed here earlier. Mutation E266D was observed in susceptible isolates (Sanglard et al., 1998) and also in resistant isolates (Kallakuri et al., 1996; Loeffler et al., 1997; Manavathu et al., 1999; Lee et al., 2004; Goldman et al., 2004). Mutation V437I was observed in fluconazole-resistant isolates (Marichal et al., 1999; White et al., 2002), while in other studies it was found in susceptible isolates (Ryder and Favre, 1997; Sanglard et al., 1998; Lee et al., 2004). These mutations were also observed in both susceptible and resistant isolates from clade II in this study.

Mutations E266 (N,T), V332 (S,L), L340 (Y,C), K342 (K,R), F380 (S,L), Y401 (T,M), V437 (I, V), N490 (T,I), D443 (M,N), K342 (K, R), M189 (I, M), L370 (S, L), P419 (P, L), V437F and L480W, P419R, F380Q observed in isolates from clade III were novel, and have never been described previously. However, mutations K342R and F380S were observed in a study by Goldman et al. (2004). The contribution of these new mutations to fluconazole resistance is at the moment unknown, and they need to be studied further. The nonsense mutation L480 (*, W), which was novel, was observed in this clade, but the contribution of this mutation to fluconazole resistance is not known and needs to be studied further.

Mutations D116E, K128T, D116 (E, D) and K128 (K, T) observed in isolates from clade III are within the first hotspot 105 to 165, mutations E266 (E, D) and E266 (N, T) are

within the second hotspot 266 to 287, mutations P419 (P, L), V437 (I, V), D443 (M,N), V437I, V437F, L480W and P419R are within the third hotspot 405 to 488 (Marichal et al., 1999). Mutations L340 (Y, C), K342 (K, R), V332 (S, L), F380 (S,L), L370 (S, L), Y401 (T, M), N490 (T,I), L340N and F380Q are outside the described hotspots. The observed novel mutations among these South African isolates, which occurred outside the previously described hotspots may warrant further study and probably a review of the hotspots, especially among South African *C. albicans* isolates.

Mutations were not associated with resistance in 2 remaining isolates from clade III, as similar mutations were observed in these matching isolates. The occurrence of similar mutations in matching fluconazole susceptible and resistant isolates in this clade suggests that resistance in these isolates was not due to these mutations in these isolates, but resistance could be due to overexpression of *ERG11* gene or drug efflux pumps genes *CDR1, CDR2* or *MDR1,* or another undescribed mechanism as has been previously suggested by Sanglard et al. (1998).

The results of the study show that in isolates from clade SA, mutations were associated with fluconazole resistance in 1(33.3%) of the 3 resistant isolates tested. Mutations were not associated with resistance in 2 remaining isolates, as similar mutations were observed in these matching isolates. Mutations D116E and K128T were observed in 1 resistant isolate (K306FR), but not in its matching susceptible isolate (K306FS). The occurrence of mutations D116E and K128T in a fluconazole-resistant isolate (K306FR) from clade SA is in agreement with the results observed in other studies, where these mutations were found simultaneously in fluconazole resistant isolates. In a study by Kallakuri et al. (1996), mutations D116E and K128T were observed in the same resistant isolate. In their study, Marichal et al. (1999) observed mutations K128T and D116E in the same 3 fluconazole resitant *C. albicans* strains from AIDS patients. In a study by Asai et al. (1999), when 2 clinical strains were characterized, these 2 mutations were observed simultaneously in these isolates**.** Manavathu et al. (1999) studied six clinical isolates of *C. albicans* with high level of resistance to fluconazole. Mutations D116E and E266D were detected in the same resistant isolate in that study.

The remaining 2 matching resistant and susceptible isolates from this clade shared the following homozygous mutations S137L, L220Y, L220I, L340I, L340C, S361R, A432L, A434P, L480I, L480W, N490T and a nonsense mutation N490*. Mutations V437I, H183T, V332S, F380S and Y401T were observed in a susceptible isolate. The occurrence of mutation V437I in a susceptible isolate in this study is in agreement with results observed in other studies. In a study by Ryder and Favre (1997), mutation V437I was observed in a fluconazole susceptible isolate characterized. Sanglard et al. (1998) observed this mutation in a fluconazole susceptible clinical isolate. In this study, this mutation was also observed in both resistant and susceptible isolates from clade II and III.

The occurrence of mutation D116 (E, D) is in agreement with results obtained in a study by Marr et al. (2001). In their study, this mutation was observed in a fluconazole clinical susceptible isolate after sequencing of *ERG11* gene and comparison with published sequence. Mutations D116 (E, D) and K128 (K, T) and S137L are within the first hotspot 105 to 165, mutations A432L, A434P, L480I, L480W and V437I are within the third hotspot. Mutations H183T, V332S, F380S, Y401T, L220Y, L220I, L340I, L340C, S361R, 490T and N490* are outside the described hotspots (Marichal et al., 1999).

Mutations S137L, H183T, L220Y, L220I, V332S, L340I, L340C, S361R, F380S, A432L, A434P, L480I, L480W, N490*, N490T and Y401T observed in isolates from this clade were novel. The presence of these novel mutations may suggest that the clinical environment in SA can select novel mutations in *C. albicans ERG11,* and it is not known whether these mutations could be responsible for fluconazole resistance. Further studies are needed to confirm this. The nonsense N490* was observed in this clade, but the contribution of this mutation to fluconazole resistance is not known and needs to be studied further.

Mutations were not associated with resistance in 2 remaining isolates from clade SA, as similar mutations were observed in these matching isolates. The occurrence of similar mutations in matching fluconazole susceptible and resistant isolates in this clade suggests

that resistance in these isolates was not due to these mutations in these isolates, but resistance could be due to overexpression of *ERG11* gene or drug efflux pumps genes *CDR1, CDR2* or *MDR1,* or another un-described mechanism as has been previously suggested by Sanglard et al. (1998).

The results of the study show that in isolates from clade SA, mutations were associated with fluconazole resistance in 1(33.3%) of the 3 resistant isolates tested. Mutations were not associated with resistance in 2 remaining isolates, as similar mutations were observed in these matching isolates. Mutations E266P, K342G, A434Q and V488L and a nonsense mutation A432* occurred in a resistant isolate K86FR.

The occurrence of mutations E266P and V488L in a resistant isolate from our study is in agreement with previous reports, where similar mutations E266D and V488I were observed in fluconazole resistant isolates. In a study by Kallakuri et al. (1996), a fluconazole-resistant clinical isolate of *C. albicans* was characterized, and mutation 266D was observed in this isolate. Loeffler et al. (1997) observed this mutation in 4 fluconazole resistant isolates when they were characterized. In a study by Ryder and Favre (1997), mutation E266D was observed in a fluconazole resistant isolate characterized.

In a study by Favre et al. (1999), 4 selected fluconazole-resistant clinical isolates were sequenced and compared with the sequence from a sensitive strain SC5314. In that study, mutation E266D was observed in 1 of these resistant isolates. When a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans* were analyzed in a study by White et al. (2002), mutation E266D was observed in 1 of the resistant isolates in that study. In their study, Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates. Mutation E266D was observed in 1 resistant isolate from that study. Lee et al. (2004) analyzed 12 resistant and susceptible clinical isolates for *ERG11* mutations by DNA sequencing. In that study, mutation E266D was observed in 2 of the resistant isolates.

The finding of mutation E266D in a fluconazole resistant isolate, which is similar to E266P found in our study, is in contrast to other studies where this mutation was found in fluconazole susceptible isolates. In a study by Franz et al. (1998), fluconazole susceptible isolates from AIDS patients with OPC were characterized, and this mutation was observed in these isolates. In their study, Marichal et al. (1999) observed mutation E266D in 2 fluconazole susceptible *C. albicans* strains from AIDS patients.

In a study by Loeffler et al. (1997), mutation V488I, which is similar to mutation V488L observed in our study, was observed in 2 fluconazole resistant isolates when they were characterized and compared with the published sequence. In their study, Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates. Mutation V488I was found in a fluconazole resistant isolate Franz et al. (1998) characterized a fluconazole resistant isolate from an AIDS patient with OPC, and mutation V488I was observed in this isolate.

The occurrence of mutations V488L and E266P in a fluconazole resistant isolate in our study, but not in its matching susceptible isolate suggests that these mutations may be responsible for resistance in this isolate.

Novel mutations K342G and A434Q and A432* were also observed in this fluconazole resistant isolate. The occurrence of these novel mutations in a resistant isolate but not in its matching susceptible isolate suggest that these mutations are involved in fluconazole resistance in this isolate. However, further studies are required to confirm this. These mutations needs to be further characterized to determine their contribution in fluconazole resistance.

The remaining 2 isolates from this clade shared homozygous mutations E266T, L220Y, V332H, Y401R, V332S, K342R, F380S, Y401T, A432L, A434P, V437F, V437I, L480W, V488F, S137L, H183T, L220Y, E266D, and a nonsense mutation L370*.

The occurrence of mutation V437I, which is similar to V437F, in both fluconazole resistant and susceptible isolates in this study is in agreement with previous studies, where this mutation was observed in both fluconazole-resistant an susceptible isolates. In a study by Ryder and Favre (1997), mutation V437I was observed in a fluconazole susceptible isolate characterized. Sanglard et al. (1998) observed this mutation in a fluconazole susceptible clinical isolate. When fluconazole resistant *C. albicans* isolates obtained from five HIV-infected patients with recurrent OPC were analyzed, Lopez-Ribot et al. (1998) observed mutation V437I in 2 of the resistant isolates. These investigators suggested that this mutation was not responsible for fluconazole resistance in these isolates, as they also detected this mutation in fluconazole susceptible isolates.

In a study of matched resistant and susceptible isolates from HIV-infected patients, Perea et al. (2001) observed mutation V437I in 2 matching susceptible and resistant isolates. These investigators concluded that this mutation was not responsible for resistance in these isolates. In their study, White et al. (2002) analyzed a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans,* and mutation V437I was observed in 2 of the resistant isolates in that study. Lee et al. (2004) analyzed 12 resistant and susceptible clinical isolates for *ERG11* mutations by DNA sequencing. In that study, mutation V437I was observed in 2 of the resistant isolates.

When Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates, mutation V437I was observed in 1 resistant isolate from that study. Park and Perlin (2005) evaluated a diverse collection of 59 clinical isolates of *C. albicans* consisting of fluconazole susceptible, dose-dependent, and resistant phenotypes by DNA sequence analysis of *ERG11* gene. In that study, mutation V437I was observed in 3 resistant and 2 susceptible isolates. These investigators concluded that this mutation was not responsible for resistance as it was also found in susceptible isolates.

The occurrence of mutation V437I, which is similar to V437F, in both fluconazole resistant and susceptible isolates in this study is in agreement with previous studies, where this mutation was observed in fluconazole-resistant isolates (Marichal et al., 1999; White et al., 2002), while in other studies it was found in susceptible isolates (Ryder and Favre, 1997; Sanglard et al., 1998; Lee et al., 2004).

Mutation S137L is within the first hotspot, mutations E266T, E266D, E266P are within the second hotspot, mutations A432*, A434Q, A432L, A434P, V437F, V437I, L480W, V488F and V488L are within the third hotspot. Mutations L220Y, V332H, L370*, Y401R, V332S, K342R, F380S, Y401T, H183T, L220Y and K342G, are outside the described hotspots.

Mutations K342G, A434Q, V488L, E266T, L220Y, V332H, L370*, Y401R, V332S, K342R, F380S, Y401T, A432L, A434P, V437F, L480W, V488F, S137L, H183T, L220Y were novel. The presence of these novel mutations may suggest that the clinical environment in SA can select novel mutations in *C. albicans ERG11,* and it is not known whether these mutations could be responsible for fluconazole resistance.

The results of this study show that fluconazole resistance was associated with mutations in 1 (33.3%) isolate from each of clades III, SA and NG. The results of the study also show that mutations were clade-related, as mutations associated with resistance were observed in clades III, SA and NG, but not in clades I and II. However, the results of our study cannot be compared with other studies regarding clade-relatedness, as this is the first study where contribution of mutations to fluconazole resistance among different clades of *C. albicans* was determined.

The absence of mutations in these isolates from clades I and II, and the remaining isolates from clades III, SA and NG could not have been caused by growing isolates in the absence of fluconazole, as these isolates were grown in the presence of highest concentration of fluconazole throughout. Fluconazole resistance in these isolates could have been due to over-expression of *ERG11* gene or drug efflux pumps genes *CDR1,*

CDR2 or *MDR1,* or some uncharacterized mechanisms as suggested by Sanglard et al. (1998). Fluconazole resistance among these isolates could also have been due to phenotypic switching, as switching has been suggested to be another mechanism by which *C. albicans* achieves resistance against antifungal agents (Soll et al., 1992).

Some of the isolates associated with fluconazole resistance were novel, these were mutations L340 (Y, C), K342 (K, R); K342G, A432* and A434Q. Other new mutations were observed in both susceptible and resistant isolates from all clades of these South African *C. albicans* isolates, and the occurrence of these mutations requires further attention. The presence of these novel mutations may suggest that the clinical environment in South Africa can select novel mutations in *C. albicans ERG11,* but it is not known whether these mutations could be responsible for fluconazole resistance. The findings of these new mutations suggest that further study of differences between different clades may be warranted, and further genetic analysis of these new mutations needs to be done to determine their significance in fluconazole resistance.

Some of these novel mutations were in the previously described hotspots, while others were outside the described hotspots. The observed novel mutations among these South African isolates, which were found to be outside the previously described hotspots, may warrant further study and probably a review of the hotspots, especially among South African *C. albicans* isolates.

4.9 Conclusion

To our knowledge, this study is the first to be performed where contribution of mutations to fluconazole resistance was determined among different clades of *C. albicans*. The results of the study show that mutations were not responsible for fluconazole resistance among isolates from clades I and II, but mutations were associated with fluconazole resistance in isolates from clades III, SA and NG. The occurrence of mutations in fluconazole resistant isolates from clades III, SA and NG, but not in clades I and II shows clade-relatedness of mutations in the *ERG11* gene. This suggests that further study of differences between different clades may be warranted.

Some of mutations observed in resistant isolates from clades III and NG were novel, and have never been described previously. Other novel mutations were also observed in both resistant and susceptible isolates from all clades. Further genetic analysis of these new mutations is required to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa.

Some of the novel mutations observed in this study were found in the previously described hotspots, while others were outside the described hotspots. These novel mutations observed outside the previously described hotspots may warrant further study and probably a review of the hotspots, especially among South African *C. albicans* isolates. The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000).

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CHAPTER FIVE: CONTRIBUTION OF OVER-EXPRESSION OF *ERG11* GENE TO FLUCONAZOLE RESISTANCE IN *CANDIDA ALBICANS* CLADES

5.1 Abstract

Background

A high increase in fluconazole resistance among clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Fluconazole acts by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase of this fungus. This enzyme is encoded by the *ERG11* gene, and is important in the synthesis of ergosterol, a primary sterol in the fungal cell membrane. Resistance to fluconazole can be caused by over-expression of *ERG11* gene, which results in increased production of lanosterol demethylase, overwhelming the capacity of the azole antifungal agent. Several studies have associated over-expression of *ERG11* gene with fluconazole resistance among *C. albicans* strains, however, nothing is known about this association among different clades of *C. albicans*, and whether over-expression is clade-related.

Objectives

To determine whether induced fluconazole resistance among *C. albicans* isolates from different clades was due to over-expression of the *ERG11* gene, and whether over-expression was clade-related.

Materials and methods

Three isolates from each of previously typed *C. albicans* clades I, II, III, SA, and NG (Blignaut et al., 2002), in which fluconazole resistance (MIC≥64µg/ml) was induced in Chapter 2, were selected. Their three matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were also included in the study. These yeast isolates were obtained from surveillance cultures on HIV/AIDS patients attending HIV/AIDS clinics in the Pretoria region. Resistant isolates were grown in Yeast extract medium containing 64µg/ml of fluconazole, while susceptible isolates were grown in Yeast extract medium without fluconazole. RNA was extracted and cDNA was synthesised from RNAs by using cDNA synthesis kit according to manufacturer's directions (Bioline, Germany). Real time PCR was performed on cDNA samples by using

Sensimix dT kit according to manufacturer's directions (Quantace, London, UK), on a Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia). Relative gene expression of *ERG11* gene among resistant isolates, relative to susceptible isolates was quantified after normalization with the *18SrRNA* house-keeping gene.

Results

When relative *ERG11* gene expression was quantified among fluconazole resistant and susceptible isolates from various clades, over-expression of *ERG11* gene was observed in 2 (66.6%) isolates from each of clades I, II and SA, and in 1 (33.3%) isolate from each of clades III and NG.

Conclusion

To our knowledge, this study is the first to be performed, where contribution of over-expression of *ERG11* gene to fluconazole resistance was determined among different clades of *C. albicans.* The results of the study show that over-expression of *ERG11* gene contributed to fluconazole resistance in isolates from all clades. However, over-expression was observed in more isolates from clades I, II and SA (66.6%) and in less isolates from clades III and NG (33.3%), showing clade-relatedness of *ERG11* over-expression. These results suggest that further study of differences between *C. albicans* clades may be warranted.

The higher occurrence of over-expression of *ERG11* gene in isolates from clades I, II, and SA, especially clade SA, which is dominant in South Africa, is a cause for concern, especially in HIV/AIDS patients with OPC, as the increased expression of *ERG11* has been reported to allow for the cells to persist within the host, which in turn leads to the subsequent development of other more stable resistant isolates. The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed.

These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level.

5.2 Introduction

An estimated 5.7 million South Africans are currently infected with (HIV) (UNAIDS/WHO report, 2008), and OPC, caused by *C. albicans* is the most common oral complication causing morbidity in these individuals (Arendorf et al., 1998). It is estimated that 80 to 95% of patients infected with HIV will experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994). Five genetic clades of *C. albicans* have been established using DNA fingerprinting methods in various locations, namely, clades I, II, III, SA and NG (Soll and Pujol, 2003), with clade SA predominating in South Africa (Blignaut et al., 2002).

Azoles such as fluconazole (oral and intravenous) are used in the treatment and/or prophylaxis of most *C. albicans* infections. While azoles have little or no toxicity, they generally lack fungicidal activity. Consequently, in AIDS patients, azoles must be administered for extended periods of time, and this could result in the increased isolation of azole-resistant strains of *C. albicans* (Rex et al., 1995). Azoles act by inhibiting the enzyme cytochrome P-450 lanosterol 14α demethylase, which is encoded by the *ERG11* gene (also described as *ERG16* or *CYP51*), and is important in the synthesis of ergosterol, a primary sterol in the fungal cell membrane. This inhibition leads to blockage of ergosterol synthesis (White et al., 1998).

