ORAL CANDIDA IN HIV POSITIVE WOMEN: INFLUENCE OF ORAL HYGIENE, CLINICAL AND SOCIAL FACTORS ON THE CARRIAGE RATES AND THE INFLUENCE OF VIRULENCE OF THE ORGANISM ON THE DEVELOPMENT OF CLINICAL INFECTION

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DECLARATION

I, Foluso John Owotade, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….. (Signature of candidate)

…… day of ……. 2014.

DEDICATION

To

Boladale, Ebunife, Fisayomi and AJ

PUBLICATIONS AND PRESENTATIONS

Poster presentation:

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ABSTRACT

Introduction

Patients with HIV infection frequently encounter oral candidiasis, caused by *Candida species*. However, factors responsible for *Candida* colonisation and development of oral candidiasis in these patients are controversial. This study investigated the effect of social and clinical factors on oral *Candida* colonisation in HIV positive women. In addition, virulence of these organisms during clinical infection, the role of non-albicans *Candida* and reinfections with *C. albicans* were investigated.

Methods

After ethical clearance, 197 HIV positive women were recruited at the Charlotte Maxeke Hospital, Johannesburg. Oral rinse samples were collected, and quantitative and qualitative analyses of *Candida* were performed. A detailed questionnaire was used to collect data on oral and personal hygiene and habits, HIV treatment and coinfections. CD4 count and HIV viral load were either determined or obtained from the clinical records. A logistic regression model was used to determine the risk factors for colonisation. *Candida* carriers were followed up for 6 months at 3 months intervals. Oral examinations were performed to detect the presence of clinical oral candidiasis, CD4 counts were noted and *Candida* was isolated and identified from the oral rinse samples at each visit. The production of proteinase and phospholipase by *Candida* isolates were measured using Bovine serum albumin agar and Egg Yolk media. The adherence ability of *Candida* species were also measured using buccal epithelial cells. *Candida* counts, virulence factors and CD4 counts were compared in patients with and without oral candidiasis using non parametric tests. Antifungal susceptibility was performed on all the *C. albicans* isolates using broth microdilution

minimum inhibitory concentration system described by Clinical Laboratory Standards Institute. Isolates of *C. albicans* from 17 patients that attended regularly over a period of 6 months and carried *C. albicans* at all the visits were subjected to ABC typing and Multilocus sequence typing (MLST) technique. The Unweighted Pair Group Method with Arithmetic Mean dendrogram (UPGMA) using cluster analysis was generated to measure genotypic similarities between the *C. albicans* strains.

Results

Candida carriage rate was 59.4% with *Candida albicans* being the predominant species. The presence of tuberculosis, diabetes mellitus ($p=0.06$), oral prostheses ($p=0.02$), caries ($p=0.02$), low CD4 counts ($p=0.02$) and absence of antiretroviral treatment ($p<0.01$) significantly increased the colonization rate. In addition, *Candida* counts were significantly higher in patients with low CD4 counts ($p<0.01$).

The virulence characteristics of *C. albicans* such as adherence to buccal epithelial cells, proteinase and phospholipase fluctuated over time but it was not significantly correlated with the CD4 count. Although the virulence factors were not significantly different in patients with and without oral candidiasis, the *Candida* counts were higher in symptomatic patients ($p<0.01$). When isolates of *C. albicans* and non-albicans *Candida* were compared, *C. albicans* produced more proteinase and phospholipase compared to non-albicans *Candida* isolated from patients with and without candidiasis ($p<0.01$). However, non-albicans *Candida* isolated from symptomatic women produced significantly high amount of phospholipase ($p=0.02$) than the controls. Nevertheless, resistance to antifungal drugs was low in *C. albicans* isolates (0.8 to 1.7%).

Although ABC genotyping showed that the same strains were carried by the majority of patients (94.1%), the more discriminatory MLST technique showed evidence of recolonisation with new strains of *C. albicans* (64.7 %) that produced significantly high amounts of proteinase. MLST also showed that microevolution occurred in the original existing oral strains. In addition, 28 previously undocumented strains of *C. albicans* that were unique to this region were identified thus expanding the MLST *C. albicans* database.

Conclusions

The findings of this study have important implications regarding *C. albicans* colonization and infection in HIV positive patients on ARV. In addition, HIV treatment maintains high CD4 counts that would reduce *C. albicans* counts and the likelihood of developing oral candidiasis. Furthermore, a decrease in *Candida* counts would collectively reduce hydrolytic enzymes that cause tissue damage. However, recolonisation by new virulent strains of *C. albicans* could occur, thus increasing the chance of candidiasis. Therefore, ARV treatment, personal hygiene and regular oral hygiene measures using antifungal compounds could reduce the risk of developing oral candidiasis.

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AAT1a	Aspartate aminotransferase
ACC1	Acetyl-coenzyme A carboxylase
ADP1	ATP-dependent permease
AEC	Acute erythematous candidiasis
AFLP	Amplified fragment length polymorphism
AIDS:	Acquired immunodeficiency syndrome
ALS:	Agglutinin-like sequence
ANOVA:	Analysis of variance
ARV	Antiretroviral therapy
ATCC:	American type culture collection
BEC:	Buccal epithelial cells
CDR	Complementarity determining region
CEC	Chronic erythematous candidiasis
CFU:	Colony forming unit
CFU/ml:	Colony forming units per millilitre
CHC	Chronic hyperplastic candidiasis
CLSI	Clinical and laboratory standards institute
CMC	Chronic mucocutaneous candidiasis
CSH:	Cell surface hydrophobicity
DMFS	Decayed missing filled surfaces
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates

DST	Diploid sequence type
EBLP	Epithelial binding lectin-like protein
ECM	Extracellular material or matrix
g	Gram
GI	Gingival index
GIT	Gastrointestinal tract
h	Hour
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
IC	Invasive candidiasis
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
kDa	kilo-Dalton
MDR	Multidrug resistant
MEE	Multilocus enzyme electrophoresis
MFC	Minimum fungicidal concentration
mg/ml	Milligram per millilitre
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
MLST	Multilocus sequence typing
mm	Millimetre
MPIb	Mannose phosphate isomerase
NCCLS	National committee for clinical laboratory

	standards
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PBS:	Phosphate buffered saline
PMC	Pseudomembranous candidiasis
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism
PFGE	Pulsed field gel electrophoresis
PI	Plaque index
PMC	Pseudomembranous candidiasis
Pr	Proteinase activity
PRP	Proline Rich Proteins
RAPD	Random amplified polymorphic DNA
REA	Restriction enzyme analysis
rep-PCR	Repetitive-sequence-based PCR
RFLP	Restriction fragment length polymorphism
RISC	Retrotransposon insertion-site context
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAP	Secreted aspartyl proteinases
SD	Standard deviation
SSDP	Sequence-specific DNA primer
ST	Sequence type
SYA1	Alanyl-RNA synthetase

TB	Tuberculosis
TBE	Tris borate EDTA
TNFα	Tumour necrosis factor alpha
µl	Microlitre
µm	Micrometre
UPGMA	Unweighted pair group method wit arithmetic averages
UV	Ultraviolet
VNTR	Variable number of short tandem repeat
VPS13	Vacuolar protein sorting protein
YCB-BSA	Yeast carbon base-bovine serum albumin
ZWF1b	Glucose-6-phosphate dehydrogenase

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

In HIV infection, oral candidiasis is the most prevalent opportunistic fungal infection, affecting up to 90% of subjects. When the infection is active it creates a significant amount of discomfort, inhibits eating, negatively impacts on quality of life and threatens the general wellbeing of HIV positive individuals (Samaranayake et al., 2009).

With the introduction of Highly Active Antiretroviral Therapy (HAART), the frequency of oral candidiasis and other opportunistic oral infections have been reduced in HIV positive patients (Ceballos-Salobrena et al., 2000). However, in Sub Saharan Africa, only a third of HIV positive individuals have access to HAART. Furthermore, oral candidiasis may occur in HIV patients on antiretroviral therapy because of poor compliance and sustenance of therapy (Thompson et al., 2010). Although oral candidiasis can be controlled with azole antifungal agents, resistance to therapy is a growing concern (Fekete-Forgacs et al., 2000; Posteraro et al., 2006) and morbidity from candidemia is still being reported (Gautam et al., 2010). In addition, the mechanism how HAART affects asymptomatic colonisation, clinical and social variables in the long term are not fully understood.

Factors that determine colonisation and virulence of *Candida* species and the development of clinical infection have been studied in HIV positive patients (Calderone and Fonzi, 2001; Cannon and Chaffin, 2001). With HIV infection, yeasts were more virulent and had greater genotypic diversity than yeasts in HIV negative individuals (Ollert et al., 1995; Sweet et al., 1995). However, it is not clear weather

yeast counts or the virulence of these organisms that predisposes to oral candidiasis. Furthermore, it is not clear whether patients carry the same strain of *Candida* or are they constantly being reinfected with new strains of *C. albicans*. In addition, the role of non-albicans *Candida* in initiating oral candidiasis is not fully elucidated (Cannon and Chaffin, 2001; Cannon et al., 1995; Samaranayake et al., 2003).

1 Literature Review

1.1 Oral candidiasis and HIV

Oral candidiasis is the clinical manifestation of infection of the oral mucosa by *Candida* species. The plural form for candidiasis is *candidiases*. Similar exchangeable terms are candidosis and candidoses for the singular and the plural form.

1.1.1 Epidemiology

In recent times, infection by pathogenic or opportunistic fungal pathogens, including *Candida* species has escalated. This may be attributed to widespread antibiotic use and immune suppression from disease or the result of therapy related to organ/ tissue transplantation (Williams et al., 2011). In colonised people, *Candida* species are opportunistic pathogens that may cause infection when there is local or systemic compromise in the immune status. *Candida* infection has thus been appropriately dubbed "the disease of the diseased" (Williams et al., 2011). The occurrence of candidiasis varies with the local or systemic condition. *Candida* associated stomatitis is present in about half of all denture wearers (Figueiral et al., 2007) and 7.2% to 52% of all cancer patients yet to resume therapy (Schelenz et al., 2011).

Colonisation with *Candida* species in the oral cavity varies widely according to age, geographic location and other local factors such as denture wearing and the systemic

health of the individuals. In presumably healthy adults, prevalence may be as low as 15.2% or as high as 75% (Odds, 1988; Yang et al., 2011). Colonisation rates are higher in individuals immunocompromised due to diabetes mellitus, cancer, cancer chemotherapy, organ transplantation and HIV infection (Schelenz et al., 2011). This pattern is supported by the work of Patel and colleagues who observed 81% prevalence in HIV positive individuals in contrast to 53% in their HIV negative counterparts (Patel et al., 2006). A similar study in South Africa among HIV positive children who were institutionalized and not receiving antiretroviral therapy had a prevalence of yeast colonisation from 37.5% to 53.3%. (Blignaut, 2007).

In HIV infected patients, 5.8% to 93% will develop an acute *Candida* infection during the course of the illness (Egusa et al., 2008). Table 1.1 shows the global prevalence of oral candidiasis in HIV infection that varies from 6% to 98% depending on the region and HIV treatment.

Table 1.1 Prevalence of oral candidiasis in HIV infection from various studies worldwide [source: Egusa et al, 2008].

Authors (Year) [Ref.]	Country (Region)	No. of Subjects (% Male)	OC (%)	PC (%)	EC (%)	AC (%)	HC (%)	AIDS Cases (%)	OI Treatment	ARV Treatment
Asia										
Umadevi, *et al*. (2007) [8]	India (South)	50 (72)	8.0	4.0	2.0	2.0	-	NA	NA	HAART (+)
		50 (80)	24.0	18.0	8.0	4.0	-	NA	NA	HAART (-)
Sharma, *et al*. (2006) [7]	India (South)	101 (72.3)	50.4	11.8	44.5	11.8	-	69.3	No	15.8%
Ranganathan, *et al*. (2006) [16]	India	1700 (74)	21	13	2	8	1	NA	Yes	Yes
Shobhana, *et al*. (2004) [156]	India (East)	410 (72)	36	-	-	-	-	NA	NA	NA
Ranganathan, *et al*. (2004) [157]	India (South)	1000 (77.4)	23.8	16.1	3.1	7.9	1.2	NA	NA	10.7%
Kerdpon, *et al*. (2004) [158]	Thailand (North)	102 (26)	25	15	18	3	-	100	Yes	Yes
Kerdpon, *et al*. (2004) [158]	Thailand (South)	135 (NA)	55	42	25	4	-	0	Yes	Yes
Nittayanata, *et al*. (2001) [159]	Thailand (North & South)	278 (82.7)	39.6	21.6	24.8	8.6	-	55.0	39.2%	6.8%
Khongkunthian, *et al*. (2001) [160]	Thailand (North)	87 (14.9)	20.7	10.3	6.9	-	-	6.9	NA	17.2%
Schmidt-Westhausen, *et al*. (2004) [9]	Cambodia	121	98.3	57.9	34.7	21.5	-	NA	Antimycotics (-)	NA
		40	70.0	45.0	25.0	20.0	-	NA	Antimycotics (+)	NA
Bendick, *et al*. (2002) [161]	Cambodia	101 (62.4)	-	52.5	22.8	12.9	-	83.2	NA	No
Pichith, *et al*. (2001) [162]	Cambodia	356 (70.2)	51.4	-	-	-	-	100	NA	NA
Liu, *et al*. (2006) [163]	China (Beijing)	77 (37.7)	28.6	14.3	13.0	1.3	-	NA	No	No
Lim AA, *et al*. (2001) [164]	Singapore	81 (92.6)	34.6	22.2	6.2	6.2	-	71.6	NA	51.9
Africa										
Tirwomwe, *et al*. (2007) [165]	Uganda	514 (25.5)	50.4	29.4	28.0	12.6	-	NA	8.6%	No
Josephine, *et al*. (2006) [10]	Cameroon	384 (38.5)	34.9	-	-	-	-	NA	NA	NA
Chidzonga MM (2003) [166]	Zimbabwe	156 (50.6)	55.1	30.8	14.7	9.6	-	NA	NA	NA
Taiwo, *et al*. (2006) [167]	Nigeria (Plateau state)	261 (40.6)	35.7	23	2.7	10	-	NA	NA	NA
Agbelusi and Wright (2005) [168]	Nigeria (Lagos)	35 (51.4)	42.9	14.3	28.6	34.3	-	NA	NA	NA
Anteyi, *et al*. (2003) [169]	Nigeria	500 (55)	49	44	4	9	-	NA	Yes	Yes
Kamiru and Naidoo (2002) [170]	Lesotho	270 (NA)	54	27	26	14	-	0	No	No
Butt, *et al*. (2007) [171]	Kenya	207 (37.7)	-	-	5	32.4	15	NA	NA	NA
Butt, *et al*. (2001) [11]	Kenya	61 (41)	80	-	-	27.9	-	NA	No	No
Latin and South America										
Ramirez-Amador, *et al*. (2003) [172]	Mexico	1000 (88)	32	16	21	-	-	NA	Yes	Yes
Pinheiro, *et al*. (2004) [12]	Brazil	161 (76)	28.6	-	-	-	-	NA	NA	70.8%
Bravo, *et al*. (2006) [13]	Venezuela	75 (81.3)	52.0	-	-	-	-	NA	NA	62.7%
Other countries										
Gileva, *et al*. (2004) [15]	Russia (Perm region)	104 (66.3)	32.7	-	-	-	-	0	NA	NA
		13 (53.8)	84.6	-	-	-	-	100	NA	NA
Kroidl, *et al*. (2005) [173]	Germany	139 (85.6)	7.2	-	-	-	-	NA	NA	HAART (92.8%)
Nicolatou-Galitis (2004) [104]	Greece	37 (NA)	35.1	24.3	10.8	-	-	48.6	No	ART (-)
		14 (NA)	28.6	14.3	14.3	-	-	28.6	No	ART (+)
		44 (NA)	18.2	11.9	7.1	-	-	65.9	No	HAART (+)
Zakrzewska and Atkin (2003) [174]	UK	358 (93.9)	29.3	9.8	8.9	5.0	5.6	NA	NA	74.3%
Shiboski, *et al*. (2001) [14]	US	294 (24.8)	5.8	-	-	-	-	NA	NA	45.6%

ABBREVIATIONS: OC (oral candidosis), **PC** (pseudomembranous candidosis), **EC** (erythematous candidosis), **AC** (angular cheilitis), **HC** (hyperplastic candidosis), **OI** (opportunistic infections), **ARV** (anti-retroviral), **HAART** (highly active anti-retroviral therapy).

Oral candidiasis is also a fairly reliable predictor of progression to AIDS. Patients with infection have a 2.5 times greater risk of progression compared with those without infection (Sharma et al., 2006). While the introduction of HAART has dramatically reduced the prevalence of oral candidiasis, the effect on oral yeast colonisation is less obvious and may be influenced by other factors (Yang et al., 2006). Furthermore, oral candidiasis is still prevalent in the era of HAART in communities without access to HAART, patients on HAART whose immune systems do not recover or become resistant to HIV drugs. In addition, some researchers have

observed a high frequency of oral candidiasis and persistent oral colonisation with *Candida* among HIV patients on HAART (Tami-Maury et al., 2011; Traeder et al., 2008). As the HIV infection progresses to AIDS, the prevalence of colonisation tends to remain as high as 81% even with HAART (Thompson et al., 2010).

Oral candidiasis may be clinically classified as *primary* or *secondary*. It is termed primary when the disease is found only in the oral and perioral structures and secondary when it manifests in the mouth as part of systemic disease. There are also lesions where oral candidiasis is present but *C. albicans* is not the sole aetiological agent. These are referred to as *Candida* associated lesions. They usually occur in patients with oral prostheses not necessarily associated with HIV infection.

1.1.2 Primary candidiasis

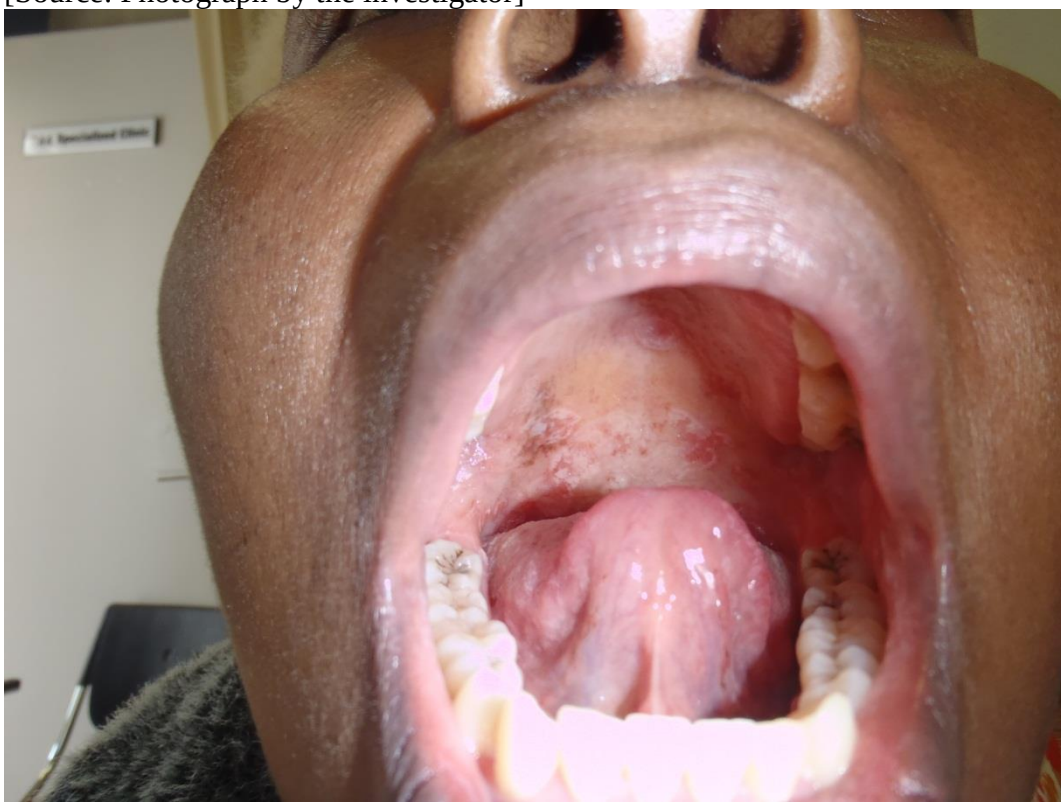
Three distinct forms of the primary candidiasis exist based on the peculiar clinical presentations (Marsh and Martin, 2009; Samaranayake et al., 2009).

Pseudomembranous candidiasis presents as a characteristic white or cream coloured plaques on the oral mucosa. Most frequent areas affected are the tongue, labial and buccal mucous membrane and hard and soft palate. The plaques can usually be scraped off leaving a red and inflamed underlying mucosa. This is usually considered diagnostic. The scraped off plaques can be stained to reveal the fungal elements and desquamated cells, blastospores, bacteria, inflammatory cells and fibrin. The patients may have problems with swallowing and a burning sensation in the mouth. Pseudomembranous and erythematous candidiases are usually acute conditions and may often be present at the same time (Samaranayake et al., 2009) (Figure 1.1).

Erythematous candidiasis is the most common type found in HIV infection. However, this candidiasis may also be associated with broad-spectrum antibiotic or corticosteroid inhaler therapy, a condition called "antibiotic sore mouth". It presents clinically with painful red patches on any part of the mucosa but chiefly on the dorsum of the tongue and palate. These lesions may be described as "kissing" when both sites are simultaneously affected. Erythematous candidiasis in HIV infection may go unnoticed because it may be asymptomatic. However, in typical antibiotic sore mouth, there is soreness and burning which may be associated with the loss of filiform papillae on the tongue (Samaranayake et al., 2009).

Hyperplastic candidiasis also known as *Candida* leukoplakia is the least commonly encountered form of primary candidiasis. It is a chronic disease that presents as white plaques of varying sizes that cannot be rubbed off unlike the lesion of the pseudomembranous variety. The lesions are usually rough and nodular on palpation and may sometimes present with red spots that are termed "speckled". The lesions usually occur at the lip commissures or the inner surface of any or both cheeks at the commissural areas and less commonly on the lateral surfaces of the tongue. Hyperplastic candidiasis is a potentially premalignant lesion and up to 15% may later undergo malignant change. A biopsy is usually performed to rule out change and to monitor the lesion. Histology reveals inflammatory exudate and *Candida* hyphae within the hyperplastic epithelium.

Figure 1.1 Pseudomembranous and erythematous candidiasis in the same patient [Source: Photograph by the investigator]



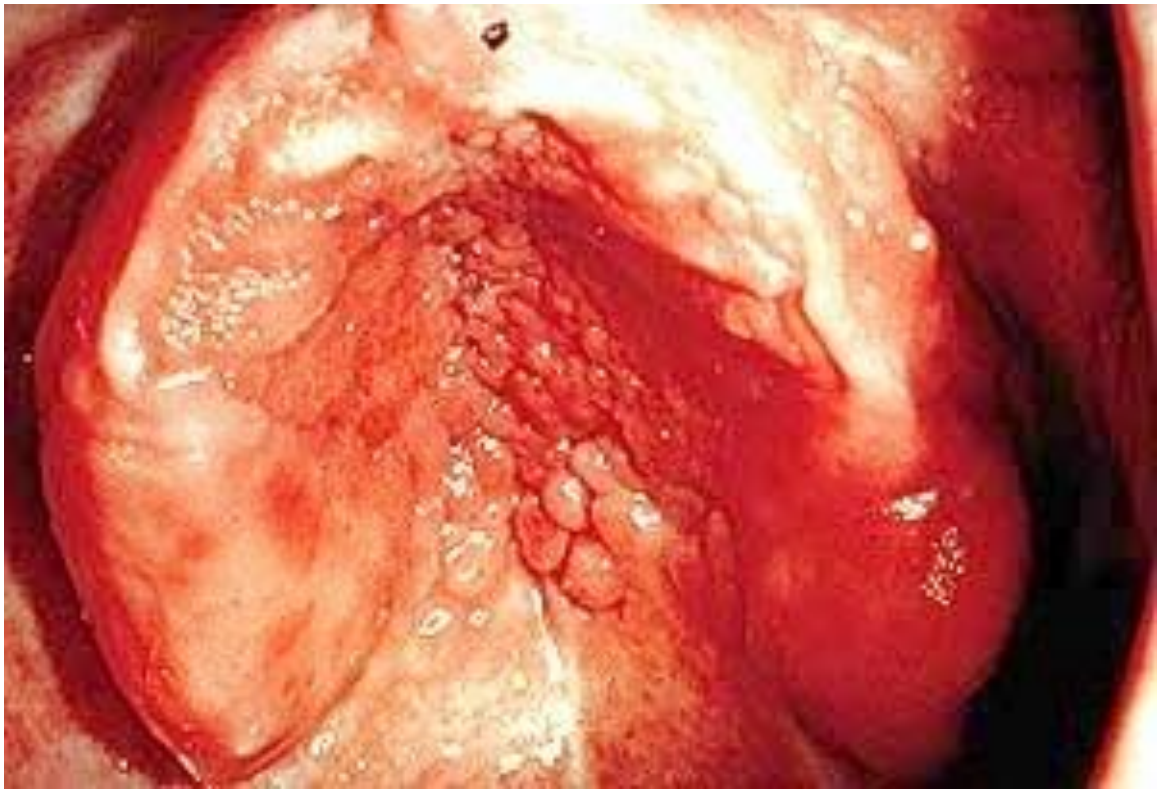
1.1.3 *Candida* associated lesions

Candida-associated denture stomatitis is also known as chronic atrophic candidiasis or denture sore mouth. Apart from the commensal *Candida* organisms, other bacterial organisms, mechanical irritation and possibly allergic reaction from the denture may be involved during the initiation stage of the infection. The causative factors lead to an overgrowth of oral *Candida* organisms in the niche between the denture surface and the palate where the saliva flow is limited. This condition may occur without symptoms or as burning sensation in the affected area. Inflammation around the commissure of the lips (angular cheilitis) may be present. Three subtypes or stages of denture sore mouth are described depending upon the severity and chronicity of the lesion. This is Newton's classification (Newton, 1962; Samaranayake et al., 2009) i.e. Type I, Patchy areas of inflammation and redness, Type II, Diffuse areas of inflammation and redness restricted to the denture bearing area and Type III, granular

inflammation with the development of papillary lesions in the palate (Figure 1.2).

Figure 1.2 Advanced (Type III) *Candida*-associated denture stomatitis

Source: http://www.dentalcare.co.uk/dental-professional-education/oralmucosal.aspx?ModuleName=coursecontent&PartID=2&SectionID=-1



In angular cheilitis, the angle of the mouth or the lip commissure is affected. The typical lesion presents as redness and fissuring in the affected area, which is painful with movement. It is similar to denture sore mouth with bacterial organisms especially *Staphylococcus aureus* being involved. Other associated factors are vitamin deficiency (mainly Vitamin B12), iron deficiency anaemia and excessive folding in the area due to ill-fitting dentures and loss of facial height in old age (Samaranayake et al., 2009).

In Median rhomboid glossitis, this previously enigmatic lesion on the dorsum of the tongue was recently associated with oral *Candida* infection even though other bacterial organisms may be involved. The usual lesion is found on the dorsum of the tongue anterior to the circumvallate papillae. It is associated with an area of depapillation that is elliptical or rhomboid in shape but may sometimes be lobulated or hyperplastic (Samaranayake et al., 2009).

Secondary *Candida* lesions occur from an early age and are usually associated with an underlying immune defect. In addition to the oral lesions, sites distant from the oral cavity such as the skin, nail and genitalia may be involved. The immunological defect may be related to systemic conditions for example HIV infection, cytotoxic therapy, haematological malignancies or may have a familial origin. Typical secondary candidiasis conditions are chronic mucocutaneous candidiasis (CMC) Syndromes. Researchers have postulated that deficient cell mediated immunity may be responsible for CMC, particularly a deranged Th2 response to yeast invasion (Puel et al., 2011). Additionally, interleukin-2, may be low or absent, gamma interferon production may be absent or increased whereas the IgG2/ IgG4 ratio is reduced in these patients (Lilic et al., 1996). Recent reports have indicated that inherited errors of interleukin-17 (IL-17) may be important in the aetiology of CMC. IL-17 is a proinflammatory cytokine that helps to recruit neutrophils and monocytes and is similar to the action of gamma interferon (Puel et al., 2011). The principal clinical feature of CMC is persistent oral candidiasis that may last for years and responds poorly to topical antifungal therapy. The white patches in the oral cavity which cannot be scraped off are hyperplastic and nodular. The lesions have a predilection for the palate and dorsum of the tongue. The

nails are usually thickened and grey and the skin may present with desquamating erythematous patches (Eyerich et al., 2010)

1.1.4 Causative organisms

Candida organisms are among the most frequently encountered fungal pathogens in human disease (Table 1.2). The term *Candida* comes from the word "*candid*" in Latin, which means white. The genus *Candida* belongs to the family *Saccharomycetaceae* with more than 350 species (Marsh and Martin, 2009). Although not many species cause disease in man, it is believed that there are many undiscovered species that includes two-thirds of *Candida* species incapable of growing at 37^0C (Schulze and Sonnenborn, 2009). While *C. albicans* is the most common aetiological agent in oral candidiasis, other *Candida* species such as *C. glabrata, C. parapsilosis* and *C. dubliniensis* have been frequently isolated from patients with candidiasis (Thompson et al., 2010). The increasing isolation of non-albicans *Candida* species may be due to increased diagnostic methods available for these organisms or their resistance to conventional antifungal agents that leads to their persistence (Gonzalez et al., 2008).

Table 1.2 *Candida* species and similar fungi implicated in human infection [Source: Marsh and Martin, 2009]

Candida species	Other uncommon fungal species
Candida albicans	*Saccharomyces* spp
Candida dubliniensis	*Cryptococcus neoformans*
Candida parapsilosis	*Histoplasma capsulatum*
Candida tropicalis	*Geotrichum* spp
Candida glabrata	*Aspergillus* spp
Candida kefyr (pseudotropicalis)	*Paracoccidiodes brasiliensis*
Candida lusitaniae	*Mucor* spp
Candida krusei	
Candida guilliermondii	
Candida utilis	
Candida lipolytica	
Candida famata	
Candida haemulonii	
Candida rugosa	

1.1.5 *Candida c*arriage

Oral colonisation (carriage) involves acquisition and persistence of a stable population at a particular site without clinical disease (Cannon and Chaffin, 1999). The interplay between acquisition, multiplication and the ability of the immune system to eliminate the organism determines whether an individual remains colonised (Cannon and Chaffin, 2001). Usually, about a third of healthy subjects are colonised by *Candida* species (van der Meer et al., 2010).

Colonizing *Candida* organisms do not exist alone in the oral cavity but live in biofilms with bacteria (Rautemaa and Ramage, 2011). This bacterial fungal

relationship determines the eventual survival and proliferation of different species. For instance, non-pathogenic commensals provide a barrier against exogenous pathogens and inhibit the transition of colonizing *Candida* to the pathogenic state (Mukherjee et al., 2005). On the other hand, the interaction of colonizing *Candida* with certain *Streptococcus* and *Pseudomonas* species may increase the resistance of the yeasts and bacteria (Wargo and Hogan, 2006). The commensal behaviour of *C. albicans* in the oral cavity is still poorly understood. It is not clear how it adapts to the changing physical and chemical conditions of the mouth such as pH and how it acquires iron from ferritin stores in the oral epithelium while other microbes do not have this ability (Martin et al., 2011).

The prevalence of yeast colonisation in the mouth varies with age, location and the immune status of the population sampled. In HIV positive patients, *Candida* carriage is higher than in HIV negative individuals (Table 1.3). For example, 80% carriage prevalence was found in HIV positive children and 57.5% of their sero-negative siblings (Cerqueira et al., 2010). Furthermore, a South African study showed a prevalence of 81.3 % in HIV positive adults compared to 63% in their HIV negative counterparts (Patel et al., 2006). The introduction of HAART has reduced the rate of oral candidiasis (Hood et al., 1998; Revankar et al., 1998) and probably the rate of colonisation as well (Cerqueira et al., 2010). However, oral candidiasis is still a major problem in patients with advanced HIV disease and those who carry strains of *C. albicans* that are resistant to antifungal drugs. Similarly, patients with non-albicans *Candida* strains who live in resource poor areas where ARVs are not available and are still vulnerable (Thompson et al., 2010). Although Wu *et al*. (2012) showed that the initiation of HAART reduced the rate of oral *Candida* colonisation (Wu et al., 2012),

Yang *et al*, (2006) showed no changes in the colonisation. They suggested that prolonged HAART might be more beneficial in reducing opportunistic yeast infections rather than yeast colonisation (Yang et al., 2006).

Table 1.3 Reports of *Candida* carriage rate in HIV-positive and HIV-negative subjects in Asia, Europe and Africa [Source: Patel et al., 2006].

Country	Sampling method	*Candida* carrier rate (%)		Reference
		HIV-positive	HIV-negative	
Thailand (adults)	Oral rinse	66·6 (n=45)	10·8 (n=74)	Teanpaisan & Nittayananta (1998)
Thailand (children)	Oral rinse/swab	70 (n=40)	40 (n=15)	Pongsiriwet *et al.* (2004)
Hong Kong	Oral rinse	54·8 (n=73)	ND	Tsang & Samaranayake (2000)
Italy	Oral rinse	61·9 (n=42)	29·3 (n=41)	Campisi *et al.* (2002)
Germany	Oral swab	73·8 (n=73)	13·8 (n=58)	Schmidt-Westhausen *et al.* (1991)
India	Swish	65·3 (n=150)	ND	Gugnani *et al.* (2003)
South Africa	Oral rinse	75 (n=28)	68 (n=28)	Hauman *et al.* (1993)

ND, Not determined.

1.1.5.1 Source of carriage

The gastrointestinal tract (GIT) is the chief source of *Candida* organisms colonizing the mouth. The GIT and the vagina to a lesser extent, serve as a pool that regularly seeds the oral cavity (Cannon and Chaffin, 2001). Other body sites that are frequently colonised by *Candida* organisms include the vagina and the skin, especially the axillary folds, the groin and the perineum (van der Meer et al., 2010).

The GIT is sterile at birth, but by the end of the first month of life, an indigenous intestinal microflora (including *Candida* organisms) is detectable. This flora becomes established by the age of 3 to 5 years and is peculiar to each individual based on compatibility with their immune system (Schulze and Sonnenborn, 2009).

Oral *Candida* may also be derived from exogenous sources such as contaminated food and contaminated hands and equipment of health workers. Other sources are

kissing or similar activities between close contacts where there is direct saliva-to-saliva contact (Cannon and Chaffin, 2001).

1.1.5.2 Sites of colonisation

In intraoral sites, *Candida* organisms are most commonly found on the dorsum of the tongue. The cheeks and other intraoral sites also harbour the organism and distribution is influenced by the availability of ligands that provide adhesion (Cannon and Chaffin, 1999). The presence and population size of colonizing *Candida* are determined by several factors. These include presence of other colonizing bacteria, use of antibiotics, presence of diabetes mellitus and oestrogens (van der Meer et al., 2010).

Other bacteria may compete for available nutrients or produce substances such as lactic acid and hydrogen peroxide that inhibit *Candida spp*. Broad-spectrum antibiotics may destroy local bacteria flora thereby provoking *Candida* overgrowth. Similarly, the high glucose content of tissues and immune deficiency in diabetics enhance yeast colonisation while oestrogens boost the glycogen content of epithelial cells thereby provide ample nutrition for yeasts to thrive (Dennerstein and Ellis, 2001).

1.1.5.3 Colonisation in other sites and invasive *Candida* infections

Candida organisms are found in other parts of the body apart from the mouth. In these sites, they behave as commensals as long as the site specific microbial populations are intact and innate immunity is not compromised (Schulze and Sonnenborn, 2009).

The vagina is the other major reservoir apart from the gut and up to 50% of women are colonised with *Candida* species (Mahmoudi Rad et al., 2011). The danger with

colonisation is the possibility of developing fungaemia and invasive candidiasis if there are overt risk factors. Colonisation itself is a known independent risk factor for fungaemia and invasive candidiasis (IC) and the number of colonised sites and intensity of colonisation are strongly predictive of IC (Magill et al., 2006; Pelz et al., 2001). Furthermore, the site of colonisation has been associated with the risk of invasive candidiasis. In a study that related the anatomic site of colonisation with the risk of IC, patients with urinary, respiratory and rectal colonisation appeared to be at the greatest risk (Magill et al., 2006). This is possible because yeasts colonizing epithelial surfaces can germinate and form hyphae which they use to invade the tissues (van der Meer et al., 2010).

C. albicans is the dominant species found at different body sites. However, the prevalence of other non-albicans *Candida* species has increased. Consequently, azole resistant *C. glabrata* is only second to *C. albicans* in the causation of IC (44%) followed by *C. tropicalis* (6%) and *C. parapsilosis* (5%). Other less commonly encountered yeasts include *Cryptococcus spp, Geotrichum spp, Rhodotorula spp, Saccharomyces spp* and *Trichosporon species* (Miceli et al., 2011).

1.1.6 Factors influencing *Candida* carriage

Several factors have been associated with oral colonisation with *Candida* organisms. These vary due to the differences in study locations and populations, sampling techniques, use and type of antiretroviral therapy and several other factors (Cerqueira et al., 2010). In addition, other associated factors are age, race, poor nutritional status, reduced salivary flow, use of antibiotics, wearing a dental prosthesis and poor oral hygiene. Underlying systemic diseases such as diabetes mellitus and disseminated

malignancy have been implicated as well as cancer chemotherapy and radiotherapy.

The extremes of age have long been associated with increased predisposition to yeast colonisation and infection. These age groups have a compromised immunity and reduced capacity to eliminate colonizing yeast organisms (Jabra-Rizk et al., 2001). In the elderly, the reasons for an increased rate of colonisation are multifactorial, i.e. compromised immunity, reduced salivary flow, nutritional deficiency and increased tendency to wear prosthesis (Darwazeh et al., 2010; Shay et al., 1997). Wearing prostheses such as dentures provide a means of adherence for *Candida* while precluding the area covered from the protective effects of saliva (Fanello et al., 2006).

Malignant conditions and their treatment with cytotoxic chemotherapy or radiotherapy, compromise cellular mediated host immunity that is needed to control fungal infections (Schelenz et al., 2011). In addition, malignant conditions and their therapy reduce the protective effects of saliva by inducing hyposalivation. The induction of mucositis from radiotherapy further compromises saliva flow and function (Suryawanshi et al., 2012). With a variable prevalence of 10.8% to 58% *Candida* colonisation in head and neck cancer, predisposition to colonisation may be a function of the type of cancer. Generally, head and neck cancers have the highest level of prevalence as radiotherapy and chemotherapy are targeted at vulnerable saliva producing tissues (Schelenz et al., 2011). In addition, the high glucose content in the tissues and impaired cellular mediated immunity in diabetics are contributing factors for yeast colonisation (van der Meer et al., 2010). Similarly, broad-spectrum antibiotics have been implicated due to destruction of the oral microbial ecosystem (Fanello et al., 2006). However, several authors did not show a statistically significant

relationship between antibiotic use and oral colonisation by yeasts (Davies et al., 2006; Schelenz et al., 2011).

Poor oral hygiene is regarded as a risk factor for oral yeast colonisation due to increased plaque accumulation that creates the ideal niche for *Candida* and other organisms. In a study among elderly hospitalised patients, poor oral hygiene was implicated after adjusting for other risk factors (Fanello et al., 2006). Conversely, healthy dentate patients did not demonstrate any significant association between poor oral hygiene (using plaque and gingival indices) and oral *Candida* colonisation. However, adults who were not flossing on a regular basis had higher colonisation rates (Darwazeh et al., 2010b).

Carious lesions serve as reservoirs for yeasts and therefore constitute risk factors in oral colonisation (Cerqueira et al., 2010). Furthermore, studies in adults and children showed that low CD4 count and high viral load were significantly associated with carriage and development of oral *Candida* infection (Duggal et al., 2010; Sharma et al., 2010). On the other hand, no relationship was found between carriage rates and CD4 count thus suggesting that other factors may influence yeast carriage and development of symptomatic infection (Barchiesi et al., 2002; Sanchez-Vargas et al., 2005b). Viral load has been independently associated with oral *Candida* infection and the association was more significant than CD4 count (Mercante et al., 2006). However, a similar study did not detect a relationship between viral load and oral colonisation with yeasts (Erkose and Erturan, 2007). In short, contributory factors for oral colonisation appear to be many and variable.

1.1.7 Treatment of oral candidiasis

Treatment of oral candidiasis involves a combination of procedures and therapy. Detailed clinical and laboratory assessments are required to identify predisposing factors and to prevent ineffective treatment and recurrence of infection. Effective oral hygiene practices must be instituted to remove *Candida* biofilms from the mouth and prosthetic appliances. Therefore, adequate attention must be paid to proper nutrition and the cessation of smoking advised in smokers. Likewise, patients on steroid inhalers must be advised to rinse their mouth with water after using the inhaler to prevent oral candidiasis. When the mouth is dry, efforts should be made to rehydrate the mouth using artificial saliva, frequent sips of water and drugs that stimulate salivary flow. The treatment of oral candidiasis includes topical or systemic drugs or a combination of both. The treatment regimen depends on factors such as the type and severity of infection, the presence of systemic illness and previous antifungal therapy. A general guide for treatment adopted from (Williams et al., 2011) is provided in Table 1.4.

1.1.7.1 Antifungal agents and their mode of action

New antifungal medications have been developed in the last decade after a long period of relative inactivity. These became imperative due to rising incidence of invasive candidiasis, infection by non-albicans *Candida*, development of resistant strains and the toxicity of the existing drugs. A wide array of antifungal therapies exists but no single agent is ideal for all the patients. Antifungal therapy is usually individualised depending on the site of the infection, other existing comorbidities and concurrent drug therapies. Drug hypersensitivity and the immune status of the patients

are other factors to consider in order to arrive at patient specific antifungal regimens (Lewis, 2011).

Six classes of antifungal drugs are available namely Polyenes, Azoles, Echinocandins, Allylamines, Pyrimidine analogues and Mitotic inhibitors. They are classified according to their mode of action (Figure 1.3). Only some of these drugs are used routinely to treat oral candidiasis (Table 1.4).