One of the major molecular mechanisms responsible for development of azole resistance in strains of *C. albicans* involves changing the target enzyme, the cytochrome P-450 lanosterol 14α-demethylase. Over-expression of *ERG11* gene has been associated with azole resistance (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Franz et al., 1998; Henry et al., 2000). This over-expression of *ERG11* gene leads to increased production of lanosterol demethylase, which overwhelms the capacity of the azole antifungal agent (White et al., 1998; Ghannoum and Rice, 1999).

Several studies have used various methods to investigate the gene expression profile associated with fluconazole resistance: Northern blot (Sanglard et al., 1995; Franz et al., 1998; Lopez-Ribot et al., 1999; Lyons and White, 2000; Perea et al., 2001; White et al., 2002), reverse transcriptase PCR (Maebashi et al., 2001), and real-time quantitative PCR (Chau et al., 2004; Goldman et al., 2004; Park and Perlin, 2005).

Quantification of mRNA by Northern hybridization has been the method of choice for analyzing gene expression in *C. albicans* (Sanglard et al., 1995; Albertson et al., 1996; Lopez-Ribot et al., 1998; Marr et al., 1998; Henry et al., 1999; Henry et al., 2000). However, this method is labor intensive, requires several steps and multiple days to complete, requires large amounts of RNA, and can lack the sensitivity required to detect small changes in gene expression (Frade et al., 2004).

Non-real-time reverse transcriptase PCR (RT-PCR) methods for analyzing gene expression improved sensitivity, but they measure amplified product at the end of the PCR, and are therefore subject to the errors caused by the plateauing effect that occurs when reagents become reduced. They also require post-PCR manipulations, which take time and increase the risk of laboratory contamination with amplified product (Souaze et al., 1996; Freeman et al., 1999; Okeke et al., 2001).

Real-time fluorescence PCR technology (Real-time RT-PCR), which couples PCR with on-line fluorescence detection of amplification products, is now the method that is widely used for gene expression quantification (Higuchi et al., 1993; Wittwer et al., 1997a; Bustin, 2000). Amplification is monitored during every cycle and enables more accurate quantification, eliminating the need for post-PCR sample processing to visualize and analyze products. This method has been successfully used for the measurement of gene expression in a variety of fields, including microbiology (Kreuzer et al., 1999; Smith et al., 2000; Okeke et al., 2001). This method has significant advantages over Northern hybridization, such as increased sensitivity for detection of low amounts of mRNA, it is most sensitive for quantification of gene expression for low abundance RNA (Lockey et al., 1998), accurate mRNA quantification (Wittwer et al., 1997b), and the ability to convert unstable RNA template into cDNA, which is less susceptible to degradation (Frade et al., 2004).

Previous work on the analysis of resistance mechanisms has routinely used matched sets of susceptible and resistant clinical isolates of the same strain (Sanglard et al., 1995; Lopez-Ribot et al., 1998; Perea et al., 2001). The use of matched sets is necessary, as *C. albicans* is mostly clonal in nature (Pujol et al., 1993), and isolates might differ considerably in their levels of expression of different genes, hence the use of matched sets in our study.

Several studies have shown that over-expression of *C. albicans ERG11* gene is associated with fluconazole resistance. Franz et al. (1998) reported that even in the absence of fluconazole, some fluconazole-resistant isolates can express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug. In another study, mRNA levels of *ERG11* were shown to be increased in several fluconazole-resistant isolates of *C. albicans* (Sanglard et al., 1995).

Over-expression of *ERG11* has been found in many other fluconazole-resistant *C. albicans* isolates compared with matched susceptible isolates (White, 1997; White et al., 1997; Cowen et al., 2000; Perea et al., 2001; Morschhauser, 2002; Lee et al., 2004; Frade et al., 2004). In a study by Henry et al. (2000), azoles were described to show increased expression of *ERG11* within a few hours of exposure, contributing significantly to the survival of azole-treated cells. A recent study showed that up-regulation of *ERG11* gene was the predominant mechanism in experimentally induced fluconazole resistance (Ribeiro and Paula, 2007).

However, other studies have shown that multiple genes are involved in conferring fluconazole resistance. In one of those studies, different genes involved in drug resistance in *C. albicans* were concomitantly over-expressed, indicating that multiple mechanisms may be operating to confer fluconazole resistance in these isolates (Albertson et al., 1996; Goldman et al., 2004).

Other studies showed that changes in expression levels of *ERG11* gene were not correlated with fluconazole resistance. One study used quantitative real-time PCR with molecular beacons to determine gene expression in fluconazole-resistant isolates. Changes in expression levels for *ERG11* were not statistically correlated with fluconazole resistance (Park and Perlin, 2005). In other studies, which did not use matched sets,

resistance was not found to correlate with over-expression of *ERG11* gene (White et al., 2002; Martinez et al., 2002).

5.3 Study problem

Although over-expression of *ERG11* gene has been reported to be responsible for fluconazole resistance among *C. albicans* isolates, nothing is known about the contribution of over-expression of this gene to fluconazole resistance among different clades of *C. albicans*, and whether over-expression will occur more in one particular clade as compared to the others. Quantification of *ERG11* gene expression in different clades of *C. albicans* isolates resistant to fluconazole may produce a better understanding of the structure of this enzyme, and may lead to the designing of new drugs, which will continue using *ERG11* gene product as the antifungal target.

5.4 Aim

To investigate the contributions of over-expression of *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades

5.5 Objectives

n) To culture matching fluconazole resistant and susceptible isolates belonging to *C. albicans* clades I, II, III, SA, and NG in YAD medium
o) To extract RNA from the isolates
p) To measure RNA concentration, purity and integrity
q) To synthesize cDNA from RNA samples
r) To quantify expression of *ERG11* gene in fluconazole resistant and susceptible isolates by performing Real-time PCR on cDNA samples
s) To compare over-expression of *ERG11* gene between resistant and susceptible isolates from different clades.

5.6 Materials and Methods

5.6.1 Isolates and culture conditions

Glycerol stocks of three isolates from each of previously typed *C. albicans* clades I, II, III, SA, and NG (Blignaut et al., 2002) with induced fluconazole resistance (MIC≥64µg/ml) were subcultured on Saboraud's Dextrose agar containing 64µg/ml of fluconazole and incubated at 35^{o}C for 48 hours. Isolated colonies were suspended and grown overnight in 5ml of YAD broth (1.5g/l Yeast Nitrogen Base, 5g/l Ammonium sulphate, 20g/l Dextrose) containing highest concentration of fluconazole at 30^{o}C with shaking. Three matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were subcultured on Saboraud's Dextrose agar and incubated at 35^{o}C for 48 hours. Isolated colonies from susceptible isolates were suspended and grown overnight in 5ml of YAD broth without fluconazole (1.5g/l Yeast Nitrogen Base, 5g/l Ammonium sulphate, 20g/l Dextrose) at 30^{o}C with shaking. Two clinical isolates with fluconazole MIC of 1:128µg/ml (FRC) and MIC of 2µg/ml (FSC) were included in the study as controls, and were treated the same as isolates.

5.6.2 RNA extraction and DNA digestion

RNA was extracted from all fluconazole resistant and susceptible isolates. Cells were first harvested from YAD cultures by centrifugation at 2500rpm for 7 minutes and washed with sterile, RNAse free water for 3 minutes. RNA was extracted from pellets by using RNAeasy mini kit according to manufacturer's directions (Qiagen, California, USA). Cells were initially homogenized with sterile, acid washed glass beads (Sigma Aldrich, Germany) in a minibead beater (Biospec Products, Inc, Oklahoma, USA), mixed for 30 seconds and put on ice for another 30 seconds. The procedure was repeated 5 times, followed by centrifugation for 5 minutes at 13000rpm. Five hundred microlitres of 70% Ethanol was added to supernatants in RNAeasy mini columns, followed by centrifugation for 15 seconds at 13000rpm.

To digest DNA, 350µl of RW1 buffer included in the RNAeasy mini kit was added to the spin column and centrifuged for 15 seconds. Ten microlitres of DNAse I stock solution was mixed with 70µl of buffer RDD and the tube was gently inverted and briefly

centrifuged. This DNAse mixture (80µl) was then added to the spin column and incubated at room temperature for 15 minutes. Another 350µl of RW1 buffer was added to the spin column and centrifuged for 15 seconds. This was followed by addition of 500µl of RPE buffer and centrifugation for 15 seconds. Another 500µl RPE buffer was added and centrifuged for 2 minutes. RNA was eluted with 80µl RNA-se free water and stored at -80^{o}C till needed.

5.6.3 Determination of RNA concentration, integrity and purity

Concentrations of all RNA samples were determined by making dilutions of 1/50 in RNAse-free water, and measuring absorbances on a Genequant spectrophotometer (Pharmacia, CA, USA) at 260nm, using RNAse-free quartz cuvettes. The concentration of RNAs was determined by using standard calculations (Absorbance x dilution factor x 40).

To verify RNA integrity, 20µg of each RNA sample was mixed with formaldehyde loading dye and 5mg/ml ethidium bromide, heated to 65^{o}C and chilled on ice for 5 min. The mixture was loaded onto a 2% formaldehyde denaturing gel, which was run at 5V/cm, stopping the run when bromophenol dye reached 2/3 or ¾ of the gel length. The gel was visualized with ultraviolet light, and the presence of intact 18S and 28S ribosomal RNA bands (semiquantatively in a ratio 2:1) was used as a criterion to determine whether RNA was degraded.

To determine the purity of RNA, RNA samples were diluted 1/50 in 10mM Tris.Cl, pH 7.5. The absorbance of RNA was measured on a Genequant spectrophotometer (Pharmacia, CA, USA) at 260nm and 280nm. The ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}) was calculated. RNA samples were considered to be pure if they had a ratio of 1.9-2.1. Only RNA samples with intact 18S and 28S ribosomal RNA bands (semi quantitatively in a ratio 2:1), and with purity of 1.9-2.1, were used for this study.

5.6.4 Synthesis of cDNA

cDNA was synthesised from RNAs by using Bioscript cDNA synthesis kit according to manufacturer's directions (Bioline GmbH, Luckenwalde, Germany). First, RNA was sensitised by mixing on ice: 1µg RNA template, 1µl Oligo $(dT)_{18}$, 10mM dNTP and DEPC-treated water up to a volume of 10µl, followed by incubation at 65°C for 10 minutes, and chilling on ice for 2 minutes. Reverse transcription was performed in a total volume of 20µl containing the sensitized RNA, 4µl of 5x BioScript Buffer, 1µl of RNase Inhibitor, 0.25µl BioScript MMLV reverse transcriptase (200u/µl) and DEPC-treated water, with incubation at 45°C for 60 minutes. The reaction was terminated by incubation at 70°C for 15 minutes, and then chilling on ice. cDNA was purified using QIAquick PCR purification kit according to manufacturer's directions (Qiagen, California, USA), and quantified by spectrophotometric measurements of the A_{260} and A_{280}. Concentrations of cDNAs were determined by using standard calculations (Absorbance x dilution factor x 33).

5.6.5 Quantitative Realtime PCR

5.6.5.1 Determination of amplification efficiency

For quantification of *ERG11* gene expression, comparative ΔCt method was used as described by Livak and Schmittgen (2001). In order to use this method, the amplification efficiency of the *ERG11* gene and the house keeping gene *18SrRNA* must be determined first, and they must be approximately equal. To determine this, 10-fold dilutions of cDNA samples were prepared, and amplifications were performed using *ERG11* and *18SrRNA* primers. The average Ct was calculated for both *ERG11* and *18SrRNA*, and ΔCt (Ct_{ERG11}-Ct_{RNA}) was determined. A plot of log cDNA dilution versus ΔCt was made. If the value of the slope was close to zero, the efficiencies of *ERG11* and *18SrRNA* were considered to be approximately equal.

Amplifications were performed using Sensimix dT kit according to manufacturer's directions (Quantace Ltd, London, UK) on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia), in a total volume of 25µl containing cDNA template, 5µM of each primer, 12.5µl of SensiMix dT (Reaction buffer, heat-inactivated Taq DNA Polymerase, dNTPs, 6mM $Mgcl_2$), 0.5µl SYBR Green and PCR grade water to make up 25µl. Five

PCR mixtures were prepared; Mixture 1 contained 50ng/µl of cDNA sample; mixture 2 contained 1µl of 1:10 dilution (5ng/µl); mixture 3 contained 1µl of 1:100 dilution (0.5ng/µl); mixture 4 contained 1µl of 1:1000 dilution (0.05ng/µl) and mixture 5 contained 1µl of 1:10000 dilution (0 005ng/µl).

The primers used in the study were house-keeping gene *18SrRNA*- Forward: 5'-CAC GAC GGA GTT TCA CAA GA-3', and *18SrRNA*-Reverse: 5'-CGA TGG AAG TTT GAG GCA AT-3' which amplified a fragment of 135bp. For *ERG11* gene, primers *ERG11*- Forward: 5'-ACT AGA TGG GAT ACT GCT GC-3' and *ERG11*-Reverse: 5'-CAT CTA TGT CTA CCA CCA CC-3', which amplified a fragment of 137bp were used. Primers were obtained from Integrated DNA Technologies, California, USA.

PCR was performed on the Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) using the following PCR conditions for *ERG11* and house keeping gene (*18SrRNA)*: the initial PCR activation step of 10 minutes at 95^{o}C, followed by 30 cycles of 95^{o}C for 5 seconds, 55^{o}C for 10 seconds, and 72^{o}C for 15 seconds, with melt of 72 ^{o}C-99 ^{o}C, rising by 1 ^{o}C, with a pre-melt of 90 seconds, with 5 seconds for each step afterwards.

5.6.5.2 Real-time PCR

When the efficiencies of house-keeping gene and genes of interest were found to be equal, Real time PCR was performed on cDNA samples by using Sensimix dT kit according to manufacturer's directions (Quantace, London, UK) on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia).

PCR was performed in a total reaction volume of 25µl containing 1µl of 50ng/µl cDNA, 5µM of each primer, 12.5µl of SensiMix dT (Reaction buffer, heat-inactivated Taq DNA Polymerase, dNTPs, 6mM $Mgcl_2$), 0.5µl SYBR Green and RNAse, DNAse-free PCR-grade water to make up the 25µl volume. PCR was performed in all samples in triplicate. PCR mix without cDNA was included in triplicate and was used as a non-template control. The primers used in the study were *18SrRNA*- Forward: 5'-CAC GAC GGA GTT TCA CAA GA-3', and *18SrRNA*-Reverse: 5'-CGA TGG AAG TTT GAG GCA AT-3' which amplified a fragment of 135bp. For *ERG11* gene, primers *ERG11*- Forward: 5'-ACT AGA TGG GAT ACT GCT GC-3' and *ERG11*-Reverse: 5'- CAT CTA TGT

CTA CCA CCA CC-3', which amplified a fragment of 137bp were used. Primers were obtained from Integrated DNA Technologies, California, USA.

The following PCR conditions were used for *ERG11* and house keeping *18SrRNA* genes-: The initial PCR activation step of 10 minutes at 95^{o}C, followed by 30 cycles of 95^{o}C for 5 seconds, 55^{o}C for 10 seconds, and 72^{o}C for 15 seconds, with melt of 72 ^{o}C-99 ^{o}C, rising by 1^{o}C, with a pre-melt of 90 seconds, with 5 seconds for each step afterwards.

To verify that the SYBR green dye detected only one PCR product, the samples were subjected to the heat dissociation protocol following the final cycle of the PCR. Heat dissociation of oligonucleotides detects differences in melting temperature and will produce a single dissociation peak for each oligonucleotide within a 2°C difference in melting temperature (Ririe et al., 1997).

5.6.5.3 Gel electrophoresis

To determine whether PCR products produced single bands, Real time PCR products of *ERG11* and house-keeping genes *18SrRNA* were resolved by electrophoresis on a 1.5% agarose gel at 100V for 1h 30 min, together with O'GeneRuler 100bp DNA ladder (Fermentas UAB, Lithuania, EU). Expected band sizes were 137bp for *ERG11* gene and 135bp for *18SrRNA* as described by the manufacturer (Integrated DNA Technologies, California, USA).

5.6.5.4 Gene expression quantification

Relative quantification was performed according to the comparative ΔCt method described by Livak and Schmittgen (2001). The difference between the mean Ct-*ERG11* and the mean Ct-*18SrRNA* was calculated (ΔCt) in order to normalise for different

amounts of cDNA. The result for the gene expression was given by a unitless value through the formula $2^{-\Delta Ct}$.

5.7 Results

5.7.1 RNA extraction and integrity

RNA was successfully extracted in all samples, and to test for integrity, RNA samples were run on 2% formaldehyde denaturing gel. The gel was visualized with ultraviolet light, and the presence of intact 18S and 28S ribosomal RNA bands (semi-quantatively in a ratio 2:1) was used as a criterion to determine whether RNA was degraded. The representative gels of these results are shown in Figure 5.1.

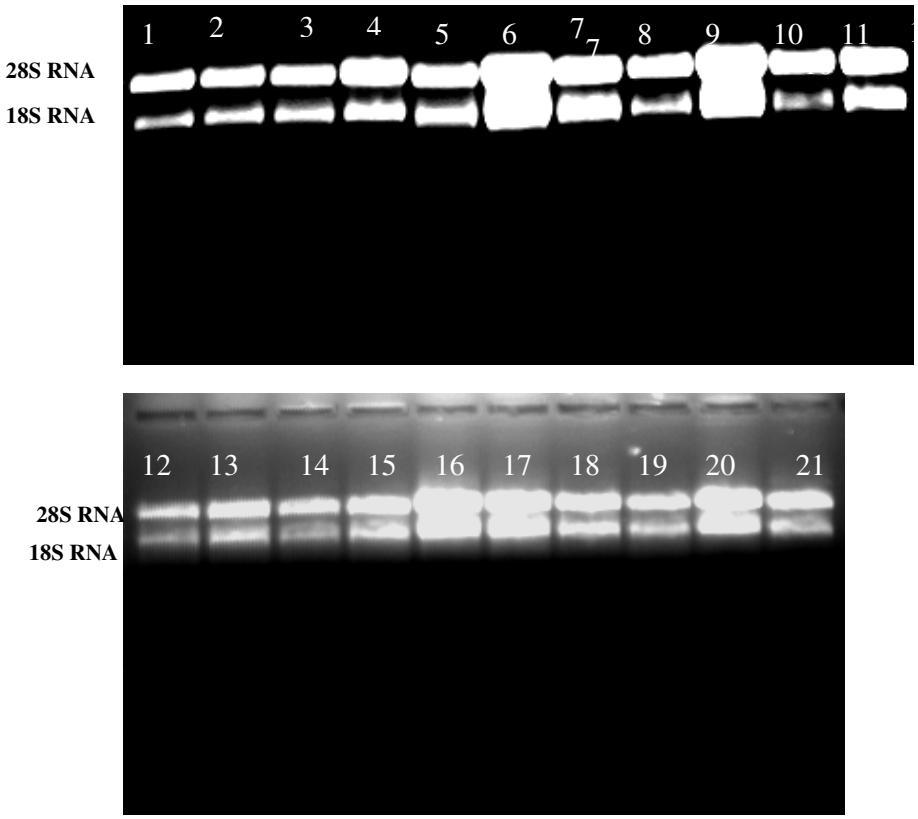

Figure 5.1: Representative gels showing RNA integrity of *C. albicans* fluconazole resistant and susceptible isolates, FRC and FSC

Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21=Fluconazole susceptible isolates; **Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20=**Fluconazole resistant isolates.

5.7.2 Real time PCR

Triplicate cDNAs from fluconazole resistant and susceptible isolates were amplified and quantified by using Real-time PCR to determine the expression of *18SrRNA* and *ERG11*. To verify that the SYBR green dye detected only one PCR product, the samples were subjected to the heat dissociation protocol following the final cycle of the PCR (Ririe et al., 1997). Dissociation of the PCR reactions produced a single peak for *ERG11* and *18SrRNA*, demonstrating the presence of only one product in the reaction. The presence of a single product was further verified by gel electrophoresis. These results are shown in Figures 5.2 and 5.3 respectively. To determine amplification efficiencies of *ERG11* gene and *18SrRNA* house keeping gene, a plot of log cDNA dilution versus ΔCt was made, and the slope of the plot was found to be approximately zero. These results are shown in Figures 5.4.

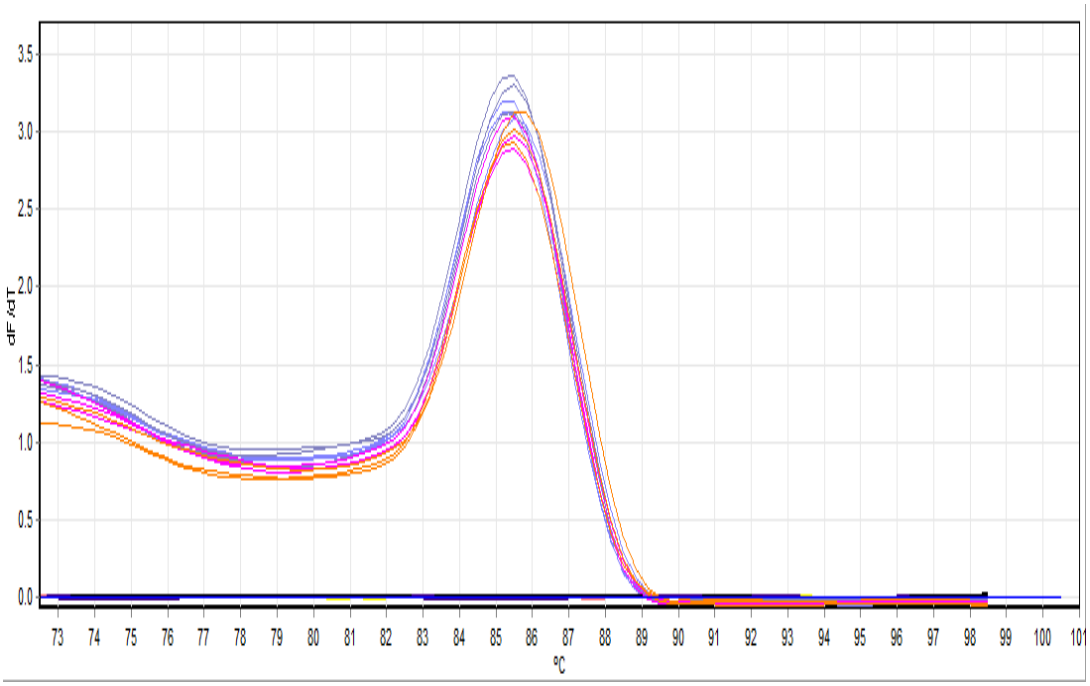

Figure 5.2: *18SrRNA* melt curve-demonstrating presence of only one product in the reaction

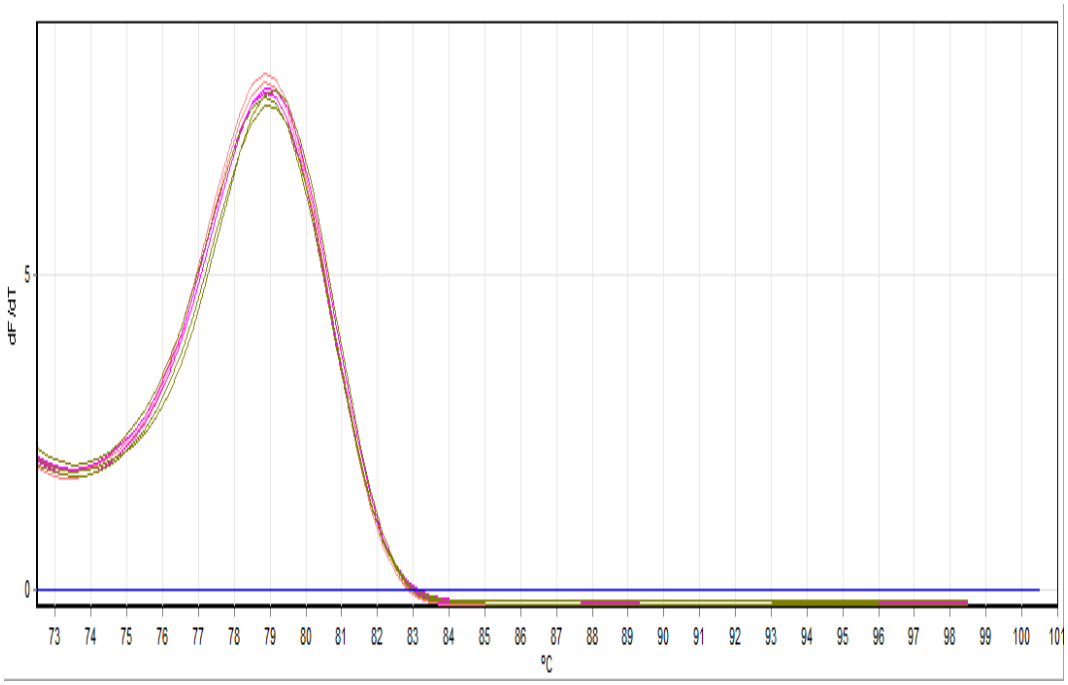

Figure 5.3: *ERG11* melt curve-demonstrating presence of only one product in the reaction

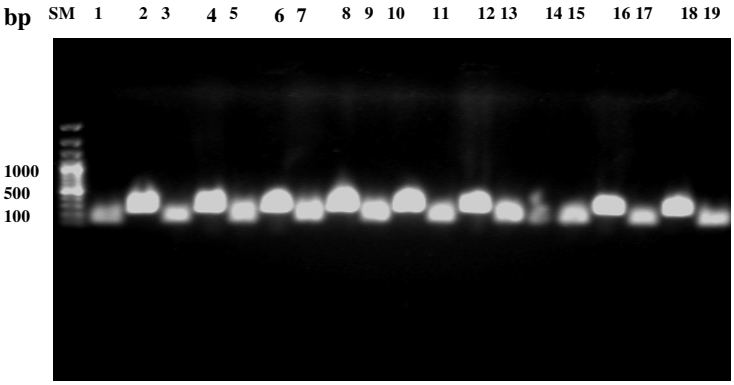

Figure 5.4: Realtime PCR products from *C. albicans* fluconazole resistant and susceptible isolates. **Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19=***18SrRNA* products; **Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18=** *ERG11* products; **SM=** O'GeneRuler 100bp DNA ladder.

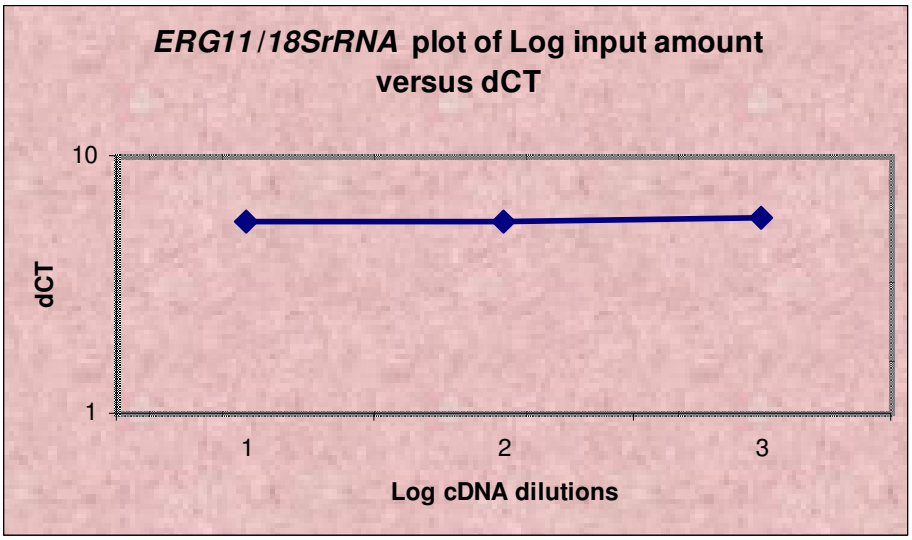

Figure 5.5: A plot of log cDNA dilutions versus **Δ**Ct showing efficiency of *ERG11* and *18rRNA* genes

5.7.3 Quantification of gene expression

5.7.3.1 Relative gene expression observed among *C. albicans* fluconazole resistant and susceptible isolates from different clades

Relative quantification was performed according to the comparative ΔCt method as described by Livak and Schmittgen (2001). The difference between the mean Ct-*ERG11* and the mean Ct-*18SrRNA* was calculated (ΔCt) in order to normalise for different amounts of cDNA. The result for the gene expression was given by a unitless value through the formula $2^{-\Delta Ct}$.

5.7.3.1a Gene expression among isolates from clade I

When relative gene expression of *ERG11* gene was compared among matching 3 fluconazole resistant and 3 susceptible *C. albicans* isolates from clade I, the following results were obtained. These results are shown in Table 5.1 and Figure 5.5.

When relative gene expression of isolate G63 fluconazole resistant isolate (G63FR) was compared with that of its matching susceptible isolate (G63FS), *ERG11* gene of G63FR was found to be over-expressed (0.062). *ERG11* gene of resistant isolate K239FR was found to be over-expressed, with a value of 0.038 as compared to its matching susceptible

isolate K239FS (0.016). No change was observed in expression of *ERG11* gene between resistant isolate K21FR and its matching susceptible isolate K21FS (0.03).

These results show that in isolates from clade I, *ERG11* over-expression was found in 2(66.6%) of the resistant isolates tested.

5.7.3.1b Gene expression among isolates from Clade II

When relative gene expression of *ERG11* gene was compared among 3 fluconazole resistant and 3 susceptible *C. albicans* isolates from clade II, the following results were obtained. These results are shown in Table 5.1 and Figure 5.5.

When relative gene expression of isolate K162FR was compared with that of its matching susceptible isolate K162FS (0.01), *ERG11* gene of K162FR was found to be over-expressed (0.04). No change was observed in expression of *ERG11* gene between resistant isolate K153FR and its matching susceptible isolate K153FS. When relative gene expression of isolate G19FR was compared with that of its matching susceptible isolate G19FS, *ERG11* gene of G19FR was found to be over-expressed (0.01).

These results show that in isolates from clade I, *ERG11* over-expression was found in 2(66.6%) of the resistant isolates tested.

5.7.3.1c Gene expression among isolates from Clade III

When relative gene expression of *ERG11* gene was compared among 3 fluconazole resistant and 3 susceptible *C. albicans* isolates from clade III, the following results were obtained. These results are shown in Table 5.1 and Figure 5.5.

No significant change was observed in expression of *ERG11* gene between resistant isolate OKP25FR (0.016) and its matching susceptible isolate OKP25FS (0.013), and between resistant isolate UP30FR (0.036) and its matching susceptible isolate UP30FS (0.034). When relative gene expression of isolate G58FR was compared with that of its matching susceptible isolate G58FS, *ERG11* gene of G58FR was found to be over-expressed (0.08).

These results show that in isolates from clade III, *ERG11* over-expression was found in 1(33.3%) of the resistant isolates tested.

5.7.3.1d Gene expression among isolates from Clade SA

When relative gene expression of *ERG11* gene was compared among 3 fluconazole resistant and 3 susceptible *C. albicans* isolates from clade SA, the following results were obtained. These results are shown in Table 5.1 and Figure 5.5.

ERG11 gene was found to be over-expressed in resistant isolate G118FR (0.1) when compared to that of its matching susceptible isolate G118FS. When relative gene expression of isolate G116FR was compared with that of its matching susceptible isolate G116FS, *ERG11* gene of G116FR was found to be over-expressed (0.03). No change was observed in expression of *ERG11* gene between resistant isolate K306FR and its matching susceptible isolate K306FS.

These results show that in isolates from clade SA, *ERG11* over-expression was found in all 2(66.6%) of resistant isolates tested

5.7.3.1e Gene expression among isolates from Clade NG

When relative gene expression of *ERG11* gene was compared among matching 3 fluconazole resistant and 3 susceptible *C. albicans* isolates from clade NG, the following results were obtained. These results are shown in Table 5.1 and Figure 5.5.

When relative gene expression of isolate G22FR was compared with that of its matching susceptible isolate G22FS, *ERG11* gene of G22FR was found to be over-expressed (0.099). *ERG11* gene of resistant isolate G6FR was found to be down-regulated (0.03) as compared to its matching susceptible isolate G6FS (0.1). No change was observed in

expression of *ERG11* gene between resistant isolate K86FR and its matching susceptible isolate K86FS. These results show that in isolates from clade NG, *ERG11* over-expression was found in 1(33.3%) of the resistant isolates tested.

5.7.3.1f Gene expression of fluconazole resistant control

ERG11 gene of fluconazole resistant control was found to be over-expressed. These results are shown in Table 5.1, Figure 5.5 and Figure 5.6.

Table 5.1: Ct values and *ERG11* expression of *C. albicans* fluconazole resistant and susceptible isolates

Isolate	Clade	MIC	*ERG11* Mean Ct values	*18SrRNA* Mean Ct values	Δ*CT*	$2^{-\Delta CT}$	Expression Level
G63FS	**I**	**0.25**	21.35	16.02	5.33	0.024	
G63FR	**I**	**1:128**	17.3	13.31	3.99	0.062	**Over-expressed**
K239FS	**I**	**0.38**	19.06	13.09	5.97	0.016	
K239FR	**I**	**1:256**	17.86	13.15	4.71	0.038	**Over-expressed**
K21FS	**I**	**0.9**	16.99	12.05	4.94	0.03	
K21FR	**I**	**≥1:256**	17.00	12.00	5	0.03	Not over-expressed
K162FS	**II**	**0.19**	19.72	13.09	6.63	0.01	
K162FR	**II**	**1:128**	17.71	13.13	4.58	0.04	**Over-expressed**
K153FS	**II**	**0.75**	18.54	14.18	4.36	0.05	
K153FR	**II**	**≥1:256**	16.99	12.16	4.83	0.03	Not Over-expressed
G19FS	**II**	**0.125**	21.02	12.09	8.93	0.002	
G19FR	**II**	**1:128**	17.42	10.9	6.52	0.01	**Over-expressed**
OKP25FS	**III**	**0.125**	19.85	13.59	6.26	0.013	
OKP25FR	**III**	**1:128**	18	12.05	5.95	0.016	Not over-expressed
UP30FS	**III**	**0.19**	18.51	13.66	4.85	0.034	
UP30FR	**III**	**≥1:256**	20	15.21	4.79	0.036	Not over-expressed
G58FS	**III**	**0.25**	20.08	13.67	6.41	0.01	
G58FR	**III**	**≥1:256**	16.29	12.78	3.51	0.08	**Over-expressed**
G118FS	**SA**	**1.0**	20.09	13.08	7.01	0.007	
G118FR	**SA**	**≥1:256**	17.02	13.73	3.29	0.1	**Over-expressed**
G116FS	**SA**	**0.5**	19.9	13.19	6.71	0.009	

G116FR	**SA**	**≥1:256**	18.11	13.07	5.04	0.03	**Over-expressed**
K306FS	**SA**	**0.75**	17.15	13.07	4.08	0.05	
K306FR	**SA**	**≥1:256**	17.99	13.74	4.25	0.05	Not Over-expressed
G22FS	**NG**	**0.25**	18.37	14.52	3.85	0.069	
G22FR	**NG**	**≥1:256**	16.20	12.87	3.33	0.099	**Over-expressed**
G6FS	**NG**	**0.38**	16.40	13.18	3.22	0.1	
G6FR	**NG**	**1:128**	17.75	12.52	5.23	0.03	Not Over-expressed
K86FS	**NG**	**0.125**	19.13	13.58	5.55	0.02	
K86FR	**NG**	**≥1:256**	18.16	12.55	5.61	0.02	Not Over-expressed
FRC		**1:128**	16	13.00	3.00	0.125	**Over-expressed**
FSC		**2**	17.90	13.00	4.9	0.03	

FR=Fluconazole resistant isolate; **FS**=Fluconazole susceptible isolate; **FRC**= Fluconazole Resistant control;

FSC=Fluconazole Susceptible Control

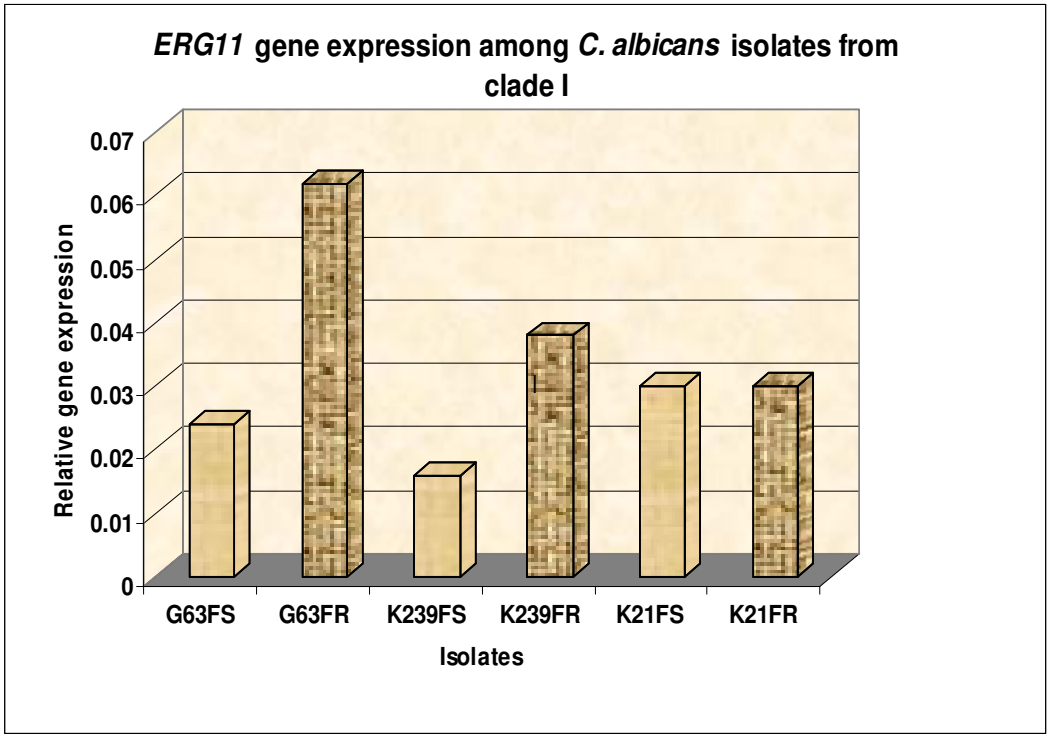

Figure 5.6: *ERG11* gene expression among *C. albicans* fluconazole resistant and susceptible isolates from clade I.