Figure 1.3 Mechanism of action of antifungal drugs [Source: Lewis RE, 2011]

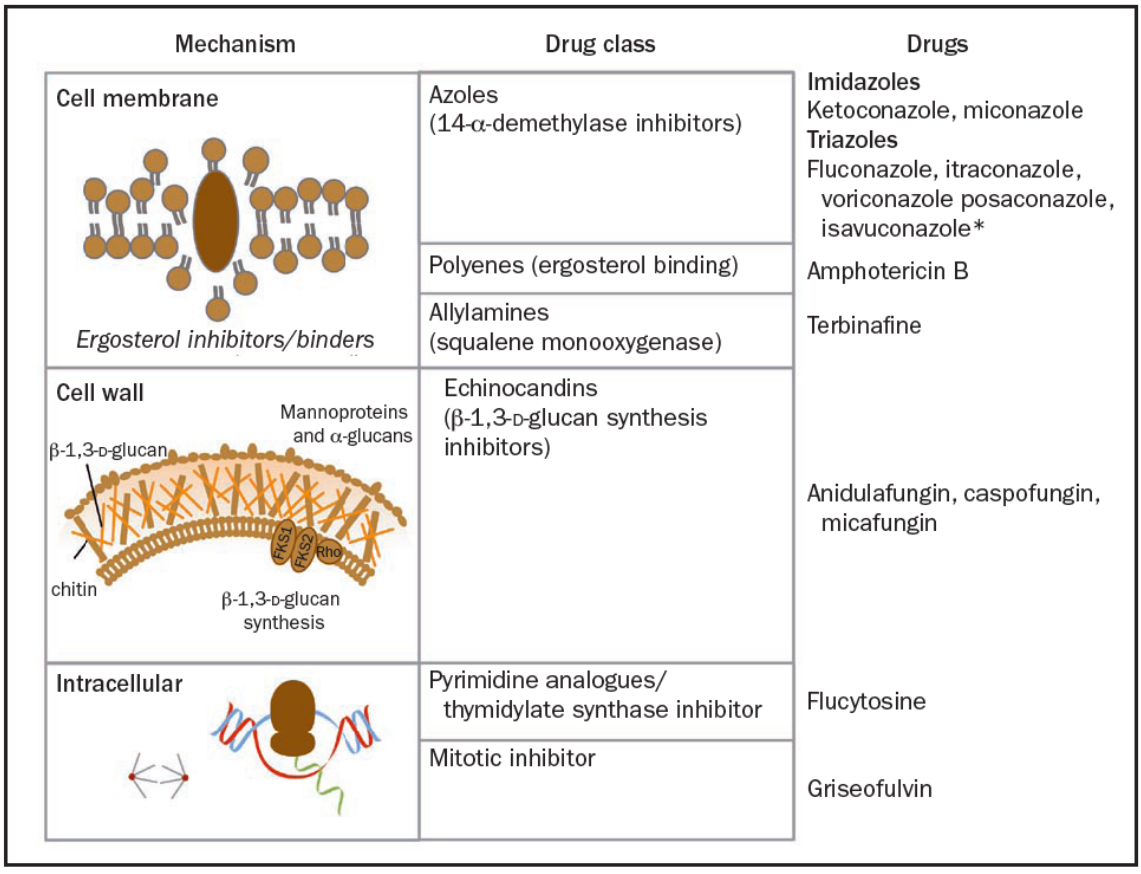


Table 1.4 Treatment guidelines for oral candidiasis [Source: Williams et al, 2011]

	PMC	AEC	CEC	CHC	Adult Dose
Topical application					
Nystatin					1 lozenge- of 100,000 units q.i.d. for 7–14 days Suspension: 500,000 units by rinse and swallow q.i.d. for 7–14 days
Amphotericin					1 lozenge (10 mg) q.i.d. for 10–15 days
Miconazole					Oral gel (24mg⁄ml); 5–10 ml q.i.d. for 7–14 days
Clotrimazole					1 lozenge (10 mg) five times per day for 7–14 days
Systemic administration					
Ketoconazole					200–400 mg⁄day for 7–14 days
Fluconazole					100 mg⁄day for 7–14 days
Itraconazole					200 mg (20 ml) suspension by rinse and swallow without food q.i.d. for 7–14 days 200 mg ⁄day (capsules taken with food) for 2– 4 weeks

PMC- Pseudomembranous candidiasis, AEC- Acute erythematous candidiasis, CEC- Chronic erythematous candidiasis, CHC- Chronic hyperplastic candidiasis

Nystatin and Amphotericin B belong to polyenes class. Amphotericin B was synthesised in 1958, and was very effective and remained the mainstay of antifungal therapy for many decades. However, toxicity to the kidneys led to the development of less toxic formulations of the same drug, namely amphotericin B lipid complex, amphotericin B colloidal dispersion and liposomal amphotericin B (Proia, 2006). Amphotericin B can be used parenterally unlike nystatin which is used topically. The polyenes act by bind to ergosterol in the cell membrane of pathogenic fungi. This causes leakage of the cell contents and death of the cell (Lewis, 2011).

Azoles belong to 2 classes: the imidazoles and the triazoles. Miconazole and ketoconazole belong to the imidazoles class of azole antifungals. They are effective against *Candida* and *Aspergillus* species although like the polyenes they are also nephrotoxic. On the other hand, triazoles have three nitrogen atoms in the azole ring that confer a greater affinity for cytochrome P450 in the yeast rather than the host cells. Therefore the triazoles have reduced toxicity unlike the imidazole and polyenes. Fluconazole (a triazole) was introduced in 1990 and showed greater efficacy and higher concentration in the tissues than the imidazoles that were already in clinical use with less side effects. However, fluconazole was not effective against *Aspergillus*, Mucorales and non-albicans *Candida*. Sensitive organisms also rapidly developed resistant strains to fluconazole and this prompted the development of the other azoles. Itraconazole, introduced in 1992, was effective against *Aspergillus* but fell into disrepute due to severe gastrointestinal (GI) side effects and unreliable absorption from the gut following oral administration. Subsequently, voriconazole was approved in 2002 for clinical use and showed better tolerability and efficacy than itraconazole. It also had excellent GI absorption following oral administration. The broad-spectrum

activity of voriconazole has an advantage as well as a disadvantage. The wide spectrum of activity led to an increase in interaction with other medication. The azoles posaconazole and isavuconazole were developed much later. Their advantage is they have much less drug interactions. Nonetheless, voriconazole is still the first line therapy in disseminated candidiasis. The azoles also act on the yeast cell membrane by inhibiting lanosterol demethylase, a cytochrome P450 dependent enzyme necessary for ergosterol synthesis (Lewis, 2011).

The introduction of semi-synthetic lipopeptides called echinocandins heralded a new phase in antifungal therapy. With a unique action on fungal cell wall, which mammalian cells do not possess, the collateral damage to host cells was reduced to a minimum. Echinocandins target the β-1,3-D-glucan synthase enzyme used for cell wall synthesis in yeast cells. With a defective cell wall, the yeast pathogens are prone to osmotic lysis and subsequent death. Capsofungin was the first to be introduced with anidulafungin and micafungin coming later. They are effective against non-albicans *Candida* and azole resistant *Candida albicans*. With few side effects and drug interactions coupled with a long half-life necessitating a once daily regimen, they appear well tolerated. Echinocandins have not showed any cross-resistance with azoles or polyenes and are useful alternatives when resistance to the other antifungals has developed (Lewis, 2011).

Terbinafine and naftifine belong to the allylamines class. These drugs target squalene monooxygenase, an enzyme necessary for ergosterol synthesis in the yeast cell membrane. Terbinafine concentrates selectively in the skin and nails after oral

administration and therefore has very few side effects. However, it is not effective for disseminated fungal infection due to the selective accumulation in the skin and nails.

Pyrimidine analogues such as flucytosine are similar to cancer chemotherapeutic drugs because they target intracellular processes in fungal cells. However, they are often ineffective as a single therapy for systemic fungal infections. The yeast enzymes cytosine permease and cytosine deaminase convert flucytosin to 5-fluorouracil which then inhibits thymidylate synthase activity and leads to miscoding of RNA. Furthermore, serious side effects such as bone marrow suppression, diarrhoea and vomiting have been reported with flucytosine therapy (Lewis, 2011).

Mitotic inhibitors, including griseofulvin act intracellularly like the pyrimidine analogues. They bind to tubulin thereby inhibiting microtubular formation in the yeast cell but they are only useful for dermatophyte infection due to selective concentration in the keratinocytes (Lewis, 2011).

1.1.7.2 Antifungal drug resistance

Antifungal resistance may be viewed from either the microbiological or clinical perspective. Microbiologic resistance is evident when the antimicrobial concentration required to inhibit the growth of an infecting organism is higher than the concentration needed to inhibit the growth of wild-type strains. On contrast, clinical resistance occurs when the inhibitory antifungal dose exceeds the normal range that can be safely administered to the patient (Turnidge and Paterson, 2007).

Resistance to conventional and newer antifungal medications is on the increase among hospitalised and immunocompromised patients. Different species of *Candida* exhibit different levels of resistance. For example *Candida glabrata* is resistant to

fluconazole and cross resistant to other azoles (Pfaller and Diekema, 2010) whereas *C. glabrata* may display multidrug resistance and several mutations in the same individual (Chapeland-Leclerc et al., 2010). The pattern of resistance and the predominant resistant organism may vary between countries and the region of studies. For example, multidrug resistant *Aspergillus fumigus*, a filamentous fungus not associated with oral candidiasis has been reported. Azole resistance was present without prior exposure to antifungal therapy which suggests that agriculturally used azoles are the likely cause of the resistant strains (Mortensen et al., 2010).

Antifungal resistance may occur through a primary or secondary mechanism, i.e. pathogenic yeasts may hinder the antifungal capacity of the drug through innate or acquired characteristics. Resistant strains from other sources may also replace sensitive indigenous flora (Pfaller, 2012). Resistance to azoles is more prevalent than any other categories of antifungal drugs. Furthermore *Candida* and other pathogenic yeasts organisms may exhibit more than one method of resistance to azole antifungals. One mechanism is the upregulation of efflux pumps that reduce the availability of the drug within the target structures (Kanafani and Perfect, 2008). As a result, the drug is not able to inhibit the enzyme lanosterol demethylase which the fungi use to synthesise cell membrane ergosterol. The *CDR* or *MDR* gene in *C. albicans* encodes the upregulation of efflux pumps and this mechanism of resistance is common to all azole drugs. *Candida* species may also become resistant to azoles due to point mutations in the *ERG11* gene that codes for the target enzyme. Alteration in the target enzymes thus inhibits their binding capacity to azoles. Furthermore, the altered target enzyme may be upregulated or the yeasts may develop alternate pathways whereby the drug is unable to interact and destroy the yeast membrane. In

addition, the *ERG3* gene is responsible for the alternative pathways (Pfaller, 2012). Resistant strains of *Candida* species have also been reported for the relatively recently developed echinocandins. Their efficacy is reduced by mutations in the gene encoding for the cell wall enzyme 1,3-β-D-glucan synthase complex targeted by the drugs (Pfaller, 2012). For these reasons, antifungal resistance raises the MICs of the drugs and worsens the prognosis of fungal infections. These observations have resulted in an increase in antifungal susceptibility testing.

In vitro susceptibility testing has helped to identify antifungal agents that may likely control an infection and have also identified agents that may not work. There are several new products that are used for susceptibility testing i.e. the Etest® and the Vitek 2® yeast susceptibility test (both bioMerieux, Inc., Marcy l' Etoile, France products) and the Sensititre YeastOne® colorimetric plate (TREK Diagnostic Systems, Inc., Cleveland, OH, USA).

1.1.7.3 Antifungal drug resistance and HIV

Antifungal drug resistance is a major clinical challenge, particularly in HIV infection where recurrent oral candidiasis may necessitate the prescription of antifungal drugs. Although azole antifungals such as fluconazole have been the predominant agents in antifungal therapy in HIV infection, azole resistance has compromised therapy. Up to 9.8% of *C. albicans* strains demonstrate resistance to fluconazole even without previous therapy and resistance could increase to 44.7% following antifungal therapy (Pfaller, 2012). Itraconazole also shows a similar pattern of resistance (Magaldi et al., 2001). In a longitudinal study of HIV positive and negative individuals, most of the resistant strains were *C. albicans* or *C. glabrata* isolated from the HIV positive group (Sanchez-Vargas et al., 2005b).

Azole antifungals are able to precipitate the growth of less susceptible strains and non-albicans *Candida* especially with prophylactic use when the CD4 count is low (Badiee et al., 2010). Non-albicans *Candida* species such as *C. krusei* and *C. glabrata* demonstrate a high levels of fluconazole resistance and are also resistant to other agents due to cross resistance (Mokaddas et al., 2007). Antifungal agents with low MICs and high activity against *Candida* species have been recommended for empirical therapy or following fluconazole resistance in HIV infection. Badiee and co-workers recommended the use of voriconazole, Amphotericin B and capsofungin due to low MICs values observed (Badiee et al., 2010). The prevention of oral candidiasis in HIV infection using mouthrinses such as triclosan/fluoride has also been suggested. These agents keep the yeast count low and stimulate salivary flow in the colonised, thereby preventing the development of clinical infection (Patel et al., 2008).

1.2 *Candida albicans*

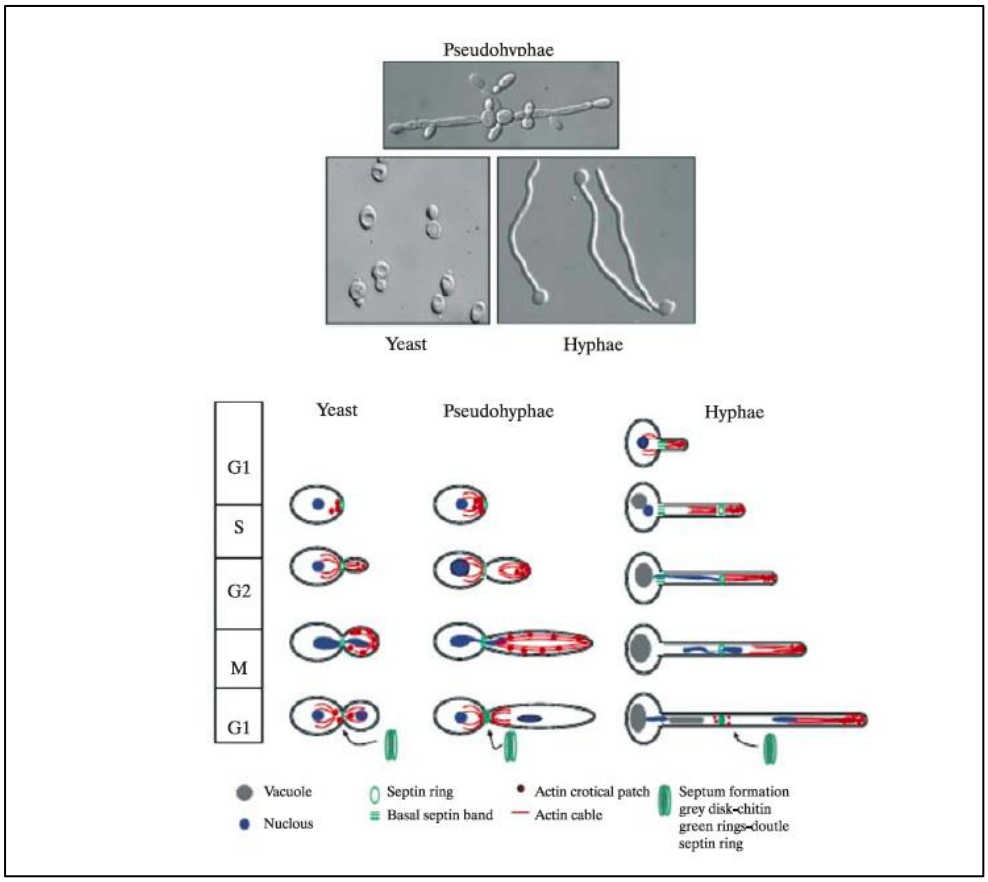
The *Candida* genus belongs to the family *Saccharomycetaceae* of which *Candida albicans* is the most common species (Shi et al., 2007). *C. albicans*, a diploid polymorphic yeast with eight chromosomes is the most common pathogenic yeast in humans (Kim and Sudbery, 2011; Schulze and Sonnenborn, 2009). The genome of *C. albicans* is 13.3-13.4 Mb encoding between 6,100 to 6,200 genes. *C. albicans* can exist in at least three distinct morphological forms- the *yeast*, the *pseudohyphae* and the *hyphae* forms (Figure 1.4). The pseudohyphae and hyphae are often collectively referred to as *filamentous*. Other forms may even occur during phenotypic switching. The filamentous forms are invasive and the ability to switch between different morphologic forms is an indication of their virulence. This virulence is enhanced by

the disseminative ability of the yeast form and the capacity of the hyphal form to invade tissues (Sudbery et al., 2004).

The morphologic differences between the yeast, the pseudohyphae and hyphae have been described (Sudbery et al., 2004). The pseudohyphae are produced from incomplete separation after the cell cycle leading to yeast cells that demonstrate polarised growth. The pseudohyphae also differ from the hyphae forms in the diameter of the filament that is not constant along the length. The pseudohyphae is constricted at the ends and wider at the centre in contrast to the hyphae that has a constant filament width.

Macroscopically, *Candida* spp. form colonies that have a cream colour and a yeasty smell following aerobic incubation at a temperature range of 20°-38°C. The pH range for growth is 2.5 to 7.5, which can be seen after 48 to 72 hours. Growth at 37°C helps to identify yeasts from clinical specimens as most pathogenic species grow readily at temperatures between 25°C and 37°C. Microscopically, *Candida* yeasts are almost identical and stain Gram-positive. However, the shape of the blastospores can vary from spherical to ovoid or elongated forms. *Candida albicans* is a dimorphic yeast, meaning that it can switch from blastospores (ovoid and budding yeast cells) to parallel-sided hyphae (Webb et al., 1998).

Figure 1.4 Morphologic forms of *Candida albicans* [Source: Kim and Sudbery, 2011]



1.2.1 Pathogenicity of *Candida albicans*

C. albicans has the ability to live as a commensal or transform to an active pathogen. The physical barrier provided by the oral epithelium, the presence of other inhibitory bacteria and an intact immune system help to maintain the commensal status of the yeast. Although some known factors aid the switch to a pathogen, the molecular mechanisms underlying yeast attachment, invasion, destruction and dissemination are not fully understood (Dalle et al., 2010).

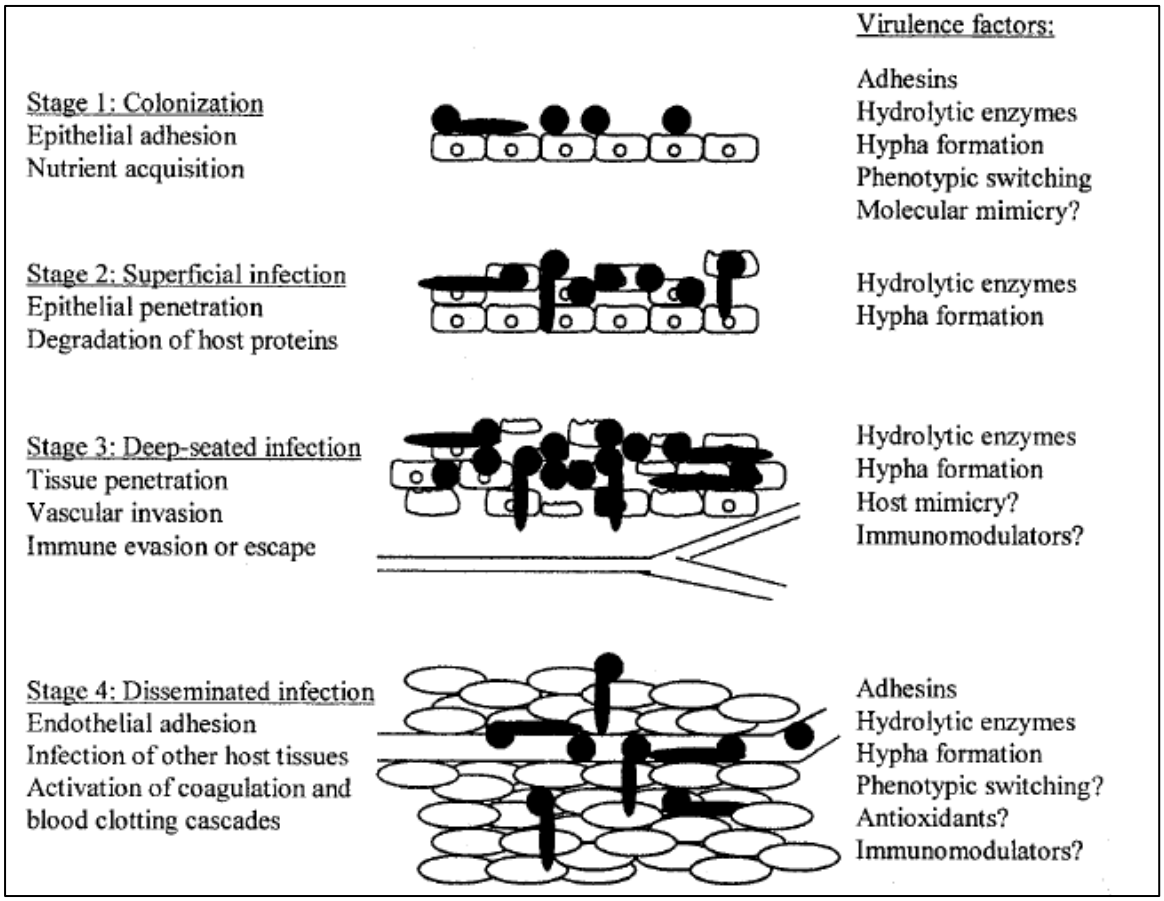
The known pathogenic characteristics involve a change in morphology from the yeast to the hyphal forms (dimorphism), phenotypic switching and the elaboration of

certain hydrolytic enzymes (Figure 1.5). These enzymes hydrolyse large substrates into smaller units for easier movement into the cell for nutritional purposes and break down host targets for colonisation and invasion. The most vigorously described are the phospholipases and proteinases (Chaffin, 2008) whereas lipases have been studied to a lesser extent (Naglik et al., 2003). Furthermore, *C. albicans* is able to haemolyse red blood cells and release haemoglobin and iron for its nutrition. This haemolytic capacity is proportional to the virulence of the yeast (Baboni et al., 2009).

Other pathogenic characteristics include the expression of factors that aid adherence, directed growth/thigmotropism, adaptation to stress and metabolic flexibility (Wachtler et al., 2011). Recent findings show that the mechanisms of invading the host epithelial cells by *Candida* include the active penetration and the induction of endocytosis (Dalle et al., 2010). With all that is known so far, it is certain that the interplay of both host and yeast factors ultimately enhance pathogenicity and the development of oral candidiasis (Martin et al., 2011).

Figure 1.5 Stages and pathogenic characteristics of *Candida albicans*

[Source: Naglik et al., 2003]



It is known that *C. albicans* isolated from HIV positive subjects produce more secreted aspartyl protease and are more adherent than isolates from health controls, which suggest they are more virulent (De Bernardis et al., 1996; Ollert et al., 1995; Sweet et al., 1995).

1.2.1.1 Adherence

The ability of *Candida albicans* to adhere to host cells, other oral bacteria and/or medical devices is the first stage in the process of infection. *C. albicans* adheres to itself by flocculation and to other organisms by coaggregation. Adherence can be specific or non-specific and enables the yeast to resists clearance from the mouth by

the cleansing action of saliva and swallowing. Specific adherence involves the interaction of cell surface proteins with specific ligands or receptors in the oral cavity. This interaction may occur between proteins or protein-sugar interactions. In contrast, non-specific adherence involves physical entrapment due to hydrophobic and electrostatic interactions with biotic and abiotic surfaces (Chaffin, 2008; Marsh and Martin, 2009).

Once the yeast makes contact with the epithelial cells, rapid formation of the hyphae and the expression of genes that mediate adherence are stimulated (Wachtler et al., 2011). The presence of specific cell wall proteins called adhesins in *Candida* species correlates with the adherence ability of the yeasts. Specific genes in the *Candida* genome code for adhesins and several types of adhesins have been described (Chaffin, 2008). The adhesins and the ligands or receptors on the host that aid adherence have been reviewed extensively (Chaffin, 2008; Chaffin et al., 1998).

Adhesins or agglutinin like proteins assists *C. albicans* to attach and form biofilms. They are glycophosphatidylinositol cell wall proteins consisting of three domains. The variable central portion of the adhesins is used to classify them into different subgroups. At least 8 genes that encode for these proteins have been described. The members of the agglutinin like sequence (ALS) gene family, *ALS1, ALS2, ALS3* are the most commonly expressed. These genes are expressed variably in different strains and clinical samples. However, upregulation of these genes have been demonstrated in patients with oral candidiasis (Zakikhany et al., 2007). Hwp1p adhesin is found on the hyphal surface and forms a covalent attachment to the host cell. Unlike the Hwp1p, Ywp1p adhesin it is a glycophosphatidylinositol cell wall protein commonly

expressed on the surface of the yeast cell. Expression is greatest towards the end of the exponential growth phase of the yeast.

Mannans and mannoproteins form part of the cell wall of the *C. albicans* and they interact with host proteins. Cell surface hydrophobicity in *C. albicans* is possible due to the existence of several proteins. These proteins enhance adherence to extracellular matrix, epithelial and endothelial cells and plastic materials in catheters and other medical devices. Several cell wall proteins and mannoproteins such as mp58 fibrinogen binding mannoprotein prove to be hydrophobic (Zakikhany et al., 2007). A similar gene *CSH1* encodes 38kDa protein that has hydrophobic properties (Singleton et al., 2001).

Filamentous, thin and long appendages on the surface of *C. albicans* called fimbriae mediate adhesive interactions with host receptors. The major component of the fimbria is a 66kDa glycoprotein which binds to glycosphingolipids on the surface of human buccal epithelial cells. The proteins in the fimbriae also have strong affinity for collagen. Epithelial binding lectin-like protein (EBLP) also contribute to the adherence of *C. albicans* to epithelial cells. The sap Protein family also called secreted aspartyl proteinases are hydrolytic enzymes implicated in *C. albicans* virulence. Apart from acting as proteinases, they also contribute to adherence (Naglik et al., 2003).

In an intact epithelium or endothelium which covers the basement membrane, the underlying connective tissue containing the extracellular matrix host proteins is inaccessible to colonizing yeasts. When this protective layer is compromised, yeasts

are able to escape into the vascular system and penetrate the deeper layers where they bind to components of the extracellular matrix. Extracellular matrix is ubiquitous and increases the potential of binding via protein-protein and protein-carbohydrate interactions (Chaffin, 2008). *C. albicans* can adhere to type I collagen, gelatin (which is denatured type I collagen) and type IV collagen. A 75kDa glycoprotein called vitronectin found in the vascular wall and serum provides a ligand for germ tubes at a greater capacity than for yeast cells. Tenascin-C is a hexameric 200 kDa protein. Like vitronectin, it also binds more readily to germ tubes than yeast cells. The entry of *C. albicans* into the blood stream via catheters or by translocation provides an avenue for the yeast to interact with proteins and other blood constituents. Proteins in serum, particularly complement fragments C3d and iC3b can bind to yeasts. Fibrinogen, C4b binding protein, factors H and FHL-1, haemoglobin and plasminogen are other serum ligands with varying degree of activity. Surfaces in the oral cavity are covered by pellicle that contains salivary components. Recently, research has shown that binding of salivary components to yeast cells is more crucial for adherence than binding to epithelial surfaces. The major salivary binding components are proline rich proteins (PRPs), which are predominantly salivary polypeptides. Statherin is another salivary polypeptide that may contribute to adherence. In contrast, salivary IgA inhibits adherence (Chaffin, 2008).

Studies have found that *C. albicans* isolated from HIV positive patients adheres more readily to oral epithelial cells than isolates from HIV negative individuals (Jain et al., 2010; Mane et al., 2012). This could be one of the reasons for the increase in colonisation and carrier rate among HIV positive patients.

1.2.1.2 Secreted aspartyl proteinases

The secreted aspartyl proteinases (SAPs) and their role in *C. albicans* virulence have been studied more comprehensively than other extracellular hydrolytic enzymes produced by *C. albicans* (Kwon-Chung et al., 1985). Ten *SAP* genes (*SAP 1-10*) that code for the protein have been identified. These genes are not peculiar to *C. albicans,* and are found in other pathogenic *Candida* species, particularly *C. dubliniensis, C. tropicalis* and *C. parapsilosis* (Fotedar and Al-Hedaithy, 2005).

The *SAP* family is divided into 4 subfamilies based on sequence homology of the *SAP* isoenzyme family. They are proteins that range from 35-50 kDa that are produced with changes in pH. This versatility allows the yeast to remain virulent in different ecological niches, for example the vagina and other mucosal surfaces, the skin and the internal organs. This virulence and capacity to cause infection is due to the ability of these enzymes to source nutrition for yeast cells, aid invasion and penetration of host tissues while evading the immune response (Naglik et al., 2003). SAPs contribute to penetration by degrading E-cadherin, an interepithelial junction protein through proteolysis (Zhu and Filler, 2010).

In human *Candida* infection, *SAP9* is the most commonly expressed *SAP* gene in wild type cells. Furthermore, there is usually an upregulation of the *SAP5* gene in active oral or vaginal infection, (Naglik et al., 2008). In addition, *C. albicans* SAP production was more intense in HIV positive individuals with oral *Candida* infection than asymptomatic HIV positive or HIV negative subjects (Mane et al., 2012). Similar high SAP production was detected in *C. albicans* infections in the vagina of HIV positive women (de Bernardis et al., 1999; Tosun et al., 2005). This infection

progresses with the advance of HIV disease and is probably induced by the HIV envelope (GP160) or other viral proteins such as Tat (Gruber et al., 2001). *SAPs* also induce inflammation via the induction of monocytes that produce proinflammatory cytokines IL-1b, IL-6 and TNFα. This pathway is totally independent of their proteolytic abilities and forms an independent pathway of interaction with host (Pietrella et al., 2010).

1.2.1.3 Phospholipases

Phospholipases are extracellular hydrolytic enzymes that enhance *Candida* species virulence. They also contribute to the pathogenesis of other fungal and non fungal organisms such as *Aspergillus fumigatus, Rhodotorula rubra, Cryptococcus neoformans, Toxoplasma gondii* and *Rickettsia rickettsii* (Niewerth and Korting, 2001).

C. albicans shows greater phospholipase activity compared to other non-albicans *Candida* species. This may be as high as 53.8% of all *C. albicans* isolates (Oksuz et al., 2007). The site of the yeast and immune status of the host influences the degree of production. Samples taken from different anatomic sites show that phospholipase production was most prominent in the mouth (59%) and faecal (42%) samples (Oksuz et al., 2007). Similarly, Price and colleagues isolated phospholipase from 55% of *C. albicans* samples from the blood, 50% from wound infection and 30% from urine samples (Price et al., 1982).

Six types of phospholipases have been described, phospholipase A, B, C and D, lysophospholipase, and lysophospholipase-transacylase. Phospholipases hydrolyse

phospholipids into fatty acids and contributes to the damage of the host cell membrane. The hydrolysed phospholipids are important cell membrane components, which are necessary for structure and function. Furthermore, phospholipases enhance adherence of yeast cells by exposing cell surface ligands and production is proportional to the degree of pathogenicity (Niewerth and Korting, 2001).

Phospholipase expression by *Candida* species is highly variable due to differences in the type, stage and site of infection in addition to the host response (Mane et al., 2011). While phospholipase production may be present in HIV infection, it may not be associated with other virulence attributes (Samaranayake et al., 2005). A study in children found slightly higher but not significantly different enzyme production in children with AIDS compared to their negative counterparts (Bosco et al., 2003). Mane et al, (2012) showed that *C. albicans* isolates from HIV positive patients with oral candidiasis produced larger amount of phospholipase compared to the isolates from HIV negative patients.

1.2.1.4 Biofilm formation

In a biofilm, yeasts and bacteria are enclosed in a matrix of polysaccharides and glycoproteins produced by the microorganisms. Mixed biofilms in the oral cavity are involved in binding of *C. albicans* to several oral microbes such as *Streptococcus oralis, Streptococcus sanguinis* and *Streptococcus gordnii* resulting in co-aggregation. The cell wall polysaccharides and proteins of the bacteria and specific *Candida* adhesins directly mediate these interactions. Indirect interactions are due to metabolic by-products and signalling molecules (ten Cate et al., 2009). Some of the extracellular signalling molecules that have been studied extensively include quorum sensing

molecules such as farnesol and tyrosol. These molecules regulate behaviour for example biofilm formation, production of virulence factors, competence and bioluminescence (Albuquerque and Casadevall, 2012).

The formation and attachment of biofilms to biotic and abiotic surfaces enable *Candida* organisms to survive and proliferate in the oral cavity. In addition, *Candida* organisms in biofilms are more resistant to antifungal agents than the planktonic forms. The molecular mechanisms mediating this resistance are yet to be fully elucidated. *In vitro* studies suggest that multidrug efflux pumps and cell wall components of *Candida* such as β-1,3-glucan are responsible for the resistance (Mukherjee et al., 2005). Biofilms have gained prominence in the last decade due to their role in infections of indwelling medical devices implanted in the body ie central venous and urinary catheters and joint prostheses. These infections respond poorly to established antifungal therapy, are associated with higher mortality rates and health care, costing more that $11 billion annually (Mukherjee et al., 2005).

The higher prevalence of *Candida* carriage in HIV infected individuals may be partly responsible for enhanced biofilm formation ability. However, a recent study did not show any significant difference in the biofilm forming ability of yeasts from HIV positive and HIV negative individuals (Jin et al., 2003). This was attributed to the possible differences between the *in vivo* situation where host immunity and other factors may play a role rather than the laboratory environment. Furthermore, higher carriage rates in HIV infection may be due to other virulence factors and host immunity rather than biofilm formation capacity (Jin et al., 2003). In addition, oral *Candida* species from patients with HIV infection had similar biofilm forming ability

to isolates from invasive C*andida* infections (Junqueira et al., 2012). This indicates that oral strains may be equally virulent if they invade the systemic circulation.

1.2.1.5 Cell surface hydrophobicity (CSH)

Hydrophobicity is a complex phenomenon that is a function of the host and the yeast characteristics. Several yeast cell surface proteins may be responsible for the hydrophobic characteristics, which include a group of low molecular weight proteins of less than 65 kDa (Chaffin et al., 1998). These hydrophobic components are more abundant in germ tubes than yeast cells.

Hydrophobic interactions aid the adherence of *Candida* organisms and biofilm formation and CSH is directly related to virulence. In view of the many adhesins in the armamentarium of *C. albicans*, CSH is not as prominent as in the non-biofilm forming *Candida* that depend more on CSH for attachment (Borghi et al., 2011). CSH differs across species with *C. dubliniensis* showing greater CSH than *C. albicans* (Borecka-Melkusova and Bujdakova, 2008). Even in *C. albicans* strains, the smooth phenotypes are associated with greater CSH compared with the wrinkled, star or ring morphologic types (Antony et al., 2007).

1.2.1.6 Phenotypic switching

Phenotypic switching, a virulence attribute in oral *Candida* that usually occurs due to the response of the yeast to environmental stresses. Such stresses induce genotypic/phenotypic changes that are reflected as differences in colony morphology. The changes occur spontaneously, reversibly and may underly the expression of other virulence attributes such as cell surface hydrophobicity, proteinase production and adherence (Antony et al., 2007).

In HIV infection, *C. albicans* strains exhibited high level phenotypic switching even before the first episode of a clinical thrush infection. This switching is also associated with a higher propensity for antifungal resistance and expression of other virulence factors (Vargas et al., 2000). The wrinkled morphology is associated with higher proteinase production compared with other morphologic types whereas the smooth variety is associated with greater CSH (Antony et al., 2007).

1.3 Oral candidiasis, HIV infection and immune response

Oropharyngeal *Candida* infection is the most common opportunistic infection in individuals with HIV infection. The severity varies from asymptomatic carrier state to disseminated and potentially fatal candidemia. The likelihood of infection is higher when the CD4 count falls below 200 cell/mm^3 or when the viral load exceeds 10,000 copies/mL (Delgado et al., 2009). Although the prevalence of oropharyngeal *Candida* infection dropped with the advent of antiretroviral therapy, infections still occur. Such infections are due to episodes of depressed immunity, non-compliance with antiretroviral therapy and the development of azole antifungal resistant strains as

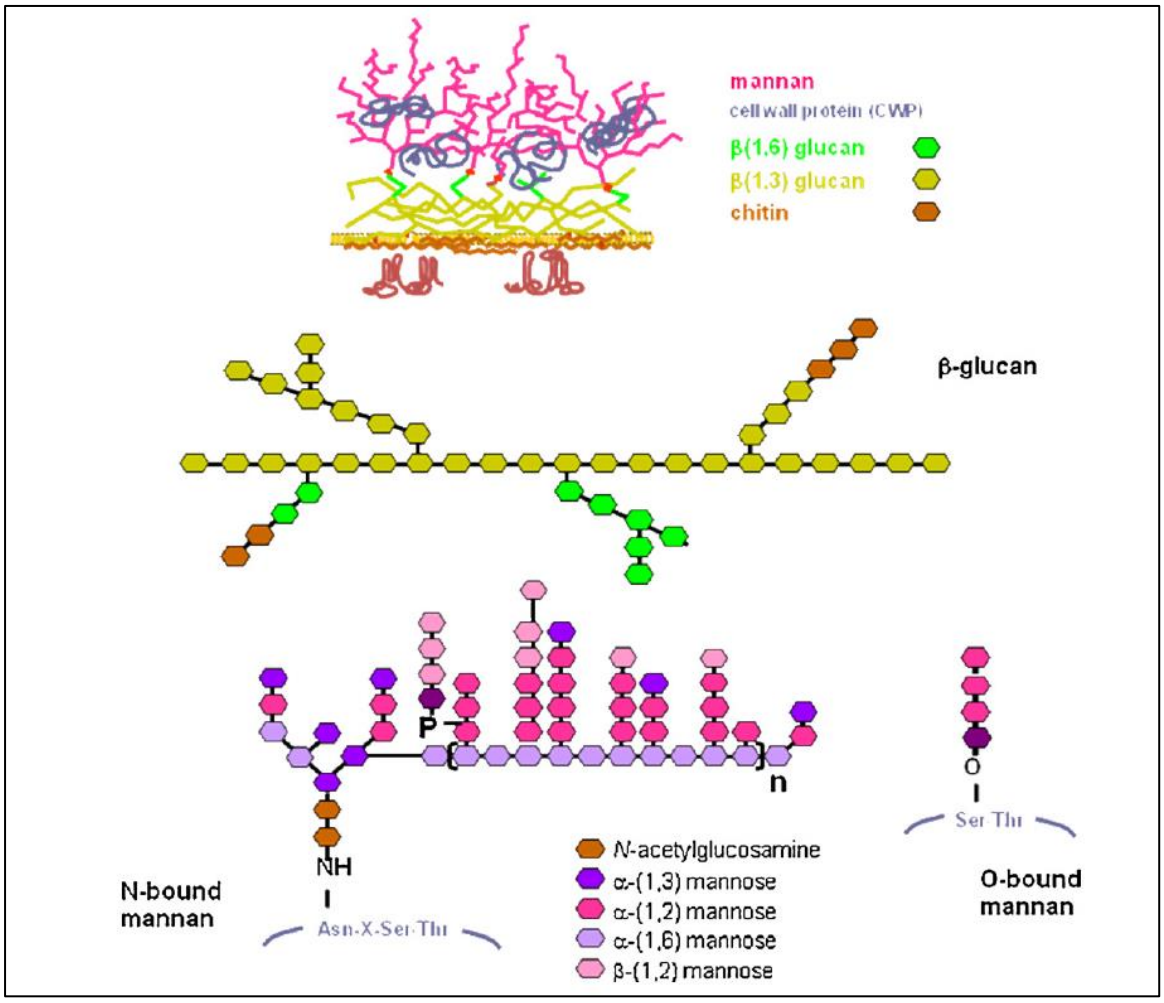
well as other factors. This justifies the need for constant monitoring of HIV positive individuals for episodes of oropharyngeal *Candida* infection (Patel et al., 2012).

The immune response to *C. albicans* is a complex phenomenon. Although the body is able to distinguish between harmless colonizing strains and exogenous pathogenic strains, the exact mechanisms are not fully understood. Pattern recognition receptors on the host interact with molecules on the surface of the yeasts known as pathogen associated molecular patterns (PAMPs) (van der Meer et al., 2010). Examples of these pattern recognition receptors are C-type lectin receptors, toll like receptors, nucleotide-binding domain leucine-rich repeat-containing receptors and the retinoic acid-inducible gene I receptors (Takeuchi and Akira, 2010).

The pathogen associated molecular patterns (PAMPs) form part of the components of the cell wall of *Candida* species (Figure 1.6). Cell wall components of *Candida* organisms that are recognised as PAMPs include chitin, the β-glucans (β-1,3 glucan and β-1,6 glucan), mannans and mannoproteins. Mannoproteins are recognized by the mannose receptors and dectin-2 whereas dectin-1 recognizes the β-glucans. The β-glucans and mannans PAMPs along with complement factor C5a induce the differentiation of T helper (T_h) lymphocytes into the T_h17 subset. The T_h17 initiate a proinflammatory cytokine response mainly involving IL-17 and IL-22, which then initiates the production of β defensins by the epithelial cells. β defensins are antimicrobial peptides with antifungal properties that are able to restrict the growth of *Candida* organisms (van der Meer et al., 2010). Failure to recognize *C. albicans* PAMPs and initiate the appropriate cytokine response due to dectin-1 deficiency is responsible for some cases of recurrent candidiasis (Ferwerda et al., 2009).

HIV infection has also revealed fresh insights into the immune response to oral *Candida* infections. The primary defence against *Candida* organisms are CD4 plus T cells. When CD4 cells are deficient as occurs in HIV infection, secondary defence mechanisms are deployed. These include $CD8^+$ T-cells, proinflammatory protective cytokines in the saliva and the defence mechanisms that are based in oral epithelial cells (Fidel, 2006). An increased understanding of these mechanisms may lead to the development of immunotherapeutic methods of curtailing infections in susceptible individuals. These individuals have a poor prognosis even in the advent of more effective antifungal therapy (Fidel, 2006; van der Meer et al., 2010).

Figure 1.6 Major pathogen associated molecular patterns on the cell wall of *C. albicans* [Source: van der Meer et al., 2010]



1.4 Genotyping of *C. albicans*

Genotyping uses differences in the DNA sequences of yeast isolates to generate strain or clone specific datasets. This helps to identify organisms at strain level i.e. below the species level. Genotyping is more accurate than phenotyping due to the poor discriminatory power, technical complexity and poor reproducibility of phenotyping methods (van Belkum et al., 2007). Phenotypes are therefore not reliable as epidemiologic markers because they do not truly reflect the genotype of an organism.

Furthermore, genotyping methods help to monitor the population and spread of yeasts in clinical and environmental settings which leads to better infection control measures. Genotyping also assist in studying colonisation patterns in individuals to determine whether the same strains or different strains are present at different times. This information makes it possible to determine risk factors for colonisation, the virulence characteristics of the strains and the evolution and relationships between strains. Such knowledge provides a basis for informed clinical decisions (Vanhee et al., 2010). The ideal genotyping method should be affordable, reproducible, easy to use and interpret. In addition, it should posses a high discriminatory power and yield rapid results.

1.4.1 Genotyping techniques

Genotyping of fungal organisms is more challenging compared to bacteria. The diploid nature of some yeasts make interpretation difficult as variable sites may show the presence of two bases. Genotyping methods for *Candida albicans* include Pulsed-field gel electrophoresis (PFGE), Restriction fragment length polymorphism (RFLP), Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP) and Amplified fragment length polymorphism (AFLP). Other available methods are Random amplified polymorphic DNA (RAPD), Retrotransposon insertion-site context (RISC), Sequence-specific DNA primer (SSDP), Repetitive-sequence-based PCR (rep-PCR), Variable number of short tandem repeat (VNTR), Electronic karyotyping/Microsatellite markers, ABC genotyping and Multilocus sequence typing (MLST). Theses different methods (Table 1.6) have their strengths and drawbacks (Boriollo et al., 2010; Garcia-Hermoso et al., 2010).

Table 1.5 Comparison of genotyping methods [Source: Vanhee et al., 2010]

Method	Reproducibility	Portability	Discriminatory Power	Ease of Use	Ease of Interpretation	Set up cost	Cost/isolate	Time to result (days)
AFLP	High	Poor	High	Moderate	Difficult	High	Moderate	2
MLST	Very high	Excellent	Moderate	Moderate	Moderate	High	High	2
PFGE	High	Poor	High	Moderate	Moderate	Moderate	Moderate	3
RAPD	Moderate	Poor	Limited	Easy	Moderate	Moderate	Low	1
RFLP	High	Poor	Moderate	Easy	Moderate	Moderate	Low	1
rep-PCR	High	Poor	Moderate	Easy	Moderate	Moderate	Low	1
SSDP	Very high	Good	Limited	Easy	Easy	Moderate	Low	1
VNTR	High	Good	HIgh	Moderate	Moderate	High	Moderate	2

Restriction fragment length polymorphism (RFLP) also known as restriction enzyme analysis (REA) involves the breakdown of DNA with restriction enzymes. The restriction fragments are then analysed using gel electrophoresis. The advantages are low cost, ease of use and rapidity of obtaining results. The main drawback is the modest discrimination, which can be improved when combined with hybridization (Vanhee et al., 2010).

Ca3 probing combines Southern blot hybridisation with the use of specific probes such as Ca3, 27a and CARE2. These probes hybridise to repetitive sequences scattered throughout the genome to identify *variability* among isolates wherever such diverse loci are found. The probes will also hybridise to *less variable* sequences thereby showing allelic polymorphism. This overcomes the problem of homoplasy,

which is a disadvantage with some genotyping techniques. They also hybridise to *hypervariable* sequences that show microevolutionary changes within a strain thus the relatedness of isolates can de demonstrated (Soll, 2000).

In Amplified fragment length polymorphism (AFLP), DNA is digested using restriction enzymes and specific adapters are joined to selected restriction sites. This is followed by 2 PCRs on the restricted template to amplify a subset of the restriction fragments. Thereafter the PCR products are separated by gel electrophoresis and banding patterns are analysed with software. It is a highly discriminatory technique. The main disadvantages are poor portability i.e. results vary with the laboratories and difficult interpretation. (Vanhee et al., 2010).

In contrast, Random amplified polymorphic DNA (RAPD), amplicons are targeted and amplified throughout the genome. Thereafter, agarose gel is used to separate the amplified products, and thereafter stained with ethidium bromide (Soll, 2000). The technique is more discriminatory than RFLP but shares the same disadvantage because it is not able to distinguish between homologous and heterozygous genotypes. This phenomenon is termed homoplasy where equal bands of electrophoretic activity may not be homologous. The other disadvantage of RAPD is low portability and low reproducibility (Vanhee et al., 2010).

Sequence-specific DNA primer (SSDP) is more stringent and overcomes the drawbacks of RFLP and RAPD. The discriminatory power is limited however it is cheap and easy to interpret.

Microsatellite length polymorphism detects the pattern of microsatellites (nucleotide sequences of short fragments on the genome of 6-10 nucleotides), which tends to repeat several times on the genome. The main disadvantage is homoplasy.

Electronic karyotyping uses chromosome length polymorphism that is common in fungi. However, the disadvantage is that homoplasy may yield results that appear similar without being ancestrally related (Vanhee et al., 2010).

In multilocus enzyme electrophoresis (MEE), the polymorphisms of housekeeping enzymes (isoenzymes) are evaluated. These enzymes are generally conserved through evolution. It has a high discriminatory power but because it is an indirect assay of genotype (using enzymes), nucleotide variation may go undetected. It is also expensive, time consuming because at least 10 enzymes must be evaluated.

In ABC genotyping, cellular DNA is digested with *Eco*RI enzyme. The PCR products of the V3 region of the 25S rRNA gene (rDNA) can be separated into 3 types based on the size of the fragments on gel electrophoresis. The predominant fragment size is then used to type the strain. The product for Genotype A is 450bp, Genotype B is 840bd whereas both fragments (450bp and 840bp) are present in Genotype C (McCullough et al., 1999).

1.4.2 Multilocus sequence typing (MLST)

This genotyping technique was introduced in 1998 to provide accurate and portable data on the biology of pathogens and to study their evolution. Originally developed for prokaryotic bacteria, it is now widely used to characterise eukaryotic organisms including *Candida* species. The differentiation of strains is based on polymorphism of the internal fragments of selected housekeeping genes. For each housekeeping locus,

the different sequences found in a bacterial or fungal species are recorgnised as distinct alleles. The alleles at each of the sequenced loci help to define an allelic profile or sequence type for each isolate. The unique sequences of a locus are assigned an allele number (Urwin and Maiden, 2003). The advantages of MLST are high discriminatory ability and easy comparison between laboratories as it allows the exchange of molecular typing data via the Internet for global epidemiology. In addition, it is reproducible and can be used to study extracted DNA or clinical specimen (Bougnoux et al., 2002).

Furthermore, MLST is a useful tool for the characterisation of *C. albicans* because it yields results that are unambiguous and is the most reliable method for differentiating *C. albicans* strains (Odds et al., 2006). In addition, it also detects slight variations in the sequences of related isolates. After extensive work on candidate housekeeping genes, MLST was used to study *Candida* organisms in 2002 and as a result, a consensus was reached using the seven most discriminatory housekeeping genes i.e. *AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13*, and *ZWF1b*. The MLST international standard for *C. albicans* is based on these genes and a central Internet database has been set up (http://calbicans.mlst.net). Thus *C. albicans* data from any country world wide can be deposited and analysed using this site (Bougnoux et al., 2003).

CHAPTER 2: STUDY DESIGN

2.1 Introduction

The factors responsible for the development of symptomatic oral candidiasis in HIV infection are controversial. Some studies reported that a decline in cellular immunity, especially CD4 counts below 200 cells/mm^3 and high viral load were associated with colonisation and candidiasis, whereas others found no relationship (Duggal et al., 2010; Erkose and Erturan, 2007; Sanchez-Vargas et al., 2005a; Sharma et al., 2010). These conflicting findings suggest that other factors may influence yeast carriage and development of symptomatic infection. Factors that may foster the development of symptomatic disease include the type and virulence of the fungi as well as antifungal resistance due to previous treatment with azoles (Loreto et al., 2010). In addition, antiretroviral treatment with protease inhibitors (PI) was associated with a decrease in opportunistic oral *Candida* infection (Diz Dios et al., 2001). On the other hand, the use of protease inhibitors did not influence *Candida* carriage despite the *in vitro* observation that PI inhibited the adherence of *Candida* (Delgado et al., 2009). Yeast counts and the expression of virulence factors tend to increase in patients with oral candidiasis. However, it is not clear whether increased yeast count or the virulence of the organism that predisposes to the development of oral candidiasis.

The persistence of the same strain in yeast colonised individuals is still contentious. While some authors identified a unique persistent strain, (Cross et al., 2004), others described multiple colonizing strain (Samaranayake et al., 2003). As a result, it is not clear whether patients colonised with *Candida* carry the same strain or get reinfected with other strains. It has been proposed that *C. albicans* isolates are highly diverse genetically in colonised individuals due to gene conversion, recombination or a

change in chromosomal ploidy. Conditions under which this occurs is poorly understood (Odds et al., 2006). To date, no longitudinal study has been undertaken to monitor strain maintenance and the acquisition of new strains of *C. albicans* in an African population.

2.2 Aim and objectives

2.2.1 Aim

The aim of this study was to investigate the effect of social and clinical factors on the *Candida* carriage in HIV positive women. In addition, the role of *Candida* counts, virulence, presence of non-albicans *Candida* and reinfections in the development of oral candidiasis was studied.

2.2.2 Objectives

a. To examine the carrier rate of *Candida* among HIV-positive women: prevalence, types and quantities of *Candida*, influence of oral hygiene, social and clinical factors.

b. To determine the virulence of *C. albicans* during the study period and the influence of quantity and/or virulence of *C. albicans* on the development of oral candidiasis.

c. To examine the occurrence and role of non-*C. albicans* yeasts in the development of candidiasis.

d. To study the antifungal sensitivity of *C. albicans* isolated from HIV positive women.

e. To determine and compare the genotypes of *C. albicans* in those colonised women at intervals during the follow up period.

2.3 Study outline

Figure 2.1 is a flow diagram depicting the study design and the layout of the thesis.

a. **To examine the carrier rate of *Candida* among HIV-positive women: prevalence, types and quantities of *Candida*, Influence of oral hygiene, social and clinical factors.**

Briefly, oral rinses from 197 HIV positive women were collected, cultured on selective media that support the growth of Candida. Thereafter, the isolates were analysed quantitatively and qualitatively. A detailed questionnaire was administered in order to collect data about oral and personal hygiene and habits, HIV treatment and coinfections. CD4 count and HIV viral load were performed or obtained from the clinical records. Descriptive statistics was applied to determine the prevalence, type and quantities of *Candida* in the study group. Chi squared statistics was used to compare proportions. The *t*-test or the non-parametric equivalent was used for continuous variables. A logistic regression model was fitted to determine the risk factors for colonisation. Results of this section of study are described in Chapter 3.

b. **To determine the virulence of *C. albicans* during the study period and the influence of quantity and/or virulence of *C. albicans* on the development of oral candidiasis.**

One hundred and seventeen women who carried *C. albicans* in their oral cavity were investigated over a period of 6 months. They were examined at

three months interval for the presences of clinical oral candidiasis and their CD4 counts were determined. In addition, oral rinse samples were collected, cultured on ChromAgar candida plates and *Candida* were isolated. Adherence ability of *C. albicans* to oral epithelial cells and production of hydrolytic enzymes are considered virulence factors in this organism. Therefore, quantitative analyses of adherence ability to oral epithelial cells, proteinase and phospholipase production by *C. albicans* were determined. The virulence properties of the yeasts isolated at the initial examination and after three months and 6 months were investigated and compared. The relationship between the quantity of *C. albicans* and their virulence scores and the development of symptoms at each stage was examined using the *t*-test or a non-parametric equivalent and correlation coefficients. Results of this section of study are described in Chapter 4.

c. **To examine the occurrence and role of non-*C. albicans* yeasts in the development of candidiasis.**

Virulence properties of all non-*C. albicans Candida* isolates were also studied. A quantitative analyses of adherence to oral epithelial cells, proteinase and phospholipase production were measured. The relationship between the virulence score and development of candidiasis was analysed using Mann Whitney tests. Results are described in Chapter 4.

d. **To study the antifungal sensitivity of *C. albicans* isolated from HIV positive women.**

The antifungal sensitivity of all the *C. albicans* isolates (122) to 9 antifungal agents was determined using the Sensititre YeastOne® colorimetric

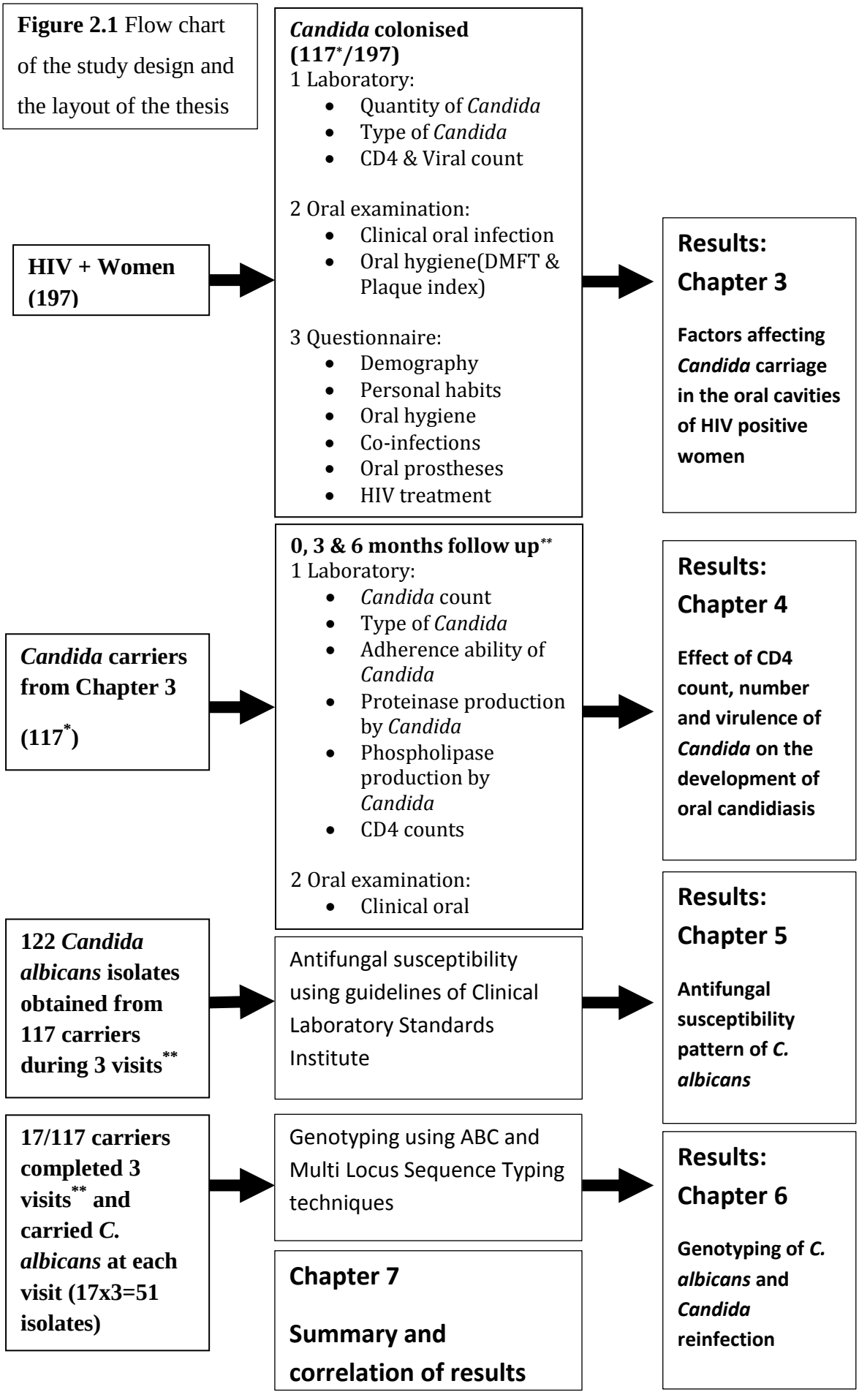
microdilution method. Minimum inhibitory concentrations were obtained and the results were interpreted using Clinical Laboratory Standard Institute revised guidelines. The results were analysed using descriptive statistics. In addition, antifungal drug resistant strains were identified. Results are described in Chapter 5.

e. **To determine and compare the genotypes of *C. albicans* in those colonised women at intervals during the follow up period.**

Maintenance and reinfection of the oral cavities with *C. albicans* strains in HIV positive women can only be measured using genotyping. Therefore, the ABC genotyping technique and multilocus sequence typing (MLST) techniques were used to genotype *C. albicans* isolated from 17 HIV positive women at three and six months follow up intervals. In ABC genotyping, the region of the 25S rRNA gene (rDNA) can be divided into 3 types based on the size of the DNA fragments. The predominant fragment size is then used to type the strain. The product for Genotype A is 450bp, Genotype B 840bp while both fragments (450bp and 840bp) are present in Genotype C (McCullough et al, 1999). MLST employs the sequencing of some selected housekeeper gene fragments (AAT1a, ACC1, ADP1, MPIb, SYA1, VPS13 and ZWF1b) and the identification of polymorphic nucleotide sites. A unique Diploid Sequence Type (DST) based on the combination of alleles at different loci was used to discriminate between *C. albicans* strains (Bougnoux et al, 2003). The genotypes of *C. albicans* strains were compared to establish the status of carriage in these women. Results are described in Chapter 6.

The findings of the above five studies are summarized in Chapter 7 and the raised questions addressed.

Figure 2.1 Flow chart of the study design and the layout of the thesis



CHAPTER 3: FACTORS INFLUENCING CARRIAGE OF *CANDIDA* IN THE ORAL CAVITIES OF HIV POSITIVE WOMEN

3.1 Introduction

Candida species usually colonise the human oropharyngeal tract and *Candida albicans* occurs most frequently. These yeasts are usually commensals but may act as opportunistic pathogens (Cannon et al., 1995). Colonisation does not always lead to infection, however, when the host immunity is compromised, the risk of a disseminated infection is high (Lott et al., 1999) and candidiasis may develop. Such infections are still a major health care challenge (van der Meer et al., 2010). For example, Das et al., (2011) reported that the incidence of candidemia was 10.9 episodes/100 000 bed-days with a crude 30-day mortality of 37% at a UK referral centre (Das et al., 2011).

There is a wide variation in the prevalence of yeast colonisation in the mouth. The variation depends on the age, location and the immune status of the population sampled. For instance, a prevalence of 80% was found in HIV positive children and in 57.5% of their seronegative siblings (Cerqueira et al., 2010). Similarly, in a previous study at Charlotte Maxeke Academic Hospital, Johannesburg, South Africa a prevalence of 81.3% was found in HIV positive adults compared with 63% in their HIV negative counterparts (Patel et al., 2006).

Higher rates of *Candida* colonisation are a consistent finding in HIV infection and this has a high predictive value for subsequent infection with oropharyngeal candidiasis (Vargas and Joly, 2002). With impaired host defence mechanisms, *C.*

albicans assumes a more pathogenic and virulent capacity that can lead to significant morbidity (Mercante et al., 2006). Many factors have been suggested as risk factors for oral colonisation; which include diabetes mellitus, head and neck cancer, smoking, oral prostheses, extremes of age, race and poor nutritional status. Others are reduced salivary flow, use of antibiotics, immunosuppressive states and presence of other locally available microflora (Ellepola et al., 2011; Jabra-Rizk et al., 2001; Schelenz et al., 2011). In HIV positive patients, the CD4 count, HIV viral load, non availability or non usage of Highly Active Antiretroviral Therapy (HAART) and the type of antiretroviral medication are possible factors that influence oral colonisation with yeasts (Back-Brito et al., 2009; Delgado et al., 2009; Wu et al., 2012). Although Wu and colleagues (2012) observed that the initiation of HAART reduced the rate of oral colonisation by *Candida*, Yang et al did not find a reduction in the colonisation of the oral cavity with HAART (Yang et al., 2006).

The variation in factors influencing oral colonisation could be attributed to differences in study populations, sampling techniques, use and type of antiretroviral therapy and other factors. Risk factors may also be peculiar to geographic settings (Cerqueira et al., 2010). For example, a study in South Africa described the prevalence of yeast carriage in paediatric HIV subjects (Blignaut, 2007) but none have investigated the risk factors for colonisation in other age groups. In addition, no study has compared the prevalence rates of *Candida* colonisation in the oral cavity pre and post HAART initiation.

This chapter summarises the results of the factors associated with oral colonisation of *Candida* species in a cohort of HIV positive women in South Africa.

3.2 Methods and materials

3.2.1 Study setting, design and subjects

The study was conducted from January to December 2011 at the HIV clinic at the Charlotte Maxeke Academic Hospital, Johannesburg, South Africa. Ethical clearance (Certificate no: M10102, appendix 9.1) was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg. Confirmed HIV positive women willing to participate were enrolled after obtaining written consent. A detailed questionnaire (appendix 9.2) was administered to collect data on demographics, personal and oral hygiene, HIV treatment, coinfections including oral infections, and previous history of oral candidiasis. The most recent CD4 count and HIV viral load (within 3 months) were obtained from the clinical records. Where such results were not available, blood samples were collected for CD4 counts and viral load.

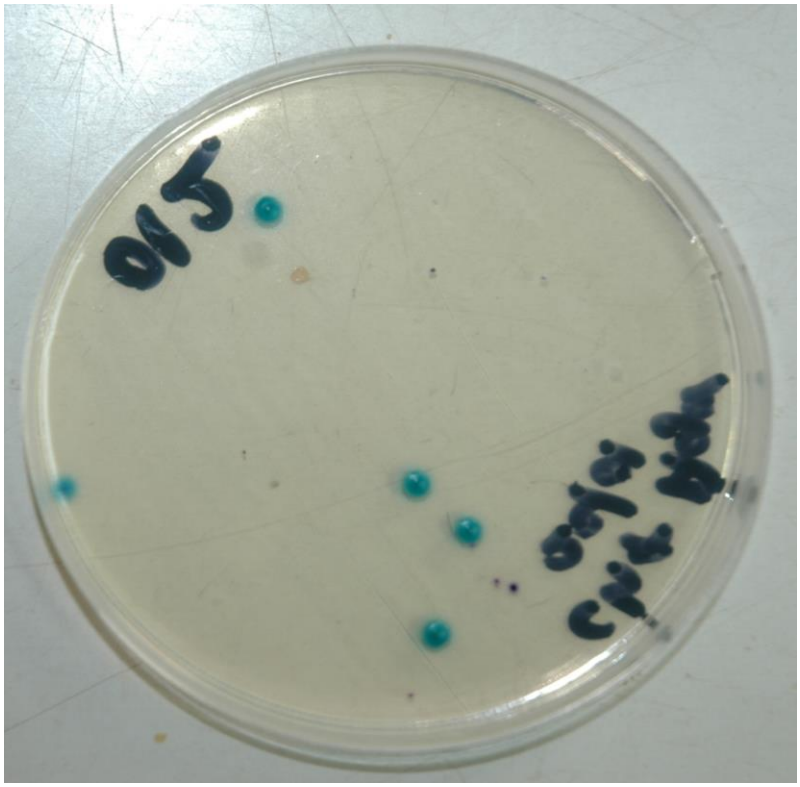
Detailed oral examination was conducted on all participants by trained and standardised oral health care personnel supervised by the author who had been trained to recognise oral aspects of HIV infections at the University of California, San Francisco, USA to screen for oral candidiasis and other HIV related oral conditions. The oral hygiene status was measured using the plaque and gingival indices (Loe, 1967) and the caries status was assessed using the number of decayed, missing and filled surfaces (DMFS) as recommended by the World Health Organization (World-Health-Organization and 1997).

3.2.2 Sample collection and laboratory techniques

A concentrated oral rinse technique (Samaranayake et al., 1986) was used to collect samples for analysis before oral examination. Patients were requested to rinse their mouth for 60 seconds with 10 millilitre of sterile distilled water and expectorate the rinse into a sterile universal container. Oral rinse samples were diluted and 1:10, 1:100, and 100 µl of the diluted samples were spread on CHROMagar *Candida* agar (CHROMagar, Paris, France) and incubated aerobically at 37°C for 48 hours. The number of colonies on the CHROMagar plates was counted and the oral yeast carriage rate was expressed as colony forming units per millilitre (cfu/ml) of saliva. CHROMagar *Candida* made it possible to identify *Candida* spp. using the growth and the colour of the yeast colonies (Figure 3.1). Single colonies were subcultured on Sabouraud dextrose agar (Oxoid, Hampshire, England) until pure cultures were obtained and were further identified.

An API 20C AUX kit (Biomerieux, France) was used to confirm presumptive identification of all the *Candida* isolates. Pure cultures of all yeast positive isolates were subcultured on Sabouraud dextrose agar (Oxoid, Hampshire, England) plates and suspended in 2ml sterile distilled H_2O equivalent to 0.5 McFarland turbidity standards. A 100µl aliquot of this preparation was inoculated into the corresponding C medium tube provided with the kit. The inoculated C medium was then transferred into cupules in a API 20C strip. Each strip was incubated at 30°C for 48-72 hours. The strips were examined for the presence of growth. Cupules with turbidity significantly greater than the negative control cupule were considered positive.

Figure 3.1 CHROMagar *Candida* plates showing green colour colonies of *Candida* species. [Source; Photographed by the investigator]



3.2.3 Statistical analysis

Continuous variables that were normally distributed were described using the mean and standard deviation, while the non-normal variables were described using the median and interquartile range. The *t*-test was used to compare means in the two groups using parametric data while the Mann-Whitney test was applied to non-parametric data. Categorical variables were compared using Chi-squared statistics or the Fisher's exact test as appropriate.

A multivariable logistic regression model was constructed with *Candida* colonisation as the outcome variable. The best-fit option was used for predictor selection. Non-parametric predictors such as CD4 count and viral load were dichotomised and

collinear predictors were dropped from the model. The DMFS was skewed but was retained as a continuous predictor after normalising with a square root transformation as suggested by the ladder of powers (Tukey, 1977). Standard procedures for model checking including Hosmer-Lemeshow goodness of fit tests were adopted and statistical significance was inferred at $p< 0.05$. Statistical analysis was performed using Stata version 12 (Statacorp, College Station, Texas, USA).

3.3 Results

3.3.1 Socio-demographic factors and oral *Candida* colonisation of HIV positive women

The results of social factors and *Candida* colonisation of the oral cavities of HIV positive women are shown in Table 3.1. Their ages ranged from 22 years to 69 years with a mean of 38 (±8.75) years. Zulu women were in the majority ie 73/199 (38.62%) out of 189 respondents while Shona were the smallest with one participant (0.53%). Most of the women were single, 137/199 (61.19%) of the 198 participants and the majority had a high school education 144/198 (72.73%). Most subjects had one to two children, 122/197 (61.93%) and the majority (144/196 subjects or 73.47%) lived in a household with at least one other member but not more than four people. Only 9 out of 198 or 4.55% admitted the regular exchange of utensils with family members while eating and only a small fraction were smokers 12/198 (6.06%). Colonisation by oral *Candida* was 59.39% (117/197).

Table 3.1 Sociodemographic factors investigated in HIV positive women colonised by *Candida*

Characteristics	**Results (n=199)***
Age in years (mean ± SD)	38 (±8.75)
range	22 - 69
Tribe – Zulu	73(38.62)
- Sotho	34(17.99)
- Pedi	6(3.17)
- Tswana	21(11.11)
- Xhosa	21(11.11)
- Swathi	8(4.23)
- Venda	3(1.59)
- Tsonga	4(2.12)
- Ndebele	13(6.88)
- Coloured	5(2.65)
- Shona	1(0.53)
Total	189(100.00)
Marital status – Married	40(20.2)
- Single	137(61.19)
- Widowed	11(5.56)
- Divorced/Separated	10(5.05)
Total	198(100)
Education – None	5(2.53)
- Elementary	21(10.61)
- High School	144(72.73)
- University	28(14.14)
Total	198(100)
Children – 0	18(9.14)
- 1-2	122(61.93)
- >2	57(28.93)
Total	197(100)
Household members – 1-4	144(73.47)
- 5-10	51(26.02)
- >10	1(0.35)
Total	196(100)
Exchange food during eating – All the time	9(4.55)
- Often	5(2.53)
- Occasionally	33(16.67)
- Never	151(76.26)
Total	198(100)
Smoke -Yes	12(6.06)
-No	186(93.94)
Total	198(100)
Colonised with *Candida* - Yes	117(59.39)
- No	80(40.61)
Total	197(100)

*Totals do not add up to 199 in some of the rows due to missing values, 2 women completed the questionnaire but did not return for the clinical examination. The total for clinical examination and all other results was 197

3.3.2 Clinical factors and oral *Candida* colonisation of HIV positive women

Clinical factors and oral *Candida* colonisation of HIV positive women are shown in Table 3.2. Oral hairy leukoplakia was the most common oral lesion, found in 27/197 or 13.71% of the patients. Oral *Candida* infection was found in 18/197 or 9.14% of the subjects. Allergies and hypertension were the most frequently encountered systemic diseases in 21.32% (42/197) and 20.81% (41/197) of the patients respectively. Active infection with tuberculosis was present in 15.75% (31/197) of the subjects while 9.14% (18/197) had one form of cancer whereas diabetes mellitus was present in only 4.59% (9/196) of the subjects. Most of the participants 132/197 (67.01%) brushed their teeth twice daily even though approximately a third 63/197 (31.98%) had ever visited a dentist. The mean Plaque Index (PI) and Gingival Index (GI) for all the subjects were 0.66 (±0.37) and 0.65 (0.42) respectively. The average DMFS for all the subjects was 23.12 (±20.93). The colonisation rate with oral *Candida* was 59.39% ie 117 of the 197 respondents carried yeasts.

Table 3.2 Clinical factors investigated in HIV positive women

Characteristics	**Frequency(%)**			
Oral lesions – *Candida* infection	18/197 (9.14)			
- Hairy leukoplakia	27/197 (13.71)			
- Kaposi sarcoma	0/197 (0)			
- Aphthous ulcer	7/197 (3.55)			
- NUP*	7/195 (3.59)			
- Papilloma	3/197 (1.52)			
- Parotid enlargement	1/197 (0.51)			
	Yes	No	Don't know	Total
Other illnesses – Hypertension	41(20.81)	155(78.68)	1(0.51)	197(100)
- Heart diseases	14(7.11)	182(92.39)	1(0.51)	197(100)
- Asthma	11(5.58)	185(93.91)	1(0.51)	197(100)
- Allergies	42(21.32)	155(78.68)	0(0)	197(100)
- Diabetes Mellitus	9(4.59)	186(94.9)	1(0.51)	196(100)
- Cancer	18(9.14)	179(90.86)	0(0)	197(100)
- Tuberculosis	31(15.74)	166(84.26)	0(0)	197(100)
Daily frequency of toothbrushing – 1	59(29.95)			
- 2	132(67.01)			
- 3	6(3.05)			
Visit dentist – Yes	63(31.98)			
- No	134(68.02)			
Plaque Index (mean ± SD)	0.66(±0.37)			
range	0-2			
Gingival Index (mean ± SD)	0.65(0.42)			
range	0-2.3			
DMFS(mean ± SD)	23.12(20.93)			
range	0-102			

* Necrotising ulcerative periodontitis

3.3.3 HIV treatment, CD4 count and viral load in HIV positive women colonised by *Candida*

The results of HIV treatment, CD4 counts, viral load and *Candida* colonisation of oral cavities of HIV positive women are shown in Table 3.3. Almost all the participants i.e. 186 of 197 (94.42%) were on antiretroviral therapy (ARV) at the time of examination. When hospital records were checked, 11/197 (5.98%) of the participants had not commenced ARVs. The prescribed ARV therapy was Highly Active Antiretroviral Therapy (HAART). The most common combination was Lamivudine (3TC), Tenofovir (TEN) and Efavirenz (EFV). Of the 184 participants with documented records of ARV therapy, 101(54.89%) were on this combination whereas in 26 participants (14.13%), Nevirapine was substituted for Efavirenz. Approximately a quarter of the patients (45/197, 24.32%) had CD4 counts below 200 cells/mm^3. A significant proportion of the patients had no results for the viral load at our initial visit because they were not tested routinely at every visit. Of the 125 subjects with data, 17 (8.63%) had a viral load of more than 1000 copies/ml.

Of the participants who carried yeast, the average count was 254.32 (±485.67), with a range of 10 - 3750 cfu/ml. *Candida albicans* was the most common species isolated ie 85 (72.65%) of the 117 participants were colonised by this yeast. Other species included *C. dubliniensis* 14 (11.97%), *C. tropicalis* 7 (5.98%), *C. famata* 6 (5.13%) and *C. glabrata* 2 (1.71%). *C. parapsilosis*, *C. krusei* and *Saccharomyces cerevisiae* were each found in 1 participant (0.85%).

Table 3.3 Clinical factors and laboratory investigations of HIV positive women colonised by *Candida*

Characteristics	**Frequency(%)**
Patients on ARV – Yes	186(94.42)
- No	11(5.58)
Total	197(100)
Time on ARV – 0 months	6(3.14)
<12 months	46(24.08)
12-24 months	42(21.99)
>24 months	97(50.79)
Total	191(100)
Current ARV treatment –	
ARV naïve	11(5.98)
3TC+TEN+EFV	101(54.89)
3TC+TEN+Aluvia	15(8.15)
3TC+TEN+NVP	26(14.13)
3TC+d4t+EFV	12(6.52)
3TC+AZT+Aluvia	4(2.17)
3TC+TEN+AZT	3(1.63)
TEN+AZT+Aluvia	4(2.17)
AZT+EFV+TEN	3(1.63)
3TC+d4t+Aluvia	2(1.09)
DDI+AZT+Aluvia	1(0.54)
EFV+TEN+Aluvia	1(0.54)
AZT+d4T+EFV	1(0.54)
Total	184(100)
CD4 count - <100	16(8.65)
(cells/mm^3) - 100-199	29(15.68)
- 200-400	59(31.89)
- >400	81(43.78)
- Not available	12(6.09)
Total	197(100)
Viral load - 0-99	96(48.73)
(copies/ml) - 100-1000	12(6.09)
- >1000	17(8.63)
Not available	72(36.55)
Total	197(100)
Candida count (n=117)– Mean ± SD	254.32(±485.67)
(cfu/ml) - Range	10-3750
No. of *Candida* species isolated (n=117)	
- *C. albicans*	85(72.65)
- *C. glabrata*	2(1.71)
- *C. tropicalis*	7(5.98)
- *C. dubliniensis*	14(11.97)
- *C. parapsilosis*	1(0.85)
- *C. krusei*	1(0.85)
- *C. famata*	6(5.13)
- *Sacharromyces cerevisiae*	1(0.85)

Table 3.4 Demographic, clinical and other characteristics of HIV positive women with and without *Candida* colonisation in the oral cavity

Characteristic	**All**	**Colonised**	**Not Colonised**	***p* value**
	N (%) 197(100)	**N(%) 117(59.4)**	**N(%) 80 (41.6)**	
Mean age in years (SD)	38.3(8.4)	37.9(8.1)	39.1(9.2)	0.45
Level of education				
None		3(60.0)	2(40.0)	
Elementary		10(47.6)	11(52.4)	
High School		86(59.7)	58(40.3)	
University		18(66.7)	9(33.3)	0.62
Smoking				
Yes		10(83.3)	2(16.7)	
No		107(57.8)	78(42.2)	0.13
Concurrent/Recent TB infection				
Present		24(77.4)	7(22.6)	
Absent		93(56)	73(44)	0.03*
Diabetes Mellitus				
Present		3 (30)	7(70)	
Absent		114(61.3)	72(38.7	0.051
Antiretroviral Therapy (ARV)				
Started		106(57)	80(43.0)	
Not started		11(100)	0(0)	0.003*
Median Time since ARV in months (IQR, min, max)		27.5(54, 0, 125)	36(53,0,133)	0.39
Use of Protease inhibitors				

Yes		17(63)	10(37)	
No		93(59.6)	63(40.4)	0.742
Wearing of dentures				
Yes		8(100)	0(0)	
No		109(57.7)	80(42.3)	0.022*
Median CD4 count in cell/mm3 (IQR, min, max)	370(364, 7, 1200)	346.5 (311, 7, 1200)	418(357, 40, 1172)	0.024*
Median viral load in copies/mm3(IQR, min, max)	25(50, 5, 315002)	40(105, 5, 315002)	5(35, 5, 14411)	0.19
Median Plaque index(IQR)	0.6(0.6)	0.60(0.59)	0.60(0.6)	0.73
Mean Gingival index(IQR)	0.58(0.5)	0.60(0.3)	0.60(0.2)	0.50
Median DMFS (IQR, min, max)	20(27,0,102)	19(27, 0,102)	16 (24,0,93)	0.07
Dry Mouth				
Present		30(51.7)	28(48.3)	
Absent		86(61.9)	53(38.1)	0.187

IQR- Interquartile range.

Table 3.4 compares *Candida* colonised participants with non colonised. The non colonised were older, mean age of 39.1 (±9.2) years compared to 37.9 (±8.1) in the colonised group. This difference was however not statistically significant. Both groups were also comparable with regards to the level of education and smoking status. However, more participants with concurrent tuberculosis (TB) were colonised by *Candida* (77.4%). This difference was significant at p=0.03. There was no statistically significant relationship between colonisation status and diabetes mellitus. All subjects who were not on ARV were colonised by yeast and this was significant at p=0.003.

The time of commencement of ARV was slightly longer in the non colonised group but this difference was not statistically significantly. The use of protease inhibitor ARV drugs had no effect. However, all the participants wearing prosthesis were colonised by yeasts and this was highly significant, p=0.022. The median CD4 count was lower in the colonised (346.5 cells/ mm^3, IQR 311) than the non colonised group (418 cells/ mm^3, IQR 357). This was significant at p=0.024. The viral load did not differ significantly in the two groups, nor did the plaque and gingival indices. The mean DMFS was higher in the colonised group 19 (IQR 27) compared to 16 (IQR 24) in the non colonised group. However this was not significantly different (p=0.07). The groups were almost equally matched regarding to the presence of a dry mouth i.e. 51.7% in the colonised and 48.3% in the non colonised group.

Table 3.5 Risk factors for oral colonisation with *Candida* in HIV positive women

	Odds Ratio	**95%CI**	**P value**
Age (in years)	0.95	0.90, 0.99	0.034*
Use of Protease inhibitors	0.78	0.25, 2.32	0.64
DMFS (Decayed, Missing, Filled surfaces)	1.30	1.07, 1.60	0.019*
Low CD4 count- $\leq$200cells/mm^3(reference CD4 count >200cells/mm^3)	1.45	0.46, 4.63	0.53
High Viral load -$\geq$10,000copies/mm^3 (reference CD4 count <10,000copies/mm^3)	5.87	0.62, 55.57	0.12
Poor oral hygiene (plaque index $\geq$1)	0.75	0.30, 1.88	0.54
Diabetes present	5.52	1.68, 18.12	0.005*
Tuberculosis present	0.34	0.11, 1.02	0.06

Multivariate logistic regression model was fitted to determine the risk factors for colonisation (Table 3.5). Younger age, odds ratio (OR) = 0.95 (95%CI=0.90,0.99; p=0.034), the dental caries status (DMFS) OR=1.30 (95%CI=1.07, 1.60; p=0.019) and diabetes mellitus OR=5.52 (95%CI=1.68, 18.12; p=0.005) were associated with colonisation. The model adjusted for CD4 count, viral load, use of protease inhibitors, oral hygiene status and the presence of tuberculosis. The dental caries status, which showed borderline association in the bivariate analysis showed significance with logistic regression. CD4 count and tuberculosis were significant in the bivariate analysis but were not significant after adjusting for other covariates in the regression output. Although age appeared to be significant, the upper confidence interval was close to 1 and was therefore disregarded.

Table 3.6 Carrier rate, quantity and variety of *Candida* species in HIV positive women

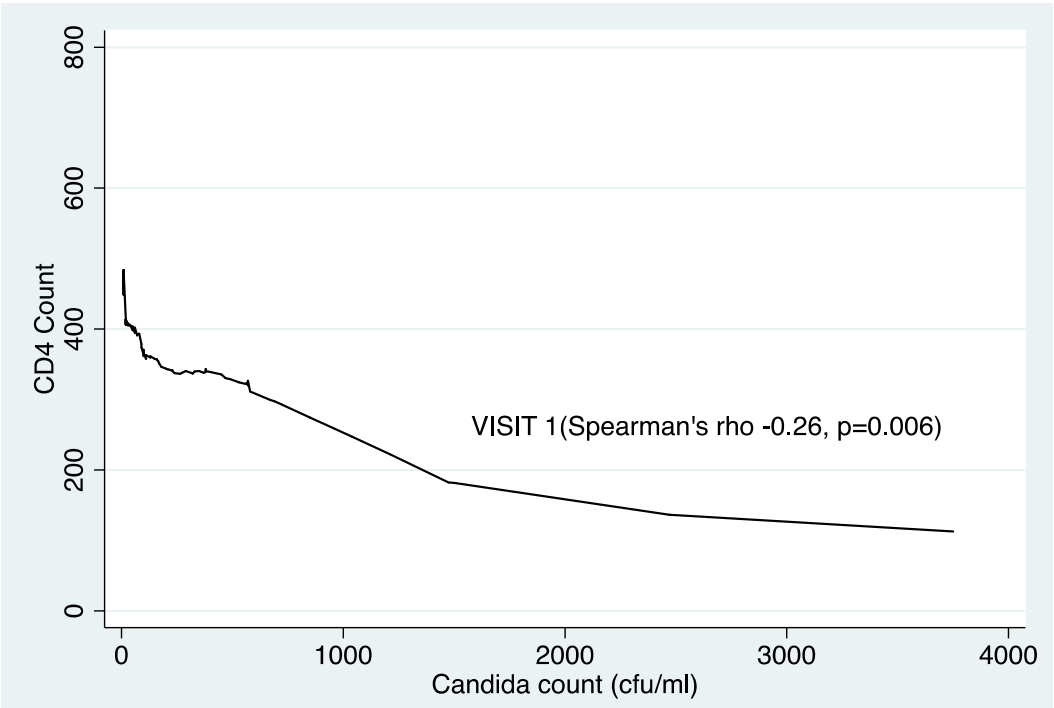
	N=117			
Carrier rate (%)	59.4			
Count prevalence (%) in cfu/ml	0	1-999	1,000-10,000	>10,000
	40.6	56.3	3.1	0
Species prevalence (%)				
C. albicans	72.7			
C. dubliniensis	12			
C. glabrata	1.7			
C. parapsilosis	0.85			
C. tropicalis	5.13			
S. cerevisiae	0.85			
C. famata	5.13			
C. krusei	0.85			

Table 3.7 Multiple *Candida* carriage in HIV positive women

No. of *Candida* species	Carriage (%) n=117
1	114 (97.4)
2	3 (2.6)
3	0

When the yeast count was broken down into categories, the majority of patients were in the lower count ranges. Only 3.1% had counts above 1000 cfu/ml while there was no participant with more than 10000 cfu/ml. The predominant species isolated was *C. albicans* followed by *C. dubliniensis*, *C. tropicalis* and *C. famata*. The yeast count was negatively correlated with the CD4 count and this was significant, p=0.006 (Figure 3.1). Only 3 participants (2.6%) of the 117 colonised women were carrying more than one species of yeast (Table 3.7).