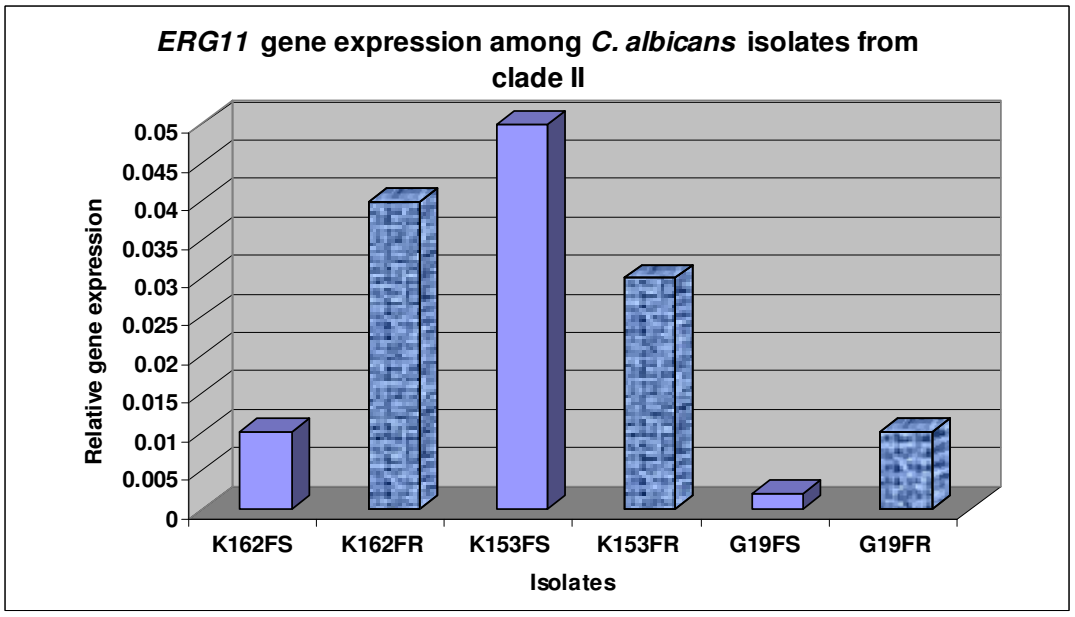

Figure 5.7: *ERG11* gene expression among *C. albicans* fluconazole resistant and susceptible isolates from clade II

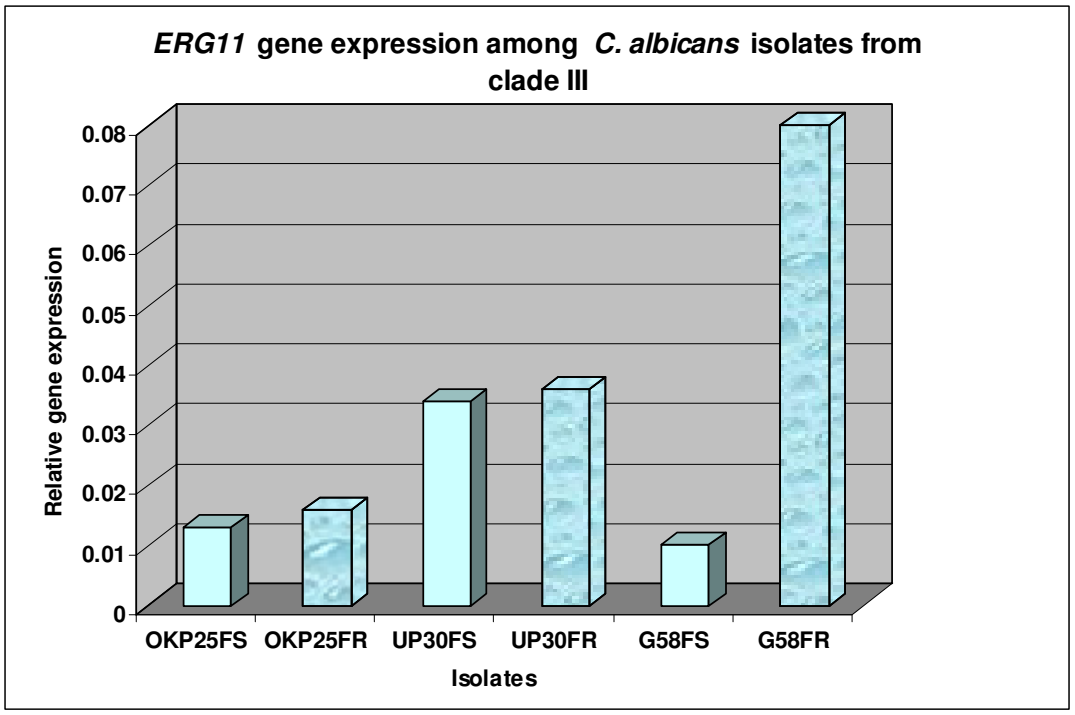

Figure 5.8: *ERG11* gene expression among *C. albicans* fluconazole resistant and susceptible isolates from clade III

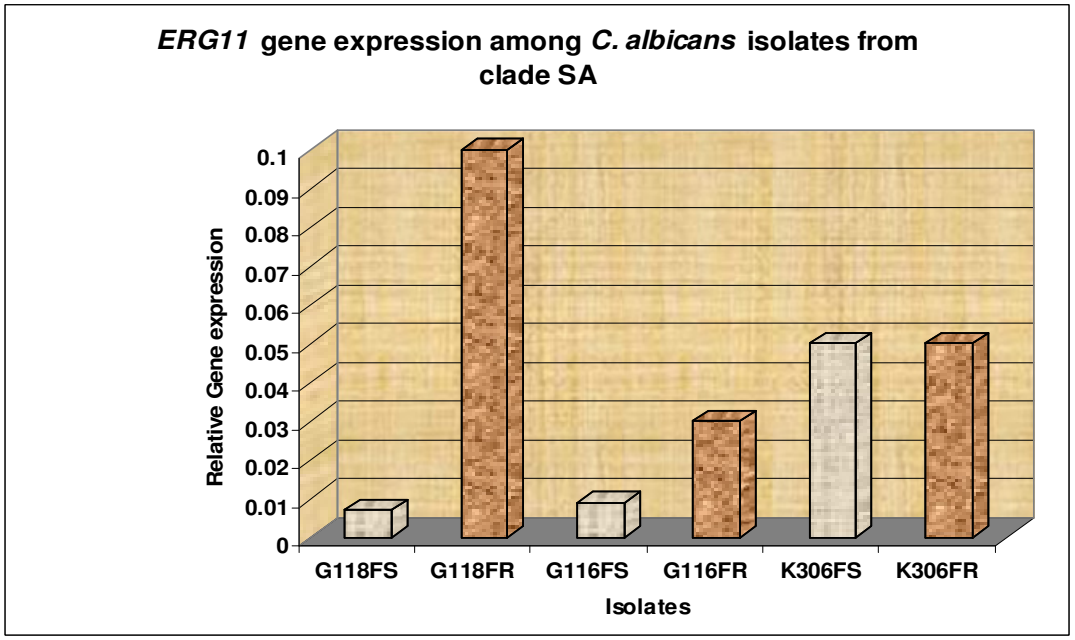

Figure 5.9: *ERG11* gene expression among *C. albicans* fluconazole resistant and susceptible isolates from clade SA

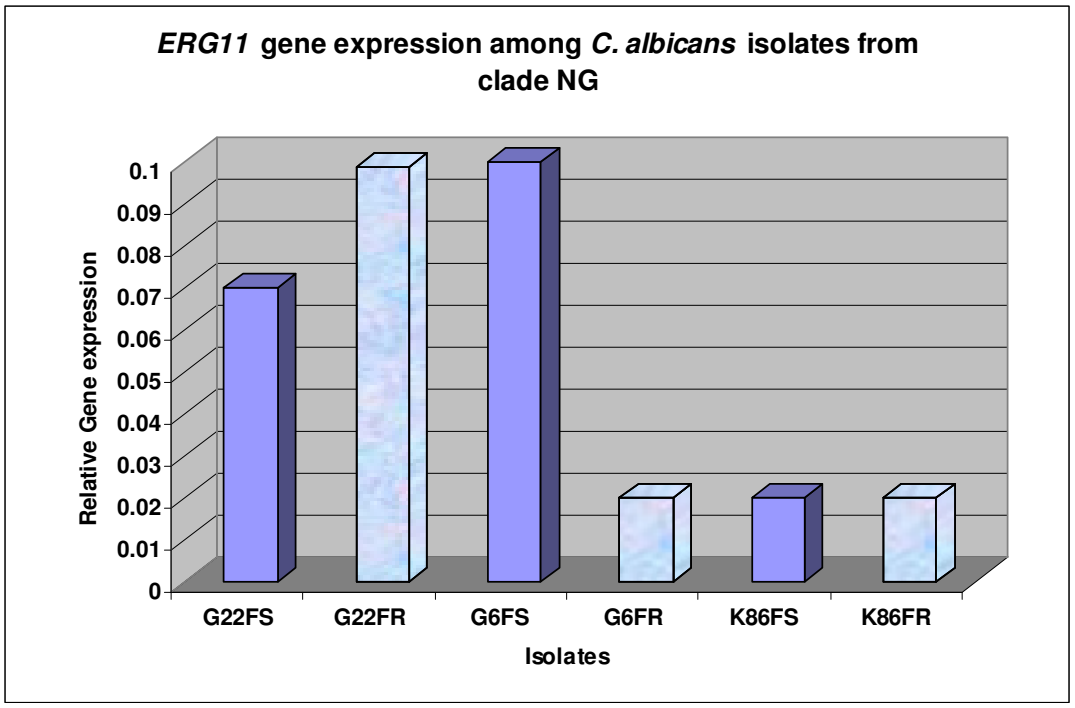

Figure 5.10: *ERG11* gene expression among *C. albicans* fluconazole resistant and susceptible isolates from clade NG

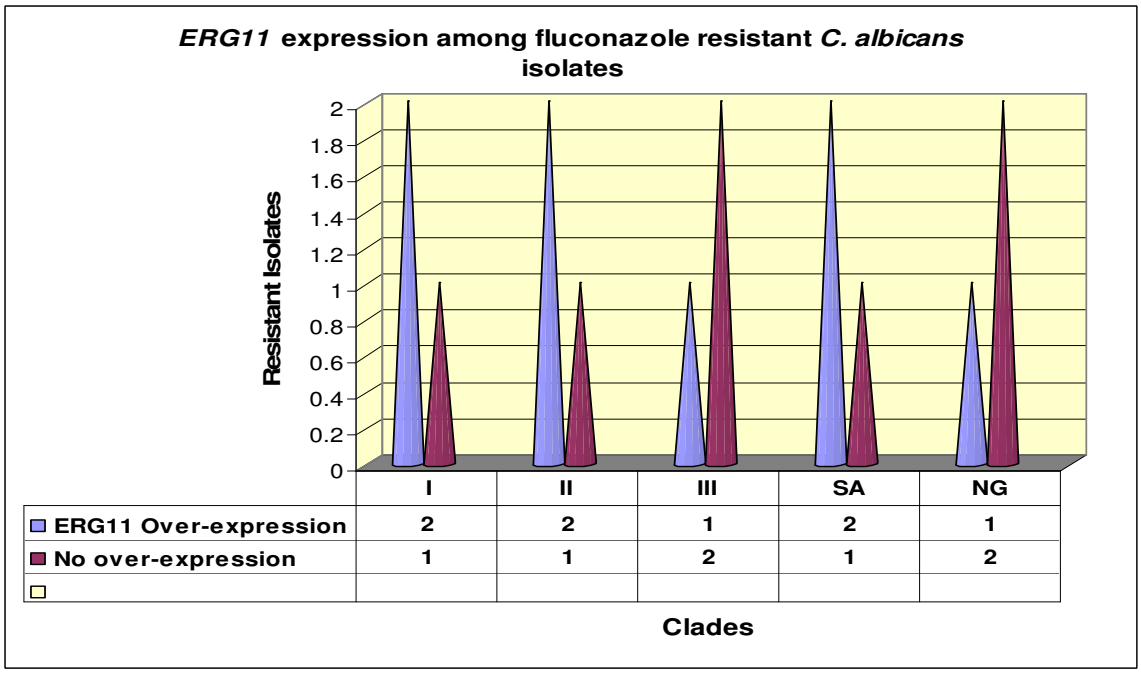

Figure 5.11: *C. albicans* fluconazole resistant isolates with over-expression of *ERG11* gene among different clades

5.8 Discussion

Over-expression of *ERG11* gene has been associated with azole resistance (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Franz et al., 1998; Henry et al., 2000), but nothing is known about the contribution of over-expression of this gene to fluconazole resistance among different clades of *C. albicans*, and whether over-expression will occur more in one particular clade as compared to the others.

Quantification of *ERG11* gene expression in different clades of *C. albicans* isolates resistant to fluconazole may produce a better understanding of the structure of this enzyme, and may lead to the designing of new drugs, which will continue using *ERG11* gene product as the antifungal target. This study was done to determine whether the induced resistance to fluconazole among *C. albicans* isolates was due to over-expression of *ERG11* gene, and whether over-expression was clade-related.

To our knowledge, this study is the first to be performed, where contribution of over-expression of *ERG11* gene to fluconazole resistance was determined among different clades of *C. albicans.* In this study, Real-time RT-PCR was used to quantify relative gene expression in matched fluconazole resistant and susceptible *C. albicans* isolates from clades I, II, III, SA and NG. This molecular method couples PCR with on-line fluorescence detection of amplification products, and is now the method that is widely used for gene expression quantification (Higuchi et al., 1993; Wittwer et al., 1997a), because of its increased sensitivity for detection of low amounts of mRNA, accurate mRNA quantification, and the ability to convert unstable RNA template into cDNA, which is less susceptible to degradation (Frade et al., 2004).

In this study, over-expression of *ERG11* gene was observed in 2(66.6%) isolates from each of clades I, II, and SA, and in 1(33.3%) isolate from each of clades III and NG. These results suggest that fluconazole resistance among these isolates from these clades was associated with over-expression of *ERG11* gene. These results of this study are in agreement with results obtained by previous investigators, where over-expression of *ERG11* gene was found to be associated with fluconazole resistance. In a study by Sanglard et al. (1995), an increase in mRNA levels of *ERG11* in several fluconazole-

resistant *C. albicans* isolates from AIDS patients was observed when Northern blotting technique was used. In their study, Albertson et al. (1996) exposed a fluconazole susceptible isolate to increasing concentrations of fluconazole, and *ERG11* gene was found to be over-expressed in this isolate after Northern blotting. A study by White (1997) investigated possible mechanisms associated with the development of drug resistance in a series of 17 clinical isolates taken from a single HIV-infected patient over 2 years. Using Northern blot analysis, increased mRNA levels of *ERG11* were observed in resistant isolates from that study.

In their study, Franz et al. (1998) showed that some fluconazole-resistant isolates can express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug. In that study, *C. albicans* was demonstrated to respond to the presence of fluconazole by enhancing the expression of the *ERG11* gene, and increased *ERG11* mRNA levels were clearly correlated with drug resistance in 1 isolate in that study. The *ERG11* gene in this isolate was constitutively expressed at higher levels than in previous isolates from the same patient even after induction of the gene by fluconazole. In a study by Henry et al. (2000), isolates exposed to azoles were described to show increased expression of *ERG11* within a few hours of exposure, contributing significantly to the survival of azole-treated cells.

Cowen et al. (2000) exposed six azole-susceptible *C. albicans* isolates for 100 days to RPMI 1640 medium with fluconazole at twice their most recently measured MIC. In that study, among the six isolates grown with fluconazole, two achieved fluconazole resistance of MIC 64μg/ml. Upregulation of *ERG11* gene was observed in the fluconazole resistant isolates in that study. In their study, Perea et al. (2001) used Northern blot technique with probes specific for the *ERG11* gene to determine *ERG11* gene expression in total RNA extracted from the different isolates growing in YEPD medium in the absence of an antifungal. Upregulation of *ERG11* genes was detected in seven isolates from five patients in that study.

Lee et al. (2004) used twelve *C. albicans* isolates; seven trailing and five susceptible dose dependent (SDD) or resistant isolates to screen for quantification of *ERG11* expression by RT-PCR using the LightCycler high-speed PCR system. Quantification of *ERG11* expression revealed that both trailing and SDD and resistant isolates were capable of *ERG11* up-regulation in response to fluconazole, although the SDD and resistant isolates showed maximal up-regulation at higher fluconazole concentrations. In a study by Frade et al. (2004), RT-LightCycler PCR was compared with Northern hybridization for quantitative analysis of gene expression in isolates with various fluconazole susceptibilities. In that study, *ERG11* gene was found to be over-expressed in 3 resistant isolates tested, and the quantification results between RT-LightCycler PCR and Northern hybridization were shown to correlate. In a study by Chau et al. (2004), Real-time quantitative PCR was used to measure *ERG11* expression levels of 38 clinical isolates, and *ERG11* gene was found to be over-expressed in 3 resistant isolates.A recent study by Ribeiro and Paula (2007) showed that over-expression of *ERG11* gene was the predominant mechanism in experimentally induced fluconazole resistance.

The results of our study also show that over-expression of *ERG11* gene was not observed in 1(33.3%) isolate from each of clades I, II and SA, and in 2 (66.6%) isolates from each of clades III and NG. These results suggest that fluconazole resistance in these isolates was not associated with over-expression of *ERG11* gene. These results are in agreement with those of previous studies, where over-expression of *ERG11* gene was not correlated with fluconazole resistance. In a study by Lopez-Ribot et al. (1998), six sequential isolates from an HIV-infected patient with OPC were analyzed by using Northern blot, and the results revealed that levels of *ERG11* mRNA remained constant for all isolates examined. These investigators suggested that it should be noted that other yet unrecognized mechanisms may be operational in this series of isolates and may have contributed to the overall decrease in fluconazole susceptibilities (Sanglard et al., 1998).