Figure 3.2 Correlation of CD4 (cells/mm^3) count with *C. albicans* count



3.4 Discussion

The innate immune system and the acquired response in a colonised healthy host help to keep *Candida* in check. The innate system comprises the polymorphonuclear leucocytes, macrophages and antimicrobial peptides while the acquired response is represented by increased circulating/mucosal IgG and mucosal IgA antibodies (Fidel, 2006). However, very early infection with HIV influences colonisation with oral *Candida* and the development of opportunistic candidiasis (Owotade et al., 2008). This is a consequence of defective Th1-type CD4 T cells and changes in saliva composition and function (Back-Brito et al., 2009).

3.4.1 Social and demographic factors

The study focused on women due to the high prevalence of HIV infection in women. More than 60% of HIV positive individuals in Sub-Saharan Africa are women (Sia et al., 2013). In addition, women also have a higher risk of acquiring HIV infection in a heterosexual relationship due to anatomical, social and economic factors(Quinn and Overbaugh, 2005). The HIV gender imbalance in South Africa may lead to males aged between 15 to 44 years outnumbering their female counterparts by the year 2020 (US-Census-Bureau, 2004).

The colonised group was younger in our study population. Although increasing age is known to predispose to colonization (Schelenz et al., 2011) the age distribution may be due to a selection bias with younger subjects more willing to participate. Most of our subjects i.e. 128/197, 65.16%, colonised or not colonised were below the age of 40 years. This is consistent with the global HIV epidemic that affects the younger age group. This is particularly noticeable in South Africa where the adult population is

declining and the elderly population is increasing. This decline in the adult age group is due to AIDS mortality and children born with HIV not attaining adulthood (Oramasionwu et al., 2011). The social implication of this is that elderly women with limited resources are compelled to cater for younger non productive adults who are too ill to work or have died (Schatz, 2007).

It may be advantageous that most of our participants 144/198 (72.73%) had high school education. This may be because adolescents who are enrolled in schools are less likely to have multiple sexual partners than uneducated adolescents. In addition, education also influences use of condom and other preventive measures because they are aware of the biology of HIV transmission (Zuilkowski and Jukes, 2012). Previous studies in South Africa show conflicting correlations between education level and sexual behavior. Some studies did not find a relationship and surmise that other factors may be more influential than just education (Dinkelman et al., 2007). Obviously, the relationship between education and sexual behavior needs further exploration.

We explored other factors, for example the number of children, household size and the frequency of exchanging food or eating utensils. The aim was to assess the influence of social status and habits that may influence transmission of yeast from colonised to uncolonised individuals. The majority of our subjects i.e. 90.8% had children. Vertical and horizontal transmission of *Candida* occurs from colonised mothers to their children. This increases the risk of invasive *Candida* infection in immunocompromised and low birth weight children (Ali et al., 2012; Bliss et al., 2008). Close social contact may also aid transmission of azole resistant strains of

Candida among family members (Muller et al., 1999). This is important considering that 144(73.47%) of our subjects lived with at least one other person. Although very few 9 (4.55%) admitted routine exchange of food or utensils when eating, the possibility of yeast transmission cannot be excluded.

3.4.2 Clinical factors

Oral lesions in HIV infection are associated with depressed immunity especially reduced CD4 counts and progression of the disease, which may indicate poor prognosis (Saini, 2011). Oral *Candida* infection and oral hairy leukoplakia are the most common oral lesions associated with HIV infection. They were the most common lesions in our volunteers constituting 9.14% and 13.71% respectively. Although a higher prevalence of oral hairy leukoplakia was found in our study, oral *Candida* infection is still the most common oral manifestation in HIV infection. Both lesions are strongly associated with HIV infection and form part of the criteria to classify HIV disease by the Center for Disease Control and Prevention (Saini, 2011).

With the introduction of ARV therapy, oral manifestations have declined although new challenges are also emerging. Oral lesions are still seen in patients with HIV infection while oropharyngeal candidiasis is the most common lesion. However the malignant potential of the oral human papilloma virus infection is gaining increasing attention recently (Patton et al., 2013).

Concurrent tuberculosis (TB) was present in 15.74% of our subjects. More colonised subjects 24/31 (77.4%) had concurrent TB infection compared to the non colonised subjects (7/31) and this was significant, p=0.03 (Tables 3.2 and 3.4). Concurrent TB in HIV infection worsens the prognosis for both diseases and increases mortality because TB compromises the immune status. In addition, previous TB infection or

anti-TB therapy may predispose to the acquisition of fluconazole resistant strains of *Candida* (Masia Canuto et al., 2000). South Africa has the third largest TB burden globally and an increasing annual rate of TB transmission. The adoption of new and targeted TB control strategies has been advocated (Wood et al., 2011). These strategies may mitigate the possible complications that could follow oral *Candida* colonisation in immune compromised subjects who have two potentially fatal conditions.

There were fewer diabetics in our subjects 9/196 (4.59%) than those with TB. However, in the multivariate analysis, diabetes mellitus (DM) was a strong risk factor for oral colonisation with *Candida* with an odds ratio of 5.52 (95%CI 1.68, 18.12). This is consistent with the findings of other authors who have established a link between DM and the risk of oral *Candida* colonisation (Goncalves et al., 2006). The high level of glucose in the tissues of diabetics and impaired cellular mediated immunity are contributing factors for yeast colonisation (Tapper-Jones et al., 1981; van der Meer et al., 2010). The low prevalence of diabetics in our study requires cautious interpretation and needs to be further investigated.

Hypertension was the predominant systemic illness in our study group in 41/197 (20.81%), while 14 subjects (7.11%) had heart disease. Cardiovascular diseases may not have any direct bearing on oral *Candida* colonisation; however, it may influence the management of HIV disease. Some ARVs selectively alter the lipid profile; however, the long-term effect on the cardiovascular status is still controversial. ARVs such as abacavir, didanosine, lopinavir/ritonavir and indinavir have been associated with the development of acute myocardial infarction (Perez-Camacho et al., 2009).

The use of ARVs with more favourable lipid profile has been recommended in HIV infection. However cardiovascular disease prevention and management programmes should be instituted similar to those in non-HIV positive individuals (Masia-Canuto et al., 2006). In the long term, the use of ARVs is of benefit because HIV worsens hypertensive states. Therefore, the long-term benefit of ARV use outweighs the marginal risk of myocardial infarction.

The oral hygiene status (measured by the plaque and gingival indices in Tables 3.4 and 3.5) and cigarette smoking did not influence the risk of colonisation. This was similar to the observations of other investigators (Darwazeh et al., 2010a; Darwazeh et al., 2010b). However, a very strong association with the dental caries was observed even after adjusting for other predictors with an odds ratio (OR) of 1.30 (95%CI 1.07, 1.60). Carious lesions are known reservoirs for yeasts and have been established as risk factors in oral colonisation (Cerqueira et al., 2010; Jacob et al., 1998). Similarly, oral prostheses are reservoirs for oral colonisation with yeasts, and our findings confirm this. All the denture wearers were yeast positive and this was significant at p=0.022 (Table 3.4). Oral prostheses provide a means of adherence for *Candida* while precluding the mucosal area below the prosthesis from the protective effects of saliva (Zadik et al., 2010).

Although there was no relationship between oral colonisation and a dry mouth, it is known that a dry mouth combines with other factors to predispose to colonisation. Malignant conditions i.e. cancer were present in 18 of our subjects (9.14%). The management of such conditions with cytotoxic chemotherapy or radiotherapy compromise the cellular mediated host immunity thereby reducing the protective

effects of saliva from hyposalivation. With increasing age, saliva production is reduced. In addition, there is a tendency to wear prostheses, be malnourished and have poor oral hygiene (Schelenz et al., 2011). These conditions may lead to yeast overgrowth and infection.

3.4.3 Antiretroviral therapy

Antiretroviral therapy was introduced in South Africa between 2004 to 2009. To date, only half of the HIV infected population has access to therapy (Webster et al., 2012). In 2006 when oral colonisation with *Candida* was investigated in our study population, ARV was not available. The prevalence of oral colonisation and the yeast count were much higher than in the present study (Patel et al., 2006). In effect, the use of ARV has reduced opportunistic oral infections (Yang et al., 2006). Therefore our observation that participants who were yet to commence ARVs were all yeast positive ($p=0.003$, Table 3.4) is consistent with other studies (Cerqueira et al., 2010). However, prolonged HAART therapy may be more beneficial in reducing opportunistic yeast infections rather than yeast colonisation as suggested by a longitudinal study (Chen et al., 2006). This study showed that ARV in the form of HAART has reduced the colonisation rate and yeast counts over time (Patel et al., 2006). Apart from being prone to yeast infection, delay in initiating ARV has been linked with increasing mortality. Strategies advocated to reduce the delay in initiating treatment include decentralising services, promptly initiating antiretroviral therapy counseling, tracing patients who default appointments while protecting patient confidentiality. Other measures are reducing user fees, and providing point-of-care tests for CD4 cell counts and TB diagnosis (Peterson et al., 2012).

Researchers have postulated that the protease inhibitor group of antiretroviral drugs inhibit *Candida* virulence by neutralising the secretory proteinase enzyme production (Migliorati et al., 2004; Perezous et al., 2005). Although 14.8% of our subjects were on lopinavir/ritonavir combination (protease inhibitors), they did not affect yeast colonisation (Table 3.5). This inability of protease inhibitors to influence yeast colonisation was corroborated by other studies (Delgado et al., 2009; Wu et al., 2012).

3.4.4 Laboratory investigations

The median CD4 count was lower in the colonised group. This difference was significant usinmg bivariate analysis, p=0.024 (Table 3.4). The difference did not attain statistical significance with regression analysis after adjusting for other variables. This may be due to confounding by other variables or residual confounding in the CD4 count as this was dichotomized in the regression analysis. Similarly, the median viral load was higher in the colonised group. This was not statistically significant using bivariate or multivariate logistic regression. The effect of these variables on oral colonisation has been controversial. Some studies observed a relationship between CD4 count <200 cells/mm^3 and oral colonisation (Delgado et al., 2009; Ohmit et al., 2003; Wu et al., 2012) while others found no relationship (Cerqueira et al., 2010). Delgado and colleagues (2009) conducted their study in a population similar to ours i.e. a cohort of HIV positive individuals who were already on ARVs. The association of oral yeast colonisation with CD4 count lower than 200 cells/mm^3 was quite strong in their study, p=0.003. This association was obtained from a bivariate analysis similar to our finding. Unlike the present study, they did not conduct a logistic regression analysis to adjust for other confounding variables.

A high viral load was associated with colonisation in several reports (Back-Brito et al., 2009; Delgado et al., 2009; Tappuni and Fleming, 2001). In contrast, other studies did not find any association (Cerqueira et al., 2010; Sanchez-Vargas et al., 2005a). Our observations regarding the CD4 count and viral load may be related to the relatively consistent recovery of the immune system and reduced viral burden in our study population. The average CD4 count and viral load for all the subjects and the period since they have been on ARVs indicate a decent immune recovery. The median CD4 count of 340 cells/mm^3 (IQR 364) coupled with a median viral load of 25 copies/ml (IQR 50) indicates a relatively acceptable average immunological and virological profile. However, this observation is mindful that a small fraction of our volunteers had very low CD4 count and high viral load. In the colonised group, the absolute yeast count was negatively correlated with the CD4 count (p=0.006, Figure 3.2) which emphasises underscores the importance of the immune system in keeping *Candida* organisms under control.

3.4.5 *Candida* species isolated

Many studies have reported the dominance of *C. albicans* in oral samples irrespective of the HIV status of the individual (Back-Brito et al., 2009; Cannon and Chaffin, 2001; Lott et al., 1999). We found that *C. albicans* had a prevalence of 72.65% which is similar to 78.6% in a study conducted previously in our centre (Patel et al., 2006). Non-*C. albicans Candida* constituted the remaining 21.4% (Patel et al., 2006). However, Delgado and colleagues (2009) found a higher *C. albicans* prevalence of 85% (Delgado et al., 2009). The prevalence of non-*C. albicans* has been on the increase with the HIV pandemic (Costa et al., 2006). A recent study in Nigeria recorded a prevalence of 55% non-*C. albicans Candida* (Nweze and Ogbonnaya,

2011). Non-*C. albicans Candida* has been implicated in severe oral and pharyngeal infections and may be resistant to azole antifungals in the HIV positive population(Costa et al., 2006). Several authors have emphasised the importance of documenting the prevalent species of yeast and their antifungal sensitivity patterns in order to give appropriate therapy when infections break out (Nweze and Ogbonnaya, 2011).

3.5 Conclusions

In conclusion, the carrier rate of *Candida species* was 59.4% in this study population. *C. albicans* was the predominant species isolated and *Candida* counts were higher in patients with low CD4 counts. Non-implementation of ARVs, wearing of oral prostheses, presence of caries, diabetes mellitus and TB infection increased the risk of oral *Candida* colonisation. Therefore, adequate oral care during HIV infection and prompt commencement of antiretroviral therapy should be emphasised to reduce the risk of *Candida* carriage and candidiasis. Efforts should also be made to increase the availability of HIV medication in Sub Saharan Africa where most of the HIV infected population resides.

CHAPTER 4: PATHOGENICITY OF *C. ALBICANS* AND NON-ALBICANS *CANDIDA* ISOLATED FROM THE ORAL CAVITIES OF HIV POSITIVE PATIENTS

4.1 Introduction

C. albicans transforms from a harmless commensal to pathogenic yeast due to the virulence factors it is capable of expressing, particularly in an immunocompromised host. These virulence factors are many and variably expressed. They include the ability to switch phenotypes and forms (yeasts to hyphae), the formation of biofilms and adherence. As a result, they adhere to and invade epithelial cells and other surfaces through the expression of adhesins and invasins (Mayer et al., 2013). The ability to haemolyse red blood cells and elaborate hydrolytic enzymes are also associated with virulence. The hydrolytic enzymes, proteinases and phospholipases assist the yeast to adhere, penetrate and invade host tissues (Mane et al., 2011). These hydrolytic enzymes also play an important role in the acquisition of nutrients from the extracellular environment (Naglik et al., 2003). In a study of *C. albicans* virulence isolated from HIV positive subjects with oral *Candida* infection, 89.7% expressed proteinase and 59.0% phospholipase enzymes (Mane et al., 2011). Apart from these virulence factors, the "fitness" attributes also contribute to the pathogenicity of *C. albicans*. These include the ability to acquire nutrition and adapt to changes in the pH of the environment, metabolic flexibility and being able to respond to stress (Mayer et al., 2013).

Unlike *C. albicans*, there is less information on the virulence characteristics of non-albicans *Candida*. However, studying the virulence attributes of non-albicans *Candida* is crucial as they are increasingly isolated in candidiasis, candidemia and

invasive candidiasis (Silva et al., 2011). Non-albicans *Candida* with pathogenic characteristics investigated recently includes *C. glabrata*, *C. tropicalis* and *C. parapsilosis*. Cell surface hydrophobicity, a virulence factor associated with adherence was observed to be similar in *C. glabrata* and *C. albicans*. Hydrophobicity fluctuates with growth conditions in *C. albicans* whereas *C. glabrata* appears to be stable. This may explain why *C. glabrata* adheres more readily to silicone and acrylic surfaces than *C. albicans* and other non-albicans *Candida* species (Hazen et al., 1986; Luo and Samaranayake, 2002; Silva et al., 2010).

In a recent study, Negri et al (2010b) found that *C. tropicalis* adhered more to the epithelium than to silicone. In addition, all strains produced biofilms and possessed haemolytic activity. Only 2 isolates were protease positive whereas one produced phospholipase (Negri et al., 2010b). Generally, the few studies on the virulence characteristics of non-albicans *Candida* indicate that they are able to produce hydrolytic enzymes though at a much reduced rate compared to *C. albicans* (Silva et al., 2011). Apart from hydrolytic enzyme production, other factors may be responsible for their virulence as virulence and pathogenicity appear to be a complex phenomenon (Cottier and Pavelka, 2012).

At present, responsible for the expression of virulence factors are not fully comprehended. This is owing to the complex and dynamic nature of the interaction between the host and yeasts. The signalling pathways initiated by the host-yeast interaction may either lead to yeast clearance or increase the elaboration of virulence factors. Commensal bacteria may either enhance host immunity or the expression of

virulence factors by yeasts. On their own, yeasts are also capable of undergoing genomic alterations which can make them more virulent (Cottier and Pavelka, 2012).

Studies that evaluated the virulence characteristics often yield different and sometimes conflicting results. This is due to the influence of many factors on the expression of virulence by *Candida*. Research has investigated a variety of subjects including healthy carriers, HIV positive individuals, cancer patients undergoing chemotherapy, denture wearers, those in Intensive Care Units and adults or neonates. In addition, samples have been taken from different sites including the mouth, vagina, rectum, bronchial secretions and blood. In addition, some studies evaluated *C. albicans*, others investigated all the *Candida* species while some restricted their investigation to non-albicans *Candida* species as a group or as individual non-albicans *Candida* species. For practical reasons, studies on the virulence of *Candida* species assessed selected virulence attributes. While some studied biofilm formation, others assessed hydrolytic enzyme production with or without biofilm formation and haemolytic properties of the yeasts (Gokce et al., 2007; Lyon and de Resende, 2007; Mane et al., 2010; Oksuz et al., 2007; Sacristan et al., 2011; Taguti Irie et al., 2006).

In addition to established virulence characteristics of *Candida* species, fresh insights have been gained about other novel virulence capacities, for example, the production of heat shock proteins (Hsps). Yeasts respond to stressful conditions such as oxidative stress, high temperature and starvation by elaborating heat shock proteins that prevent protein unfolding and aggregation that can lead to their death. An example is Hsp90, which is associated with virulence and drug resistance in *C. albicans* (Singh et al., 2009).

This increase in knowledge makes it imperative to study the expression of virulence factors of *Candida* species and the relationship of those factors to clinical infection. Furthermore, the roles of these factors that may be peculiar to yeasts in the environment need further investigation. The present chapter investigated the virulence properties i.e. proteinase and phospholipase production, and adherence to oral epithelial cells of *C. albicans* and non-*C. albicans Candida* isolates obtained from HIV positive women. Patients with oral candidiasis and asymptomatic carriers were followed up for a period of six months. In addition, relationship between virulence factors, *Candida* counts and CD4 counts were investigated.

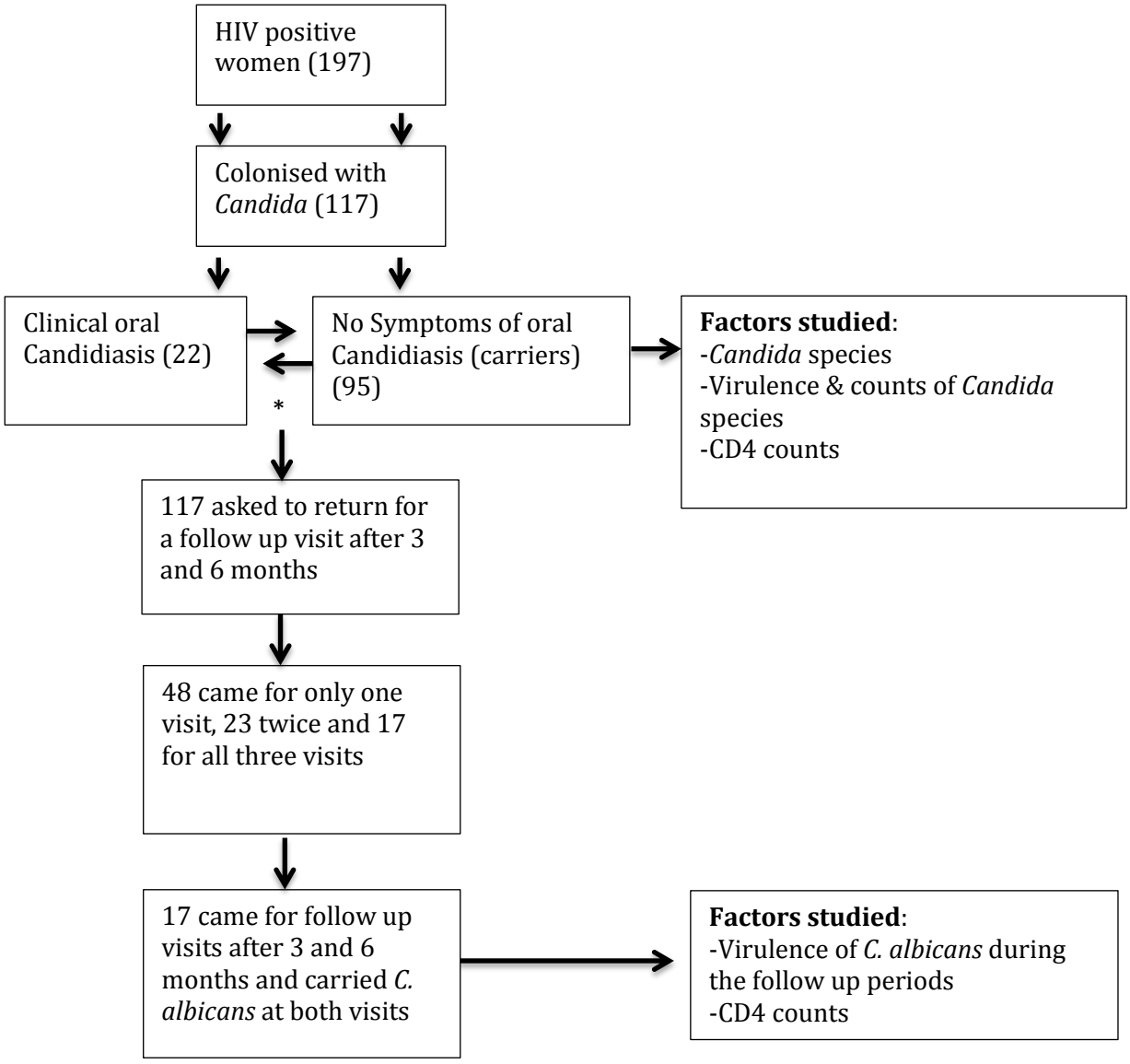
4.2 Methods and materials

4.2.1 Sample collection

The study was undertaken at the HIV clinic at the Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa. The subjects were 197 HIV-positive women who agreed to participate (also described in Chapter 3, section 3.2.1) after signing a written consent form (Figure 4.1). Ethical clearance was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg. Data on HIV treatment, previous history of oral candidiasis and the most recent CD4 counts within the past 3 months were obtained from the clinical records of the patients. Trained oral healthcare personnel screened patients for the presence of oral candidiasis. If present the lesions were categorized using the location and the clinical type as described (Samaranayake et al., 2009). Oral rinse samples were collected from 117 patients who were colonised with *Candida species* (see chapter 3). Thereafter, they were asked to return after 3 and 6 months and further samples were obtained and processed.

The samples were transferred to the laboratory in sputum jars and 100 µl of rinse sample were inoculated onto CHROMagar *Candida* plates (CHROMagar Paris, France) and incubated at 37 °C for 48 hours. When growth was observed, colonies were counted and subcultured on Sabouraud dextrose agar (Oxoid, Hampshire, England) for 48 hours.

Figure 4.1 Study population and design



*: Groups compared

4.2.2 Yeast identification

Cultures were identified using the API 20 C AUX system ® (bioMérieux). Yeast cells were inoculated into 2 mls of distilled water and concentrated to 2 McFarland of turbidity. 100 µl of the suspension was transferred to the C medium supplied with the API 20 C AUX and gently homogenised with the pipette to avoid the formation of bubbles. The homogenised mix was then transferred to the cupules in the test strip. Care was taken not to overfill or underfill the cupules. The test strips were then placed in a tray of appropriate size that had been lined with a moist paper towel. The tray was covered and incubated at 30°C for 48 hours. The yeast identification was carried out based on the presence of turbidity in the cupules compared with the first control cupule as described by the manufacturers. A seven-digit numerical profile based on the positive reactions was then uploaded to the supplier's website for definitive yeast identification.

4.2.3 Adherence assay

An adherence assay was performed using a technique previously described (Ghannoum et al., 1986) with modifications. *Candida* isolates from the subjects were cultured in Sabouraud dextrose broth (Oxoid, Hampshire, England) and incubated at 37°C for 2 hours while shaking at 60rpm. Yeast cells were harvested by centrifugation, washed three times with sterile distilled water and resuspended into 2 mls sterile distilled water and adjusted to obtain 10^7 cells/ml. Buccal epithelial cells (BEC) were harvested from healthy non-*Candida* carriers using sterile cotton buds by rubbing the inner lining of the cheeks firmly thereafter rinsing with 4mls of sterile distilled water in blue capped tubes until it was sufficiently turbid. The BEC were washed twice and reconstituted in 2 mls sterile distilled water.

The BEC and the *Candida* cells were then mixed and incubated for 3 hours at 37°C while shaking. The mixture was then filtered through a 20µm nylon filter to remove non-adherent *Candida* cells. The epithelial cells that remained on the filter were harvested, washed twice with distilled water and suspended in distilled water. A drop of these cells was mounted on a glass slide, air dried, heat fixed and stained with crystal violet for 1 minute. The number of adherent yeast cells per 100 BEC was counted and reported as the adherence assay.

4.2.4 Proteinase assay

The method described by Aoki and colleagues for assessing proteinase production was used with modifications (Aoki et al., 1990). The method employs bovine serum albumin agar plates. *Candida* strains were cultured on fresh Sabouraud dextrose agar (Oxoid, Hampshire, England) for 48 hours. An inoculum of yeast cells was suspended in sterile distilled water and ten microlitres of *Candida* culture suspension with a negative control were then placed on paper discs which had been adequately spaced on the bovine serum albumin agar plates. *C. albicans* ATCC 90028 was used as positive control. The inoculated plates were incubated at 37° C for 14 days. After incubation, the colony diameters were measured before staining with Coomassie brilliant blue. The plates were flooded with the staining solution and incubated for 20 minutes. The staining solution was discarded and a destaining solution was applied for the same period of time. Finally the plates were rinsed with water and allowed to dry. The clear zones were measured and the proteinase activity (Pr) expressed as the ratio of the colony diameter and the clear zone.

4.2.5 Phospholipase assay

The phospholipase assay was undertaken using the egg yolk technique (Ghannoum, 2000; Samaranayake et al., 2005). *Candida* strains were cultured on fresh Sabouraud dextrose agar (Oxoid, Hampshire, England) for 48 hours. An inoculum of yeast cells was made in sterile distilled water and ten microlitres of the culture suspension and a negative control with no yeast cells were suspended on paper discs that were placed adequately apart on the egg yolk medium. *C. albicans* ATCC 90028 was used as positive control. The plates were incubated for 4 days at 37°C and the colony diameter and precipitation zone around the discs were measured. Phospholipase activity (designated as Pz) was determined using the ratio of the diameter of the colony to the diameter of the colony plus the surrounding precipitation zone.

4.2.6 Statistical analysis

Variables that were analysed using proportions, mean, median and appropriate measures of variability included *Candida* counts, virulence factors and CD4 counts. They were significantly skewed and therefore compared in patients with and without oral candidiasis using non parametric tests (Mann-Whitney two-sample statistic). A non-parametric correlation (Spearman correlation) was used to explore the relationship between the virulence factors and the CD4 count. Statistical significance was inferred at $p < 0.05$

4.3 Results

4.3.1 Demography and oral candidiasis

One hundred and ninety seven women were screened with a mean age of 38.3±8.4 years, 117 (59.4%) were colonised with *Candida* species. At the first visit, oral

candidiasis was present in 15 (12.8%) of those colonised, 10 (66.7%) were cases of pseudomembranous candidiasis while erythematous type and angular cheilitis accounted for 4 cases (26.7%) and one case (6.7%) respectively. At the second visit, the 3 who were previously infected still had persistent infection whereas one had persistent infection at the third visit. The total cases of oral candidiasis were 19 for all visits.

4.3.2 Proteinase and phospholipase production and adherence to oral epithelial cells assay

One hundred and ninety seven HIV positive women were screened for the *Candida* carriage. Only 117 women were colonised with *Candida* species in their oral cavity at their first visit. Fifty four of these women were asked to come back for follow up visits after 3 and 6 months. Forty eight women came only once and 23 twice during the 6 months study period. Only 17 patients came for all three visits and carried *C. albicans* in their oral cavity at each visit (Figure 4.1). If the remaining women came more than once, they carried non albicans *Candida*. The results of the virulence factors produced by these *C. albicans* isolates are shown in Table 4.1.

Table 4.1 Production of proteinase, phospholipase and adherence to oral epithelial cells by *C. albicans* isolated from HIV positive women over a six month period

	Adherence (Adherent *Candida* count/ 100 epithelia cells)			**Proteinase (Pr) (Colony diameter/ precipitation diameter)**			**Phospholipase (Pz) (Colony diameter/ precipitation diameter)**		
Study ID	**0 Baseline**	**3 months**	**6 months**	**0 Baseline**	**3 months**	**6 months**	**0 Baseline**	**3 months**	**6 months**
2	1032	1958		.605	.812		.59	1	
3	1221	1502	1664	.6577	.902	.99	.481	.988	1
5	1796			.564			.58		
6	568	1428	1388	.966	.877	1	.79	.979	1
10	822	1588	1532	.924	.934	.94	.83	.903	1
11	918	2184		.769	.978		.976	.998	
12	989	1408	1110	.948	.965	.927	.962	.899	1
16	1480			.802			.61		
24	273	1725		.93	.994		.55	.933	
25	610			.968			.789		
26	1286			.875			.696		
27	770	1922	1000	.944	.946	1	.65	1	.763
28	538			.93			.887		
32	1233	1778		.971	.808		.889	.968	
34	1027			.937			.877		
35	1167	1718	1254	.958	.986	1	.951	.926	1
37	2066	1494		.983	.915		.958	1	
38	2142			.943			.982		
41	1372	1398	1768	.655	.996	.88	.965	.976	1
42	2226	1030	376	.785	.964	1	.99	.99	1
45	1816	1408	1040	.822	.998	1	.994	.913	1
46	1560			.854			.966		
47	1662			.779			.986		
51	2237	1920		.786	.913		.993	.99	
54	2625			.864			.913		
55	1402			.866			.995		
59	1663			.841			.999		
62	956	1840		.784	.894		.935	.943	
64	1554		2584	.798		1	.873		.521
68	1560		2114	.86		.85	.924		.99
71	1666			.755			.983		
74	1996	1352		.611	.899		.892	1	
77	1404			.725			.7		
78	1628	908	2310	.707	.956	.93	.94	.99	1
80	2085			.742			.794		
81	2844	1884		.8	.975		.88	.99	
82	775	1654	570	.671	.837	1	.858	1	1

84	1614		1686	.5		1	.98		1
86	1278			.82			.97		
89	2140			.656			.76		
91	720			.79			.975		
98	2468			.972			.971		
99	1138			.972			.972		
100	2146	560		.953	1		.964	1	
104	2546		692	.809		1	.986		1
105	1702			.917			.933		
107	926			.929			.832		
110	1525		2142	.904		1	.975		1
117	2038	1768		.676	1		.973	1	
119	1403	1222		.785	.977		.976	.99	
120	1360			.782			.991		
121	2508			.989			.941		
125	1522			.625			.972		
132	1920	2309	2135	.988	.748	.849	.988	1	1
134	1430			.99			1		
138	1735			.816			.986		
140	1330	1508	1608	.872	1	.95	.948	1	1
141	1324			.872			.89		
143	2026	1863		.9	1		.94	1	
144	1700	1812		.872	.898		.942	.95	
149	1503			.873			.927		
156	884			.955			.955		
164	1606		1532	.868		.91	.99		1
165	1356	1948		.937	.924		.936	1	
166	1542	1302	974	.843	.941	1	.986	1	.99
167	1430	1470		.833	.892		.975	1	
168	962	2989		.845	1		.989	.853	
169	1622			.956			.718		
170	1584	1750		.842	.895		.905	1	
173	1558	2440	1810	.889	1	1	.972	1	.683
176	1788			.886			.872		
178	1901			.873			.897		
179	1675			.764			.865		
180	2108			.906			.922		
182	1114			.926			.885		
183	1782			.863			.815		
185	1344	2060	1118	.963	.923	.886	.91	1	1
186	1420			.899			.969		
187	1626			.779			.716		
188	1176			.962			.89		
190	1338			.905			.868		
191	1434	2178		.783	.846		.918	1	

193	854	1254	570	.893	.94	1	.952	1	.595
196	1432	1817		.91	1		.766	1	
197	1556			.949			.904		

Lower Pr and Pz values are indicative of higher phospholipase production and 1 indicates no production.
Highlighted patients kept their appointments and carried *C. albicans* at all three visits.
Candida counts and CD4 counts are provided in the raw data in the attached CD-ROM.

Of the 117 subjects who were colonised with *Candida* from our sample of 197 participants, 85 were positive for *C. albicans* and were followed up for 6 months. However, only 17 of those who reported for follow up visits were consistently *C. albicans* positive at the follow up visit. These are highlighted in the Table 4.1.

Table 4.2 Virulence of *C. albicans* during the 6 months follow up period

Virulence Test	**Mean ± SD (n=17)**		
	0 months	**3 months**	**6 months**
Adherence *	1288.35 (460.09)	1578.65 (411.34)	1307.47 (544.24)
Proteinase**	0.852 (0.116)	1 (0)	1 (0)
Phospholipase***	0.622 (0.425)	1 (0)	1 (0)

*: Adherent *C. albicans* count per 100 epithelial cells
** & ***: Ratio of diameter of colony and the diameter of the colony plus the precipitation zone expressed (1 = no activity and < 1 = enzyme production).

The average virulence characteristics for all the *C. albicans* positive subjects who were available for follow up for six months (highlighted in Table 4.1) indicates that the average values fluctuated from visit to visit. Adherence was 1288.35 ±460.09 at baseline visit, increased to 1578.65 (±411.34) after 3 months and dropped to 1307.47

(±544.24) at 6 months. The mean proteinase level was 0.852 (±0.116) at the baseline visit whereas no enzymes were produced at 3 and 6 months. Similarly, the result for phospholipase was 0.622 (±0.425) at baseline but showed no enzymatic activity after 3 months and 6 months (Table 4.2).

4.3.3 Relationship between CD4 counts and virulence factors of *C. albicans* isolated from HIV positive women over 6 months.

The results of CD4 counts of the HIV positive women and the virulence factors are shown in Table 4.3. The CD4 count for the 85 subjects who were *C. albicans* positive and for the 17 subjects who were available for the follow up visit and carried *C. albicans* for the period are highlighted. A non parametric correlation of the virulence factors was undertaken using Spearman's correlation (Table 4.4). A negative correlation between CD4 count and adherence assay was observed (-0.0519) indicating that adherence increased with declining CD4 count. However, this correlation did not reach statistical significance. There was a significant positive correlation between proteinase and phospolipase assays (Spearman's rho 0.2140, $p<0.05$). This indicates there is a strong tendency its assay was elevated when the other was also high.

Table 4.3 CD4 counts and virulence factors by *C. albicans* isolated from HIV positive women over 6 months**.**

	CD4 Count (cells/mm3)			Adherence (Adherent Candida count/ 100 epithelia cells)			Proteinase (Pr) (Colony diameter/ precipitation diameter)			Phospholipase (Pz) (Colony diameter/ precipitation diameter)		
StudyID	0 Baseline	3 months	6 months	0 Baseline	3 months	6 months	0 Baseline	3 months	6 months	0 Baseline	3 months	6 months
2	546	490		1032	1958		.605	.812		.59	1	
3	347		701	1221	1502	1664	.6577	.902	.99	.481	.988	1
5	250			1796			.564			.58		
6	196			568	1428	1388	.966	.877	1	.79	.979	1
10	418		533	822	1588	1532	.924	.934	.94	.83	.903	1
11	567			918	2184		.769	.978		.976	.998	
12	571	378		989	1408	1110	.948	.965	.927	.962	.899	1
16	104			1480			.802			.61		
24	1200			273	1725		.93	.994		.55	.933	
25	595			610			.968			.789		
26	398			1286			.875			.696		
27	370	490	380	770	1922	1000	.944	.946	1	.65	1	.763
28	393			538			.93			.887		
32	156	561		1233	1778		.971	.808		.889	.968	
34	27			1027			.937			.877		
35	821			1167	1718	1254	.958	.986	1	.951	.926	1
37	626	496		2066	1494		.983	.915		.958	1	
38	246			2142			.943			.982		
41	1023	1200	821	1372	1398	1768	.655	.996	.88	.965	.976	1
42	462		350	2226	1030	376	.785	.964	1	.99	.99	1

45	35	35	29	1816	1408	1040	.822	.998	1	.994	.913	1
46	166			1560			.854			.966		
47	194			1662			.779			.986		
51	555	769		2237	1920		.786	.913		.993	.99	
54	86			2625			.864			.913		
55	302			1402			.866			.995		
59	19			1663			.841			.999		
62	202	389		956	1840		.784	.894		.935	.943	
64	58		503	1554		2584	.798		1	.873		.521
68	640			1560		2114	.86		.85	.924		.99
71	505			1666			.755			.983		
74	699	510		1996	1352		.611	.899		.892	1	
77	564			1404			.725			.7		
78	307			1628	908	2310	.707	.956	.93	.94	.99	1
80	321			2085			.742			.794		
81	295	580		2844	1884		.8	.975		.88	.99	
82	34		312	775	1654	570	.671	.837	1	.858	1	1
84	701		682	1614		1686	.5		1	.98		1
86	301			1278			.82			.97		
89	108			2140			.656			.76		
91	273			720			.79			.975		
98	167			2468			.972			.971		
99	242			1138			.972			.972		
100	400	296		2146	560		.953	1		.964	1	
104	485		456	2546		692	.809		1	.986		1
105	400			1702			.917			.933		

107	260			926			.929			.832		
110	280		319	1525		2142	.904		1	.975		1
117	410	412		2038	1768		.676	1		.973	1	
119	187			1403	1222		.785	.977		.976	.99	
120	355			1360			.782			.991		
121	228			2508			.989			.941		
125	225			1522			.625			.972		
132	78	63		1920	2309	2135	.988	.748	.849	.988	1	1
134	748			1430			.99			1		
138	19			1735			.816			.986		
140	584	467	480	1330	1508	1608	.872	1	.95	.948	1	1
141	146			1324			.872			.89		
143	109	154		2026	1863		.9	1		.94	1	
144	581	485		1700	1812		.872	.898		.942	.95	
149	388			1503			.873			.927		
156	398			884			.955			.955		
164	464		466	1606		1532	.868		.91	.99		1
165	163			1356	1948		.937	.924		.936	1	
166	350	372		1542	1302	974	.843	.941	1	.986	1	.99
167	588	544		1430	1470		.833	.892		.975	1	
168	926	885		962	2989		.845	1		.989	.853	
169	236			1622			.956			.718		
170	400	583		1584	1750		.842	.895		.905	1	
173	469	348		1558	2440	1810	.889	1	1	.972	1	.683
176	377			1788			.886			.872		
178	396			1901			.873			.897		

179	241			1675			.764			.865		
180	193			2108			.906			.922		
182	270			1114			.926			.885		
183	141			1782			.863			.815		
185	9	69		1344	2060	1118	.963	.923	.886	.91	1	1
186	770			1420			.899			.969		
187	300			1626			.779			.716		
188	190			1176			.962			.89		
190	253			1338			.905			.868		
191	363	387		1434	2178		.783	.846		.918	1	
193	600	527	793	854	1254	570	.893	.94	1	.952	1	.595
196	380			1432	1817		.91	1		.766	1	
197	107			1556			.949			.904		

Where lower Pr and Pz values are indicative of higher phospholipase production and 1 represents no production.