In their study, Martinez et al. (2002) determined *ERG11* gene expression in an isolate from a patient with AIDS who was receiving continuous oral fluconazole for treatment of OPC. In that study, fluconazole resistance was not found to correlate with over-expression of *ERG11* gene. White et al. (2002) used Northern blotting analysis to quantify *ERG11* gene expression in azole resistant isolates. Although considerable variation in the levels of *ERG11* expression was observed within this collection of

isolates, the level of expression did not appear to be related to the azole resistance of the isolates.

In a study by Rogers and Barker (2002), no change in over-expression of *ERG11* gene was observed in resistant isolates, which is in accordance with results from our study. These investigators suggested that the low expression of *ERG11* gene among resistant isolates in their study may have been due to choice of controls used for normalization of gene expression. Most of the previous studies used *ACT1* gene as a control gene, while these investigators used *18SrRNA* gene, and they suggested that it was possible that differences in the expression of *ACT1* between resistant and susceptible isolates could count for this discrepancy. This could have been the case with our study, since we also used *18SrRNA* as a normalization gene. Our results also correlate with those of a study by Park and Perlin (2005), where quantitative real-time PCR with molecular beacons was used to determine gene expression in fluconazole-resistant isolates. Changes in expression levels for *ERG11* were not statistically correlated with fluconazole resistance in that study.

The results of our study show that over-expression of *ERG11* gene was not observed in some of the resistant isolates tested. Maximal induction of the *ERG11* promoter by azoles is said to occur not during logarithmic growth but after the diauxic shift and requires azoles to be present throughout logarithmic growth. In a study by Henry et al. (2000), azoles were shown to increase expression of *ERG11* within a few hours of azole exposure, contributing significantly to the survival of azole-treated cells. That same report also found that *ERG11* expression quickly decreases upon removal of drug exposure to the yeasts.

However, the absence of *ERG11* over-expression in the isolates from our study could not have been caused by growing isolates in the absence of fluconazole, as these isolates

were continuously grown in the presence of highest concentration of fluconazole. One explanation could be that fluconazole resistance in these isolates could have been due to over-expression of efflux pumps such as *CDR1, CDR2 or MDR1,* or due to another un-described mechanism as suggested by Sanglard et al. (1998). Fluconazole resistance among these isolates could also have been due to phenotypic switching, as switching has been suggested to be another mechanism by which *C. albicans* achieves resistance against antifungal agents (Soll et al., 1992). Another explanation could be the choice of controls used for normalization of gene expression in our study. Most of the previous studies used *ACT1* gene as a control gene, while we used *18SrRNA* gene, and the differences in the expression of *ACT1* and *18SrRNA* between resistant and susceptible isolates could have counted for this discrepancy.

The results of our study also show that over-expression of *ERG11* gene was clade-related, as over-expression was observed in more isolates from clades I, II and SA (66.6%), and less in isolates from clades III and NG (33.3%). These results suggest that further study of differences between *C. albicans* clades may be warranted. The higher occurrence of over-expression of *ERG11* gene in isolates from clades I, II, and SA, especially clade SA, which is dominant in South Africa, is a cause for concern, especially in HIV/AIDS patients with OPC, as the increased expression of *ERG11* has been reported to allow for the cells to persist within the host, which in turn leads to the subsequent development of other more stable resistant isolates. However, the results of our study could not be compared to other studies as far as clade-relatedness is concerned, as this study is the first to be performed, where contribution of over-expression of *ERG11* gene to fluconazole resistance was determined among different clades of *C. albicans*.

5.9 Conclusion

To our knowledge, this study is the first to be performed, where contribution of over-expression of *ERG11* gene to fluconazole resistance was determined among different clades of *C. albicans.* The results of the study show that over-expression of *ERG11* gene contributed to fluconazole resistance in isolates from all clades. However, over-expression was observed in more isolates from clades I, II and SA and in less isolates from clades III and NG, showing clade-relatedness of *ERG11* over-expression. These results suggest that further study of differences between *C. albicans* clades may be warranted.

The higher occurrence of over-expression of *ERG11* gene in isolates from clades I, II, and SA, especially clade SA, which is dominant in South Africa, is a cause for concern, especially in HIV/AIDS patients with OPC, as the increased expression of *ERG11* has been reported to allow for the cells to persist within the host, which in turn leads to the subsequent development of other more stable resistant isolates.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level (Pfaller, 2000).

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CHAPTER SIX: RELATIONSHIP BETWEEN PHENOTYPIC SWITCHING AND FLUCONAZOLE RESISTANCE AMONG *CANDIDA ALBICANS* CLADES

6.1 Abstract

Background

Increased resistance to fluconazole has been reported both *in vivo* and *in vitro* due to increased use of this antifungal in treatment of *C. albicans* infections in AIDS patients. Several mechanisms have been shown to be involved in the rapid adaptation of *C. albicans* to different environments, and phenotypic switching is one of these mechanisms. *C. albicans* is capable of high-frequency, phenotypic switching, distinguishable by colony morphology. There is evidence to suggest that this phenotypic switching plays a role in fluconazole resistance. Although relationship between phenotypic switching and fluconazole resistance has been studied in different strains of *C. albicans*, nothing is known about this relationship among different clades of *C. albicans*.

Objectives

To determine whether the induced fluconazole resistance among *C. albicans* isolates belonging to different clades was related to phenotypic switching, and whether switching was clade-related.

Materials and Methods

Three isolates from each of previously typed *C. albicans* clades I, II, III, SA, and NG (Blignaut et al., 2002), in which fluconazole resistance (MIC≥64µg/ml) was induced in Chapter 2, were selected. Their three matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were also included in the study. These yeast isolates were obtained from surveillance cultures on HIV/AIDS patients attending HIV/AIDS clinics in the Pretoria region. Primary and secondary cultures were prepared by inoculating cells on Lee's medium agar supplemented with arginine and zinc, and containing phloxine B. The plates were incubated at 35°C for 48hrs, and then further at

25°C for 10 days for colony formation and switching. The switched colonies were counted and colony morphologies viewed and photographed. Phenotypic switching behavior and different colony morphologies obtained between the resistant and susceptible isolates from different clades were compared. Switch phenotypes among fluconazole resistant isolates in different clades were compared. Switch phenotypes and MIC levels among fluconazole resistant isolates from different clades were compared.

Results

In this study, eleven colony switch phenotypes were generally observed among fluconazole resistant isolates tested. These included ring; smooth white; irregular wrinkle; stipple; star; switch from ring to irregular wrinkle; smooth with sector; switch from ring to stipple; ring with sector; rough and heavily myceliated.

In isolates from clade I, phenotypic switching was related to resistance in 2 (66.6%) of the resistant isolates tested. The switch phenotypes observed among resistant isolates from this clade were ring with sector; smooth sector; switch from ring to wrinkle; stipple and ring. In isolates from clade II, phenotypic switching was related to resistance in 2 (66.6%) of the resistant isolates tested. Switch phenotypes observed among resistant isolates from this clade were ring with sector; smooth sector; switch from ring to wrinkle; stipple; ring and smooth.

In isolates from clade III, phenotypic switching was related to resistance in 2 (66.6%) of the resistant isolates tested, as switching occurred only in resistant isolates, but not in their matching susceptible isolates. Switch phenotypes observed among resistant isolates from this clade were stipple; ring; irregular wrinkle and rough. In isolates from clade SA, phenotypic switching was related to resistance in 1(33.3%) of the resistant isolates tested. Switch phenotypes observed among resistant isolates from this clade were stipple and irregular wrinkle. In isolates from clade NG, phenotypic switching was related to resistance in 1(33.3%) of the resistant isolates tested, as a different switch phenotype

occurred in this resistant isolates when compared to its matching susceptible isolate. Switch phenotype observed among resistant isolates from this clade was stipple.When the switch phenotypes and MIC levels of resistant isolates from different clades were compared, stipple was the most common switch phenotype observed in all clades, and it was associated with the highest fluconazole MIC levels among isolates from all clades.

Conclusion

To our knowledge, this study is the first to be performed on the assessment of phenotypic switching in *C. albicans* isolates representing the various clades. The results of the study show that phenotypic switching was related to fluconazole resistance among isolates from clades I, II, III, SA and NG. However, switching was observed in more isolates from clades I, II and III (66.6%) and in less isolates from clades SA and NG (33.3%), showing clade-relatedness of switching. These results suggest that further study of differences between *C. albicans* clades may be warranted.

The results show that isolates from clade II had the highest number of switch phenotypes. The high number of switch phenotypes observed in clade II of these fluconazole resistant isolates, as compared to other clades in this study may suggest that *C. albicans* isolates belonging to this clade may survive better under adverse conditions than isolates from other clades, and that isolates from this clade need to be studied further. The dominant phenotype observed in all clades of these fluconazole resistant, South African *C. albicans* isolates was stipple, which was associated with highest fluconazole MICs levels. These results suggests that the antifungal susceptibility of the stipple phenotype requires attention in HIV/AIDS patients, especially in patients who do not respond clinically to fluconazole treatment or in cases of life-threatening *C. albicans* infections of these immuno-compromised hosts.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level.

6.2 Introduction

Oropharyngeal candidiasis (OPC), caused by *C. albicans,* is a significant cause of morbidity and mortality in immunosuppressed individuals infected with HIV (Barchiesi et al., 1996). It is estimated that 80 to 95% of patients infected with HIV will experience at least one episode of OPC during the course of their illness (Sangeorzan et al., 1994). Increased resistance to fluconazole has been reported both *in vivo* and *in vitro* due to increased use of this antifungal in treatment of *C. albicans* infections in AIDS patients (Laguna et al., 1997; Maenza et al., 1997), and there is evidence to suggest that phenotypic switching may play a role in this resistance (Vargas et al., 2000; Kiraz et al., 2000).

Several mechanisms have been shown to be involved in the adaptability of *C. albicans* to different environments, including the bud-hypha transition, point mutations and high-frequency phenotypic switching (Soll et al., 1991). Previous studies have demonstrated that most of *C. albicans* strains and related species have the capacity to switch spontaneously, at high frequencies and reversibly between different cell types that are distinguishable by the appearance of the resulting colonies on agar plates with low zinc concentrations (Slutsky et al., 1985; Slutsky et al., 1987; Gil et al., 1988; Soll, 1992; Hellstein et al., 1993; Soll, 2000).

In previous studies done, switching was shown to play an important role in *C. albicans* pathogenesis, by regulating several phase-specific genes involved in pathogenesis (Soll, 1997; Soll, 2000). In other studies, switching was shown to regulate *C. albicans* antigenicity (Anderson et al., 1990), constraints on the bud-hypha transition (Anderson et al., 1993), sensitivity to neutrophils and oxidants (Kolotila and Diamond, 1990), adhesion (Vargas et al., 1994), secretion of aspartyl proteinase (Morrow et al., 1992), and virulence in a mouse model (Kvaal et al., 1999).

In addition, switching has been shown to occur at sites of infection. In a study of a single recurrent vaginitis patient, it was demonstrated by DNA fingerprinting that the same strain was responsible for three successive infections and that phenotypic switching

occurred between episodes. The results of that study suggested that each switching phenotype of the infecting strain may exhibit a specific drug susceptibility profile (Soll et al., 1989). High-frequency switching, in addition to other mechanisms, is suggested to be another mechanism by which *C. albicans* achieves resistance against antifungal agents (Soll et al., 1992). In one study, switched *C. albicans* isolates from HIV positive individuals were found to be more resistant to a number of antifungal drugs including amphotericin B and fluconazole, than isolates from HIV-negative controls (Vargas et al., 2000).

Results from several studies have demonstrated that infecting isolates switch at significantly higher frequencies than commensal isolates (Gallagher et al., 1992; Hellstein et al., 1993), and that isolates causing deep mycoses switch more frequently than isolates causing superficial mycoses (Jones et al., 1994). Switching may provide *C. albicans* with mechanisms for adapting to different environments, for evading host defense mechanisms and for adhering to different types of surfaces (Vargas et al., 1994).

Switching is usually distinguished by colony morphology and in some cases by the phenotype of cells in the budding phase (Anderson and Soll, 1987; Anderson et al., 1989; Vargas et al., 2000). It has been suggested that switching in *C. albicans* involves differential gene expression (Kvaal et al., 1999; Sonneborn et al., 1999; Srikantha et al., 2000), therefore it is possible that different phenotypic variants possess different physiologic and virulent traits. Previous studies have also demonstrated that switch phenotypes of *C. albicans* differ in their adhesion to epithelia (Kennedy et al., 1988).

Three different systems of phenotypic switching have been described in *C. albicans*; the white–opaque transition; the 3153A-type switching and the unmyceliated-heavy myceliated system:

In the white–opaque transition, first described in the strain WO-1 (Slutsky et al., 1987; Rikkerink et al., 1988), cells switched between a form that gives rise to hemispherical, creamy white colonies (white phase) and a form that produced flat, gray colonies (opaque phase). In that study, variant colony phenotypes showed the following white-opaque transition: switch from white to opaque; switch from opaque to white; white colony with an opaque sector; opaque colony with a white sector; irregular wrinkled colony with a

white sector; irregular wrinkled colony next to white colony with an opaque sector; medusa colony; opaque colony with a medusa sector; switch from opaque to white and medusa; fried-egg and opaque colonies and fried-egg sector in an opaque colony (Slutsky et al., 1987).

The other switching system, 3153A-type switching, was described in cultures of the standard laboratory strain 3153A (Slutsky et al., 1985), and it has also been observed in other laboratory strains and clinical isolates (Pomes et al., 1985; Soll et al., 1987). This type of switching was different from the white– opaque transition, and produced at least seven different colony morphologies when plated on an amino acid-rich agar that has low zinc concentrations, then incubated at 25°C. These phenotypes included "smooth", "star", "ring", "irregular wrinkle" "stipple", "fuzzy" and reverant smooth (Slutsky et al., 1985; Ramsey et al., 1994). The variant colony phenotypes in strain 3153A were found to be due partially to the proportion and distribution of budding cells, hyphae, pseudohyphae, and branched hyphae in the colony dome (Slutsky et al., 1985; Pomes et al., 1985). The third system, unmyceliated-heavy myceliated includes strains that switch between colonies with and without dense myceliation (Soll et al., 1987).

Limited studies on the relationship between phenotypic switching and antifungal resistance of *C. albicans* have been done. In one study, the different colony variations *in vitro* were shown to be able to generate phenotypes with stable, reduced azole susceptibility without prior exposure to azoles (Gallagher et al., 1992). In their study, Velegraki et al. (1996) isolated different phenotypes of *C. albicans* strains from neutropenic patients who had no prior prophylactic therapy. In that study, the smooth, irregular, fuzzy and stipple phenotypes were obtained, and the stipple phenotype was found to have consistently higher MIC levels as compared to other phenotypes (Velegraki et al., 1996).

Kiraz et al. (2000) showed that switched phenotypes from HIV positive individuals were more resistant to fluconazole and voriconazole than those from HIV negative individuals. These investigators reported that fluconazole susceptibility of the stipple phenotype showed statistically significant difference from other phenotypes. In another study, the fluconazole, voriconazole and itraconazole MIC mean levels of the stipple phenotype were found to be higher than that of smooth, irregular, star, fuzzy and ring (Cetinkaya and Kiraz, 2005).

6.3 Study problem

Several studies have demonstrated that the expression of specific virulence and pathogenesis factors of *C. albicans* are correlated with phenotypic switching (White et al., 1993; Miyasaki et al., 1994; White and Agabian, 1995; Vargas et al., 2000), therefore the importance of phenotypic switching in the *C. albicans* disease in humans is significant, and needs to be studied further. In addition, there is evidence to suggest that phenotypic switching may play a significant role in the ability of *Candida* strains to survive under adverse conditions and perhaps cause more severe forms of disease in the immunocompromised host (Vargas et al., 2004).

Only limited studies on the relationship between phenotypic switching and fluconazole resistance of *C. albicans* have been done, and nothing is known about this relationship among different clades of *C. albicans*. It is not known whether a particular switch phenotype will be more common in one particular clade, especially the unique South African clade (SA), and whether switching is clade-related. Knowledge in this regard would contribute to a better understanding of fluconazole resistance and subsequently to improved management of South African patients infected with fluconazole resistant *C. albicans*.

6.4 Aim

To investigate the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades.

6.5 Objectives

a) To prepare a primary culture of matching fluconazole resistant and susceptible isolates belonging to *C. albicans* clades I, II, III, SA, and NG on Lee's medium.
b) To prepare a secondary culture by spreading equal number of cells from isolates on Lee's medium.
c) To count the number of colonies and switched colonies by using a colony counter.
d) To assess and compare phenotypic switching between fluconazole resistant and susceptible isolates from different clades.
e) To compare switch phenotypes from fluconazole resistant isolates among different clades
f) To compare observed switch phenotypes and MIC levels among fluconazole resistant isolates

6.6 Materials and Methods

6.6.1 Isolates and primary culture

Glycerol stocks of three isolates from each of previously typed *C. albicans* clades I, II, III, SA, and NG (Blignaut et al., 2002) with induced fluconazole resistance (MIC≥64µg/ml), and their 3 matching fluconazole susceptible (MIC≤8µg/ml) isolates (parent strains) from the same clades were selected for assessing phenotypic switching. *C. albicans* strain 3153A was included in the study as a control. Fifty microlitres of stored isolates were spread in agar plates containing the nutrient components of the defined medium of Lee (Lee et al., 1975), supplemented with 70µg/ml of arginine and 0.1µM of zinc sulphate (Bedell and Soll, 1979). To visualize sectoring, 5µg/ml of phloxine B was added to the medium (Anderson and Soll, 1987). The plates were incubated at 35°C for 48hrs. After incubation, plates were covered with parafilm and incubated again at 25°C for 10 days in the dark.

6.6.2 Secondary culture of cells

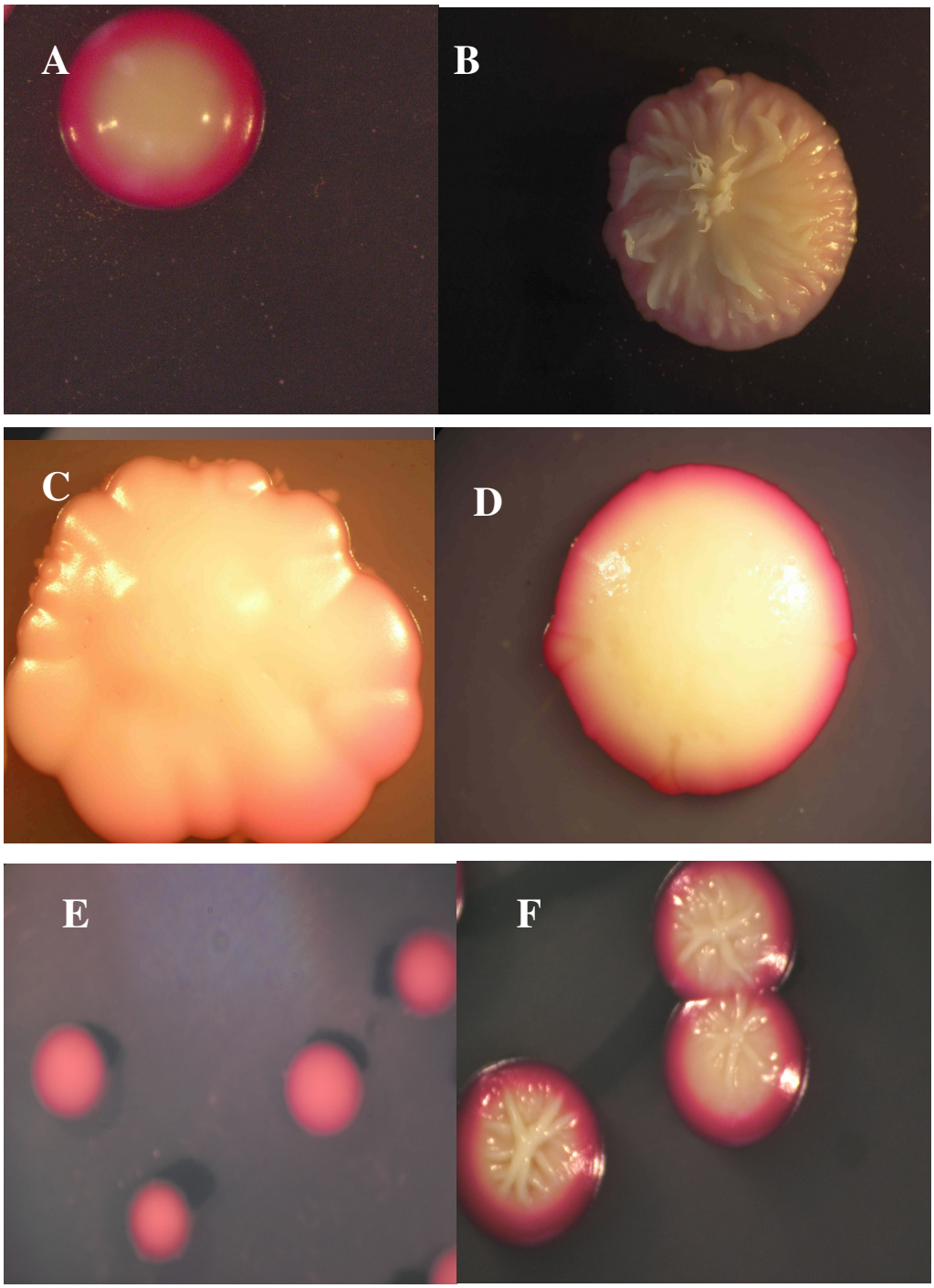
Isolated colonies obtained from the primary cultures were suspended in 1000µl of sterile water, counted with a counting chamber (Hausser Scientific, Horsham, PA, USA) on a light microscope, and serially diluted in sterile water to a final concentration of 10^3cells/ml. A 100µl volume containing 100 cells was spread on 10 agar plates containing supplemented Lee's medium. Plates were incubated at 35°C for 48hrs. After incubation, plates were covered with parafilm and incubated again at 25°C for 10 days in the dark.

6.6.3 Counting of colonies and switched colonies

The total number of colonies and switched colonies were counted using a Stuart colony counter (Stuart Scientific Co. LTD, UK). Different colony morphologies were viewed with Olympus microscope (Olympus Optical Co., Tokyo, Japan), and photographed. When all colonies were identical, it was assumed that phenotypic switching had not occurred. When more than one phenotype was seen, the most frequent phenotype was assumed to be the native type and all others were presumptive switched phenotypes (Jones et al., 1994). Phenotypic switching behavior and different colony morphologies obtained between the resistant and susceptible isolates from different clades were compared. Switch phenotypes and MIC levels among fluconazole resistant isolates from different clades were compared.

6.7 Results

In this study, when *C. albicans* isolates with induced fluconazole resistance and their matching susceptible isolates were grown on Lee's medium, eleven colony phenotypes were generally observed. These phenotypes included ring; smooth white; irregular wrinkle; stipple; star; switch from ring to irregular wrinkle; switch from ring to stipple; ring with sector; rough; smooth with sector and heavily myceliated. *C. albicans* strain 3153A, which was used as a control in this study, exhibited these switch phenotypes: smooth, star, ring, irregular wrinkle and stipple. Examples of these colony phenotypes are shown in Figure 6.1.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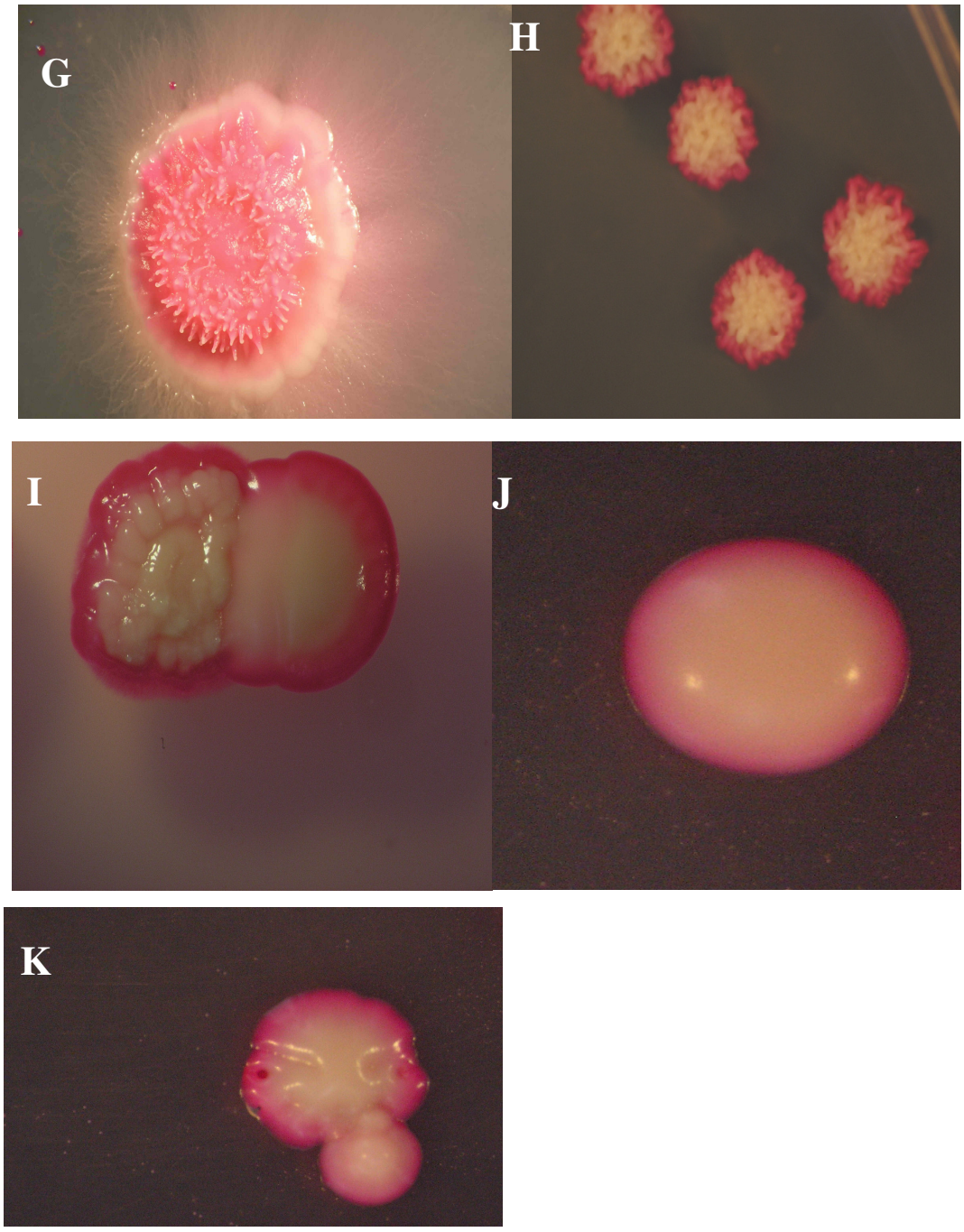

Figure 6.1: Examples of switch phenotypes originating from fluconazole resistant and susceptible *C. albicans* isolates. (A) Ring; (B) Star; (C) Stipple; (D) Smooth with sector; (E) Rough; (F) Switch from ring to wrinkle; (G) Heavily myceliated star; (H) Irregular wrinkle; (I) Ring with sector; (J) Smooth; (K) Switch from ring to stipple.

6.7.1 Phenotypic switching among fluconazole resistant and susceptible isolates from different *C. albicans* clades

Secondary cultures were prepared from isolated colonies obtained from primary cultures on Lee's medium, and incubated at 25°C for 10 days in the dark. Switched colonies were counted and photographed. When all colonies were identical, it was assumed that phenotypic switching had not occurred. When more than one phenotype was seen, the most frequent phenotype was assumed to be the native type and all others were presumptive switched phenotypes (Jones et al., 1994). When a resistant isolate exhibited the same phenotype as its matching susceptible isolate, the resistant isolate was reported as "not switched". Phenotypic switching behavior and different colony morphologies obtained between the resistant and susceptible isolates from different clades were compared.

The following results were obtained among isolates from various clades. These results are shown in Table 6.1 and Figure 6.2

6.7.1.1 Switching among isolates from clade I

When phenotypic switching was assessed among 3 fluconazole resistant isolates and their matching 3 susceptible isolates from this clade, the following results were obtained:

No switching behaviour was observed in fluconazole resistant (G63FR) and its matching susceptible isolate (G63FS), as they both exhibited the same irregular wrinkle phenotype. No switching behaviour was observed in a susceptible isolate K239FS, while its matching resistant isolate K239FR exhibited ring with sector, smooth with sector and ring phenotypes. No switching behaviour was observed in a susceptible isolate K21FS, while its matching resistant isolate K21FR exhibited switch from ring to irregular wrinkle and stipple phenotypes.

These results show that in isolates from clade I, phenotypic switching was related to resistance in 2 (66.6%) of the resistant isolates tested.

6.7.1.2 Switching among isolates from clade II

When phenotypic switching was assessed among 3 fluconazole resistant isolates and their matching 3 susceptible isolates from this clade, the following results were obtained:

Susceptible isolate K162FS exhibited switching phenotype star, while its matching resistant isolate K162FR exhibited these different switch phenotypes: switch from ring to stipple; switch from ring to irregular wrinkle; ring and smooth white. Switching was observed in this resistant isolate as it exhibited different phenotypes from its matching susceptible isolate. Susceptible isolate G19FS exhibited smooth white and ring with sector phenotypes, while no switching behaviour was observed in its matching resistant isolate G19FR. Susceptible isolate K153FS exhibited switching phenotypering with sector, while its matching resistant isolate K153FR exhibited smooth with sector and stipple phenotypes. Switching was observed in this resistant isolate as it exhibited a different phenotype from its matching susceptible isolate.

These results show that in isolates from clade II, phenotypic switching was related to resistance in 2 (66.6%) of the resistant isolates tested.

6.7.1.3 Switching among isolates from clade III

When phenotypic switching was assessed among 3 fluconazole resistant isolates and their matching 3 susceptible isolates from this clade, the following results were obtained:

Susceptible isolate OKP25FS exhibited no switching phenotype, while its matching resistant isolate OKP25FR exhibited ring phenotype. Susceptible isolate UP30FS exhibited no switching phenotype, while its matching resistant isolate UP30FR exhibited rough, stipple and irregular wrinkle phenotypes. Susceptible isolate G58FS exhibited stipple switching phenotype, while its matching resistant isolate K153FR exhibited no switching behaviour.

These results show that in isolates from clade III, phenotypic switching was related to fluconazole resistance in 2 (66.6%) of the resistant isolates tested.

6.7.1.4 Switching among isolates from clade SA

When phenotypic switching among 3 fluconazole resistant and 3 susceptible isolates from this clade was compared, the following results were obtained.

Susceptible isolate G116FS exhibited switching phenotypes heavily myceliated star and irregular wrinkle, while its matching resistant isolate G116FR exhibited irregular wrinkle phenotype. No switching was observed in this resistant isolate as it exhibited the same phenotype as its matching susceptible isolate. Susceptible isolate G118FS exhibited stipple phenotype, while its matching resistant isolate G118FR exhibited the same phenotype. Therefore no switching was observed in this resistant isolate. No switching was observed in a susceptible isolate K306FS, while its matching resistant isolate K306FR exhibited irregular wrinkle and stipple phenotypes.

These results show that in isolates from clade SA, phenotypic switching was related to fluconazole resistance in 1 (33.3%) of the resistant isolates tested.

6.7.1.5 Switching among isolates from clade NG

When phenotypic switching was assessed among 3 fluconazole resistant isolates and their matching 3 susceptible isolates from this clade, the following results were obtained:

Susceptible isolate G22FS exhibited switching phenotype ring, while its matching resistant isolate G22FR exhibited stipple phenotype. Susceptible isolate G6FS exhibited switching phenotype stipple, while its matching resistant isolate G6FR exhibited no switching behaviour. No switching was observed in both susceptible isolate K86FS and its matching resistant isolate K86FR

These results show that in isolates from clade NG, phenotypic switching was related to resistance in 1 (33.3%) of the resistant isolates tested.

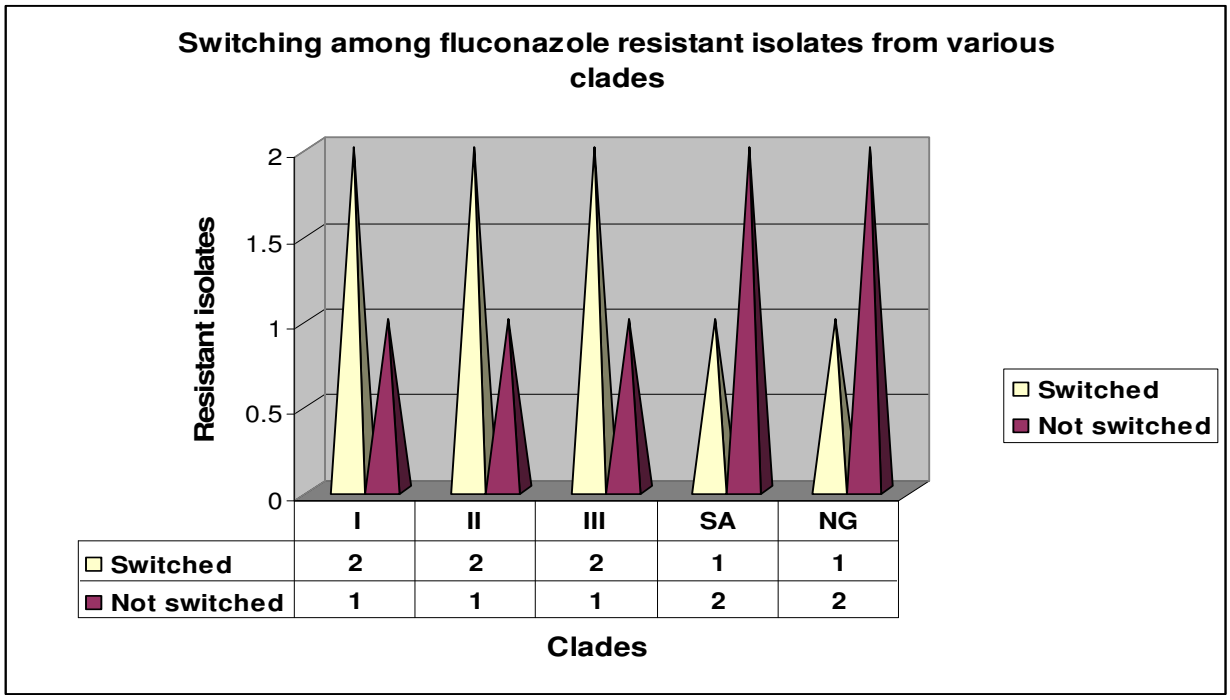

Figure 6.2: Fluconazole resistant isolates with switching among various *C. albicans* clades

Table 6.1: Switching and switch phenotypes among fluconazole resistant and susceptible *C. albicans* isolates from different clades

Isolate	Clade	*MIC*	Original phenotype in primary cultures	Switch phenotypes in secondary cultures	Final switching result in resistant isolates
G63FS	**I**	**0.25**	**Irregular wrinkle**	**No switching**	
G63FR	**I**	**1:128**	**Irregular wrinkle**	**No switching**	**No switching**
K239FS	**I**	**0.38**	**Irregular wrinkle**	**No switching**	
K239FR	**I**	**1: 256**	**Smooth white**	**Ring with sector smooth with sector Ring**	**Switching**
K21FS	**I**	**0.19**	**Smooth myceliated**	**No switching**	
K21FR	**I**	**≥1:256**	**Ring with "pimples"**	**Switch from ring to irregular wrinkle; stipple**	**Switching**
K162FS	**II**	**0.19**	**Irregular wrinkle**	**Star**	
K162FR	**II**	**1:128**	**Rough ring**	**Switch from ring to stipple; switch from ring to irregular wrinkle; ring ; smooth white**	**Switching**
G19FS	**II**	**0.125**	**Ring**	**Ring with sector; smooth white**	
G19FR	**II**	**1:128**	**Smooth white**	**No switching**	**No switching**
K153FS	**II**	**0.75**	**Ring**	**Ring with sector; smooth white**	
K153FR	**II**	**≥1:256**	**Ring**	**Smooth with sector ; Stipple; smooth white**	**Switching**
OKP25FS	**III**	**0.125**	**Ring**	**No switching**	
OKP25FR	**III**	**1:128**	**Smooth white**	**Ring**	**Switching**
UP30FS	**III**	**0.19**	**Irregular wrinkle**	**No switching**	

UP30FR	**III**	**≥1:256**	**Smooth white**	**Rough, stipple, irregular wrinkle**	**Switching**
G58FS	**III**	**0.25**	**Ring**	**Stipple**	
G58FR	**III**	**≥1:256**	**Smooth white**	**No switching**	**No switching**
G116FS	**SA**	**0.5**	**Smooth white**	**Heavily myceliated star; irregular wrinkle**	
G116FR	**SA**	**≥1:256**	**Smooth white**	**Irregular wrinkle**	**No switching**
G118FS	**SA**	**1.0**	**Smooth white**	**Stipple**	
G118FR	**SA**	**≥1:256**	**Smooth white**	**Stipple**	**No switching**
K306FS	**SA**	**0.75**	**Smooth white**	**No switching**	
K306FR	**SA**	**≥1:256**	**Smooth white**	**Irregular wrinkle; stipple**	**Switching**
G22FS	**NG**	**0.25**	**Smooth white**	**Ring**	
G22FR	**NG**	**≥1:256**	**Smooth white**	**Stipple**	**Switching**
G6FS	**NG**	**0.38**	**Smooth white**	**Stipple**	
G6FR	**NG**	**1:128**	**Smooth white**	**No switching**	**No switching**
K86FS	**NG**	**0.125**	**Ring**	**No switching**	
K86FR	**NG**	**≥1:256**	**Smooth white**	**No switching**	**No switching**

6.7.2 Switch phenotypes observed among fluconazole resistant isolates from different clades

When switched phenotypes from secondary cultures of fluconazole resistant isolates were compared between the clades, the following results were observed. These results are shown in Figure 6.3.