Table 4.4 Correlation of CD4 count with virulence factors of *C. albicans* in HIV positive women over six months (n=85)

Correlation Factor	CD4 count	Adherence	Proteinase	Phospholipase
CD4 count	1.0000			
Adherence	-0.0519	1.0000		
Proteinase	0.0615	-0.1427	1.0000	
Phospholipase	0.1645	0.1429	0.2140*	1.0000

Spearman's rho in boxes *p<0.05

4.3.4 Production of proteinase, phospholipase and adherence to oral epithelial cells by non-*albicans Candida*

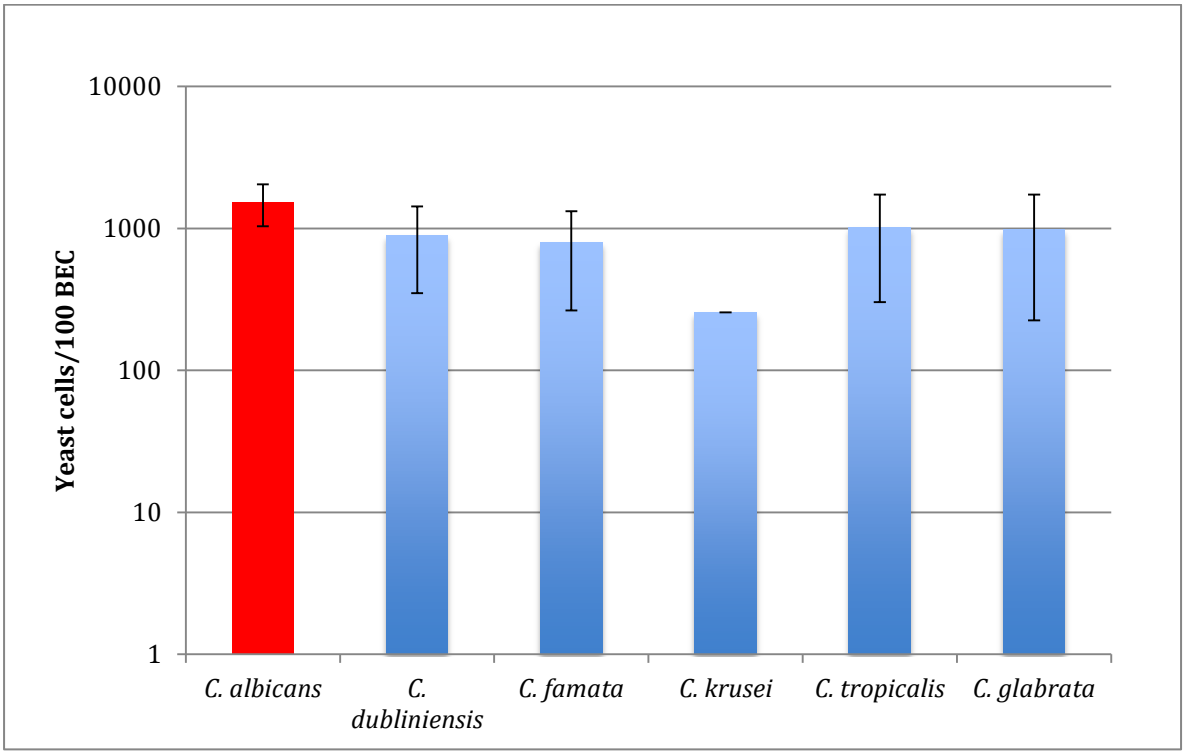
The results of the production of proteinase, phospholipase and adherence to oral epithelial cells by non-*albicans Candida* are shown in Table 4.5 and summarised in Figure 4.2 and 4.3.

Table 4.5 Production of proteinase, phospholipase and adherence to oral epithelial cells by non-*albicans Candida* isolated from HIV positive women

		Adherence			Proteinase			Phospholipase		
Study ID	**Yeast**	**Baseline**	**3 months**	**6 months**	**Baseline**	**3 months**	**6 months**	**Baseline**	**3 months**	**6 months**
1	*C. famata*	1498			**1**			**0.82**		
5	*C. dubliniensis*		1404			0.96			1	
7	*C. dubliniensis*	1190			1			0.94		
14	*C. dubliniensis*	1172			0.99			0.61		
30	*C. dubliniensis*	800			0.864			0.942		
32	*C. dubliniensis*			972			0.88			0.85
36	*C. tropicalis*	582			0.945			0.5		
46	*C. neoformans*									
58	*C. tropicalis*	1828			0.98			0.996		
61	*C. tropicalis*	2444			0.98			0.55		
64	*C. tropicalis*		1280			1			0.61	
69	*C. tropicalis*	810			0.98			0.45		
77	*C. famata*		72			0.88			0.79	
84	*C. pelliculosa*									
87	*C. famata*	1292			0.95			0.51		
90	*C. dubliniensis*	938			0.98			1		
99	*C. dubliniensis*		1042			1			0.76	
101	*C. dubliniensis*	216			0.99			0.93		
108	*C. dubliniensis*	310			0.99			1		
109	*C. dubliniensis*	1188			1			0.5		
110	*C. tropicalis*		860			0.95			0.75	
115	*C. tropicalis*	204			0.99			0.46		
116	*C. krusei*	256			1			0.99		
124	*C. dubliniensis*	302			0.97			1		
126	*C. tropicalis*	556			0.97			0.42		
127	*C. dubliniensis*	564			0.96			0.91		
128	*C. dubliniensis*	1162			1			1		
129	*C. famata*	922			0.9			0.86		
130	*C. famata*	740			0.97			0.51		
133	*C. dubliniensis*	668			1			1		
136	*C. parapsilosis*									
141	*C. dubliniensis*		88			1			0.77	

146	*C. famata*	1148			1			0.7		
155	*C. famata*	70			1			0.75		
157	*C. glabrata*	1514			1			1		
158	*C. glabrata*	446			0.91			1		
162	*S. cerevisiae*									
164	*C. dubliniensis*		1570			0.98			0.89	
170	*C. famata*			620			0.99			0.57
175	*C. dubliniensis*	1378			0.98			0.97		
178	*C. dubliniensis*		270			0.96			0.59	
181	*C. dubliniensis*	408			0.91			0.64		
198	*C. dubliniensis*	2174			0.93			0.52		
199	*C. tropicalis*	582			0.96			0.52		

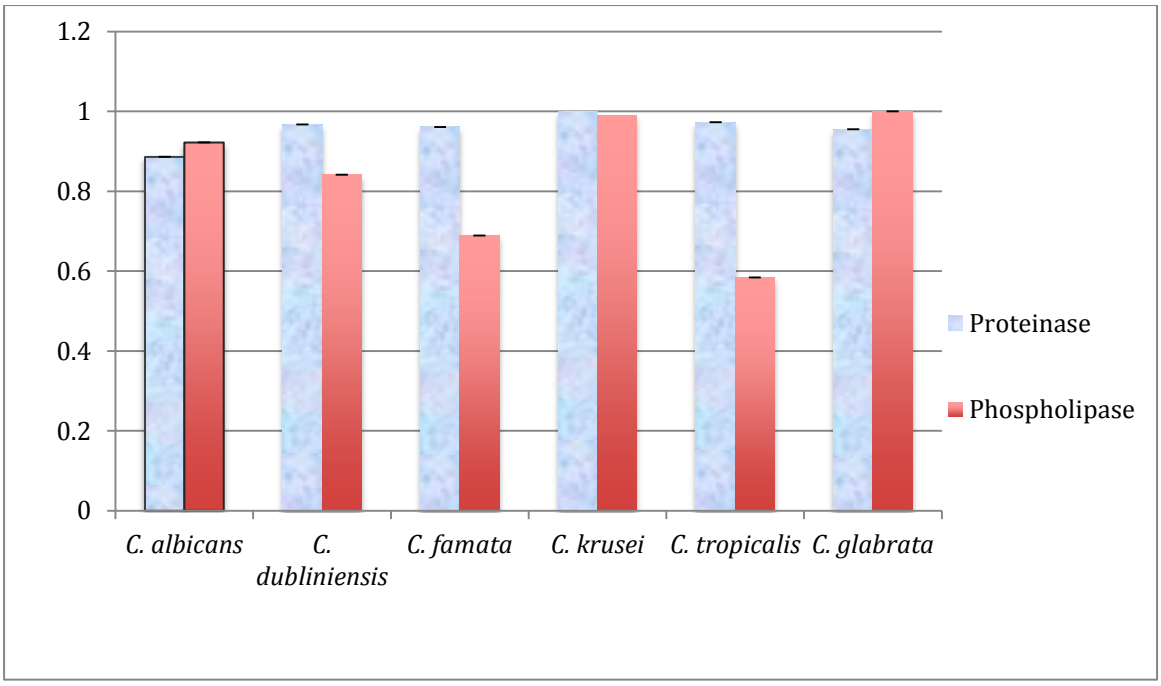
Figure 4.2 Adherence to oral epithelial cells by *C. albicans* and non-*albicans* *Candida* isolated from HIV positive women



None of the non-albicans *Candida* showed as much adherence as *C. albicans* ie mean of 1540 yeast cells/100 BEC) as indicated in Figure 4.2 as a reference (red bar). Non-albicans *Candida* species that showed the most adherence was *C. tropicalis* with 1016

yeast cells/100 BEC. It was followed by *C. glabrata* with 980 yeast cells/100 BEC and *C. dubliniensis* with 890 yeast cells/100 BEC. *C. famata* and *C. krusei* produced the least adherence with 795 and 256 yeast cells/100 BEC respectively.

Figure 4.3 Production of proteinase, phospholipase by *C. albicans* and non-*albicans Candida* isolated from HIV positive women **(**Virulence of 1 indicates no enzyme production and < 1 indicates enzyme production)



None of the non-albicans *Candida* species produced as much proteinase as *C. albicans* with an average of 0.886 (Lower number indicates greater production, 1 represents no production). Of the non-albicans *Candida* species, *C. glabrata* had the highest proteinase production with an average of 0.955. This was followed by *C. famata* (0.961) and *C. dubliniensis* (0.967) and *C. tropicalis* (0.973) respectively. *C. krusei* hardly produced any enzyme and had an average of 1. Phospholipase production was highest with *C. tropicalis* (0.584) and this was much higher than the

average phospholipase production by *C. albicans* (0.922). *C. famata* and *C. dubliniensis* followed with 0.689 and 0.841 respectively. *C. glabrata* and *C. krusei* were not producing the enzyme with 1 and 0.99 respectively.

4.3.5 Comparison of production of proteinase, phospholipase and adherence to oral epithelial cells by *C. albicans* and non-*albicans Candida*

When the production of virulence factors of *C. albicans* and non-albicans *Candida* was compared (Table 4.6), *C. albicans* showed a much higher adherence rate 1540.6±42.3 compared to the non albicans strains with 888.5±90.5. This was highly significant at $p<0.001$. Likewise, the *C. albicans* yeasts showed greater proteinase activity with an average of 0.89±0.009 compared to 0.97±0.005 in the non-albicans *Candida*. This was also significant at $p<0.001$. Conversely, the non-albicans yeasts showed more phospholipase activity with an average of 0.76±0.032 compared to 0.92±0.009 in *C. albicans*. This was also significant at $p<0.001$. (Table 4.6)

Table 4.6 Comparison of average virulence factors of *C. albicans* and non-albicans *Candida*

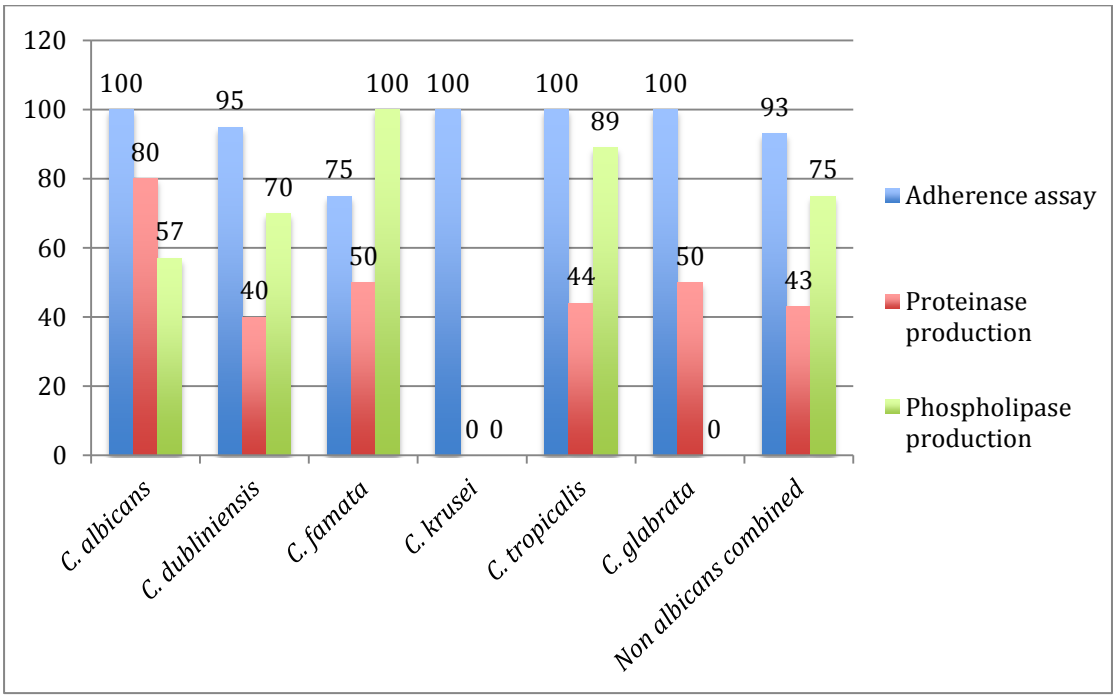
Virulence Factor	***C. albicans* (N=145)**	**Non-albicans *Candida* (N=40)**	**p value**
Adherence (mean±SD)	1540.6±42.3	888.5±90.5	<0.001*
Proteinase (mean±SD)	0.89±0.009	0.97±0.005	<0.001*
Phospholipase (mean±SD)	0.92±0.009	0.76±0.032	<0.001*

Table 4.7 Comparison of the proportion of *C. albicans* and non-albicans *Candida* producing virulence factors

Species	Adherence assay No. of isolates (%)	Proteinase production No. of isolates (%)	Phospholipase production No. of isolates (%)
C. albicans	145/145 (100%)	116/145(80%)	83/145(57%)
C. dubliniensis	19/20(95%)	8/20(40%)	14/20(70%)
C. famata	6/8(75%)	4/8(50%)	8/8(100%)
C. krusei	1/1(100%)	0/1(0%)	0/1(0%)
C. tropicalis	9/9(100%)	4/9(44%)	8/9(89%)
C. glabrata	2/2(100%)	1/2(50%)	0/2(0%)
Non- albicans combined	37/40(92.5%)	17/40(42.5%)	30/40(75%)

The average values of the virulence factors are given in Table 4.6; this is based on the data generated by the *Candida* producing virulence factors. Not all the *Candida* produce the virulence factors, Table 4.7 and Figure 4.3 shows the percentage of yeasts producing the virulence factors. All the *C. albicans* strains adhered to BEC, 80% produced proteinase and 57% phospholipase. Adherence of the non-albicans *Candida* was lower i.e. with 92.5%, proteinase production 42.5% and phospholipase production 75%. The percentage of different *Candida* species producing virulence factors is displayed graphically for easier comprehension (Figure 4.4).

Figure 4.4 Proportion of *Candida species* producing virulence factors



4.3.6 Virulence factors and *Candida* count in patients with and without oral candidiasis

Twenty two of the 197 women were clinically diagnosed with oral candidiasis at the first visit (11.2% prevalence). No yeasts were cultutered from three on further laboratory investigations and were therefore not included in the statistiscal analysis. This explains why there were only 19 with oral candidiasis (Tables 4.9 and 4.10). However, not all the patients with candidiasis at the initial visit returned for follow up visits. A comparison of the virulence factors of the isolates showed there was no significant difference between patients with clinical infection and their counterparts with no infection but may have been colonised (Table 4.8). However, much higher yeast counts were related to clinical infection. The median yeast count in patients with oral candidasis was 280 cfu/ml compared to 70 cfu/ml in patients without candidiasis. This was significant at p=0.003. The median count was used for comparison because the variable was not normally distributed.

Table 4.8 Relationship of virulence factors and yeast count to oral candidiasis

Factor (median value)	**Infection Present**	**Infection Absent**	**p value**
Adherence	1408	1431	0.4130
Proteinase	.944	.93	0.9476
Phospholipase	.952	.965	0.4319
Candida count	280	70	0.003*

*All the patients carried yeasts at all the visits

4.3.7 Relationship of *Candida* species to oral candidiasis

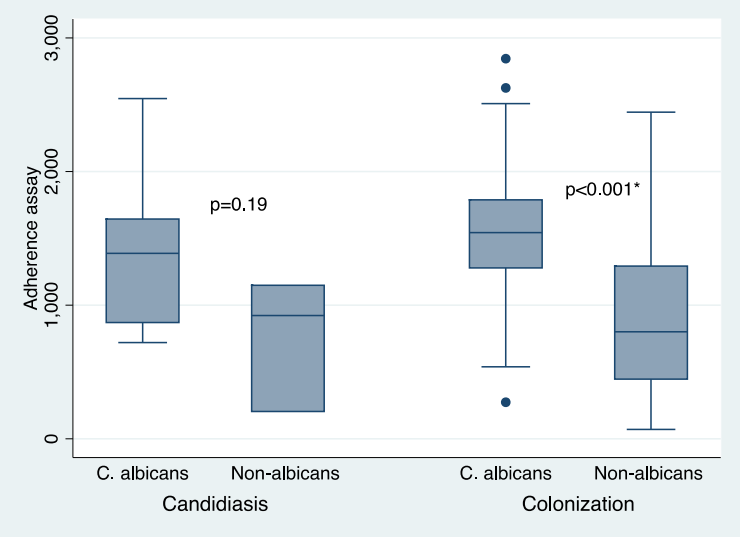
When the presence of *C. albicans* and non-*C. albicans* in the symptomatic and asymptomatic state was compared (Table 4.9), the results showed that the majority of the infection (78.95%) was caused by *C. albicans*. The non-albicans *Candida* only accounted for 21.05%. However, this difference was not statistically significant.

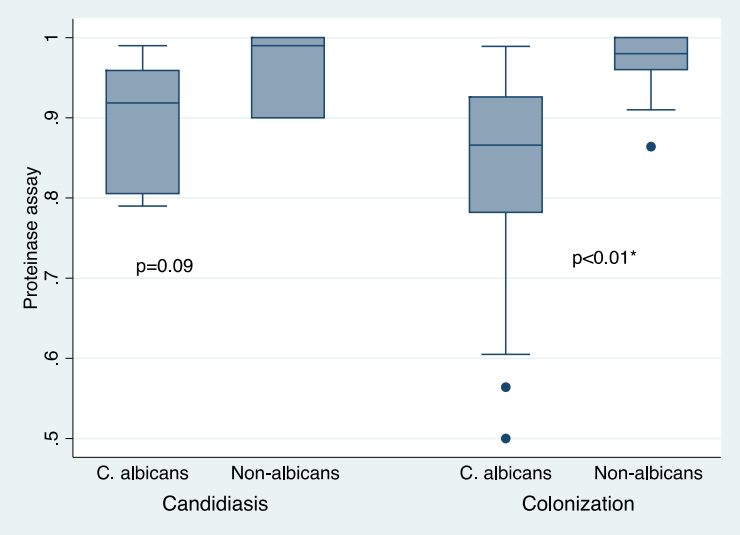
Table 4.9 Relationship of *Candida* species to oral candidiasis (*Candida* positively identified, all visits)

Type of *Candida*	**Candidiasis present N(%)**	**Candidiasis absent N(%)**	**Total (%)**
C. albicans	15 (78.95)	130 (69.52)	145 (70.39)
Non-*C. albicans Candida*	4 (21.05)	57 (30.48)	61 (29.61)
Total	19 (100)	187 (100)	206 (100)

Fishers exact =0.598, percentages reflected are column percentages

Figure 4.5 Comparison of virulence characteristics of *Candida* species with non-albicans *Candida* in those with and without oral candidiasis (colonisation)





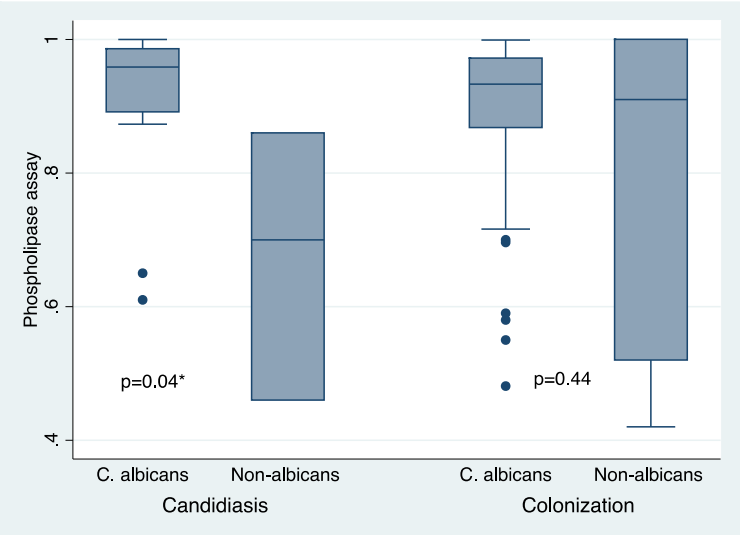


Table 4.10 Comparison of virulence attributes of *Candida species* isolated from HIV positive women with and without candidiasis

Subjects	**Isolates (n)**	**Mean ± SD**					
		Adherence		**Proteinase**		**Phospholipase**	
		Yeast no./100BEC	p	Pr	p	Pz	p
All	*C. albicans* (145)	1540.6±42.3	<0.01*	0.89±0.01	<0.01*	0.92±0.01	<0.01*
	Non-albicans (40)	888.5±90.5		0.97±0.01		0.76±0.03	
With Oral candidiasis	*C. albicans* (15)	1407.6±509.8	0.19	0.899±0.07	0.09	0.9164±0.12	0.02*
	Non-albicans (4)	961±571.7		0.968±0.04		0.7275±0.19	
No oral candidiasis	*C. albicans* (130)	1555.9±509.4	<0.01*	0.885±0.10	<0.01*	0.923±0.11	0.44
	Non-albicans (36)	880.4±580.3		0.967±0.04		0.7685±0.21	

Adherence: No. of Yeast cells/100 epithelial cells.
Pr: ratio of the colony diameter and the clear zone.
Pz: ratio of the colony diameter and the precipitation zone.
*Significant

The virulence of *C. albicans* and non-albicans *Candida* isolated from patients with oral candidiasis and without (colonised) is shown in Figure 4.5 and Table 4.10. The virulence properties of *C. albicans* were consistently higher than the non-albicans group irrespective of the development of clinical infection. In the group without oral candidiasis, the mean adherence of *C. albicans* was 1555.9±509.4 compared with 880.4±580.3 for non-albicans *Candida*, $p<0.01$. In the group with oral candidiasis who carried *C. albicans*, adherence was also higher with a mean of 1407.6±509.8 compared to 961±571.7 for non-albicans *Candida*. This difference did not attain statistical significance, $p=0.19$. A similar trend was observed with proteinase assay, enzyme production was higher with *C. albicans* in patients with or without oral

candidiasis. In patients who did not develop oral candidiasis, *C. albicans* produced more enzyme than the non-albicans *Candida*. However, this was not statistically significant (mean of 0.899±0.07 in *C. albicans* compared with 0.968±0.04 in the non-albicans group, p=0.09).

The trend was opposite in patients with oral candidiasis. Phospholipase production was much higher in the non-albicans group with a mean of 0.7275±0.19 compared to 0.9164±0.12 and this difference was significant at p=0.02.

4.4 Discussion

4.4.1 Virulence of *C. albicans* in HIV infection

Opportunistic oral candidiasis was prominent as an indicator of the presence and progression of HIV infection from the onset. Oral candidiasis was adduced to compromise in host immunity present in HIV infection. The immune compromise is mainly in the depletion Th17 subset of the CD4 cells, a situation that allows *Candida* organisms to cause mucosal disease (Cassone and Cauda, 2012). At a point, it was proposed that oral candidiasis was initiated by more virulent strains replacing the commensal strains rather than just the deficient immunity. This assertion prompted investigations into the virulent characteristics of *Candida* species in HIV infection (Sweet et al., 1995). Research thus confirmed greater adherence to buccal epithelial cells in HIV infected subjects compared to HIV negative controls (Sweet et al., 1995).

In our study, all the isolates of *C. albicans* had adherence, 80% produced proteinase and 57% produced phospholipase (Table 4.7) thus indicating that the isolates were capable of initiating infection. Furthermore, *Candida* species obtained from HIV positive subjects with TB infection had more virulence attributes (phospholipase

enzyme production) than HIV and TB negative controls (Ramesh et al., 2011). In addition, *C. albicans* strains from HIV infected patients were more adherent, and produced more haemolysins, proteinases and phospholipases than the HIV negative controls (Mane et al., 2012). Conversely, yeasts from HIV positive subjects did not differ in biofilm forming ability (a virulence factor) compared with HIV negative controls. This is in spite of increased yeast carriage in the HIV positive group. This was attributed to the presence of other virulence determinants (Jin et al., 2003). Unquestionably, there is evidence is that *Candida* organisms are more able to express their virulence characteristics in an immune depleted host such as in HIV infection. The principal mechanisms are the increased expression of SAPs and growth of the *Candida* hyphae (Heilmann et al., 2011). In addition, the HIV virus interacts with *Candida* organisms whereby the envelope proteins of HIV such as GP160 and Tat are able to induce SAP production by *Candida* (Gruber et al., 2001).

4.4.1.1 Adherence assay

Our findings confirm the presence of virulence attributes in the *Candida* species isolated from HIV positive women in Tables 4.1 and 4.2. The average value of the adherence assay in this study is larger than other studies. We found an average of 1288.35 (±460.09) yeast cells/BEC compared with the maximum range of 989 in a similar study in HIV positive subjects (Mane et al., 2011). This difference may be due to differences in strain variation because the expression of virulence factors are strain specific (Ghannoum, 2000). Adherence assays are also sensitive to differences in technique and buccal epithelial cells vary in their affinity for yeast cells (Sandin et al., 1987). Apart from the average adherence values, the proportion of the yeasts expressing particular virulence characteristics also indicates the degree of virulence.

All the *C. albicans* yeasts and 92.5% of the non-albicans *Candida* in our study showed adherence ability (Table 4.9). This is similar to the finding of Mane and co-workers who showed that 95% of isolates were able to adhere (Mane et al., 2011). The high level of adherence in our study and others confirm the ability of *Candida* to adhere to the epithelial cells in HIV infection. This is usually a precursor to invasion and subsequent infection.

4.4.1.2 Proteinase production

The average proteinase value of 0.89 (±0.009) for *C. albicans* (Table 4.7) is slightly higher (less proteinase production) than 0.765(±0.107) reported by Mane and colleagues. A larger proportion of *C. albicans* in their study (89.7%) were proteinase positive compared to 80% in this study (Table 4.7). Their *C. albicans* strains showed more proteinase activity probably because they took samples from HIV subjects who had active oral *Candida* infection whereas not many of our subjects had oral candidiasis. Furthermore in another study, in HIV positive subjects with cancer, 83.6% of all *Candida* species and 94.1% of *C. albicans* were proteinase positive (Kumar et al., 2006). Although proteinase activity is associated with virulence, not all SAPs are needed for invasion of the epithelium, and only SAP5 gene expression may aid invasion (Lermann and Morschhauser, 2008). In our study, proteinases were not differentiated and the detection is often technique sensitive.

4.4.1.3 Phospholipase production

Phospholipase production was relatively low in our subjects. The average of 0.92 (±0.009) in *C. albicans* was much higher (indicating low enzyme activity) than what was obtained in similar studies. For instance, Ramesh and colleagues found an

average of 0.48 (±0.007) and Mane and co-workers reported 0.710 (±0.109). This may be due to the differences in the sample population as they studied individuals who were more immunocompromised (Mane et al., 2011; Ramesh et al., 2011). In terms of the proportion of yeasts showing phospholipase activity, our samples were only 51% positive for *C. albicans* (Table 4.7). Other studies have varied from as high as 100% (Kumar et al., 2006) to 73.3% (Tay et al., 2011), 60% (Gokce et al., 2007) and 59% (Mane et al., 2011). A significant observation in the present study is that phospholipase production was higher in non-albicans *Candida* compared to *C. albicans* isolates.

4.4.1.4 CD4 count and virulence

A low CD4 count along with other risk factors predict the development of oropharyngeal candidiasis (Chattopadhyay et al., 2004). We monitored the CD4 changes in our subjects during the follow up period to see if there was any correlation with the virulence factors (Table 4.4). There was a weak negative correlation between adherence and CD4 count. The proteinase and phospholipase levels were positively correlated with the CD4 count; however, these did not attain statistical significance. While a low CD4 count has been conventionally associated with the development of clinical *Candida* infection, the relationship with virulence factors is less well defined. In a study on the adherence properties of *C. albicans* in HIV positive children with HIV negative controls, there was no significant correlation with the CD4 count and the viral load. This is in spite of a greater adherence of *C. albicans* isolated from the HIV positive children (Portela et al., 2010). Of note is the statistically significant correlation of 0.2140 between proteinase and phospholipase assays. A similar correlation between virulence factors has been observed in other surveys. Mane and

colleagues reported a significant correlation between adherence and proteinase assay (p=0.01) and between proteinase and phopspolipase (p=0.002). They stated the *p* values but did not specify the correlation coefficients (Mane et al., 2011). Correlation of virulence factors indicate the variety of pathogenic abilities of *Candida* yeasts.

Unlike other studies that were purely cross sectional, our subjects were followed up for 6 months and the trend in virulence characteristics over time was captured. The virulence characteristics varied over time and many factors may be responsible. Yeast strains may be different at follow up visits; which may be due to exogenous strains or mutations of endogenous strains. The host's potent innate and adaptive immune responses that influence the virulence of this organism may also change with time. This fluctuation did not result in clinical infection in the follow up period. Scientific debate on the establishment of oral candidiasis has revolved around the balance of the pathogenic ability of the yeast versus the host immune capabilities. The factors necessary for the establishment of oral candidiasis in our study population appears to go beyond the virulence factors that we studied.

4.4.2 Comparison of virulence and clinical infection in *C. albicans* and non-albicans *Candida*

The non-albicans *Candida* in our subjects had virulence characteristics i.e. adherence, proteinase and phospholipase production (Tables 4.5, 4.6 and Table 4.7). Non-albicans species express virulence factors with varying ability according to the species. In their study, Silva and colleagues demonstrated biofilm formation in non-albicans species, *C. tropicalis* and *C. parapsilosis* showed a greater capacity than *C.*

glabrata (Silva et al., 2011). Similarly, *C. glabrata* exhibited a higher capacity for adherence than other non-albicans *Candida* yeasts (Negri et al., 2010a).

Compared with *C. albicans*, there were significant differences in the virulence characteristics of the non-albicans *Candida*. In many studies, *C. albicans* demonstrated greater virulence characteristics compared with non-albicans *Candida.* The increased adherence of *C. albicans* (Table 4.7, Figure 4.2 and Figure 4.4) is similar to Irie and colleagues' observation where *C. albicans* from vaginal specimens showed greater adherence than non-albicans *Candida* (Taguti Irie et al., 2006). In the same study, *C. albicans* from blood culture samples showed greater virulence properties compared with non-albicans *Candida* yeasts. Proteinase production was present in 89.7% and 25.8% respectively. Furthermore, a similar study reported phospholipase production of 60% in *C. albicans* and 0% in non-albicans *Candida* yeasts (Gokce et al., 2007). In a study in blood culture samples, proteinase production by *C. albicans* species was 93.7% i.e. higher than 88.5% in non-albicans *Candida* yeasts. In addition, phospholipase was 73.3% in *C. albicans* compared to 7.7% in non-albicans *Candida*. This was statistically significant (Tay et al., 2011). Our findings followed a similar trend with 80% of *C. albicans* yeasts producing proteinase compared with 43% in the non-albicans *Candida* yeasts (Table 4.7). However, a higher proportion of the non-albicans *Candida* yeasts produced phospholipase i.e. 75% versus 57% in *C. albicans*. The reason for this variation is not immediately clear and requires further investigation. However, it has been documented that phospholipase production may be influenced by the predominant *Candida* strains (Ghannoum, 2000).

C. albicans and non-albicans *Candida* yeasts are all capable of causing oral candidiasis. Although we found a higher proportion (78.95%) of *C. albicans* in patients with infection (Table 4.9), non-albicans *Candida* were present and they expressed virulence factors (Table 4.10). This suggests that they may play a role in the development of infections and indeed non-albicans *Candida* related infections are becoming more prevalent. In a survey of hospital infections caused by *Candida* species, non-albicans *Candida* yeasts were significantly more predominant and their biofilms showed a higher metabolic activity compared to *C. albicans* yeasts (Ferreira et al., 2013).

4.4.3 Virulence of *Candida* species and oral *Candida* infection

We reported a very small prevalence of oral *Candida* infection (9.2% all visits) in Table 4.9. This is consistent with the era of widely available ART where the prevalence of opportunistic *Candida* infection has declined. A recent survey of HIV positive subjects with the majority already on ART showed a prevalence of 27% with oropharyngeal *Candida* infection (Patel et al., 2012). The expression of virulence factors was not significantly different in our subjects with oral candidiasis compared with those subjects that were colonised but without infection. However, the yeast count was much higher in those with infection ($p=0.003$). This is to be expected as previous studies have associated an increased yeast count with clinical infection (Altarawneh et al., 2013). *Candida* yeasts that express virulence factors are capable of causing infection, although the combination of several virulence factors and other underlying variables are involved in the establishment of clinical *Candida* infection (Sacristan et al., 2011). In addition, a cumulative effect of the small amount of hydrolytic enzymes produced by a high number of *C. albicans* can cause pathological

changes. The elevated yeast count related to oral candidiasis may be due to a quantitative effect of the virulence factors. If yeasts are able to multiply, the ability to cause tissue damage and infection is much greater. Furthermore, as virulence factors are expressed in the milieu of a host environment, the effect of investigating the virulence factors in a culture medium rather than in the host requires further investigation. However, practically all the published studies on virulence characteristics are *in vitro* studies similar to ours in view of the difficulty in conducting *in vivo* studies.

As shown in Table 4.2, the number and virulence of *Candida* fluctuate over time due to the immune response and many other factors. Studies have shown high levels of virulence factors in isolates of *C. albicans* from the oral cavities of HIV positive patients compared to HIV negative patients. From our study it is apparent that the presence and quantity of virulence factors alone is not responsible for the infection. This suggests that *C. albicans* isolates may be more virulent but if their count is very low, infection may not occur. This suggests it is important to keep the number of *Candida* in the oral cavity as low as possible to prevent the occurrence of infection. Maintaining a high CD4 count and optimum oral hygiene with antifungal mouthrinses can achieve this goal. Triclosan and fluoride containing mouthrinses can reduce the *Candida* count in oral cavity (Patel et al., 2008). Virulence of *C. albicans* can also be reduced using therapeutic agents (Gauwerky et al., 2009).

The difficulty of constructing an acceptable model for the development of clinical yeast infection has been emphasised. This is owing to the complex interaction between the yeast in a commensal and pathologic state, the host and other

microorganisms. Continuous research incorporating microbiological, immunological and evolutionary genomics are needed to further clarify this complex process (Cottier and Pavelka, 2012).

4.5 Conclusions

Virulence factors of *C. albicans* isolated from all the HIV positive women who were available for six months follow up fluctuated from visit to visit. This fluctuation showed no correlation with CD4 counts. Colonised women carried *C. albicans* and non-albicans *Candida* in their oral cavity and these *Candida* species showed virulence factors, which suggests potential to cause infection. However, there was no difference in the proportion of *C. albicans* and non-albicans *Candida* in patients with oral candidiasis and with no infection. This study also showed that there was no significant difference in the measured virulence factors of *C. albicans* isolated during the infection and colonisation state. However the *C. albicans* counts were significantly high in the oral cavities of patients with candidiasis.

The prevalence of oral *Candida* infection was low (9.2%) which is consistent with the era of widely available antiretroviral therapy. The yeast count was significantly associated with clinical infection but not the virulence characteristics of adherence, proteinase and phospholipase. This implies that the establishment of infection is a complex process and virulence characteristics need to be further evaluated in relation to clinical infection. However, a large number of *C. albicans* collectively would produce a large amount of virulence factors, which would trigger the development of infection.

CHAPTER 5: ANTIFUNGAL SUSCEPTIBILITY OF *CANDIDA ALBICANS* ISOLATED FROM HIV POSITIVE WOMEN

5.1 Introduction

Prior to the introduction of HAART, and to a lesser extent in the era of HAART, recurrent oral candidiasis still occurs. Development of antifungal resistance has been associated with antifungal agents used to treat recurrent candidiasis in HIV infection. In these situations, the appropriate doses prescribed for the usual duration become ineffective (Hamza et al., 2008).

5.1.2 Antifungal testing methods

The Clinical and Laboratory Standards Institute (CLSI) M27-A3 protocol, formerly called the National Committee for Clinical Laboratory Standards (NCCLS) has been the reference standard method for *in vitro* testing of conventional antifungal agents since 1997 (Espinel-Ingroff et al., 1999). The protocol uses either broth microdilution (BMD) or agar based techniques to evaluate amphotericin B, azole antifungals, echinocandins and 5-flucytosin. Some of the challenges associated with the method include the lack of efficiency and convenience for routine laboratory testing. With increasing demand for routine laboratory testing of antifungal agents, more efficient, simple, ready to use and reproducible methods such as the Etest® and the Sensititre YeastOne® Colorimetric Panel (TREK Diagnostic Systems, Cleveland, OH, USA) have been developed (Lu et al., 2004). The Sensititre YeastOne® is a 96-well colorimetric microdilution panel that uses a colour change to indicate growth. The wells contain serially arranged 2-fold dilutions of the antifungal agents (Figure 5.1). The panel evaluates anidulafungin, micafungin, caspofungin, 5-flucytosine,

posaconazole, voriconazole, itraconazole, fluconazole and amphotericin B (Pfaller, 2008).

Studies have compared the degree of agreement between the CLSI M27-A3 protocol and the Sensititre YeastOne® Colorimetric Panel. A recent study found 70.6% to 92.2% for essential agreements. Within categories, an agreement of 94.1% was obtained for 5-flucytosin and 72.6% for Amphotericin B. Consequently, the Sensititre YeastOne® Colorimetric was judged to be reliable for the *in vitro* evaluation of antifungal agents (Bertout et al., 2011). A similar study comparing the YeastOne® method with the CLSI method for testing the antifungal sensitivity of *Candida* species to the 3 echinocandins showed 99% reproducibility and 100% essential agreement between the two methods (Pfaller et al., 2008).

5.1.3 Antifungal susceptibility patterns

Candida species have a very high level of variability in the pattern of sensitivity to antifungal agents (Bertout et al., 2011). This variability can be attributed to the resistance capacity of the species studied or previous use of antifungal agents. Furthermore, the source of the yeast and the immune status of the individual may also play a role (Badiee et al., 2010; Sanchez-Vargas et al., 2005b).

In Africa, routine antifungal susceptibility testing is not undertaken becuase oral thrush infection is treated with empirical antifungal agents or local herbs such as lemon juice (Wright et al., 2009). Consequently, data on the antifungal susceptibility profile of *Candida* species from South Africa is still very sparse. The last study on the antifungal susceptibility profile of oral isolates was published more than 10 years ago, before fluconazole was widely available (Blignaut et al., 2002a). Since then,

antifungal testing has become standardized globally and recommended to detect susceptibility patterns and resistant strains which is a growing problem (Pfaller, 2012). It is therefore important to characterise the pattern of antifungal susceptibility of *Candida* species in the era of moderate availability of antifungal agents. This knowledge will serve as a guide for antifungal therapy and can also help to predict the outcomes of therapeutic interventions.

5.2 Methods and materials

5.2.1 Sample collection

An oral rinse with 10 mls of sterile distilled water was used to collect samples from the participants (117 HIV positive women whose oral cavities were colonized with *C. albicans* as described in Chapter 3, section 3.3). The samples were transferred in sputum jars and 100 µl of rinse sample inoculated onto CHROMagar® Candida plates (CHROMagar Microbiology) and incubated at 37 °C for 48 hours. Where there was growth, colonies were counted and subcultured on Sabouraud dextrose agar (Oxoid, Hampshire, England) for 48 hours.

5.2.2 Yeast identification

This was undertaken using the API 20 C AUX system ® (bioMérieux). Yeast cells from a fresh yeast culture were inoculated into 2 mls of distilled water and concentrated to 2 McFarland of turbidity. A100 µl was transferred from the suspension to the C medium supplied with the API 20 C AUX and gently homogenised with the pipette care was taken to avoid the formation of bubbles. The homogenised mix was transferred to the cupules in the test strip taking care not to overfill or underfill the cupules. The test strips were then carefully placed in a tray of

appropriate size that had been lined with a moist paper towel. The tray was covered and incubated at 30°C for 48 hours. The yeast identification was carried out based on the presence of turbidity in the cupules compared to the control cupule. A seven-digit numerical profile based on the positive reactions was then uploaded to the website for definitive yeast identification.

5.2.3 Determination of antifungal susceptibility

Antifungal susceptibility tests were performed using the principles of the Broth Microdilution minimum inhibitory concentration system described by Clinical Laboratory Standards Institute (CLSI, 2008a). Sensititre YeastOne® microdilution colorimetric microtitre plates (Figure 5.1) containing twofold diluted and dehydrated 9 antifungal agents were obtained (Separation Scientific Pty Ltd). The antifungal agents were Anidulafungin, Micafungin, Caspofungin, 5-Flucytosine, Posaconazole, Voriconazole, Itraconazole, Fluconazole and Amphotericin B. The panels also contains AlamarBlue®, an established cell viability indicator. It converts resazurin to the fluorescent molecule, resorufin by exploiting the natural reducing power of living cells. The active ingredient of AlamarBlue® (resazurin) is a blue nontoxic and nonfluorescent compound that can permeate the cell. On entering the cells, resazurin is reduced to resorufin, a compound which produces very bright red fluorescence. Fresh yeast cultures were inoculated into normal saline and adjusted to a turbidity of 0.12 McFarland standards. 20µl from the inoculum was then added to the Sensititre YeastOne® broth and gently vortexed (Figure 5.2). From the inoculated broth, 100 µl was added to each well of the Sensititre YeastOne® panel using a multichannel pipette and incubated at 35 °C for 24 hours. The results were read after incubation and the minimal inhibitory concentration was determined as the lowest concentration that

prevented growth (the first blue well). The wells with growth turned red (Figure 5.3). Control strains of *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were tested based on CLSI guidelines.