6.7.2.1 Switch phenotypes among resistant isolates from clade I

Switch phenotypes observed among resistant isolates from this clade were ring with sector, which occurred in 1(33.3%) isolate; smooth with sector in 1(33.3%) isolate; switch from ring to wrinkled in 1 (33.3%) isolate; stipple in 1(33.3%) isolate and ring in 1 (33.3%) isolate.

These results show that there was no dominating phenotype in resistant isolates from clade I.

6.7.2.2 Switch phenotypes among resistant isolates from clade II

Switch phenotypes observed among resistant isolates from this clade were smooth, which occurred in 2(66.6%) isolates; ring in 1(33.3%) isolate; stipple in 1(33.3%) isolate; switch from ring to wrinkle in 1(33.3%) isolate; switch from ring to stipple in 1(33.3%) isolate; smooth with sector in 1(33.3%) isolate.

These results show that in resistant isolates from clade II, phenotype smooth was the most common phenotype observed.

6.7.2.3 Switch phenotypes among resistant isolates from clade III

Switch phenotypes observed among resistant isolates from this clade were wrinkle, which occurred in 1(33.3%) isolate; ring in 1(33.3%) isolate; stipple in 1(33.3%) isolate and rough in 1(33.3%) isolate.

These results show that there was no dominating phenotype in resistant isolates from clade III.

6.7.2.4 Switch phenotypes among resistant isolates from clade SA

Switch phenotypes observed among resistant isolates from this clade were wrinkle, which occurred in 1(33.3%) isolate, and stipple in 1(33.3%) isolate. These results show that there was no dominating phenotype in resistant isolates from clade SA.

6.7.2.5 Switch phenotypes among resistant isolates from clade NG

Stipple was the only switch phenotype observed among resistant isolates from this clade and it occurred in 1 (33.3%) isolate.

When switch phenotypes observed in resistant isolates were compared among all clades, stipple was the most common phenotype observed in all clades (I, II, III, SA and NG), followed by ring, which was found in clades I, II and III. Phenotypes ring with sector; smooth sector and switch from ring to wrinkle were all found in clades I and II. Phenotype irregular wrinkle was found in clades III and SA. Phenotype smooth was found only in clade II, while rough was found only in clade III. Isolates from clade II had the highest number of switch phenotypes. These results are shown in Figure 6.3.

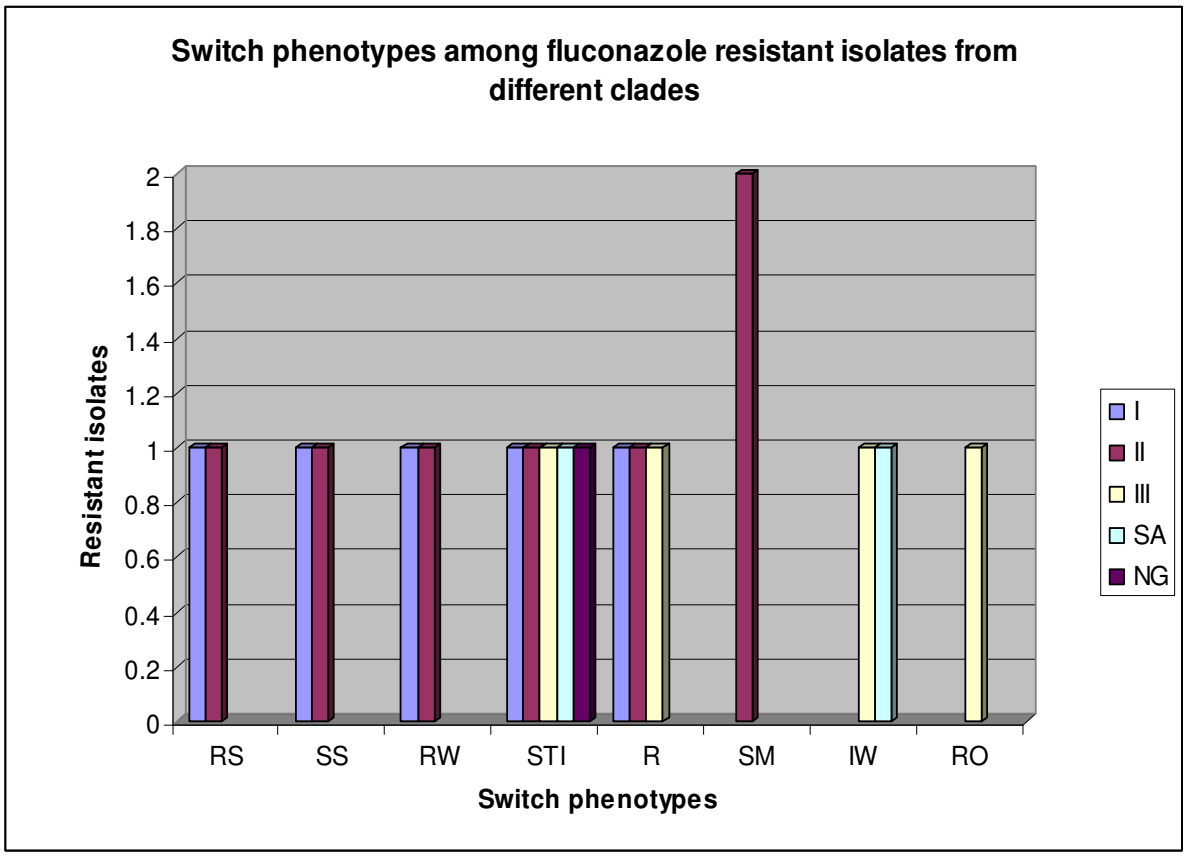

Figure 6.3: Switch phenotypes observed among fluconazole resistant isolates from various *C. albicans* clades. **RS**: ring with sector; **SS**: smooth sector; **RW**: switch from ring to wrinkle; **STI**: stipple; **R**: ring; **SM**: smooth; **IW**: irregular wrinkle; **RO**: rough

6.7.3 Comparison of switch phenotypes with MIC levels among fluconazole resistant isolates

When the MIC levels of fluconazole resistant isolates (determined in Chapter 2) were compared with the switch phenotypes, the following results were observed. These results are shown in Table 6.1.

6.7.3.1 Switch phenotypes and MIC levels among isolates from clade I

Isolate G63FR with MIC 1:128, showed no switching behaviour. Isolate G19FR with MIC 1:128, had the following switch phenotypes: Ring with sector; smooth with sector and ring. Isolate K153FR with MIC ≥1:256, had the following switch phenotypes: switch from ring to irregular wrinkle and stipple.

6.7.3.2 Switch phenotypes and MIC levels among isolates from clade II

Isolate K162FR with MIC 1:128, had the following switch phenotypes: smooth; ring; stipple; switch from ring to wrinkle; switch from ring to stipple. Isolate G19FR with MIC 1:128 showed no switching behaviour. Isolate K153FR with MIC ≥1:256, had the following switch phenotypes: smooth with sector and stipple.

6.7.3.3 Switch phenotypes and MIC levels among isolates from clade III

Isolate OKP25FR with MIC 1:128 had ring phenotype. Isolate UP30FR with MIC ≥1:256 had these switch phenotypes: wrinkle, stipple and rough. Isolate G58FR with MIC ≥1:256 showed no switching behaviour.

6.7.3.4 Switch phenotypes and MIC levels among isolates from clade SA

Isolate G116FR with MIC ≥1:256 had irregular wrinkle phenotype. Isolate G118FR with MIC ≥1:256 had stipple phenotype. Isolate K306FR with MIC ≥1:256 had wrinkled and stipple phenotypes.

6.7.3.5 Switch phenotypes and MIC levels among isolates from clade NG

Isolate G22FR with MIC ≥1:256 had stipple phenotype. Isolate G6FR with MIC 1:128 showed no switching behaviour. Isolate K86FR with MIC ≥1:256 had no switching behaviour.

These results show that the stipple phenotype was the most common phenotype observed in resistant isolates from all clades, and was mostly associated with MIC of ≥1:256, which was the highest MIC among these isolates.

6.8 Discussion

C. albicans has the ability to cause superficial and systemic infections, and is capable of invading almost any body location (Soll et al., 1991). This is due to its ability to change its virulence in order to maximize its survival in any given host environment. Phenotypic switching is one of the several mechanisms by which this adaptability may occur (Soll, 1992; Soll, 2002). *C. albicans* can switch spontaneously and reversibly between different cell forms, giving this organism the ability to adapt to different host niches and to evade the host defense mechanisms (Hellstein et al., 1993; Soll, 2000), and this switching has also been associated with a decrease in susceptibility to antifungals (Soll, 2002).

Although relationship between phenotypic switching and fluconazole resistance has been studied in different strains of *C. albicans*, nothing is known about this relationship among different clades of *C. albicans,* and more specifically the unique South African clade. This study was done to determine the relationship between phenotypic switching and fluconazole resistance among different clades of *C. albicans*, and whether switching was clade-related. To our knowledge, this study is the first to be performed on the assessment of phenotypic switching in *C. albicans* representing the various clades.

In this study, phenotypic switching among fluconazole resistant and susceptible isolates from clades I, II, III, SA and NG was determined by growing isolates on Lee's medium agar supplemented with arginine and zinc, and containing phloxine B. The plates were incubated at 35°C for 48hrs, covered with parafilm and incubated again at 25°C for 10 days in the dark.

In this study, a total of 11 switch phenotypes were observed, and these included ring; smooth white; smooth with sector; irregular wrinkle; stipple; star; switch from ring to irregular wrinkle; switch from ring to stipple; ring with sector; rough and heavily myceliated. The occurrence of these different switch phenotypes in different clades may serve different roles in providing adaptability and survivability to *C. albicans* under differing conditions, as suggested by Vargas et al. (2004).

The results of this study show that phenotypic switching was related to resistance in 2 (66.6%) isolates from each of clades I, II and III, and in 1(33.3%) isolate from each of clades SA and NG. These results are in agreement with those from few previous studies, where phenotypic switching was found to be associated with fluconazole resistance. A study by Vargas et al. (2000) showed that switched phenotypes from HIV positive individuals were more resistant to fluconazole and voriconazole than those from HIV negative individuals. In that study, the phenotypes observed included smooth white, irregular wrinkle, star, ring, myceliated and heavily myceliated.

In a study by Vargas et al. (2004), four switch phenotypes from a single strain of *Candida albicans* isolated from an HIV-positive individual were exposed to high concentrations of fluconazole, and these investigators observed that smooth white and very wrinkled phenotypes grew significantly faster than either ring or heavily myceliated and were most drastically affected by 4X and 6X MIC concentrations of fluconazole over a 24 hour period. These investigators concluded that different switch phenotypes from *C. albicans* may serve different roles in providing adaptability and survivability under differing conditions.

The results of our study also show that the stipple phenotype was consistently observed among the isolates with the highest MIC levels of ≥1:256 among isolates from clades I, II, III, SA and NG. The occurrence of the stipple phenotype in this study is in agreement with reports by previous studies, where the stipple phenotype was found to be associated with high fluconazole MIC levels. In a study by Velegraki et al. (1996), different phenotypes of *C. albicans* strains from neutropenic patients were isolated. In that study, the smooth, irregular, fuzzy and stipple phenotypes were obtained, and the stipple phenotype was found to have consistently higher MIC levels as compared to other phenotypes, which is in agreement with our results.

In their study, Kiraz et al. (2000) investigated the relationship between phenotypes of *C. albicans* strains isolated from clinical specimens and the susceptibility of the strains to

three antifungal agents, fluconazole, amphotericin B and flucytosine. These strains were found to express one of the six phenotypes: smooth, fuzzy, irregular, star, ring and stipple. The mean MICs of fluconazole were consistently higher for *C. albicans* strains expressing the stipple phenotype.

In a study by Cetinkaya and Kiraz (2005), the relationship between phenotypes of *C. albicans* strains isolated from clinical specimens of non-neutropenic patients, and the susceptibility of the strains to three antifungal agents, fluconazole, itraconazole and voriconazole were investigated. These strains were found to express one of the six phenotypes: smooth, fuzzy, irregular, star, ring and stipple. The mean MICs of fluconazole were consistently higher for *C. albicans* strains expressing the stipple phenotype.

The association of the stipple phenotype with high fluconazole MIC levels observed in our study suggests that the antifungal susceptibility of the stipple phenotype requires attention in HIV/AIDS patients, especially in patients who do not respond clinically to fluconazole treatment or in cases of life-threatening *C. albicans* infections of these immuno-compromised hosts.

The results of our study also show that switching among fluconazole resistant isolates was clade-related, as switching was observed in more isolates from clades I, II and III (66.6%), and in less isolates from clades SA and NG (33.3%). However, we cannot compare our results regarding clade-relatedness as this study is the first to be performed on the assessment of phenotypic switching in *C. albicans* isolates representing the various clades.

The results of our study also show that there was a high number of switch phenotypes observed among these fluconazole resistant isolates obtained from HIV/AIDS individuals. These results are in agreement with those obtained by Vargas et al. (2000), where high frequency switching was observed among HIV positive individuals as compared to HIV-negative individuals, although the isolates in that study were not genotyped into clades. The occurrence of different switch phenotypes in our study is in accord with a report by Pomes et al. (1985), where *C. albicans* was reported to possess multiple switching systems differing in their phenotypic repertoire.

The results of the study show that a high number of switch phenotypes was observed in clade II of these fluconazole resistant isolates, as compared to other clades. These results may suggest that *C. albicans* isolates belonging to this clade may survive better under adverse conditions than isolates from other clades, as different switch phenotypes from *C. albicans* may serve different roles in providing adaptability and survivability under differing conditions (Vargas et al., 2004). These results suggest that further study of differences between different *C. albicans* clades may be warranted, and that isolates from this clade need to be studied further.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level.

6.9 Conclusion

To our knowledge, this study is the first to be performed on the assessment of phenotypic switching in *C. albicans* isolates representing the various clades. The results of the study show that phenotypic switching was related to fluconazole resistance among isolates from clades I, II, III, SA and NG. However, switching was observed in more isolates from clades I, II and III (66.6%) and in less isolates from clades SA and NG (33.3%), showing clade-relatedness of switching. These results suggest that further study of differences between *C. albicans* clades may be warranted.

Various switch phenotypes were observed in resistant isolates from different clades in our study. The occurrence of these different switch phenotypes may provide these *C. albicans* strains with the ability to adapt and survive better under various conditions, as compared to other strains with no switching. The high number of switch phenotypes observed in clade II of these fluconazole resistant isolates, as compared to other clades in this study may suggest that *C. albicans* isolates belonging to this clade may survive better under adverse conditions than isolates from other clades. These results suggest that isolates from this clade need to be studied further.

The dominant phenotype observed in all clades of these fluconazole resistant, South African *C. albicans* isolates was stipple, which was associated with highest fluconazole MICs levels. These results suggests that the antifungal susceptibility of the stipple phenotype requires attention in HIV/AIDS patients, especially in patients who do not respond clinically to fluconazole treatment or in cases of life-threatening *C. albicans* infections of these immuno-compromised hosts. These findings suggest that the evaluation of colony phenotypes and their antifungal susceptibilities in *C. albicans* isolates may be useful in therapy.

The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and that a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is needed. These findings also stress the importance of identifying pathogens that can potentially infect HIV-infected individuals to subspecies level.

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WHITE, T. C. & AGABIAN, N. (1995) *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *J Bacteriol,* 177**,** 5215-21.

CHAPTER SEVEN: COMBINED DISCUSSIONS, CONCLUSIONS, RECOMMENDATIONS, LIMITATIONS

7.1 Combined Discussions

Azoles and polyenes are antifungal agents used for treatment and/or prophylaxis of *C. albicans* infections, and a high increase in antifungal resistance in clinical isolates of *C. albicans* in HIV/AIDS patients has been reported. Although 5 genetic clades were described among *C. albicans* isolates, little is known about their phenotypic characteristics, and not much is known about antifungal resistance with regard to each of these clades. The widespread use of fluconazole for treatment of OPC among HIV/AIDS patients has led to the worldwide increased resistance to this antifungal. Resistance to fluconazole can be caused by point mutations in the *ERG11* gene or over-expression of this gene, however, not much is known about the contribution of these mutations and over-expression to fluconazole resistance among different clades of *C. albicans*, and whether mutations or over-expression are clade-related.

Several studies have demonstrated that the expression of specific virulence and pathogenesis factors of *C. albicans* are correlated with phenotypic switching (White et al., 1993; Miyasaki et al., 1994; White and Agabian, 1995; Vargas et al., 2000), and there is evidence to suggest that phenotypic switching may play a significant role in the ability of *Candida* strains to survive under adverse conditions and perhaps cause more severe forms of disease in the immunocompromised host (Vargas et al., 2004). Only limited studies on the relationship between phenotypic switching and fluconazole resistance of *C. albicans* have been done, and not much is known about this relationship among different clades of *C. albicans*.

7.1.1 Induction of antifungal resistance

The induction of antifungal resistance among South African *C. albicans* isolates belonging to different clades was investigated by exposing 20 *C. albicans* isolates from each of clades I, II, III, SA and NG (n=100) in increasing concentrations of polyenes (AmB and nystatin) and azoles (fluconazole and miconazole).

The results of the study showed that resistance to polyenes (AmB and nystatin) could readily be induced in isolates from all clades, which is in agreement with previous studies (Athar and Winner, 1971; Broughton et al., 1991; Haynes et al., 1996; Kelly et al., 1997; Barker et al., 2004). The ease with which polyene resistance could be induced in this study can have serious implications, especially for patients with systemic *C. albicans* infections, in whom AmB is mostly used, as AmB has remained a "gold standard" for the treatment of life-threatening systemic fungal infections (Ellis, 2002).