Figure 5.1 96-well Sensititre YeastOne® panel containing antifungal agents

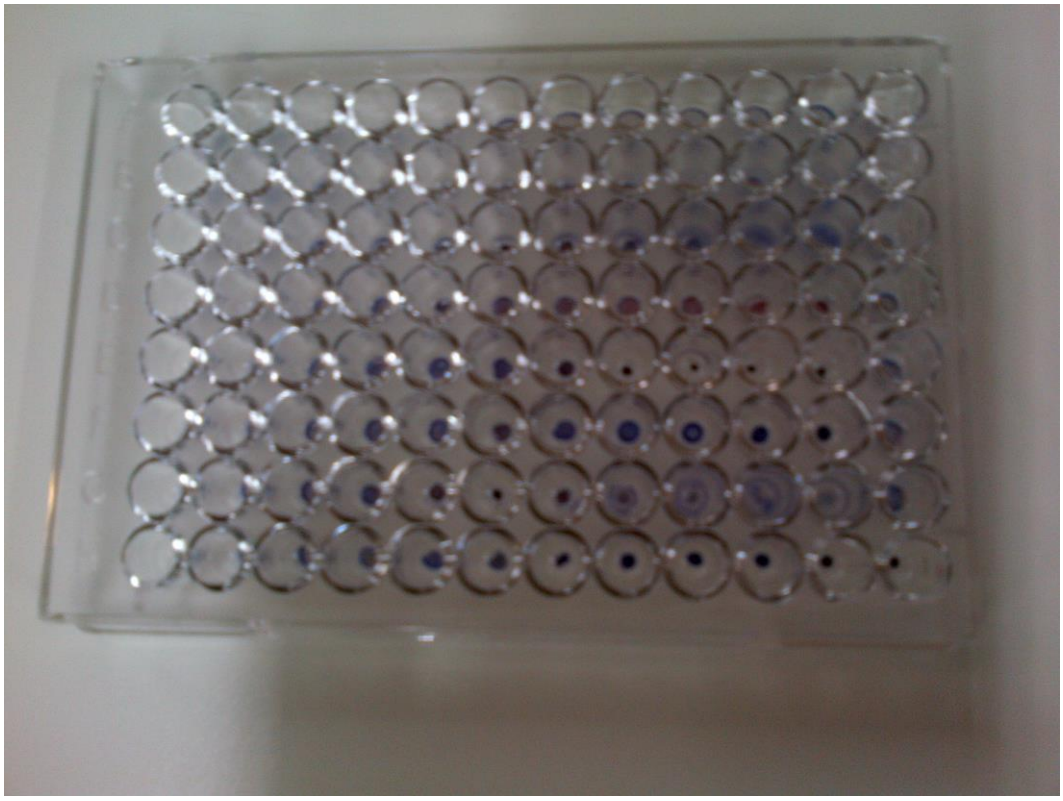
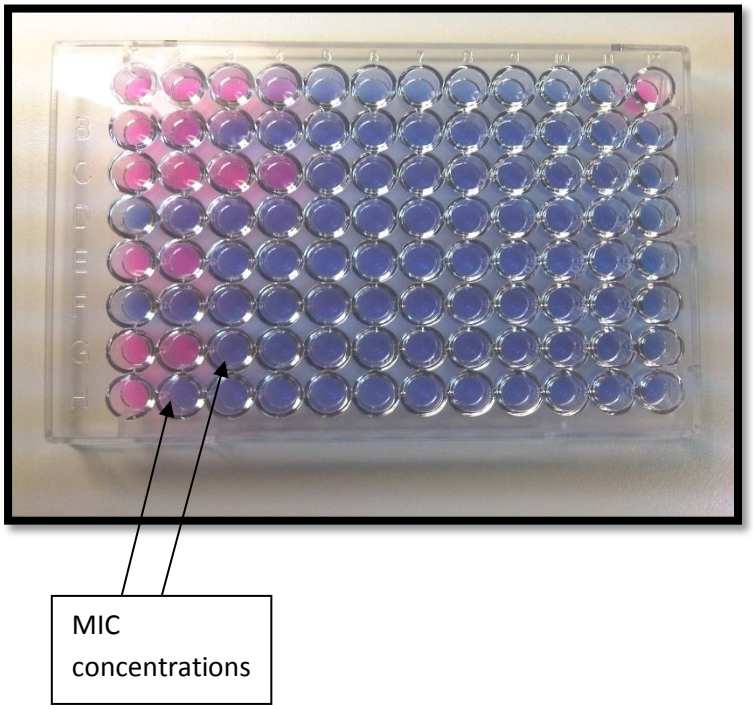


Figure 5.2 Sensititre YeastOne® broth after inoculation

Figure 5.3 Sensititre YeastOne® microdilution colorometric microtitre plate after incubation showing MIC values



5.2.4 Determination of clinical breakpoints and epidemiologic cutoff values

The broth microdilution method that the Sensititre YeastOne® Colorimetric Panel uses has been standardised for *Candida* species by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The methods from both bodies are similar. Recently, the CLSI developed a revised standard with the following advantages over the previous standard. Firstly, antifungal sensitivity reading after 24 hours has been validated. Secondly, epidemiologic cutoff values (ECV) have been established for the various *Candida* species and the clinical breakpoint (CBP) for each antifungal has been determined for each species (Pfaller and Diekema, 2012). Previously, clinical breakpoints and epidemiologic cutoff values were determined irrespective of the *Candida* species, which failed to distinguish wild type yeasts from non-wild types ie mutant and resistant yeasts. This had significant clinical implications (Pfaller et al., 2012). The clinical breakpoints of the CLSI and the EUCAST were used to characterise the sensitivity profile of the *Candida* yeasts (Arendrup et al., 2011; Lass-Florl et al., 2011; Pfaller and Diekema, 2012).

Important terminologies for interpreting antifungal sensitivity data are defined (EUCAST, 2013) and the revised standards can be found in Tables 5.1.

Clinically Susceptible (S)

This is antimicrobial activity level with a high likelihood of therapeutic success. The microorganism is categorised as susceptible (S) by using the appropriate breakpoint in a defined phenotypic test system. This breakpoint can be altered.

Clinically Intermediate (I)

In this category, the antimicrobial agent activity level is associated with uncertain therapeutic effect. Infection due to the organism may be appropriately treated in the sites of the body where the drugs are physically concentrated. Furthermore, high drug dosages can be used. A buffer zone to prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations is also indicated. The predetermined breakpoint is used to categorise the organism as intermediate (I). This cutoff may be modified when legitimate changes in circumstances demand.

Clinically Resistant (R)

Antimicrobial activity level in this category is associated with a high likelihood of therapeutic failure. The organism is categorised as resistant (R) using the appropriate breakpoint, which may be legitimately changed when the circumstances demand.

Clinical breakpoints are defined as S<x mg/L; I>x, <y mg/L; R>y mg/L

Wild type (WT)

Wild type microorganism species do not exhibit acquired and mutational resistance mechanisms to a particular drug. The organism is categorised as the wild type with the application of a defined epidemiologic cutoff value that can be changed when the circumstances require. The wild type strains may or may not show a clinical response to antifungal therapy.

Non-Wild Type (NWT)

The non-wild type (NWT) species have acquired or innate mutational resistance mechanism to a particular drug, which is categorized using a defined epidemiologic cutoff value. This can change as the circumstances require. The non-wild type strains may or may not show a clinical response to the antifungal therapy.

The epidemiological cutoff value is therefore an MIC threshold value that allows a discrimination of wild type strain from a non-wild type strain for each particular antifungal and species combination.

The wild type is presented as MIC ≤ ECV and non-wild type as MIC > ECV.

Table 5.1 Clinical breakpoints (CBP) for the interpretation of Sensititre YeastOne® Colorimetric Panel for *C. albicans*

	CBP (µg/mL)				ECV (µg/mL)	
Antifungal	**S**	**SDD**	**I**	**R**	**WT**	**Non-WT**
AND	≤ 0.25		0.5	≥ 1	≤ 0.12	> 0.12
MF	≤ 0.25		0.5	≥ 1	≤ 0.03	> 0.03
CAS	≤ 0.25		0.5	≥ 1	≤ 0.12	> 0.12
FC	≤ 4		8-16	≥ 32	≤ 0.5	> 0.5
PZ	< 0.06			> 0.06	≤ 0.06	> 0.06
VOR	≤ 0.12		0.25-0.5	≥ 1	≤ 0.03	> 0.03
IZ	≤ 0.12	0.25-0.5		≥ 1	≤ 0.12	> 0.12
FZ	≤ 2	4		≥ 8	≤ 0.5	> 0.5
AB					≤ 2	> 2

CBP- clinical break point, ECV- epidemiological cutoff values, S-sensitive, SDD,-sensitive dose dependent, I-intermediate, R- resistant, WT-wildtype, Non-WT- non wild type, AND- anidulafungin, MF-micafungin, CAS-caspofungin, FC-5-flucytosin, PZ- posaconazole, VOR- voriconazole, IZ-itraconazole, FZ-fluconazole, AB-amphotericin B.

5.3 Results

One hundred and twenty two isolates identified as *C. albicans* were tested. They were made up of 72 isolates from the first visit, 29 from the second and 21 from the third visit. The results are presented in Tables 5.2 and 5.3.

The majority of the isolates were sensitive to the test antifungals with posaconazole showing the highest prevalence of resistant strains (1.7%). All the echinocandins demonstrated 0.8% resistance similar to the other azole antifungals apart from posaconazole. No strains were resistant to amphotericin B and the clinical break points for 5-flucytosin is yet to be determined. However, 5-flucytosin had the most non-wild type strains.

Table 5.2 Antifungal susceptibility profiles of *Candida albicans* isolates

Antifungal	**CBP (µg/mL)**				**ECV (µg/mL)**	
	S	**SDD**	**I**	**R (%)**	**WT**	**Non-WT**
AND	121			1 (0.8)	121	1
MF	121			1(0.8)	120	2
CAS	120			1(0.8)	120	1
FC	ND	ND	ND	ND	115	7
PZ	120			2(1.7)	120	2
VOR	121			1(0.8)	121	1
IZ	121			1(0.8)	121	1
FZ	121			1(0.8)	119	3
AB	122			0(0)	122	0

CBP- clinical break point, ECV- epidemiological cutoff values, S-sensitive, SDD,-sensitive dose dependent, I-intermediate, R- resistant, WT-wildtype, Non-WT- non wild type, AND- anidulafungin, MF-micafungin, CAS-caspofungin, FC-5-flucytosin, PZ- posaconazole, VOR- voriconazole, IZ-itraconazole, FZ-fluconazole, AB-amphotericin B. ND- not defined yet

Table 5.3 Antifungal susceptibility profiles of *Candida albicans isolates* showing the MICs

Antifungal	MIC(µg/mL)		
	Range	MIC_{50}	MIC_{90}
AND	0.015-8	0.03	0.03
MF	0.08-8	0.08	0.08
CAS	0.015-8	0.015	0.06
FC	0.06-64	0.06	0.25
PZ	0.008-8	0.008	0.015
VOR	0.008-8	0.008	0.008
IZ	0.015-16	0.015	0.003
FZ	0.12-128	0.12	0.12
AB	0.12-0.5	0.12	0.12

MIC- minimum inhibitory concentration

5.4 Discussion

The majority of yeasts in our study population were *C. albicans*, which is confirmed by other reports. It is also the reason why the sensitivity profile of *C. albicans* has been described most often (Hamza et al., 2008). As with other previous studies in Africa, the proportion of resistant strains of *Candida* to antifungal agents is also low, although the echinocandins were not tested in earlier studies (Blignaut et al., 2002a; Hamza et al., 2008). In our study only 0.8% of *C. albicans* isolates were resistant to echinocandins. A very recent study from Ethiopia that tested for antifungal resistance found 2% resistance to echinocandins (Mulu et al., 2013). This is certainly due to the recent introduction of the echinocandins, which are not often used in Africa. Of all the echinocandins (anidulafungin, micafungin, and caspofungin) that were tested in our study, the resistant strains were less than 1% (Tables 5.2 and 5.3). This is consistent

with other reports that documented a range between 95% to 99% susceptibility to echinocandins (Badiee et al., 2010; Garcia-Agudo et al., 2012; Mulu et al., 2013). It is not certain why we observed a case of resistance to echinocandins but this has been confirmed in other samples (Pfaller, 2012). Although *C. parapsilosis* and *C. glabrata* exhibited a high resistance to echinocandins, they remain the drugs of choice as alternatives to azoles and in cases of invasive candidiasis (Garcia-Agudo et al., 2012).

The range of azole resistance in our subjects was also low, ranging from 0.8% to 1.6% in *C. albicans* yeasts (Table 5.2). This is just a marginal increase from the 0.4% reported in 2002 when azoles were first introduced in South Africa (Blignaut et al., 2002a). It appears that more than one decade of azole therapy has not had a significant impact on the development of azole resistant strains of *C. albicans*. The study by Blignaut et al (2002) and our study were conducted on isolates from the northern part of South Africa. A recent study from the southern parts of South Africa has reported fluconazole resistance of 50% (Abrantes et al., 2013). Higher percentage of azole resistant *C. albicans* strains has also been reported from Iran (10%) and India (12.5%). However, these studies were conducted in settings where the patients were HIV positive and had been exposed to repeated therapy with azole antifungals (Badiee et al., 2010; Mane et al., 2010). More recently, 32% of *Candida* isolates were found to be resistant to multiple antifungals (Mulu et al., 2013). However, in this study over half the resistant strains were non-albicans *Candida* species, which reflects the increasing role of these yeasts in antifungal resistance (Mulu et al., 2013). This high level of resistance may also be due to the subjects, the majority of whom had been exposed to repeated azole therapy due to recurrent oropharyngeal candidiasis. In addition, they were all late presenting AIDS patients (Mulu et al., 2013). This finding,

however, points to the danger that may ensue if antifungal therapy becomes indiscriminate and use repeated as the authors also observed a shift to resistant non-albicans *Candida* in the same subjects.

With regards to non-albicans *Candida*, 5% fluconazole resistance to *C. glabrata* was documented in recent reports from Tanzania and 50% from India (Hamza et al., 2008; Mane et al., 2010). We isolated non-albicans *Candida* including *C. glabrata* but did not subject them to antifungal sensitivity tests because very few (6 types of non-albicans *Candida* in total 26 isolates, Chapter 3, Table 3.6) non-albicans *Candida* were isolated. This would not have given meaningful results. However, azole resistance to *C. glabrata* has been sufficiently reported as a serious concern. The clinical breakpoints for *C. glabrata* regarding fluconazole have the highest cuttoffs (>64 μg/mL). The yeast is known to possess a high ability to mutate in a single individual and azoles or amphotericin B are not able to control this change. For this reason, institutions need to be on the lookout for multidrug resistant *C. glabrata* (Pfaller, 2012).

The previous use of any azole antifungal is a risk factor for azole resistance. Furthermore, azoles share cross-resistance as previous use of one azole may predispose to resistance to another. A study in Tanzania found a significant relationship between previous use and the development of resistant *Candida* species strains with fluconazole and itraconazole (Hamza et al., 2008). Prior treatment with fluconazole was also found to be a strong predictor for the development of candidaemia (Garnacho-Montero et al., 2010). Every subject in our study was asked for a history of previous antifungal use; however, the majority could not indicate

which drugs they had taken previously. From the records it was discovered that the same subject (Study ID 10) carried yeast that were resistant to all the azole antifungals. In her history, she did not reveal that she had taken antifungal agents previously, even though she carried *C. albicans* and had an HIV positive partner. It is possible that she was on antifungal previously but did not understand what treatment she was receiving. It is also possible that she acquired azole resistant strains from her partner because she did not have candidiasis when she was examined originally. If she develops candidiasis, azole antifungals will be ineffective if prescribed. This case typifies the danger of multiple resistance to azoles.

No case of resistance to amphotericin B was documented, unlike 8.4% resistance found previously (Blignaut et al., 2002a). This was probably because amphotericin B and nystatin were the antifungal drugs generally available at the time of the study. In addition, azoles were relatively new and echinocandins were not available in this part of the world when the study was conducted. However, the maximum MIC of 0.5 μg/mL reported in *C. albicans* in this study is below the limit of 2 μg/mL where treatment with other antifungal agents is recommended (Pfaller and Diekema, 2012). This is consistent with reports from across the world where *Candida* species generally have low MICs to amphotericin B and the drug recommended as an effective therapy (Brito et al., 2011; Hamza et al., 2008).

The need for guidelines for testing antifungal susceptibility has been emphasised (Table 5.3) and a guide provided (Pfaller and Diekema, 2012). Antifungal susceptibility testing should not be performed for the routine treatment of oral candidiasis except when the response to therapy is inadequate. However,

determination of the antifungal sensitivity profile of *Candida* yeasts from time to time is necessary to guide empirical therapy and acts as a surveillance measure to detect the development of resistance.

Table 5.4 Guidelines for the use of antifungal susceptibility testing [Source: Pfaller and Diekema, 2012]

Clinical scenario	**Recommendation (s)**
Routine	• All *Candida* isolates from deep sites (blood, normally sterile body fluids, tissues, abscesses) must be identified to the species level • Sensitivity testing of fluconazole and an echinocandin against *C. glabrata* from deep sites recommended • Sensitivity testing of fluconazole and an echinocandin against other species of *Candida* may be helpful but susceptibility usually predictable by species • Apply the CBPs or ECVs to interpret results as appropriate (Table 5.1) • Completion of cross-resistance between fluconazole and all other azoles to be must be considered for *C. glabrata* • Create an antifungogram
Mucosal candidiasis	• Determination of azole susceptibility not routinely necessary • Susceptibility testing of azoles may be useful for patients unresponsive to therapy
Species with high rates of ***intrinsic*** or ***acquired*** resistance causing infection	• Susceptibility testing not necessary when intrinsic resistance is known o *C. lusitaniae* and amphotericin o *C. krusei* and fluconazole, 5-flucytosine o *C. guilliermondii* and echinocandins • With high rates of acquired resistance, monitor closely for signs of failure and perform susceptibility testing o *C. glabrata* and fluconazole, amphotericin B, and echinocandins o *C. krusei* and amphotericin B o *C. guilliermondii* and amphotericin B o *C. rugosa* and amphotericin B, fluconazole, and echinocandins

Unusual species or new treatment options (e.g., echinocandins, voriconazole, posaconazole)	• Susceptibility of *Candida* spp. to echinocandins may be assumed unless initial response is suboptimal • Susceptibility testing warranted if there was prior exposure to echinocandins or fluconazole • Selection of therapy based on published consensus guidelines and review of survey data on the organism-drug combination in question • Susceptibility testing may be helpful when patient is not responding to what should be effective therapy
Clinical response in patients despite being infected with an organism later found to be resistant	• Best approach not clear. Take into account severity of infection, patient immune status, consequences of recurrent infection, etc. • Consider alternative therapy for infections with isolates that appear to be highly resistant to initial therapy

Choice of susceptibility testing	• CLSI methods
	  o Broth based, M27-A3
	  o Agar based, M44-A2
	• EUCAST EDef 7.1
	• Commercial methods
	  o Etest
	  o Sensititre YeastOne
	  o Vitek 2

5.5 Conclusions

With the low prevalence of antifungal resistance in *C. albicans* isolated from our subjects, the likelihood of response to therapy with any conventional azole, amphotericin B or echinocandins is very high. This is assuming that the other factors determining response to therapy remain favourable. The factors include the immune response of the host, the virulence of the organism, drug pharmacodynamics and pharmacokinetics. All these factors contribute to how an individual will respond to an antifungal agent (Pfaller, 2012; Pfaller and Diekema, 2012). Certainly our data is a valuable addition to the baseline data on the antifungal sensitivity profile of yeasts in our study population. This should be undertaken on a regular basis and not every decade.

CHAPTER 6 GENOTYPING OF *C. ALBICANS* ISOLATED FROM THE HIV POSITIVE WOMEN OVER 6 MONTHS' FOLLOW UP PERIOD

6.1 Introduction

6.1.1 Genotyping of *C. albicans*

Genotyping of yeasts evolved with the development of more accurate and portable methods which enables results to be compared across the globe. Furthermore, it aids in deciphering the epidemiological distribution of yeasts, identifies the predominant strains in individuals and associated factors with colonisation. Genotyping is more accurate in the study of strain colonisation in individuals to deteremine persistence of the same strain and acquisition of exogenous strains. Genotyping is also used in monitoring outbreaks of infection (Vanhee et al., 2010). However, knowledge on genotyping is scarce.

Knowledge of *C. albicans* strain diversity and evolutionary changes over time are relatively scanty in the global populaton (Pires-Goncalves et al., 2007). Data is lacking from some countries and individuals have to be followed up by conducting serial genotyping of isolates. This genotyping may yield more than one strain per individual. Consistently taking samples from individuals over time and sampling several strains to determine the molecular epidemiology requires considerable time and money. In addition, genotyping techniques are many and varied, and results may not be comparable across laboratories and countries. Consequently, different genotyping methods are still being evaluated to identify their strengths and drawbacks (Vanhee et al., 2010).

The population structure of *C. albicans* strains are usually consistent in the same person. In addition, colonising strains are related with respect to age, same site in the body, family members, geographic location and subjects with similar conditions such as HIV infection. However, geographic strain epidemiology is becoming less distinct due to human travel. Furthermore, more than one genotype can exist in the same body part or individual thereby indicating the dynamic nature of colonisation (Clemons et al., 1997; Kleinegger et al., 1996; Odds, 2010; Xu et al., 1999).

6.1.2 ABC Genotyping

ABC genotyping is one method of genotyping *C. albicans*. It offers a simple way of genotyping *C. albicans* in spite of the poor discriminatory ability relative to some other methods. ABC genotyping is used alone or as an adjunct to other genotyping methods (Abdulrahim et al., 2013; Chaves et al., 2012; Kumar et al., 2006). It is useful where sequence typing facilities and other more sophisticated genotyping methods are not available (Kumar et al., 2006). However, ABC genotyping alone is not recommended when the pleomorphism in the strains differ by more than a single nucleotide (Chaves et al., 2012). To illustrate this, ABC genotyping was combined with microsatellite typing to monitor strain epidemiology in subjects with candidemia. In the study, similar ABC genotypes were found to be present in different anatomic sites in subjects who developed candidemia in contrast to a diveristy of strains in subjects without candidemia (Chaves et al., 2012). A comparable investigation combined ABC genotyping with MLST (Multilocus Sequence Typing) genotyping to monitor *C. albicans* involvement in leukoplakia. Enrichment of genotype C was found in leukoplakia (Abdulrahim et al., 2013).

6.1.3 Multilocus Sequence Typing (MLST)

This highly discriminatory genotyping method is used to characterise the molecular epidemiology of *Candida* species. The polymorphisms of seven housekeeping genes are used to characterise the strains. Details of the housekeeping genes for *C. albicans* MLST is illustrated in Table 6.1 (Spampinato and Leonardi, 2013).

Table 6.1 Housekeeping genes for *C. albicans* MLST [Source: Spampinato and Leonardi, 2013]

Locus	Chromosome	Gene product	Primers	Sequenced fragment size (bp)
Ca*AAT1a*	2	Aspartate aminotransferase	F: ACTCAAGCTAGATTTTTGGC R: CAGCAACATGATTAGCCC	349
Ca*ACC1*	R	Acetyl-coenzyme A carboxylase	F: GCAAGAGAAATTTTAATTCAATG R: TTCATCAACATCATCCAAGTG	407
Ca*ADP1*	1	ATP-dependent permease	F: GAGCCAAGTATGAATGATTTG R: TTGATCAACAAACCCGATAAT	443
Ca*PMI1b*	2	Mannose phosphate isomerase	F: ACCAGAAATGGCCATTGC R: GCAGCCATGCATTCAATTAT	375
Ca*SYA1*	6	Alanyl-RNA synthetase	F: AGAAGAATTGTTGCTGTTACTG R: GTTACCTTTACCACCAGCTTT	391
Ca*VPS13*	4	Vacuolar protein sorting protein	F: TCGTTGAGAGATATTCGACTT R: ACGGATGGATCTCCAGTCC	403
Ca*ZWF1b*	1	Glucose-6-phosphate dehydrogenase	F: GTTTCATTTGATCCTGAAGC R: GCCATTGATAAGTACCTGGAT	491

F and R indicate forward and reverse primers, respectively.

The combination of the allele numbers of the seven genes is used to derive a unique sequence type (ST or DST number) for every strain. Currently on the MLST database (http://calbicans.mlst.net/), there are 2142 sequence types for *C. albicans* (as at 17th October 2013). This is the largest global database of strain type information for *C. albicans*. DST 69 is the most common globally and 290 distinct DSTs are from oral isolates. The DSTs representing the strains are also categorised into 24 clonal complexes by the eBURST algorithm to describe relatedness. Clonal complex 1

(CC1) is the most common. Furthermore, MLST classified *C. albicans* yeasts into 5 major and many minor clades. The major clades are 1, 2, 3, 4 and 11 (Odds, 2010).

Recent updates on the *Candida* MLST database include renaming of one of the *C. albicans* housekeeping genes MPIb to PMIb. Typing schemes for other important *Candida* yeasts such as *C. glabrata* and *C. tropicalis* have also been developed on the MLST database. Unlike *C. albicans*, six housekeeping genes are used to type both yeasts. Similar gene sets have also been described for *C. krusei and C. dubliniensis* (Spampinato and Leonardi, 2013). The major advantages of MLST include having a high discriminatory ability, high portability (similar results are obtainable across laboratories) and the database is available on the Internet for global accessibility.

6.1.4 Carriage of *Candida* strains

C. albicans is a diploid yeast without a sexual cycle. Mating is extrememly rare and occurs without meiosis. Furthermore, *C. albicans* yeasts show a clonal pattern of reproduction but are able to shuffle chromosomes leading to duplicate genes and reassortment of genes. Consequently, *C. albicans* strains tend to reveal a high genetic diversity from clonal reproduction and a very high rate of recombination events (Bougnoux et al., 2008b; Odds et al., 2007). The eBURST software on the MLST database show clonal clusters of related yeasts from the same clone with a lot of singleton strains. Clonal relationship is determined by similarity of six out of the seven housekeeping genes on the MLST database.

As similar or closely related strains persist in individuals, the distinction between microevolution of a colonising strain and a replacing strain may be difficult to

determine because of the significant genetic relatedness. Differentiation is only possible when the replacing strain is markedly different but this is not always the case. However, it is important to determine the difference between colonising and exogenous strains of *C. albicans* strains due to their implications in pathogenesis. On the other hand, few individuals carry more than one predominant strain and replacement of a strain is not always from an exogenous source (Odds, 2010). The correct identification of a colonising strain versus an exogenous (reinfecting) strain is important in order to determine the virulence of the strain which relates to the ability to cause infection.

Studies that follow up subjects to determine the yeast strain epidemiology over time are few and none has characterised the *C. albicans* strains from the oral cavity using MLST in South Africa. With the highest HIV burden in the world, it is important to deteremine if the strains present in HIV infected individuals in South Africa are colonising strains or exogenous infecting strains because they tend to have recurrent candidiasis. Furthermore, conditions that predispose to microevolution of colonising strains are also poorly understood (Odds et al., 2006) and to date, no study has monitored strain maintenance or occurrence of new strains of *C. albicans* in a longitudinal study in an African population. The objective of this chapter was to determine and compare the genotypes of *C. albicans* in colonized women at intervals during the follow up period to detect the acquisition of exogenous strains using ABC genotyping and MLST techniques.

6.2 Methods and materials

6.2.1 Subjects, sample collection and *Candida* species isolation

One hundred and seventeen HIV positive women carried *Candida* in their oral cavity (Chapter 3) and they were asked to come for follow up visits after 3 and 6 months. Only 17 women kept their appointments and carried *C. albicans* at all 3 visits (0, 3 and 6 months). Oral rinse samples were collected, cultured and *C. albicans* was identified using the API technique (full method described in Chapter 3). The samples from 17 women who carried *C. albicans* (17 x 3 = 51 isolates) at all the three visits were subjected to ABC genotyping and MLST technique.

6.2.2 DNA extraction

Genomic DNA was extracted from *C. albicans* samples using the technique previously described with modifications (De Baere et al., 2002). One colony from each sample was inoculated into 1.5ml Eppendorf tubes containing sterile distilled water, boiled for 6 minutes, centrifuged for 4 minutes at 14680 rpm and placed on ice. The supernatant was collected, DNA concentration was confirmed with the NANODROP 2000® (Thermo Scientific, Wilmington, DE, USA) and the samples stored at -75°C until required. The samples had an average DNA material of 38.7 ng/µl.

6.2.3 ABC genotyping

A 50 µl PCR mix was prepared using the primers described for the technique (McCullough et al., 1999). From the 2 primers CA-Int L (5/-ATAAGGGAAGTCGGCAAAATAGATCCG TAA-3/) and CA-Int R (5/-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3/), 0.5 µl of each primer was

added to the mix. The other components of the mix were 10 µl of Taq buffer, 10 µl of $MgCl_2$, 2 µl of dNTPs, 0.5 µl of Taq polymerase enzyme and 5 µl of DNA from respective samples and 21.5µl of sterile distilled water. Amplification was carried out using the MJ Mini Personal Thermal Cycler® (BIO-RAD Hercules, CA, USA) with the protocol – initial denaturation, 95°C for 2 minutes and subsequently 30 seconds, annealing 57 °C for 1 minute, extension 72 °C for 2 minutes and the cycle repeated for 34 times with final temperature of 72°C for 10 minutes. Agarose gel electrophoresis was used to separate and demonstrate the PCR products. The gel was constituted with 0.3 g agarose powder and 30mls of 1x TBE (Tris borate EDTA) buffer. The loading buffer (100 µl) was mixed with 4 µl of ethidium bromide. A drop of this mix was then added to 10 µl of PCR product from each sample. A 1kb ladder was used with every gel. The electrophoresis was run for one hour at 300 mA and 120 V in a tank with TBE buffer. The gel was viewed under UV illumination and the images captured and analysed using the VersaDoc QuantityOne machine® (BIO-RAD Hercules, CA, USA). The genotype of each sample was determined by the size of the fragments, A=450bp, B=840bp, C=450bp & 840bp and categorised using the ABC scheme.

6.2.4 MLST genotyping

The 50 µl PCR mix contained 0.5 µl of the forward and 0.5 µl of the reverse primers of each of the housekeeping genes (Table 6.1). Each mix also contained 10 µl of Taq buffer, 10 µl of $MgCl_2$, 2 µl of dNTPs, 0.5 µl of Taq polymerase enzyme and 5 µl of DNA from respective samples and 21.5 µl of distilled water. Each sample was subjected to seven PCR reactions, one for each gene. Agarose gel electrophoresis

described in 6.2.4 was performed using 10 µl of the PCR product. The remaining amplified gene content (40 µl) was subjected to DNA sequencing.

6.2.5 Sequencing

DNA sequencing for the MLST samples was undertaken using the ABI Big Dye V3.1 on the ABI 3500XL machines as per manufacturer instructions.

6.2.6 Genotyping and strain identification using the MLST database

The forward and reverse sequences of each of the housekeeping genes from every sample were subjected to further analysis using the Geneious 6R software (Biomatters, http://www.geneious.com). A consensus sequence conforming to the number of base pairs for each gene was obtained by aligning and trimming the forward and reverse sequences for each gene sequence. The trimmed sequences were compared with the existing allele sequences on the MLST database. Conformity with existing allele sequence produced the allele number and when there is no perfect agreement the MLST database indicated the closest allele and percentage closeness. Allele numbers for the seven genes were then used to generate a diploid sequence type (DST) number unique to that strain. Existing DST numbers were noted. Sequence data were sent to the MLST curator at the Institut Pasteur (Paris) to assign new numbers for the alleles and DSTs that were novel.

Using the Geneious® 6R software (Biomatters, http://www.geneious.com), dendrograms were generated from the sequence data of respective alleles. The unweighted pair group method with arithmetic averages (UPGMA) algorithm was used. Tree gap alignment option was 65% similarity and the gap open penalty was 12.

Using cluster analysis, another UPGMA dendrogram was generated in Stata® 12 (StataCorp, College Station, Texas) from all the alleles and for all the visits per subject. The method used to interpret the data was based on the similarity coefficient as previously described. The algorithm computed a distance between isolated strains from which a number was generated, 0 representing no relationship and 1 indicating identical strains. From 0.9 to 1 indicated microevolution, 0.8-0.9 indicated moderate relationship and less than 0.8 was unrelated. A matrix of similarities was generated for all the observations. The correlation option was used for the analysis rather than the Euclidian distance as similarities was easier to interpret (Boriollo et al., 2006; Soll, 2000).

6.2.7 Association between virulence and genotypes

A repeated measures analysis of variance was used to determine the variation in the virulence factors during the follow up visit after categorising into two groups, those reinfected with different strains and those carrying the same strain at all visits.

6.3 Results

6.3.1 ABC genotyping

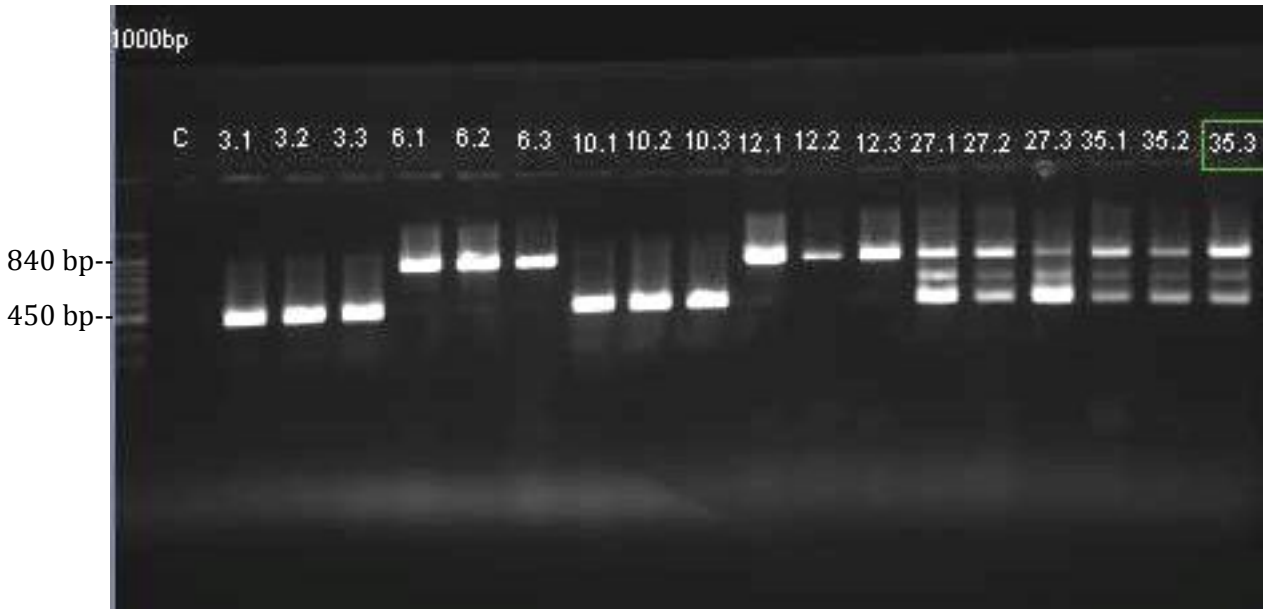
ABC genotyping revealed a predominance of the genotype B in 31 (60.8%) of the 51 strains. This was followed by genotype A in 14 (27.4%) and genotype C in 6 (11.8%). Sixteen (94.1%) out of the 17 women followed up carried the same ABC genotype for all the three visits (Table 6.2). Figure 6.1 shows the gel samples for subjects 3, 6, 10, 12, 27 and 35. Subjects 3 and 10 carried genotype A consistently for all visits. Likewise, Subjects 6 and 12 carried genotype B consistently for all visits and Subjects 27 and 35 were colonised with the same genotype C at all visits.

Table 6.2 Strain characteristic using V3 region of the 25S rRNA gene of *C. albicans* isolated from 17 patients (ABC genotyping)

	C. albicans strains genotypes (A=450bp, B=840bp, C=450bp & 840bp)			
No.	**Baseline**	**3 months**	**6 months**	**Comment**
003	A	A	A	Consistent
006	B	B	B	Consistent
010	A	A	A	Consistent
012	B	B	B	Consistent
027	C	C	C	Consistent
035	C	C	C	Consistent
041	A	A	A	Consistent
042	B	B	B	Consistent
045	B	B	B	Consistent
078	B	B	B	Consistent
082	A	A	B	Inconsistent
132	B	B	B	Consistent
140	B	B	B	Consistent
166	B	B	B	Consistent
173	B	B	B	Consistent
185	B	B	B	Consistent
193	A	A	A	Consistent

Total- Genotype A=14(27.5%), Genotype B=31(60.8%), Genotype C=6 (11.7%)

Figure 6.1 PCR products of the V3 region of the 25S rRNA of *C. albicans*.



[1000bp molecular weight marker, C: negative control, Subject 3 & 10: genotype A (450 bp), Subjects 6 & 12: genotype B (840bp), Subjects 27 & 35: genotype C (450 bp & 840 bp)]

6.3.2 MLST genotyping

Some of the amplified PCR products of test genes are shown in figure 6.2 to 6.7. The results of all seven alleles tested and the DST numbers generated in MLST technique are shown in Table 6.3.

Figure 6.2 PCR products of the AAT1a gene of *C. albicans* using MLST technique.

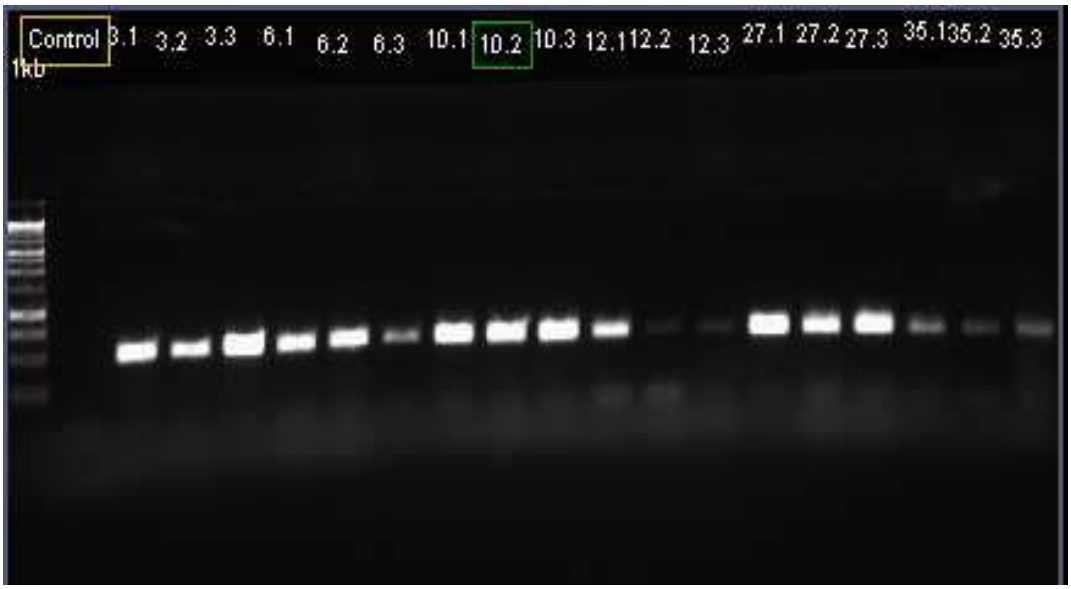


Figure 6.3 PCR products of the ADP1 gene of *C. albicans* using MLST technique

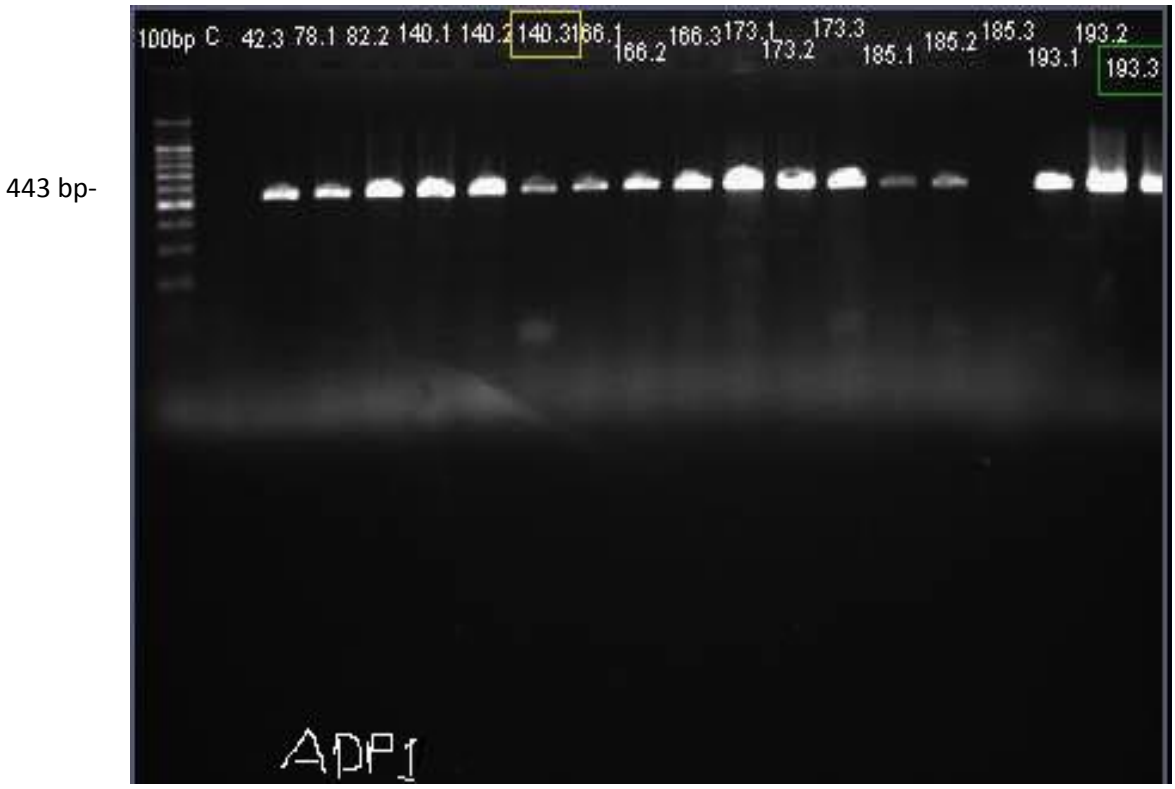


Figure 6.4 PCR products of the MP1b gene of *C. albicans* using MLST technique

Figure 6.5 PCR products of the SYA1 gene of *C. albicans* using MLST technique

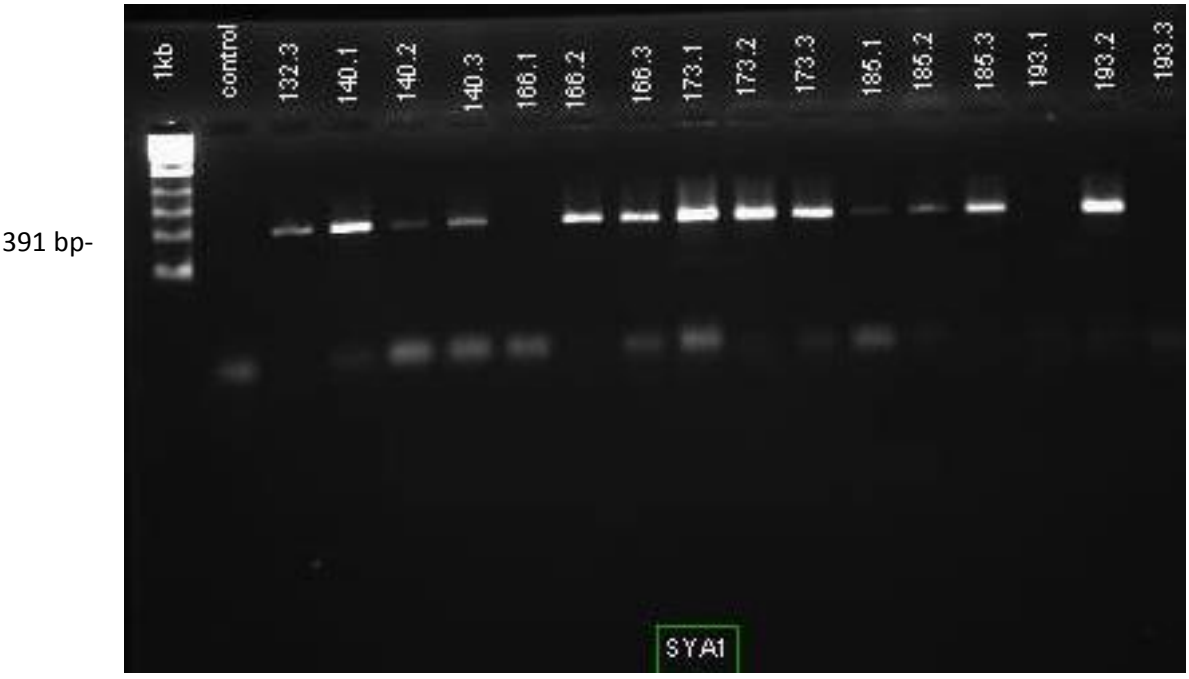


Figure 6.6 PCR products of the VPS13 gene of *C. albicans* using MLST technique

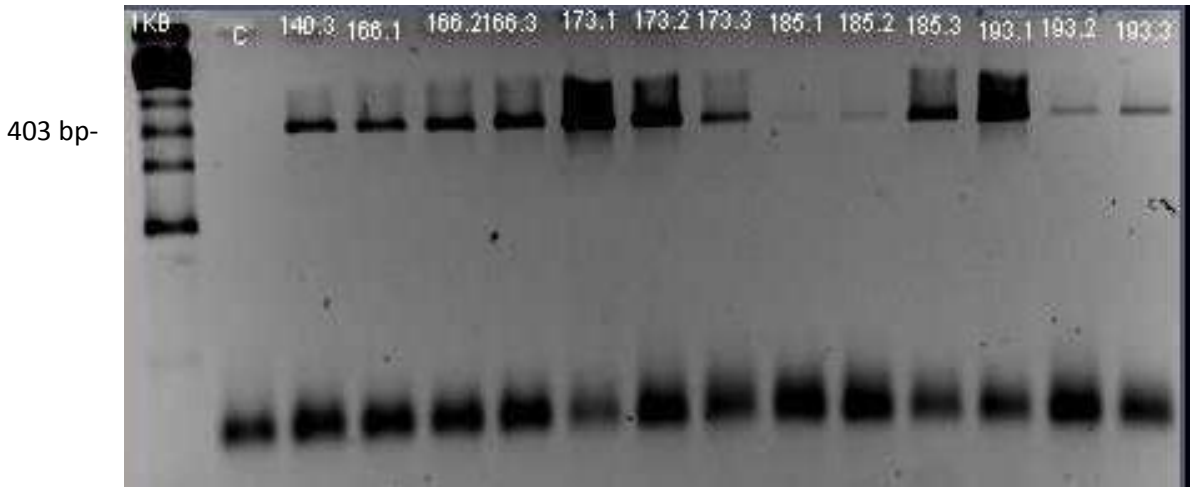


Figure 6.7 PCR products of the ZWF1b gene of *C. albicans* using MLST technique

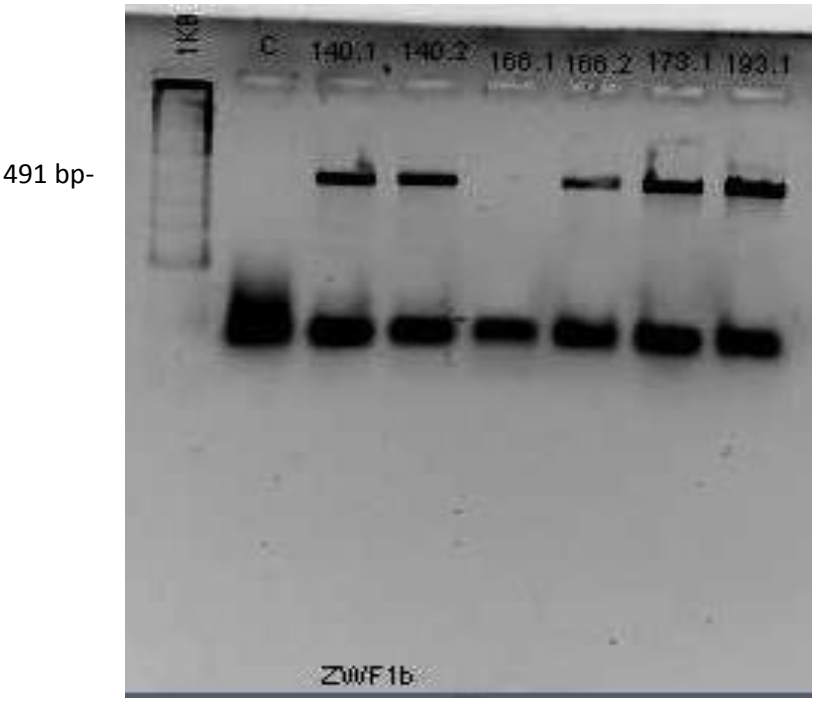


Table 6.3 Strain characteristics of *C. albicans* isolated from HIV positive women over 6 months follow up period using MLST

STUDYID	VISIT	AAT1a	ACC1	ADP1	MPIb	SYA1	VPS13	ZWF1b	DST
3.1	1	31	3	2	2	2	24	12	2164
3.2	2	35	4	4	4	34	4	4	808
3.3	3	31	91	2	2	2	24	12	2177
6.1	1	143	14	8	4	7	10	8	2178
6.2	2	143	92	8	4	7	10	8	2179
6.3	3	52	14	8	4	7	10	8	2165
10.1	1	31	2	5	2	2	24	241	2182
10.2	2	31	2	5	2	2	24	25	2166
10.3	3	31	2	5	2	2	24	25	2166
12.1	1	8	7	8	4	2	10	22	2167
12.2	2	144	7	8	4	2	10	22	2180
12.3	3	70	7	8	4	2	10	22	2168
27.1	1	8	14	125	4	7	10	8	2183
27.2	2	8	14	8	4	7	10	8	124
27.3	3	8	14	8	4	7	10	8	124
35.1	1	8	3	8	4	7	13	8	210
35.2	2	8	3	8	4	186	13	8	2186
35.3	3	8	3	6	4	7	13	8	2187
41.1	1	8	14	8	4	7	3	8	95
41.2	2	8	14	8	4	7	3	22	1909
41.3	3	8	14	8	4	7	3	22	1909
42.1	1	70	14	6	4	84	10	8	2169
42.2	2	145	14	6	4	76	10	8	2181
42.3	3	70	14	6	4	84	10	8	2169
45.1	1	14	14	8	4	50	10	8	2170
45.2	2	14	14	8	4	50	10	8	2170
45.3	3	14	14	30	4	50	10	8	2171
78.1	1	14	3	6	4	2	10	8	2172
78.2	2	70	3	6	4	2	10	8	2173
78.3	3	14	3	6	4	2	10	8	2172
82.1	1	35	4	4	4	185	26	4	2185
82.2	2	4	4	4	4	34	26	4	646
82.3	3	8	7	8	4	7	26	4	2174
132.1	1	54	93	10	36	184	113	242	2188
132.2	2	53	31	10	36	83	113	111	1019
132.3	3	53	31	10	36	83	113	111	1019
140.1	1	8	3	6	4	7	10	8	915
140.2	2	70	3	6	4	7	10	8	619
140.3	3	70	3	6	4	7	10	8	619
166.1	1	8	7	8	4	7	10	8	144
166.2	2	70	7	8	4	7	10	8	661
166.3	3	63	7	8	4	7	10	8	2175
173.1	1	70	14	8	4	7	249	8	2189
173.2	2	70	14	8	4	7	10	8	656
173.3	3	8	14	8	4	7	10	8	124
185.1	1	8	3	8	4	7	10	8	392
185.2	2	8	3	8	4	7	10	8	392
185.3	3	8	94	8	4	7	10	8	2184
193.1	1	31	5	5	12	2	21	5	2176
193.2	2	31	95	5	12	2	21	5	2190
193.3	3	31	5	5	2	2	21	5	375

New alleles and DSTs are highlighted in red

Fourteen undocumented alleles and 28 diploid sequence types (DSTs) unique to this study group were identified and added to the *C. albicans* MLST database by the curator. New allele numbers and DSTs are highlighted in red (Table 6.3). Strain

consistency (indicated by the DST number) was less distinct from visit to visit in the same person unlike ABC genotyping which showed a more consistent pattern. This data was simplified by separating the DST numbers at each visit (Table 6.4). Nine subjects (52.9%) carried the same strain at 2 visits, and 8 subjects (47.1%) carried different strains at all visits (Table 6.4). It should be noted that genetically similar strains might have divergent DST numbers. The more sensitive polymorphism of the alleles was used to assess strain relatedness in Figures 6.8, 6.9 and Table 6.5.

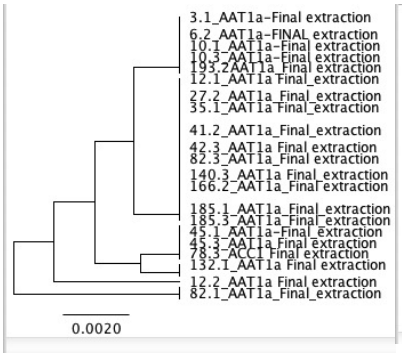
Table 6.4 Genotypes with DST numbers of *C. albicans* isolated from the HIV positive women over 6 months

No.	**Patient**	**DST of *C. albicans* at various visits**			**Same DST at 2 visits**	**Same DST at 3 visits**	**Different DST at all visits**
1	3	2164	808	2177			X
2	6	2178	2179	2165			X
3	10	2182	2166	2166	X		
4	12	2167	2180	2168			X
5	27	2183	124	124	X		
6	35	210	2186	2187			X
7	41	95	1909	1909	X		
8	42	2169	2181	2169	X		
9	45	2170	2170	2171	X		
10	78	2172	2173	2172	X		
11	82	2185	646	2174			X
12	132	2188	1019	1019	X		
13	140	915	619	619	X		
14	166	144	661	2175			X
15	173	2189	656	124			X
16	185	392	392	2184	X		
17	193	2176	2190	375			X
					9/17 (52.9%)	**0/17 (0%)**	**8/17 (47.1%)**

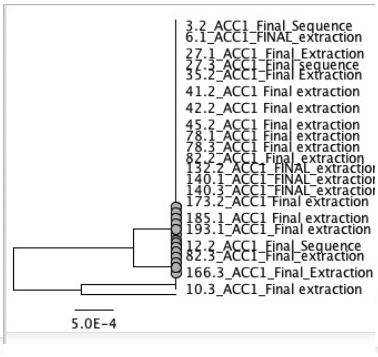
New DSTs Unique from this study are highlighted

Figure 6.8 UPGMA Dendrogram to show relatedness of the *C. albicans* alleles

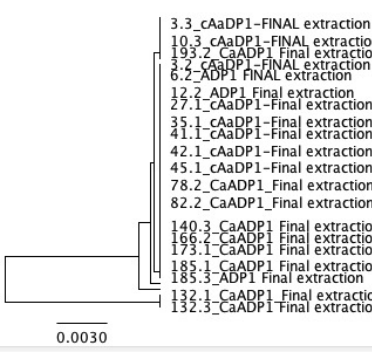
AAT1a



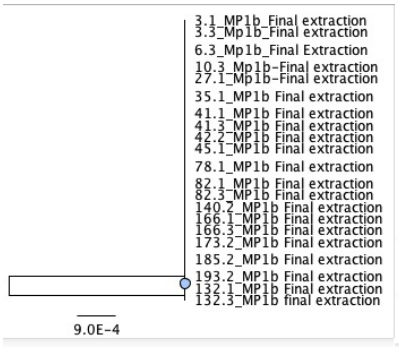
ACC1



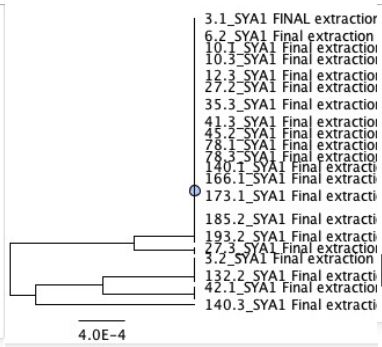
CaADP1



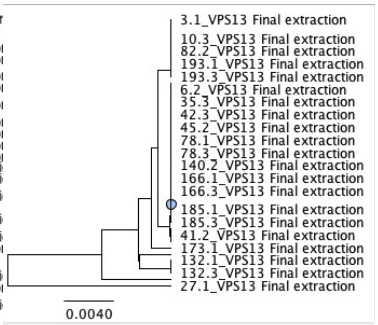
MP1b



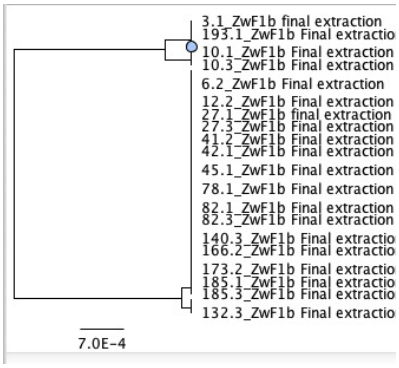
SYA1



VPS13

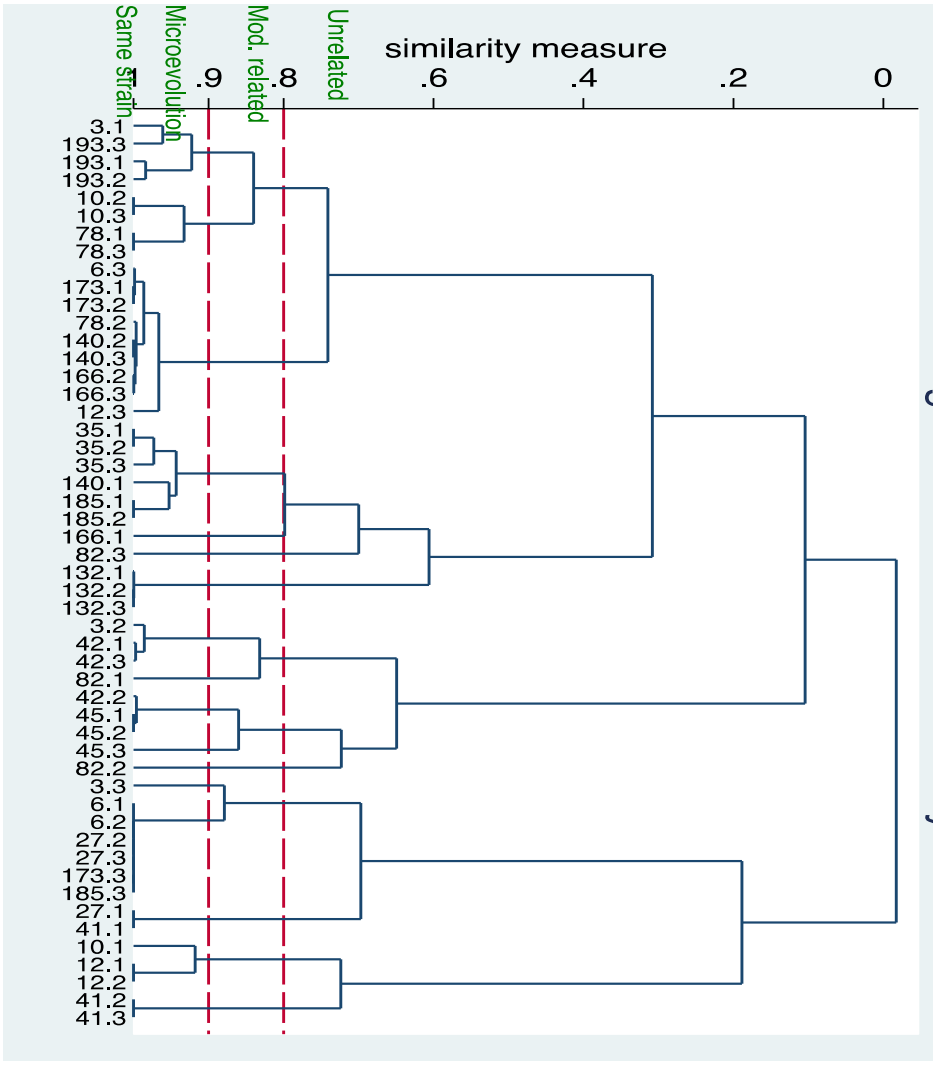


ZWF1b



Where 3.1, 3.2 & 3.3 means patient number 3 and isolate of *C. albicans* at visit 1, visit 2 and visit 3 respectively. This is applicable to all the strains described in this dendrograms

Figure 6.9 UPGMA Dendrogram to show relatedness of the *C. albicans* DST genotyping



*Mod. related= moderately related

Where 3.1, 3.2 & 3.3 means patient number 3 and isolate of *C. albicans* at visit 1, visit 2 and visit 3 respectively. This is applicable to all the strains described in this dendrograms

Table 6.5 Similarity matrix table and percentage similarities for all *C. albicans* strains

No.	Patient	Parameters	Comparison of strains			Final interpretation
			Visit 1 & 2	**Visit 1 & 3**	**Visit 2 & 3**	
1	3	Isolate	3.1 to 3.2	3.1 to 3.3	3.2 to 3.3	Reinfection
		% Similarity	<80	<80	<80	
		Interpretation	different	different	different	
2	6	Isolate	6.1 to 6.2	6.1 to 6.3	6.2 to 6.3	Reinfection
		% Similarity	100	<80	<80	
		Interpretation	same	different	different	
3	10	Isolate	10.1 to 10.2	10.1 to 10.3	10.2 to 10.3	No reinfection
		% Similarity	81-90	81-90	100	
		Interpretation	Moderately related	Moderately related	same	
4	12	Isolate	12.1 to 12.2	12.1 to 12.3	12.2 to 12.3	Reinfection
		% Similarity	100	<80	<80	
		Interpretation	same	different	different	
5	27	Isolate	27.1 to 27.2	27.1 to 27.3	27.2 to 27.3	Reinfection
		% Similarity	<80	<80	100	
		Interpretation	different	different	same	
6	35	Isolate	35.1 to 35.2	35.1 to 35.3	35.2 to 35.3	No reinfection
		% Similarity	100	91-99	91-99	
		Interpretation	same	Micro evolution	Micro evolution	
7	41	Isolate	41.1 to 41.2	41.1 to 41.3	41.2 to 41.3	Reinfection
		% Similarity	<80	<80	100	
		Interpretation	different	different	same	
8	42	Isolate	42.1 to 42.2	42.1 to 42.3	42.2 to 42.3	Reinfection
		% Similarity	<80	91-99	<80	
		Interpretation	different	Micro evolution	different	
9	45	Isolate	45.1 to 35.2	45.1 to 35.3	45.2 to 45.3	No reinfection

		% Similarity	100	81-90	81-90	
		Interpretation	same	Moderately related	Moderately related	
10	78	Isolate	78.1 to 78.2	78.1 to 78.3	78.2 to 78.3	No reinfection
		% Similarity	81-90	100	81-90	
		Interpretation	Moderately related	same	Moderately related	
11	82	Isolate	82.1 to 82.2	82.1 to 82.3	82.2 to 82.3	Reinfection
		% Similarity	<80	<80	<80	
		Interpretation	different	different	different	
12	132	Isolate	132.1 to 132.2	132.1 to 132.3	132.2 to 132.3	No reinfection
		% Similarity	100	100	100	
		Interpretation	same	same	same	
13	140	Isolate	140.1 to 140.2	140.1 to 140.3	140.2 to 140.3	Reinfection
		% Similarity	<80	<80	100	
		Interpretation	different	different	same	
14	166	Isolate	166.1 to 166.2	166.1 to 166.3	166.2 to 166.3	Reinfection
		% Similarity	<80	<80	100	
		Interpretation	different	different	same	
15	173	Isolate	173.1 to 173.2	173.1 to 173.3	173.2 to 173.3	Reinfection
		% Similarity	100	<80	<80	
		Interpretation	same	different	different	
16	185	Isolate	185.1 to 185.2	185.1 to 185.3	185.2 to 185.3	Reinfection
		% Similarity	100	<80	<80	
		Interpretation	same	different	different	
17	193	Isolate	193.1 to 193.2	193.1 to 193.3	193.2 to 193.3	No reinfection
		% Similarity	91-99	91-99	91-99	
		Interpretation	Micro evolution	Micro evolution	Micro evolution	
Summary:		**11 (64.7%) –reinfection, 6 (35.3%) – no reinfection (microevolution/same strain)**				

Where 3.1, 3.2 & 3.3 means patient number 3 and isolate of *C. albicans* at visit 1, visit 2 and visit 3 respectively. This is applicable to all the strains described in this dendrograms

Analysis of the individual alleles showed that they were closely related and not more than 5 clusters per alleles were present in Figure 6.8. This group of dendrograms shows the relationship of the individual alleles for subjects. The very few clusters present show the distinct relatedness. Compared with the dendrogram in Figure 6.9, there is less variability, which underscores the importance of strain typing using more than one locus. Some patients are not shown in some dendrograms due to the algorithm used to generate the dendrogram.

The dendrogram in Figure 6.9 from the combination of all the alleles in all the subjects showed 4 distinct clusters which has the highest Duda/Hart coefficient of 0.99. At 80% similarity (broken line above with the value of 0.8), 11 clusters are apparent and 17 clusters at 90% similarity. The largest cluster contained 17 isolates from 9 subjects, followed by 14 isolates from 8 subjects. The other clusters had 11 isolates from 6 subjects and 9 isolates from 4 subjects. Strains from individual subjects were found in more than 1 cluster. A correlation matrix was generated from the dendrogram that showed the relatedness of the strains (Table 6.5). The strain relatedness in Table 6.3 was confirmed with the dendrogram. Similar strains are grouped (for instance, 10.2 and 10.3 have the same DST number and have 100% similarity. The detailed correlation (similarity) between the strains is displayed in the correlation matrix table (Table 6.5). In this table, 100% similarity between strains were interpreted as same strains, >90% similarities as microevolution, >80% similarities as moderately related and <80% similarities as different strains (Boriollo et al., 2006; Soll, 2000). These results showed that in 11 subjects (64.7%) reinfection with different strains occurred in the follow up period while 6 (35.3%) had no

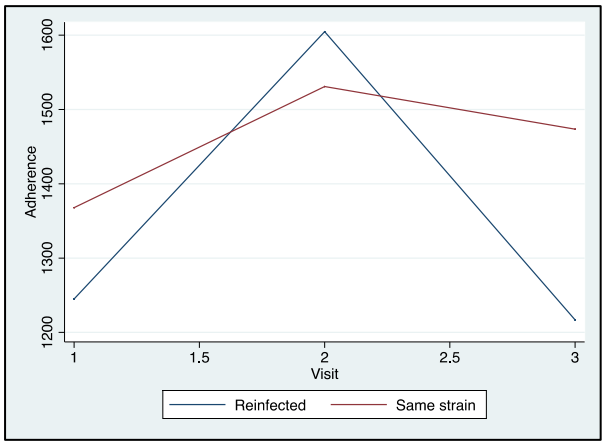
reinfection. These subjects either carried the same strains all the time or the strains at follow up visits were closely related including microevolution.

6.3.3 Association between virulence and genotypes

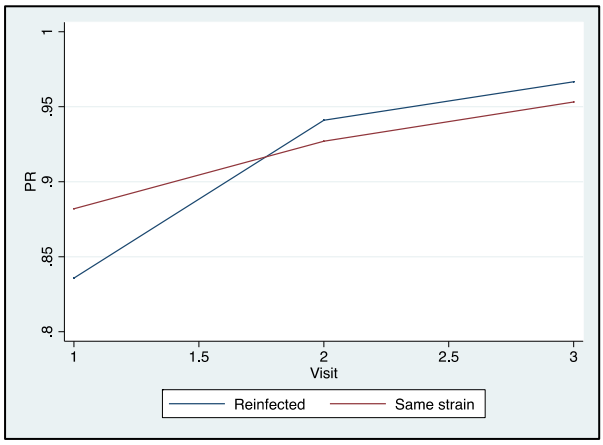
An attempt was made to investigate an association between the virulence of *C. albicans* isolated from the women who were reinfected with other strains during the study period and women who carried the same strain throughout the study period. The results are shown in Figure 6.10 and Table 6.6. Proteinase production was significantly high in *C. albicans* isolated from the women who were reinfected with other strains ($p=0.0033$) compared to the women who carried the same strains of *C. albicans* for 6 months. There was no difference in the adherence property and phospholipase production.

Figure 6.10 Virulence properties of *C. albicans* on follow up visits comparing isolates from women reinfected with other strain and women who carried the same strain

Adherence ability



Proteinase production



Phospholipase production

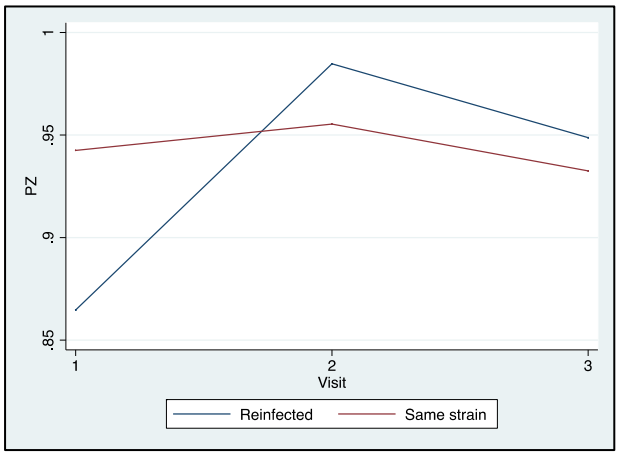


Table 6.6 Virulence properties of *C. albicans* isolated from the women reinfected with other strains and women who carried the same strain throughout the study period.

Category	Virulence factor	Mean Square	F	p value
Reinfected with different strains	Adherence	514425.545	2.57	0.1013
	Proteinase	.052885627	7.71	0.0033*
	Phospholipase	.041711997	3.08	0.0683
Same strain all visits	Adherence	41021.5556	0.20	0.8222
	Proteinase	.007774386	0.99	0.4057
	Phospholipase	.000786055	0.07	0.9373

6.4 Discussion

6.4.1 Comparison of genotyping methods

ABC genotyping is not the gold standard for *Candida* genotyping; however, it enabled the comparison of genotypes from visit to visit in our study population. Overall, the most predominant genotype was the B type in 31 strains (60.8%) in Table 6.2. The genotype B was also prevalent in HIV infected individuals unlike genotype A, which is the most predominant in the general population and patients with candidiasis (Vidotto et al., 1999). Furthermore, ABC genotyping revealed a predominance of genotype A in HIV infected and cancer patients (Kumar et al., 2006); however, there was no association between the ABC genotype and antifungal resistance. The predominance of genotype B requires further investigation to determine if this is a general trend in our study population or peculiar to HIV patients. This is necessary as an association was found between genotype B and vulvovaginal

candidiasis and itraconazole resistance (Li et al., 2008; Liu et al., 2009). A similar study found a predominance of genotype C in oral leukoplakia, especially the potentially premalignant *Candida* leukoplakia (Abdulrahim et al., 2013). Therefore there is a need to determine association of the predominant genotype with unusual virulence and antifungal resistance in our population.

In spite of the limitations, ABC genotyping can be used to monitor strain types at different body sites in critically ill patients. The same ABC strain colonising multiple sites was found to predict the development of candidemia (Chaves et al., 2012). However, ABC genotyping is not the most sensitive technique and therefore it may not have fulfilled our objective of determining strain persistence.

6.4.2 Carriage and strain diversity

Using more than one genotyping method allowed comparison of the discriminatory abilities of ABC and MLST techniques. ABC genotyping showed a consistent *C. albicans* strain in 16 of the 17 subjects followed up for 6 months. On the contrary, consistent strain carriage was not present with MLST genotyping using the DST numbers. Only 6 subjects carried the same strain in all the visits, the majority 11 or 64.7% were reinfected with other strains (Table 6.5). This finding is not surprising as MLST uses a scheme involving more areas of polymorphism unlike ABC genotyping that employs one.

The observed reinfection in the majority of women could have been due to the following reasons. Firstly, it may be due to gene re-assortment and duplication of a colonising strain. It is known that succeeding clones may acquire an alteration in

genetic information. The genotyping techniques would interpret this as either microevolution or closely related. Secondly, exogenous strains may account for the differently isolated strains in multiple visits and thirdly, a different strain may be sampled at subsequent visits when multiple colonising strains are present. The determination of the mechanism at play is not an easy task (Odds, 2010). A predominant strain is usually present in the same individual and may colonise multiple sites (Odds et al., 2006; Verma et al., 2003) or multiple colonising strains may be present in the same individuals (Samaranayake et al., 2003). The differences in the observations might have arisen from the very dynamic nature of strain evolution. In addition, infection and other environmental factors may trigger microevolution of colonising strains. Apart from microevolution, individuals may acquire strains from the environment, family members, from hospital staff and other patients in a hospital setting. Multiple strains may also be present in the same site or in different body sites in the same person, (Kam and Xu, 2002; Taylor et al., 2003).

From the clonal pattern of our isolates in the dendrograms in Figure 6.8 and 6.9, there were less than 4 distinct clonal patterns with many strains belonging to the branch of a tree. It may be inferred that the closely related but different strain types found in our population may reflect the ability of the yeast to shuffle chromosomes due to the need to adapt to changing environmental conditions. This usually ensures adaptation and survival. As indicated, a clonal and diploid organism like *C. albicans* yields a greater variety of chromosomal patterns compared with a purely clonal and haploid organism (Odds, 2010). The probable scenario in our subjects is that both microevolution of colonizing strains and reinfection with different strains are occurring.

Microevolution or microvariation has been previously defined as a difference of only one allele of the seven MLST genes when comparing two strains (Jacobsen et al., 2008). It may arise due to changes in karyotype or through a loss of heterozygosity. These changes, which result from stimuli within the environment lead to rearrangement of genomic sequences. Microevolution can occur in either the state of colonisation or infection and it not only fosters environmental adaptation, it also diversifies the strain molecular characteristics (Bougnoux et al., 2007).

Microevolution of yeast strain may also indicate a transition to a pathogenic state or a change towards antifungal resistance. Some workers have suggested that strain or clade type may be related to virulence (Tavanti et al., 2005). Our results showed that the newly acquired oral cavity strains produced large amount of proteinase. Although there was no development of infection in these subjects during this follow up period, these women may be at the risk of developing infection, particularly if other risk factors permit.

A previous South African study on strains isolated from patients with clinical infection did not show any strain being specifically related to oropharyngeal infection (Blignaut et al., 2002b). The previous use of antifungal agents was subjectively determined in our study population and most of the subjects were not certain about taking antifungal agents and the type of these agent. However, a study that investigated antifungal resistance with *C. albicans* genotype did not find any relationship and many studies have yielded inconsistent findings (Girish Kumar et al., 2006; Liu et al., 2009). On the other hand, other workers have observed that Clade 1 in the MLST database has demonstrated a resistance to flucytosin (Odds and

Jacobsen, 2008). The role of such strain diversity in the light of the other multiple host factors that may lead to clinical infection in our study population would require further studies to address such objectives. This is in light of the low prevalence of antifungal resistance in our study population (described in Chapter 5) and the differences in antifungal use and prescription.

Reinfection involves colonisation by a strain from another body site or other individuals. This has been demonstrated in immune compromised subjects such as HIV infected, neonates and cancer patients. The danger is that such strains may be more virulent or more resistant to antifungal drugs thereby predisposing the carrier to mucosal or disseminated infections. Disseminated infections may sometimes be fatal (Bliss et al., 2008; Bougnoux et al., 2008a; Cliff et al., 2008). In our HIV infected subjects, their fluctuating immune status is a cause for concern. The danger of reinfection, particularly with highly virulent and resistant strains is high. This may occur when the immune status is low and requires constant vigilance and monitoring.

The eBURST has been used previously to determine the phylogeny of *C. albicans* yeast strains. The knowledge of the ancestry of colonizing strains is important from epidemiologic and pathogenic viewpoints (Tavanti et al., 2005). However, eBURST analysis of our samples on the MLST website could not be performed because it was not functional. Several messages to the primary curator at the Imperial College in London have not yielded results. It should be noted, however, that the reliability of the eBURST algorithm in predicting ancestry and correctly grouping genetically similarly strains into clades has been questioned (Turner et al., 2007). It appears to perform better for mutations rather than recombination. In *C. albicans*, recombination

occurs more frequently (Turner et al., 2007). For these reasons the UPGMA dendrogram was used to analyse and interpret our results.

From earlier studies, some of the previously typed strains in our subjects belonged to clade 4, which carried the most frequently occurring clonal complex of 124 (Alastruey-Izquierdo et al., 2013). The relationship between the strains sampled at different visits is particularly striking in Subjects 27 and 173 where the strains at all visits belonged to the same clonal cluster and clade even though the DSTs varied slightly (Table 6.3). The new DSTs are yet to be fully characterised since they are yet to be isolated in other locations. The determination of their characteristics requires further investigation. To reduce the possibility of missing multiple colonising strains, multiple yeast colonies need to be sampled and patients followed up for a longer period of at least 12 months (Samaranayake et al., 2003). While this is possible, the logistics required are enormous besides the difficulty of keeping contact with subjects to arrange a follow up visit. Genotyping and sequencing requires considerable amount of time and money. Perhaps, it is for this reason that many studies (including ours) on strain distribution and evolution in *C. albicans* only sampled single colonies (Odds, 2010; Pires-Goncalves et al., 2007).

In spite of the relationship noted in the strains in our subjects, scientists globally realise that the whole exercise of DNA typing to identify strains is evolving. The only foolproof means to conclude that two strains are related is to compare whole genome sequences (Soll, 2000).

6.4.3 Newly identified strains and demographic characteristics

A study (Ca3 technique) on oral *C. albicans* strains from South Africa revealed a peculiar clade restricted to South Africa. It was surmised that the strains were established commensals restricted to the location where they were isolated (Blignaut et al., 2002b). In that study, samples were taken once and patients were not followed up to monitor changes. Since then, no study has genotyped oral *Candida* yeast samples to determine the prevalent strains in that area and the persistence of such strains. It was difficult to confirm if the strains in our subjects were related to the *C. albicans* clade isolated previously in South Africa since the genotyping methods were different. The basis for strain typing in Ca3 probing is the variability of the major repeat sequences on the *Candida* genome, while MLST employs single nucleotide polymorphism of selected housekeeping genes. Some workers have however identified a moderate correlation in the clades identified by both methods in spite of the differences in technique (Odds et al., 2007; Odds and Jacobsen, 2008; Tavanti et al., 2005). The advantages of MLST genotyping compared to Ca3 probing include lower cost of processing, detection of genetic variations from single nucleotide pleomorphisms in the same subjects followed up over time (which Ca3 cannot detect). The MLST global database is also considerably larger (Chowdhary et al., 2006).

Our study confirmed that there are native strains that have not been isolated previously using MLST. While the new strains are oral strains, some of the previously identified strains we found in the mouth have been previously isolated from blood samples taken from South African subjects. The details of the global and South African strain types on the MLST database is shown in Appendix 9.5 and 9.6. To date, 74 strains of *C. albicans* from South Africa are on the MLST database and 28 of

these are the newly isolated strains from our samples and they constitute the only oral strains. Obtaining the same strain in multiple body sites is an independent risk factor for invasive candidiasis in immunocompromised subjects (Chaves et al., 2012). The need to maintain adequate immune competence becomes very crucial in our study population to obviate colonisation of multiple body sites by the same strains.

The distribution of *C. albicans* in Appendix 9.5 shows a global distribution. The genetic diversity is as varied as the distribution. This pattern may have arisen due to recent acquisition of the yeast by human beings, which has compelled adaptation to different environments. Alternatively, it is possible that the yeast has been evolving with humans right from inception (Odds, 2010). Distinct strains have been tied to particular geographic regions, although several strains have been found in multiple locations (Takakura et al., 2008). Some of the previously documented strains present in our subjects have been found elsewhere. DST 95 is the most ubiquitous, found in UK, Switzerland, India, Pakistan, Australia, Morocco, France, and Austria. DST 124 has been previously isolated in the UK, Israel, France, USA, Morocco, Austria (Appendix 9.8). Geographic localisation of *C. albicans* strains has become more difficult due to extensive human travel and interaction. It is therefore necessary to further evaluate the newly identified strains to determine distributional and virulence characteristics. This should be a continuous exercise in order to monitor evolutionary changes.

One major disadvantage of the MLST system is that the current scheme does not include any gene from chromosome 3 and 5 of the *Candida* genome. An ideal MLST system would be to have representative genes from both ends and centre of each of

the eight chromosomes, thereby requiring 24 housekeeping genes for MLST typing. While that may be more discriminatory than the present scheme, the future of genotyping for any organism is whole genome sequencing. For a yeast like *C. albicans*, single nucleotide pleomorphisms on all the eight chromosomes could serve as the basis for strain typing in the near future (Odds, 2010).

6.4.4 Conclusion

MLST genotyping identified *C. albicans* unique to this region with previously undocumented DSTs and alleles in our study population. Although ABC genotyping showed that the majority of the subjects carried the same genotype for all three visits, MLST technique identified a mixture of genetically related and unrelated strains in follow up visits. This indicates microevolutionary changes and reinfection with different strains occurring in our subjects. Newly acquired strains produced more proteinase, suggesting that reinfection with different strains could be one of the reasons for the reoccurrence of candidiasis. Therefore, the factors that are responsible for these changes and the implications of virulence and clinical infection require further investigation. Apart from newly identified strains, some of the strains identified in oral samples have been previously isolated from blood samples. The need to further evaluate and monitor strain epidemiology becomes more paramount as the immune status of individuals is constantly changing. In addition, the role of human travel and other factors that may predispose to development of infection also require further evaluation.

CHAPTER 7: SUMMARY AND CORRELATION OF RESULTS

7.1 Summary of results

Oral candidiasis (OC) has been associated with the HIV pandemic and it still poses a threat to the life and well being of an HIV positive individual. When it occurs in HIV infection, it is often due to severely depressed immunity or failure of treatment in patients on antiretroviral therapy. Apart from the diagnostic and prognostic importance of oral candidiasis, colonised individuals harbouring opportunistic pathogens like *Candida* species can progress from mucosal infection to invasive and life threatening disseminated infections (van der Meer et al., 2010).

With the introduction of highly active antiretroviral therapy (HAART) in 1996, the prevalence of oral candidiasis abated but the condition did not disappear. Furthermore, HAART has not significantly changed the unusual high prevalence of oral colonisation by *Candida* in HIV positive subjects (Yang et al., 2006). In addition, in sub Saharan Africa many HIV positive patients are still prone to OC due to inadequate HAART coverage which is almost 50% of the 23.2 million infected (Decroo et al., 2013). Furthermore, some HIV positive subjects on HAART may not comply with antiretroviral therapy for a variety of reasons (dos Reis HL et al 2011). As a result, opportunistic infections such as OC often occur and impact negatively on the quality of life and increase the likelihood of unfavourable outcomes (dos Reis HL et al 2011).

The factors that predispose to oral colonisation and oral candidiasis are still very controversial. The exact role of the virulence and type of *Candida* organism, and the host immune status remain unresolved. Similarly, the molecular epidemiology of *Candida* yeasts; their virulence and antimicrobial characteristics are poorly understood in the HIV positive population in Africa.

The study, therefore, investigated the relationship between carriage rates, virulence and genotypic diversity of *Candida*, social and clinical factors and the development of clinical infection. A series of objectives were drawn to investigate these factors. A summary of results is depicted as a flow diagram (Figure 7.1) and described here.

1. To examine the carrier rate of *Candida* among HIV-positive women: prevalence, types and quantities of *Candida*, influence of oral hygiene, social and clinical factors (Chapter 3).

Of the 197 women screened, 117 (59.39%) were colonised with yeasts. Colonisation rate has dropped from 81.3% reported in a previous study undertaken in the same setting (Patel et al., 2006). Oral hairy leukoplakia was the most common oral manifestation of HIV infection, in 27 (13.71%), followed by oral candidiasis in 15 (12.8%) of those colonised. Without doubt, the lower rate of oral colonisation and candidiasis may be due to the introduction of HAART because the majority of the women 186 (94.42%) were on antiretroviral therapy at the time of recruitment. In the 117 colonised with yeasts, 85 (72.65%) were colonised with *C. albicans* which shows that *C. albicans* is still the predominant species. *C. dubliniensis* was the most common non-albicans *Candida* in 14 subjects (11.97%), which is higher than elsewhere (Owotade et al., 2013). While it is beneficial that the prevalence of oral colonisation and candidiasis has reduced, the potential for recurrent mucocutaneous

and invasive infection is still a cause for concern. *Candida* with its repertoire of pathogenic capacity has not been completely eliminated and its presence in immune suppressed HIV positive individuals is not reassuring (Anwar et al, 2012).

Principal factors associated with colonisation by *Candida* yeasts include concurrent infection with tuberculosis (p=0.03), diabetes mellitus (p=0.005) and not being on antiretroviral therapy (p=0.003). This is of concern because South Africa has one of the highest estimated TB rates in the world. In addition, *Candida* colonisation could be due to reduced immune response since low CD4 counts were also associated with the colonisation (p=0.022) and higher *Candida* counts (r= -0.26, p=0.006). However, increased HAART coverage has been proposed as a means of stemming the rising incidence and mortality of HIV related TB in South Africa (Pretorius et al 2014), which will have additional benefits for other opportunistic infections such as oral candidiasis.