In this study, induction of resistance against nystatin and AmB was found not to be clade-related, as resistance could be induced equally among isolates from all clades. However, our results could not be compared with results from other studies regarding clade-relatedness, as this study was the first where induction of AmB and nystatin resistance was done in isolates belonging to different clades.

Resistance against azoles (fluconazole and miconazole) was successfully and readily induced in all 20 *C. albicans* isolates from each of clades I, II, III, SA and NG after exposure to increasing concentrations of these antifungals, and induction was not clade-related. This ready induction of azole resistance is in agreement with results from previous studies, where fluconazole resistance was successfully induced (Hernandez et al., 1995; Albertson et al., 1996; Calvet et al., 1997; Cowen et al., 2000; Marr et al., 2001; Ribeiro and Paula, 2007; Angiolella et al., 2008).

The ease with which azole resistance could be induced in our study can have serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles are mostly used for *C. albicans* infections. This ease with which resistance against azoles could be induced may also explain the widespread increase in azole resistance among clinical isolates, as described by several investigators (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002). The results of our study confirmed that continuous exposure of isolates to azoles will finally enable isolates to become resistant to these antifungals. Induction of azole resistance was found not to be clade-related, as resistance was induced equally among isolates from all clades. However, we could not compare these results to other studies concerning clade-relatedness, as this is the first study where induction of azole resistance was done in different clades of *C. albicans.*

To investigate the survival and retention of resistance among antifungal resistant isolates after long-term storage at -80^{o}C, the stored isolates were grown on RPMI agar containing highest concentration of each antifungal, and on RPMI agar without antifungals, according to a method described by Patterson et al. (1996).

To our knowledge, this study was the first in which survival and retention of resistance among azole and polyene resistant isolates from different *C. albicans* clades was investigated after long-term storage in freezing conditions. The results of this study showed that 95-100% of azole resistant isolates survived and retained their resistance, as compared to polyene resistant isolates (35-75%). The high survival and resistance retention rates of azole resistant isolates could be attributed to the fact that these isolates have intact ergosterol due to possible over-expression of *ERG11* gene, allowing these isolates to grow and survive. This is in agreement with results observed in a study by Henry et al. (2000), where isolates grown in presence of fluconazole were observed to have intact ergosterol and over-expression of *ERG11* gene.

Ergosterol is important for the fluidity and integrity of *C. albicans* membrane, and for the proper functioning of many membrane-bound enzymes (White et al., 1998). Over-expression of *ERG11* gene results in increased production of lanosterol demethylase, an enzyme responsible for ergosterol synthesis, leading to increased synthesis of ergosterol and contributing significantly to the survival of azole-treated cells (Henry et al., 2000). The high survival and resistance retention rates of of azole resistant isolates could explain the widespread reported increase in azole resistance among clinical isolates due to increased and extended use of azoles (Redding et al., 1994; Sanglard et al., 1995; Franz et al., 1998; Franz et al., 1999; Martinez et al., 2002).

Survival and retention of resistance among azole resistant isolates was found to be not clade-related, as isolates from all clades survived and retained their resistance equally. These results could not be compared with other studies, as this was the first study where survival and retention of resistance among *C. albicans* resistant isolates after storage at -80^{o}C has been done.

The survival and resistance retention rates among polyene (AmB and nystatin) resistant isolates were found to be low as compared to that of azoles, with survival and resistance retention rates ranging from 35-75%. The reduced survival and resistance retention rates of polyene resistant isolates could be attributed to the fact that these isolates have decreased ergosterol content in their membranes, or have sterol-like compounds (Ellis, 2002; Lupetti et al., 2002), which is one of the mechanisms they use to develop resistance. Therefore their membranes are not as intact as those of azoles, and they therefore die easily and survive less.

The decreased ergosterol content in AmB resistant isolates was demonstrated in several previous studies. In a study by Haynes et al. (1996), an isolate with induced AmB resistance was found to have decreased ergosterol content. In their study, Kelly et al (1997) induced AmB resistance after experimental exposure of susceptible isolates to increasing concentrations of fluconazole. These isolates were found to have sterol-like compounds instead of ergosterol. A study by Barker et al. (2004) exposed a susceptible isolate to increasing concentrations of AmB, and the isolate developed resistance to AmB. This AmB resistant isolate was found to have sterol intermediates eburicol and lanosterol, instead of ergosterol. The decreased ergosterol and the replacement of ergosterol by sterol intermediates lead to weak cell wall of *C. albicans*, which then lead to fungal low survival rate.

The results of our study also showed that the survival and resistance retention rates of polyene resistant isolates was clade-related, with AmB resistant isolates from clade NG surviving and retaining their resistance better (60%) than isolates from other clades, and those from clade SA surviving and retaining their resistance the least (30%). Nystatin resistant isolates from clade NG survived and retained their resistance better (75%) than isolates from other clades, and those from clade II survived and retained their resistance the least (40%).

The reduced survival and resistance retention rates of AmB resistant isolates from clade SA was in support of the results of the study by Blignaut et al. (2005), where a high natural resistance to AmB was observed in isolates from clade SA. The high survival and resistance retention rates of AmB and nystatin resistant isolates from clade NG, and the low survival and resistance retention rates of nystatin resistant isolates from clade II suggested that further study of differences between different *C. albicans* clades may be warranted, and that isolates from these clades need to be studied further.

7.1.2 Contribution of mutations, over-expression and phenotypic switching

To investigate the contributions of mutations in the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, 30 isolates were used. DNA was extracted from the isolates, PCR was performed and PCR products were sequenced. Obtained sequences were compared with the published *ERG11* sequence from a wild-type, fluconazole-susceptible *C. albicans* strain (Lai and Kirsch, 1989).

To investigate the contributions of over-expression of the *ERG11* gene to fluconazole resistance among *C. albicans* isolates belonging to different clades, 30 isolates were used. RNA was extracted, cDNA synthesized and Real time PCR performed on a Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia). Relative gene expression of *ERG11* gene among resistant isolates, relative to susceptible isolates was quantified after normalization with the *18SrRNA* house-keeping gene.

To investigate the relationship between fluconazole resistance and phenotypic switching among *C. albicans* isolates belonging to different clades, 30 isolates were used. Primary and secondary cultures were prepared on Lee's medium agar supplemented with arginine and zinc, and containing phloxine B. The switched colonies were counted and colony morphologies viewed and photographed. Phenotypic switching behavior and different

colony morphologies obtained between the resistant and susceptible isolates from different clades were compared. Switch phenotypes among fluconazole resistant isolates in different clades were compared. Switch phenotypes and MIC levels among fluconazole resistant isolates from different clades were compared.

In overall, when the results obtained from investigations of contributions of mutations and over-expression of the *ERG11* gene, and relationship between phenotypic switching and fluconazole resistance among *C. albicans* isolates belonging to different clades were compared, the following were observed:

In fluconazole resistant isolates from clades **I and II,** no mutations associated with fluconazole resistance were observed in the *ERG11* gene. However, in each of these clades, 33.3% of the isolates exhibited *ERG11* over-expression only, 33.3% phenotypic switching only and 33.3% both *ERG11* over-expression and phenotypic switching. These results show that *ERG11* over-expression and/or phenotypic switching were the mechanisms responsible for fluconazole resistance in isolates from these clades.

Although no previous studies have been done were both *ERG11* over-expression and phenotypic switching were studied in the same isolates, our results are in agreement with previous studies, where *ERG11* over-expression was shown to be responsible for fluconazole resistance. In studies by Sanglard et al. (1995), Albertson et al. (1996), White (1997) and Perea et al. (2001), upregulation of *ERG11* in fluconazole-resistant *C. albicans* isolates after using Northern blotting technique was observed. Franz et al. (1998) showed that some fluconazole-resistant isolates can express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug. Studies by Henry et al. (2000) and Cowen et al. (2000) exposed isolates to azoles and increased expression of *ERG11* within a few hours of exposure was observed , contributing significantly to the survival of azole-treated cells.

Lee et al. (2004) used the LightCycler high-speed RT-PCR system to quantify *ERG11* expression, and *ERG11* up-regulation was observed in response to fluconazole. A study by Frade et al. (2004) compared RT-LightCycler PCR with Northern hybridization for quantitative analysis of gene expression in isolates with various fluconazole susceptibilities. In that study, *ERG11* gene was found to be over-expressed in 3 resistant

isolates tested, and the quantification results between RT-LightCycler PCR and Northern hybridization were shown to correlate. Chau et al. (2004) used Real-time quantitative PCR to measure *ERG11* expression levels of 38 clinical isolates, and *ERG11* gene was found to be over-expressed in 3 resistant isolates. A recent study by Ribeiro and Paula (2007) showed that over-expression of *ERG11* gene was the predominant mechanism in experimentally induced fluconazole resistance.

The results of the study also showed that phenotypic switching was the only mechanism associated with fluconazole resistance in 33.3% of the isolates from each of clades I and II. These results are in agreement with previous studies where phenotypic switching was found to be associated with fluconazole resistance. In a study by Vargas et al. (2004), phenotypic switching was observed when isolates were exposed to fluconazole. In studies by Velegraki et al. (1996), Kiraz et al. (2000) and Cetinkaya and Kiraz (2005) the stipple switch phenotype was found to be associated with consistently higher fluconazole MIC levels as compared to other phenotypes. However, it cannot be concluded with certainty that switching was the only mechanism responsible for fluconazole resistance in those studies, as neither mutations nor *ERG11* over-expression were simultaneously studied in those studies.

In fluconazole resistant isolates from **clade III,** 33.3% of the isolates exhibited *ERG11* over-expression and 33.3% phenotypic switching. Previous studies where *ERG11* over-expression was shown to be responsible for fluconazole resistance have been described above (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Perea et al., 2001; Henry et al., 2000; Cowen et al., 2000; Lee et al., 2004; Chau et al., 2004; Ribeiro and Paula, 2007). Previous studies where phenotypic switching was found to be associated with fluconazole resistance are described above (Velegraki et al., 1996; Kiraz et al., 2000; Vargas et al., 2004; Cetinkaya and Kiraz, 2005).

However, 33.3% of the resistant isolates from clade III exhibited both mutations (L340 (Y, C), K342 (K, R), D116E, K128T and E266D) and phenotypic switching. Although no previous studies have been done were both mutations and phenotypic switching were studied simultaneously in the same isolates, previous studies have been done where mutations were shown to be responsible for fluconazole resistance.

In a study by Kallakuri et al. (1996), mutations D116E and K128T were observed in the same resistant isolate. In their study, Marichal et al. (1999) observed mutations K128T and D116E in the same 3 fluconazole resitant *C. albicans* strains from AIDS patients. In a study by Asai et al. (1999), when 2 clinical strains were characterized, these 2 mutations were observed simultaneously in these isolates**.** Manavathu et al. (1999) studied six clinical isolates of *C. albicans* with high level of resistance to fluconazole. Mutations D116E and E266D were detected in the same resistant isolate in that study.

The occurrence of mutation E266D in resistant isolate in our study is in agreement with previous studies, where this mutation was observed in fluconazole resistant isolates. In a study by Kallakuri et al. (1996), mutation E266D was observed in a resistant isolate when it was characterized. Loeffler et al. (1997) characterized fluconazole resistant isolates, and mutation E266D was observed in 4 of the resistant isolates. In a study by Ryder and Favre (1997), mutation E266D was observed in a fluconazole resistant isolate characterized. In their study, Manavathu et al. (1999) investigated six clinical isolates of *C. albicans* with high level of resistance to fluconazole, and mutation E266D was observed in 2 of the isolates.

In a study by Favre et al. (1999), 4 selected fluconazole-resistant clinical isolates were sequenced and compared with the sequence from a sensitive strain SC5314. In that study, mutation E266D was observed in 1 of these resistant isolates. When a collection of unmatched fluconazole resistant and susceptible clinical isolates of *C. albicans* were analyzed in a study by White et al. (2002), mutation E266D was observed in 1 of the resistant isolates in that study. In their study, Goldman et al. (2004) sequenced 20 *C. albicans* isolates from AIDS patients, which included 9 fluconazole resistant, 6 susceptible-dose dependent and 5 susceptible isolates. Mutation E266D was observed in 1 resistant isolate from that study. Lee et al. (2004) analyzed 12 resistant and susceptible

clinical isolates for *ERG11* mutations by DNA sequencing. In that study, mutation E266D was observed in 2 of the resistant isolates.

The occurrence of two or more amino acid substitutions appears to be common in *ERG11* gene from fluconazole-resistant strains as has been observed in this study and previous studies discussed above. It is unclear as yet whether all identified mutations are important for conferring reduced affinity for azoles. In their study, Sanglard et al. (1998) found by introducing some amino acid substitutions in *ERG11* gene from a sensitive strain that double point mutations usually have a synergistic effect over single amino acid substitution, explaining the apparent natural occurrence of more than one mutation in azole-resistant *ERG11* gene.

Mutations L340 (Y, C), K342 (K, R) observed in resistant isolate from this clade were novel, and have never been described previously. The presence of these novel mutations may suggest that the clinical environment in South Africa allow the appearance of novel mutations in *C. albicans ERG11* gene. Further characterization of these new mutations is needed to determine their contribution to fluconazole resistance in *C. albicans* isolates.

In fluconazole resistant isolates from **clade SA,** 66.6% of the isolates exhibited *ERG11* over-expression only and 33.3% both phenotypic switching and mutations (D116E and K128T). These results show that *ERG11* over-expression was the most predominant mechanism responsible for fluconazole resistance among isolates from this clade. These results are in agreement with above-mentioned studies, where *ERG11* over-expression was found to be responsible for fluconazole resistance (Sanglard et al., 1995; Albertson et al., 1996; White, 1997; Perea et al., 2001; Henry et al., 2000; Cowen et al., 2000; Lee et al., 2004; Chau et al., 2004). In particular, a study by Ribeiro and Paula (2007) found *ERG11* over-expression to be the predominant mechanism in experimentally induced fluconazole resistance.

In isolates from **clade NG,** 33.3% of the isolates exhibited both *ERG11* over-expression and phenotypic switching, and 33.3% exhibited mutations (E266P, K342G, A434Q, V488L, A432*) only. Thirty-three percent of the isolates exhibited neither mutations, nor switching nor *ERG11* over-expression. These results show that mutations were found to be the only mechanism responsible for fluconazole resistance in at least 33.3% of isolates from this clade. Previous studies where mutations were shown to be responsible for fluconazole resistance have been described above (Kallakuri et al., 1996; Marichal et al., 1999; Asai et al., 1999; Manavathu et al., 1999; Loeffler et al., 1997; Ryder and Favre, 1997; Favre et al., 1999; White et al., 2002; Goldman et al., 2004; Lee et al., 2004).

Thirty-three percent of the isolates from this clade exhibited none of the mechanisms studied here, suggesting that resistance could have been due to over-expression of drug efflux pumps genes *CDR1, CDR2* or *MDR1,* or another undescribed mechanism as has been previously suggested by Sanglard et al. (1998). Other studies have shown that multiple genes are involved in conferring fluconazole resistance.

The results of this study confirm previous reports that multiple mechanisms may be operating to confer fluconazole resistance in *C. albicans* isolates (Albertson et al., 1996; Goldman et al., 2004).

7. 2 Combined Conclusions

- Resistance to polyenes and azoles could readily be induced in *C. albicans* isolates from all clades, and induction was not clade-related.
- Azole resistant isolates survived and retained their resistance better than polyene resistant isolates after long-term storage at -80^{o}C.
- Survival and retention of resistance among azole resistant isolates was not clade-related, while that among polyene resistant isolates was clade-related. The ease with which azole resistance could be induced in this study can hold serious implications, especially in HIV/AIDS patients who are already immuno-compromised, and in whom azoles are mostly used for *C. albicans* infections.

- Fluconazole resistance among isolates from clades I and II was not due to mutations, but due to either phenotypic switching only, *ERG11* over-expression only or a combination of phenotypic switching and *ERG11* over-expression.
- Fluconazole resistance among isolates from clades III was due to either phenotypic switching only, *ERG11* over-expression only or a combination of phenotypic switching and mutations.
- *ERG11* over-expression was the most predominant mechanism responsible for fluconazole resistance among isolates from clade SA.
- Fluconazole resistance among isolates from clade NG was due to mutations only, or a combination of phenotypic switching and *ERG11* over-expression. The mechanism responsible for fluconazole resistance in 33.3% of the isolates from this clade is unknown, as none of the mechanisms studied here were exhibited in this isolate.
- Novel mutations were observed in this study, and their contribution to fluconazole resistance is at this stage not known. Genetic analysis of these mutations needs to be done to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa.
- Some of the novel mutations observed in this study were found outside the previously described hotspots. These novel mutations may warrant further study and probably a review of the hotspots, especially among South African *C. albicans* isolates.
- The occurrence of over-expression of *ERG11* gene in fluconazole resistant isolates from various clades is a cause for concern, especially in HIV/AIDS patients with OPC, as the increased expression of *ERG11* allows for the cells to persist within the host, which in turn leads to the subsequent development of other more stable resistant isolates.

- A high number of switch phenotypes occurred more in isolates from clade II as compared to others. This suggests that isolates belonging to this clade may survive better under adverse conditions than isolates from other clades.
- The stipple phenotype was found to be the most dominant in fluconazole resistant isolates from all clades, and this phenotype was found to be associated with the highest fluconazole MICs levels. These results suggests that the antifungal susceptibility of the stipple phenotype requires attention in HIV/AIDS patients, especially in patients who do not respond clinically to fluconazole treatment or in cases of life-threatening *C. albicans* infections of these immuno-compromised hosts.
- The combined results of this study confirm previous reports that multiple mechanisms may be operating to confer fluconazole resistance in *C. albicans* isolates (Albertson et al., 1996; Goldman et al., 2004).

7.3 Recommendations

- The findings of this study demonstrate that *C. albicans* clades do differ phenotypically, and therefore a continued analysis of clade-specific phenotypic characteristics of *C. albicans* isolates is recommended.
- Pathogens that can potentially infect HIV-infected individuals need to be studied to subspecies level in order to improve treatment of these patients.
- Continued antifungal surveillance is needed to predict the evolution of resistance in a particular population and to take timely measures.
- Evaluation of colony phenotypes and their antifungal susceptibilities in *C. albicans* isolates is recommended as this may be useful in therapy.
- Genetic analysis of the novel mutations is recommended to determine their significance to azole resistance, especially in *C. albicans* isolates from HIV/AIDS patients in South Africa.
- Further study and probably a review of the mutation hotspots is recommended, especially among South African *C. albicans* isolates.

7.4 Limitations of the study

- Although resistance against polyene antifungal agents was induced in these isolates, mechanisms involved in this resistance were not addressed in this study; this will also be covered in a prospective study.
- Cross-resistance between azole and polyenes has been reported in previous studies, but our study did not address this aspect. A prospective study will be done to see if there was any cross-resistance, and whether it was clade-related.

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