Other associated factors with colonisation were the wearing of dentures (p=0.022) and having a high DMFT (p=0.019). Adherence ability of *Candida* species to oral prostheses and association with dental caries is well described (Raja et al., 2010; Witzel et al., 2012) and this may explain the findings in this study. Although 86.87% of patients were educated only 67% claimed to brush their teeth twice a day. However, only 32% visited dentists, which is necessary for the diagnosis and treatment of dental caries (Table 3.1, 3.2). This may be the probable reason why the median DMFS (Decay Missing Filled Surfaces) was 20 with IQR 27 and range 0-102 (Table 3.4). Consequently, 30/148 surfaces either had caries, restorations or were missing. If these patients were not visiting dentists regularly, these surfaces probably

became decayed and may have been colonized by *Candida* as well (Witzel et al., 2012).

Results in this section stress the importance of implementing HAART to improve the immune response and maintain lower counts of *Candida* in the oral cavity. These results also highlight the importance of oral hygiene and monitoring of carious status. These measures should reduce the chances of recurrent oral candidiasis.

2. To determine the virulence of *C. albicans* during the study period and the influence of quantity and/or virulence of *C. albicans* on the development of oral candidiasis during the study period and to examine the occurrence and role of non-*C. albicans* yeasts in the development of candidiasis in the study period (Chapter 4).

The virulence characteristics of the yeasts isolated from the *Candida* carriers fluctuated and did not correlate with the CD4 counts in the 6 months follow up period. In addition, virulence factors did not differ in patients with oral candidiasis and those without infection. However, the yeast count was higher in the infected group. These results suggest that a high count without a commensurate increase in the virulence may cause infection due to a cumulative effect. These results stress the importance of maintaining low counts of *Candida* in the oral cavity.

In spite of the lower prevalence of non-albicans *Candida* yeasts (21%) in the oral cavity, their role in oral candidiasis cannot be excluded. Therefore, virulence characteristics of *C. albicans* and non-albicans *Candida* in the colonisation state and during active infection were compared. Virulence properties were significantly higher in *C. albicans* isolated from colonised and infected individuals. *C. albicans* is an aggressive yeast and produce invasive hydrolytic enzymes when conditions are

favourable (Ells et al, 2014). However, non-albicans *Candida* isolated from the patients with oral candidiasis produced a significantly larger amount of phospholipase. In addition, this enzyme is responsible for tissue damage and is clearly implicated in the pathogenesis of oral candidiasis due to non-albicans *Candida*. In the oral cavity, non-albicans *Candida* and *C. albicans* usually reside together. For this reason, their individual role in pathogenesis cannot be determined. However non-albicans *Candida* yeasts are capable of causing systemic infections (Al-Rawahi and Roscoe, 2013) which establishes their role in the pathogenesis of infection.

Clinical infection is a complex process due to many other factors apart from the virulence of the yeasts. Although we could not relate the limited virulence characteristics (adherence ability, proteinase and phospholipase production) with infection, their role, fitness attributes of the yeasts and host factors in initiating clinical infection need further investigation. As our study follow up time is limited, the likelihood of developing clinical infection in our population is very likely to occur beyond the study period as yeasts constantly elaborate virulence factors while host immunity fluctuates. In addition, the current antifungal armamentarium is still limited; hence, new antifungal strategies are being developed to target the virulence properties of yeasts (Peirce and Lopez-Ribot 2013). This confirms that the study of the virulence attributes of the yeasts remains relevant.

Suppression of *C. albicans* with antifungal drugs sometimes causes over growth of intrinsically resistant non-albicans *Candida* (Kamikawa et al., 2014). In addition, the regular use of antifungal agents for recurrent oral candidiasis favours the development of drug resistant strains of *C. albicans* (Bondaryk et al., 2013). For this reason, the

antifungal sensitivity of *C. albicans* isolated from our study population was investigated.

3. To study the antifungal sensitivity of *C. albicans* isolated from HIV positive women (Chapter 5).

Antifungal resistance was low in the study population. The prevalence of resistance to posaconazole was 1.7% (2/120), while it was 0.8% i.e. 1/120 of the strains were resistant to echinocandins and the azole antifungals, fluconazole, itraconazole and voriconazole. Posaconazole resistance is a problem because it is a second-generation extended-spectrum triazole and is recommended for prophylaxis of invasive fungal diseases in immunocompromised hosts (Lyseng-Williamson KA, 2011). No antifungal resistance to amphotericin B was observed. This is contrary to the finding of Blignaut et al. (2002) who reported 8.5% resistance to this drug. Large studies are required to confirm these findings. Azole resistance was only found in one subject which supports the theory that multi-azole resistance is probably due to previous repeated antifungal exposure (Pfaller, 2012). Antifungal resistance was low in our study population, nonetheless, the isolation of strains resistant in particular to echinocandins is a cause for concern because it is a new drug. Furthermore, this resistance could be the cause poor outcomes and increased fatality (Parmeland et al, 2013). Furthermore, a single resistant strain of *C. albicans* in an immune compromised individual can rapidly progress from thrush to fatal candidemia (Gautam et al, 2010). The risk factors for antifungal resistance in our study population needs to be investigated further. In addition, azole non-responsive patients should be treated with other types of antifungal drugs.

4. To determine and compare the genotypes of *C. albicans* in those colonised women at intervals during the follow up period (Chapter 6).

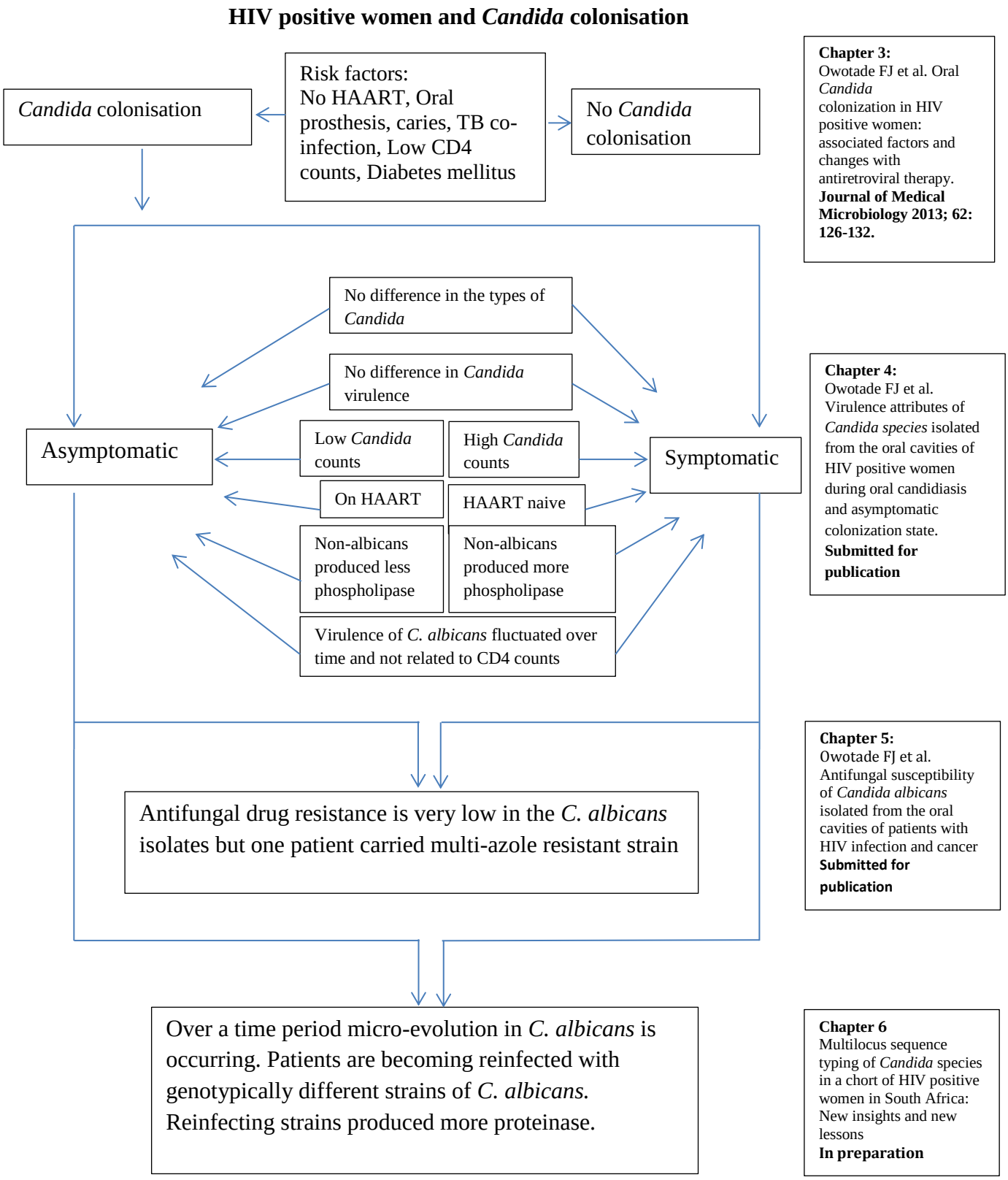
The present study has limitations because the study investigated a very dynamic and complex process with constantly variable host and yeast factors. Furthermore, these factors are constantly interacting at the molecular, microbial and subclinical level (Thein et al., 2009).

ABC genotyping revealed a predominance of genotype B in 31(60.8%) of the 51 strains isolated from the 17 women sampled at three visits followed by genotype A i.e. 14 (27.4%) and genotype C in 6 (11.8%). Sixteen (94.1%) out of the 17 women carried the same ABC genotype for all the three visits and showed the less discriminatory nature of this technique. However MLST, a more discriminatory technique showed that only 6/17 (35.3%) women carried the same strains at all three visits whereas 11 subjects (64.7%) were reinfected with different strains. Further analysis showed that both microevolution and reinfection with other strains was occurring in our study population. The source of reinfection could be colonization from other parts of body, the living environment or family members. In our study population 99% of patients had families of between 1-10 members. In addition, 24% of patients shared their food during meal times (Table 3.1). This may have been the reason why large number of patients 11/17 (64.7%) were reinfected with different strains over a 6 month period. This observation may be significant because virulence activity and antifungal resistance have been associated with particular strain types and their source (Nawrot et al, 2008). Reinfection may lead to recurrent oral candidiasis if highly virulent or drug resistant strains are acquired. Although the correlation between virulence factors and clinical infection was not established (Chapter 4, Table

4.8), newly acquired *C. albicans* strains during the follow up period produced large amount of the tissue damaging proteinase (Table 6.6 and Figure 6.10) which suggests possible initiation of pathogenic activity. Highly virulent newly acquired strains of *C. albicans* may even lead to severe local and disseminated infection in HIV positive patients if the immune status becomes severely compromised.

In the present study, 14 previously undocumented alleles and 28 diploid sequence types (DSTs) unique to our study population and region were identified and added to the *C. albicans* MLST database by the curator. As previously shown by Blignaut et al, (2002) these results confirmed that oral cavity isolates of *C. albicans* in the African population are different from those documented elsewhere and many native strains are yet to be discovered.

Figure 7.1 Illustrative summary of principal findings



In this study, colonised women were distinctly different from those who did not carry yeasts. In addition, they were more likely to be more immune compromised because they were not on HAART, had tuberculosis, diabetes mellitus and a low CD4 count. The low prevalence of clinical infection and contributing factors to being symptomatic were less distinct. However, the absence of HAART and a high yeast count contributed to the development of clinical infection. Obviously, the compromised immune status aided yeast multiplication leading to infection. Although the virulence of the yeasts did not increase during infection, other virulence factors or fitness attributes of the yeasts may have contributed to yeast infection. Furthermore, the study captured a brief part of a continuum where infection may supervene if the circumstances are favourable. Although the non-albicans *Candida* yeasts were not as aggressive as *C. albicans,* their role in the development of infection needs to be evaluated further.

The process of infection is complex and involves many factors. The level of virulence versus the state of immunity required to initiate infection has not been determined. In this study both fluctuated throughout the follow up period without definite correlation. With the present state of knowledge, it is extremely difficult to produce a model to explain when the interaction between the host immune status and a commensal yeast will lead to clinical infection (Cottier and Pavelka, 2012).

The genotyping of the yeast further revealed an on-going dynamic process in the subjects. Most subjects appear to be reinfected with other strains, which may play a role in colonisation and infection. Although resistance to antifungal therapy remains low, with reinfection and microevolution occurring, the low resistance pattern may change and the few resistant individuals may develop life threatening candidemia.

With this complex and dynamic process, constant monitoring of the intervening factors is not just necessary but highly recommended.

7.2 Recommendations

- Efforts should be made to improve HAART coverage and retain enrolment in African subjects. This can be further achieved by going into the communities and not waiting for the patients to come to tertiary hospitals such as Charlotte Maxeke (Decroo et al., 2013).
- It is important to keep the yeast count low in the colonised HIV positive subjects. Simple mouthwashes such as triclosan are effective (Patel et al., 2008). This is particularly crucial for patients wearing dentures and other forms of prosthesis.
- Oral examinations should be conducted with every clinic visit of HIV positive patients. Attention should be paid to other factors that may encourage oral yeast colonisation such as caries and immune status as reflected by the CD4 count. Early access to oral care to prevent complications is advocated in HIV infection (Liberali et al., 2013).
- The antifungal resistance profile of *Candida* in colonised patients should be constantly monitored and guidelines strictly followed when prescribing antifungal drugs.
- Attention should be paid to personal and oral hygiene in order to minimise reinfection (Muzurovic et al., 2012).

7.3 Limitations of the study

- Genotyping and virulence of the strains could not be undertaken for more than one colony due to the complexity of logistic requirements ie 117 patients X 3 visits X 2 to 5 colonies: identification, virulence properties, genotyping with 7 genes and sequencing reverse and forward. It is possible that the same strain or new strains may have been missed in the follow up visit. Furthermore, the cost of investigating is an additional limiting factor.
- The role of virulence in infection is ideally studied in the host environment where the immune and other host factors directly interact with the virulence of the organism. Apart from animal models of *Candida* infection, studying the virulence of *Candida* species in human volunteers is extremely difficult. Most studies still rely upon *in vitro* snapshot observations.
- Even when strains are totally identical using current molecular methods, it cannot be assumed that strains are the same. This will remain a limitation until the whole genome sequencing is available for biological species.
- Follow up is difficult in any cohort of HIV positive individuals. Uncertain social and psychological status, unforeseen illnesses, complications and even death are reasons for attrition. Therefore out of 117 eligible subjects, only 17 women were available at follow up visits and carried *C. albicans*. A larger group may have yielded more information.

- The role of other possible contributory factors to colonisation, such as history of previous antifungal use or previous *Candida* infection could not be accurately determined. Most of the subjects were not able to accurately recall information that was not in their case notes. Even the information they gave may be biased.
- *C. albicans* is a commensal carried by women in their oral cavities, vagina and gut. Ideally all these cavities should have been sampled to isolate *C. albicans* and genotype. This would have clarified the source of transmission of *C. albicans* between body cavities of an individual. However, it was impossible to collect samples from these cavities for many reasons. Therefore patients classified as being reinfected with new strains of *C. albicans* in Chapter 6 may have acquired these strains from other body cavities.

7.4 Future Research

- The epidemiology of oral candidiasis in HIV infection should be further explored with larger regional surveys that include rural hospitals and communities. Thus risk factors and other yeasts may be further clarified compared to data from subjects attending a tertiary level clinic such as the Charlotte Maxeke Hospital.
- The role of the risk factors should be assessed in males and children to determine if the pattern is similar in all three groups.

- Other virulence and fitness factors of *C. albicans* not evaluated here for example elaboration of heat shock proteins should be studied to determine their role in oral infection.
- Mother-child pairs who are HIV positive should be studied to determine whether they carry the same strains and have acquired resistant strains from their mothers.
- The role of oral strains that may cause disseminated infection in our study population needs to be further explored. Studies show that disseminated infection can occur without mucosal infection because oral strains have been isolated from blood samples.
- Studies are needed to explore the demographic and pathogenic characteristics of the new genotypic strains of *C. albicans* isolated in our study.
- Antifungal susceptibility profile of *Candida* organisms from other setting should be performed to determine whether the resistance level is similar.
- The role of non-albicans *Candida* in colonisation and oral infection needs to be clarified.

7.5 Conclusion

In conclusion, in our study population of HIV positive women prevalence of oral candidiasis and colonization with *Candida* was low. Factors associated with oral *Candida* colonisation were tuberculosis, diabetes mellitus, low CD4 count, dental caries, wearing oral prosthesis and not being on HAART. Production of proteinase and phospholipase, and the adherence ability *Candida* isolated from these patients fluctuated over time. Although there was no relationship between virulence properties of *C. albicans,* CD4 count and symptomatic infection, there was an increase in the yeast count during symptomatic candidiasis. In addition, *C. albicans* produced significantly higher levels of virulence factors than non-albicans *Candida* isolated from both symptomatic and asymptomatic women. *C. albicans* isolates from these women were sensitive to commonly used antifungal agents. However, microevolution was evident in *C. albicans* isolates. The majority of women were became reinfected with extraoral or exogenous strains that produced more proteinase. In addition, new genotypic strains of *C. albicans* unique to this region were identified and placed in the MLST *C. albicans* database.

CHAPTER 8: REFERENCES

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CHAPTER 9: APPENDIX

9.1 Ethical clearance certificate

,UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Drs M Patel/F Owotade

CLEARANCE CERTIFICATE	M10102
PROJECT	Oral Candida in HIV Positive Women: Influence of Oral Hygiene, Clinical and Social Factors on the Carriage of Rates and the Influence of Virulence of the Organism on the Development of Clinical Infection (revised title)
INVESTIGATORS	Drs M Patel/F Owotade
DEPARTMENT	Clinical Microbiology & Infectious Diseases
DATE CONSIDERED	29/01/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 12/02/2014 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr M Patel

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

9.2 Questionnaire

A. BASELINE EXAMINATION FOR ALL PARTICIPANTS

DATE: **HOSPITAL NO:**

Willing to participate: Yes No

Available for 6 months: Yes No

Included in the study: Yes No

Contact details: Tel no:…….

Home Address:……

A**. BIODATA/DEMOGRAPHICS**

1. Study ID……. 2. Age last birthday (years)……

3. Nationality…….. 4. Tribe/Ethnic group…….

5. Marital Status- (1) Married☐ (2) Single☐ (3) Widow ☐ (4) Divorced/Separated☐

6. Highest Educational Attainment- (1)No formal schooling☐ (2).Elementary school☐

(3)High school☐ (4)College/University☐

7. Occupation……. 8. Annual Income …….

9. Number of children……

10. Ages of children…….

B. LIVING CONDITIONS/HABITS.

1. Area of residence……. 2. Household size…….

3. Number of rooms……

4. How often do you eat together from the same plate?

(1) All the time ☐ (2) Often ☐ (3) Occasionally☐ (4) Never☐

5. Do you routinely exchange utensils while eating?

All the time☐ Often ☐ Occasionally☐ Never☐

6. Do you premasticate (chew food in your mouth for your child and then feed them) food for your children before they have teeth? Yes☐ No☐

7. Are you currently breastfeeding a child? Yes☐ No☐

8. Are you a smoker? Yes☐ No☐

9. If your response to B9 was yes, how many packs of cigarette do you smoke in a day?……..

10. For how long have you been smoking (years) ?.....

11. How often do you brush your teeth daily? Once ☐ Twice☐ More than 2 times ☐

12. Have you ever being to a dentist before? Yes☐ No☐

13. Do you have a regular dentist? Yes☐ No☐

C. MEDICAL HISTORY☐

1. Have you been hospitalized before? Yes☐ No

2. Do you suffer from any of the following?

	Yes	No
High blood pressure/Hypertension		
Heart disease other than Hypertension		
Asthma		
Allergies		
Diabetes		
Liver disease		
Bleeding disorder		
Kidney disease		
Cancer		
Malaria		

Tuberculosis		

3. Are you currently on any medications? Yes☐ No☐

4. If yes, please list.

Steroids ☐ Antihypertensive ☐ HAART ☐ Others (List) ☐

5. Time since HIV Diagnosis (Months)……

6. Time since the commencement of HAART (Months)……

7. Current HAART Medications with PI?

1……. 2…….

3……. 4……..

8. Is partner or significant other also HIV positive? Yes☐ No☐ Don't know☐

9. Have you ever had oral thrush since HIV diagnosis? Yes☐ No☐ Don't know☐

10. If yes to C9, How many times?.....times.

11. Have you ever taken medication for oral thrush? Yes☐ No☐ Don't know☐

12. Have you ever had to change your medication for oral thrush? Yes☐ No☐ Don't know☐

13. Do you currently have oral thrush? Yes☐ No ☐ Don't know☐

14. Do you currently have vaginal thrush? Yes☐ No☐ Don't know☐

15. Are you currently taking any medication for thrush? Yes☐ No☐

16. Current thrush medication

1.Fluconazole☐ 2Nystatin☐ 3Miconazole☐ 4Ketoconazole☐ 5 Itraconazole☐

0. None☐

D. ORAL EXAMINATION (Baseline)

1. Oral Hygiene using Plaque Index……

2. Dental Caries status using DMFT…….

3. Oral *Candida* Infection present? Yes☐ No. ☐

4. If present, location (using the attached chart, Appendix A)…….

5. Type of candidiasis- (1)Erythematous☐ (2)Pseudomembranous☐ (3) Angular cheilitis☐

6. Any other oral lesion? Yes☐ No☐.

7. If yes, tick the appropriate

	Hairy Leukoplakia
	Papilloma/Wart
	Kaposi Sarcoma
	Ulcer
	NUP
	Parotid enlargement
	Other

8. Any oral prosthesis such as dentures and bridges? Yes☐ No☐

9. Is mouth abnormally dry? Yes☐ No☐

E. LABORATORY VALUES (Baseline)

1. CD4 count……. 4. Viral Load……..

F. Baseline Oral rinse collected: yes☐ No☐

Travel and meal allowance given: R…….

Signature of participant:……

Next appointment given:…….Date:……

NOTE- TO BE RECALLED IF COLONISED WITH CANDIDA. (RESULTS IN SECTION G, PAGE 8)

D. ORAL EXAMINATION (3 months) DATE:

1. Oral Hygiene using Plaque Index……

2. Dental Caries status using DMFT…….

3. Oral *Candida* Infection present? Yes☐ No☐

4. If present, location (using the attached chart, Appendix A)…….

5. Type of candidiasis- 1.Erythematous☐ 2.Pseudomembranous☐ 3.Angular cheilitis☐

6. Any other oral lesion? Yes ☐ No☐.

7. If yes, tick the appropriate

	Hairy Leukoplakia
	Papilloma/Wart
	Kaposi Sarcoma
	Ulcer
	NUP
	Parotid enlargement
	Other

8. Any oral prosthesis such as dentures and bridges? Yes☐ No☐

9. If yes to D8, time since wearing prosthesis (years)……..

10. Is mouth abnormally dry? Yes☐ No☐

E. LABORATORY VALUES (3 Months)

1. PCV……..% 2. HCT……g/dl

3. CD4 count……cells./ml^3 4. Viral Load……..copies/ml

F. 3 months oral rinse collected: yes☐ No☐

Travel and meal allowance given: R…….

Signature of participant:……

Next appointment given:…….Date:……

D. ORAL EXAMINATION (6 Months) DATE:

1. Oral Hygiene using Plaque Index……

2. Dental Caries status using DMFT…….

3. Oral Candida Infection present? Yes☐ No☐.

4. If present, location (using the attached chart, Appendix A)…….

5. Type of candidiasis- Erythematous☐ Pseudomembranous☐ Angular cheilitis☐

6. Any other oral lesion? Yes ☐ No☐.

7. If yes, tick the appropriate

	Hairy Leukoplakia
	Papilloma/Wart
	Kaposi Sarcoma
	Ulcer
	NUP
	Parotid enlargement
	Other

8. Any oral prosthesis such as dentures and bridges? Yes☐ No☐

9. If yes to D8, time since wearing prosthesis (years)……..

10. Is mouth abnormally dry? Yes☐ No☐

E. LABORATORY VALUES (6 Months)

1. PCV……..% 2. HCT……g/dl

3. CD4 count……cells/ml^3……. 4. Viral Load……copies/ml

F. 6 months Oral rinse collected: yes☐ No☐

Travel and meal allowance given: R…….

Signature of participant:……

Do not forget to thank the participant.

G. (Laboratory Results) Study No:

I Baseline

1. Candida count……..cfu/ml

2. Yeast identification: Number (using API 20c sheet)

3. Adherence to oral epithelial cells:…….yeast cells/100 epithelial cells

4. Proteinase:…….mg/ml (Pr)

5. Phospholipase…….Pz

6. Genotype of *C. albicans:*……(Assign a number for each)

7. Sensitive to Amphotericin B☐, Fluconazole☐, Ketoconazole☐, Itraconazole☐ Nystatin☐

II 3 months

1. Candida count……..cfu/ml

2. Yeast identification:……

3. Adherence to oral epithelial cells:…….yeast cells/100 epithelial cells

4. Proteinase:…….mg/ml

5. Phospholipase…….Pz

6. Genotype of *C. albicans:*…….

3. Adherence to oral epithelial cells:…….yeast cells/100 epithelial cells

4. Proteinase:…….mg/ml

5. Phospholipase…….Pz

6. Genotype of *C. albicans:*…….

7. Sensitive to Amphotericin B❒, Fluconazole❒, Ketoconazole❒, Itraconazole❒ Nystatin❒

III 6 months

1. Candida count……..cfu/ml

2. Yeast identification:……

3. Adherence to oral epithelial cells:…….yeast cells/100 epithelial cells

4. Proteinase:…….mg/ml

5. Phospholipase…….Pz

6. Genotype of *C. albicans:*…….

7. Sensitive to Amphotericin B❒, Fluconazole❒, Ketoconazole❒, Itraconazole❒ Nystatin❒

9.3 Raw data

Table 9.3.1 Social factors investigated in HIV positive women colonised with *Candida*

StudyID	Age	Tribe	Marital Status	Education	Childrenn	Household size	Exchange food	Smoker	Colonised
1	34	Swathi	Single	High School	1	2	Never	No	Yes
2	38	Zulu	Single	High School	1	2	Never	No	Yes
3	46	Zulu	Single	High School	1	2	Never	No	Yes
4	56	Zulu	Single	High School	4	4	Never	No	No
5	58	Xhosa	Single	High School	3	3	Never	No	Yes
6	38	Zulu	Married	High School	1	2	Never	No	Yes
7	34	Zulu	Married	High School	3	8	Never	No	Yes
8	36	Tswana	Single	High School	0	3	Never	No	No
9	41	Sotho	Married	High School	2	5	Occasionally	No	No
10	38	Venda	Single	High School	3		All the time	No	Yes
11	34	Tsonga	Single	High School	1	6	Never	No	Yes
12	34	Sotho	Single	High School	2	9	Never	No	Yes
13	39	Zulu	Married	University	3	3	Never	No	No
14	46	Sotho	Single	High School	4	3	Never	No	Yes
15	52	Zulu	Single	High School	2	4	Never	No	No
16	27	Tswana	Single	High School	0	1	Never	Yes	Yes
17	39	Sotho	Single	High School	2	1	Never	No	No
18	36	Zulu	Single	High School	3	4	Never	No	No
19	57	Zulu	Single	Elementary	2	5	Never	No	No
20	34	Tswana	Single	High School	0	4	Never	No	No
21	34	Zulu	Single	High School	2	4	Never	No	No
22	32	Zulu	Single	High School	1	3	Occasionally	No	No
23	34	Zulu	Married	High School	3	6	All the time	No	No
24	34	Zulu	Single	High School	4	5	Never	No	Yes
25	25	Zulu	Single	High School	2	4	Occasionally	No	Yes
26	25	Ndebele	Single	High School	3	4	Occasionally	No	Yes
27	38	Tswana	Single	Elementary	1	1	Never	No	Yes
28	51	Zulu	Single	High School	3	10	Occasionally	Yes	Yes
29	26	Zulu	Single	University	2	4	Never	No	No
30	40	Tswana	Single	High School	2	2	All the time	No	Yes
31	28	Zulu	Single	High School	1	4	Often	No	No
32	40	Pedi	Single	Elementary	0	1	Never	No	Yes
33	38	Zulu	Single	Elementary	1	4	All the time	No	No

34	42	Xhosa	Single	High School	2	2	Never	No	Yes
35	41	Xhosa	Single	High School	1	3	Never	No	Yes
36	45	Xhosa	Divorced/Separated	Elementary	3	5	Never	No	Yes
37	38	Zulu	Single	University	1	2	Never	No	Yes
38	22	Zulu	Single	High School	1	3	Never	No	Yes
39	58	Tswana	Single	Elementary	3	3	Never	No	No
40	38	Zulu	Single	High School	2	3	Never	No	No
41	41	Tswana	Married	University	2	4	Occasionally	No	Yes
42	31	Zulu	Single	None	2	2	Never	No	Yes
43	51	Zulu	Married	High School	2	4	Never	No	No
44	32	Sotho	Single	High School	0	3	Never	No	No
45	30	Pedi	Single	University	2	3	Occasionally	No	Yes
46	30	Tswana	Single	Elementary	2	4	Never	No	Yes
47	36	Ndebele	Single	High School	2	3	Never	No	Yes
48	33	Zulu	Married	High School	2	4	Often	Yes	No
49	50	Xhosa	Single	High School	1	2	Never	No	No
50	27	Sotho	Single	High School	1	2	Never	No	No
51	34	Zulu	Single	High School	1	2	Never	No	Yes
52	37	Sotho	Single	High School	0	1	Never	Yes	No
53	60	Swathi	Single	Elementary	1	1	Never	No	No
54	44	Ndebele	Single	High School	3	5	Never	No	Yes
55	49	Zulu	Divorced/Separated	High School	4	6	Never	No	Yes
56	34	Zulu	Single	High School	2	1	Never	No	No
57	37	Zulu	Single	High School	0	1	Never	No	No
58	33	Zulu	Single	High School	2	4	Occasionally	No	Yes
59	31	Zulu	Single	University	2	3	Never	No	Yes
60	32	Sotho	Married	High School	3	5	Occasionally	No	No
61	42	Sotho	Married	High School	1	8	Never	No	Yes
62	43	Sotho	Single	High School	3	4	Occasionally	Yes	Yes
63	63	Ndebele	Single	Elementary	2	4	Never	No	No
64	34	Zulu	Single	High School	2	3	Never	No	Yes
65	32	Tswana	Single	High School	2	4	Never	No	No
66	29	Tswana	Married	University	1	2	Never	No	No
67	29	Xhosa	Single	High School	2	8	Occasionally	No	No
68	33	Zulu	Single	High School	2	3	Never	No	Yes
69	39	Ndebele	Single	University	2	4	Never	No	Yes
70	25	Ndebele	Single	High School	1	4	Never	No	No
71	43	Venda	Single	High School	2	3	Never	No	Yes

72	32	Coloured	Married	High School	2	4	Occasionally	No	No
73	41	Tswana	Single	High School	1	1	Never	No	No
74	41	Xhosa	Single	High School	4	6	Occasionally	No	Yes
75	51	Tswana	Widowed	High School	3	2	Never	No	No
76	37	Zulu	Single	None	3	3	Never	No	No
77	48	Zulu	Single	High School	2	1	Never	No	Yes
78	35	Tsonga	Married	High School	2	5	Never	No	Yes
79	38	Zulu	Married	Elementary	2	5	Never	No	No
80	51	Swathi	Widowed	High School	3	5	Never	No	Yes
81	26	Xhosa	Single	High School	0	1	Never	No	Yes
82	28	Swathi	Single	None	1	1	Never	No	Yes
83	29	Sotho	Single	High School	1	2	Never	No	No
84	38	Xhosa	Single	High School	1	8	Never	No	Yes
85	37	Sotho	Married	High School	4	3	Never	No	No
86	38	Xhosa	Single	High School	2	3	Never	No	Yes
87	34	Tswana	Single	University	0	3	Never	No	Yes
88	52	Sotho	Married	High School	2	4	Never	No	No
89	41	Tswana	Widowed	High School	3	4	Never	No	Yes
90	26	Xhosa	Single	Elementary	1	2	Never	No	Yes
91	39	Tswana	Single	High School	3	2	Occasionally	No	Yes
92	23	Ndebele	Single	High School	1	4	Never	No	No
93	37	Zulu	Married	High School	3	2	Occasionally	No	No
94	40		Married	University	2	4	Never	No	No
95	42	Zulu	Single	High School	1	3	Never	No	No
96	43	Zulu	Single	High School	2	4	Never	No	No
97	42	Zulu	Single	Elementary	4	6	Occasionally	No	No
98	34	Xhosa	Single	High School	1	2	Occasionally	No	Yes
99	64	Tswana	Single	Elementary	3	5	Never	No	Yes
100	33	Zulu	Single	High School	2	1	Never	No	Yes
101	35	Xhosa	Married	High School	2	12	Never	No	Yes
102	40	Ndebele	Single	High School	3	4	Occasionally	No	No
103	47	Sotho	Widowed	University	2	5	Occasionally	No	No
104	45	Sotho	Single	High School	2	3	Never	No	Yes
105	24	Coloured	Single	High School	3	8	Never	Yes	Yes
106	29	Xhosa	Single	High School	2	8	Never	No	No
107	31		Single	High School	2	5	Never	No	Yes
108	40	Zulu	Single	High School	4	1	Never	No	Yes
109	28	Xhosa	Single	High School	2	6	Never	No	Yes

110	42	Xhosa	Single	High School	2	1	Never	No	Yes
111	27	Ndebele	Single	High School	1	3	Never	No	No
112	31	Zulu	Single	High School	2	2	Never	No	No
113	45	Zulu	Single	High School	2	6	Occasionally	No	No
114	55	Sotho	Widowed	Elementary	4	7	Never	No	No
115	69	Zulu	Widowed	High School	3	5	Never	Yes	Yes
116	38	Sotho	Married	High School	3	7	Often	No	Yes
117	42	Zulu	Single	High School	3	2	Never	No	Yes
118	47	Zulu	Single	High School	3		Never	No	No
119	39		Widowed	High School	2	3	Never	No	Yes
120	46	Zulu	Single	High School	4	3	Often	No	Yes
121	46	Xhosa	Single	High School	2	3	Never	No	Yes
122	40	Sotho	Single	University	3	4	Never	No	No
123	30	Zulu	Single	High School	1	3	Never	No	No
124	33	Zulu	Married	High School	4	5	Never	No	Yes
125	25	Sotho	Single	High School	0	3	Never	No	Yes
126	32	Xhosa	Single	University	1	4	Never	No	Yes
127	29	Zulu	Married	High School	1	3	Never	Yes	Yes
128	37	Tswana	Single	High School	2	7	Never	No	Yes
129	28	Ndebele	Married	High School	2	3	Never	No	Yes
130	37	Sotho	Single	High School	4	3	Never	No	Yes
131	46	Zulu	Married	University	1	3	Occasionally	No	No
132	30	Zulu	Single	High School	0	2	Never	No	Yes
133	28	Zulu	Single	High School	3	6	Never	No	Yes
134	31	Coloured	Single	High School	2	3	Never	Yes	Yes
135	31	Sotho	Single	High School	0	2	Never	No	No
136	29	Xhosa	Married	University	3	5	Occasionally	No	Yes
137	27	Coloured	Single	High School	3	4	Occasionally	No	No
138	53	Sotho	Divorced/Separated	University	4	5	Occasionally	No	Yes
139	54	Sotho	Divorced/Separated	High School	3	2	Never	No	No
140	35	Zulu	Single	High School	2	6	Occasionally	No	Yes
141	29	Sotho	Married	High School	1	4	Never	Yes	Yes
142	43	Zulu	Single	Elementary		1	Occasionally	No	No
143	28	Zulu	Divorced/Separated	High School	2	1	Never	No	Yes
144	42	Sotho	Single	High School	1	4	Never	Yes	Yes
145	30	Zulu	Single	High School	1	2	Never	No	No
146	39	Ndebele	Widowed	High School	3	5	All the time	No	Yes

147	50	Zulu	Divorced/Separated	University	4	1	Never	No	No
148	32	Zulu	Single	High School	5	4	All the time	No	No
149	40	Tswana	Widowed	High School	2	3	Never	No	Yes
150	47	Sotho	Married	High School	2	4	Never	No	No
152	36	Zulu	Married	Elementary	4	4	Occasionally	No	No
153	30	Tswana	Married	High School	2	4	All the time	No	No
154	39	Sotho	Single	High School	2	3	Never	No	No
155	47	Zulu	Married	High School	1	2	Never	No	Yes
156	36	Sotho	Married	University	2	4	Never	No	Yes
157	49	Coloured	Divorced/Separated	High School	2	1	Never	Yes	Yes
158	45	Sotho	Single	High School	3	7	Never	No	Yes
159	47	Sotho	Married	Elementary	1	6	Never	No	No
160	30	Zulu	Single	High School	2	3	Never	No	No
161	32	Zulu	Single	High School	1	3	Never	No	No
162	30	Zulu	Single	High School	0	2	Occasionally	No	Yes
163	45	Zulu	Married	High School	1	4	Never	No	No
164	46	Swathi	Divorced/Separated	High School	1	3	Often	No	Yes
165	40	Sotho	Single	University	2	2	Occasionally	No	Yes
166	34	Zulu	Single	High School	2	5	Never	No	Yes
167	38	Swathi	Married	High School	2	3	Never	No	Yes
168	41		Single	High School	2	3	Never	No	Yes
169	32	Sotho	Single	High School	1	1	Never	No	Yes
170	48	Pedi	Single	High School	3	3	Never	No	Yes
172	38		Married	High School	3	5	Never	No	No
173	43		Single	University	1	2	Never	No	Yes
174	27	Zulu	Single	High School	0	8	Never	No	No
175	27	Ndebele	Single	Elementary	1	6	Never	No	Yes
176	32	Tsonga	Married	High School	2	3	Occasionally	No	Yes
177	56	Pedi	Single	None	4	5	Occasionally	No	No
178	30	Xhosa	Single	Elementary	0	5	Never	No	Yes
179	32		Single	University	2	3	Occasionally	No	Yes
180	29		Single	High School	1	4	Never	No	Yes
181	52	Zulu	Single	Elementary	1	1	Never	No	Yes
182	26	Ndebele	Single	High School	1	2	Never	No	Yes
183	32		Single	University	0	6	Never	No	Yes
184	42	Zulu	Single	High School	1	3	Never	No	No
185	39	Pedi	Single	University	3	4	Never	No	Yes
186	33	Xhosa	Widowed	High School	2	3	Never	No	Yes

187	33	Zulu	Single	High School	1	2	Never	No	Yes
188	53	Tswana	Widowed	None	4	2	Never	No	Yes
189	40	Venda	Single	University	4	6	Never	No	No
190	37	Swathi	Married	University	2	4	Never	No	Yes
191	48	Zulu	Married	Elementary	3	5	Never	No	Yes
192	36	Shona	Single	High School	1	2	Occasionally	No	No
193	30	Swathi	Divorced/Separated	High School	0	1	Never	No	Yes
194	41	Pedi	Single	High School	1	2	Never	No	No
195	46	Zulu	Divorced/Separated	High School	1	6	Never	No	No
196	37	Sotho	Single	University	1	5	Never	No	Yes
197	35	Zulu	Single	High School	2	3	All the time	No	Yes
198	37	Sotho	Married	High School	2	6	Never	No	Yes
199	26	Tswana	Married	University	0	2	Never	No	Yes

Table 9.3.2 Clinical factors investigated in HIV positive women colonised with *Candida*

StudyID	Candida Infection	HL	KS	Aphthous Ulcer	NUP	Papilloma	Parotid swelling	Hypertension	Heart Disease	Asthma	Allergies	DM	Cancer	TB	Daily brush times	Seen Dentist	PI	GI	DMFS	Colonised
1	No	No	No	No	No	No	No	Don't know	Yes	No	No	No	No	No		Yes	.9	1.8	28	Yes
2	No	No	No	No	No	No	No	No	No	No	No	No	No	No	3	No	.5	1.4	43	Yes
3	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.6	1.2	76	Yes
4	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	1	1.1	35	No
5	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	No	.5	.9	102	Yes
6	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.3	.6	77	Yes
7	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	.5	.8	10	Yes
8	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No	No	4	No	.5	1.4	11	No
9	No	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.08	.9	13	No
10	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.5	1.1	9	Yes
11	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.7	.8	6	Yes
12	Yes	No	No	Yes	No	No	No	No	No	No	No	No	No	No		Yes	1	1.5	6	Yes
13	No	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No		No	.5	.4	0	No
14	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.2	.5	24	Yes
15	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes	No	4	No	.9	.8	30	No
16	Yes	No	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.7	.6	29	Yes
17	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	1	No	.5	.7	34	No
18	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.1	.6	7	No
19	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No		No	.4	.3	35	No
20	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.5	.5	15	No
21	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.1	.4	30	No
22	No	No	No	No	No	No	No	No	No	No	No	No	No	No	3	No	.8	.6	6	No
23	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	No	.1	.6	42	No
24	No	No	No	No	No	No	No	No	No	Yes	No	No	No	Yes		No	.5	.5	5	Yes
25	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1.2	.9	36	Yes

26	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1	1.4	18	Yes
27	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.3	.7	20	Yes
28	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.2	.6	33	Yes
29	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.4	.5	2	No
30	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No		No	.2	.3	11	Yes
31	No	Yes	No	No	No	No	No	No	No	Yes	Yes	No	No	No	4	No	.5	1	2	No
32	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No		Yes	.2	.5	32	Yes
33	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.1	.8	21	No
34	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	No	1.3	.6	37	Yes
35	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	1	.7	41	Yes
36	No	No	No	No	No	No	No	Yes	No	No	No	No	No	Yes		Yes	.4	1.1	18	Yes
37	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No	No		No	.5	.3	16	Yes
38	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.3	.4	7	Yes
39	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No	No		No	1	.6	42	No
40	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	No	.5	.6	7	No
41	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.4	.01	15	Yes
42	No	No	No	No	No	No	No	No	Yes	No	No	No	Yes	No		No	.83	.7	16	Yes
43	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No	No		Yes	1.08	1.08	49	No
44	No	No	No	No	No	No	No	No	No	No	Yes	No	No	Yes	1	No	.7	.3	14	No
45	No	No	No	Yes	No	Yes	No	No	No	No	No	No	No	No		Yes	.5	0	8	Yes
46	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.4	.8	16	Yes
47	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.3	.5	11	Yes
48	No	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No		Yes	.3	.5	3	No
49	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	1	No	.6	1.1	17	No
50	No	No	No	No	No	No	No	No	No	No	No	No	No	No	0	Yes	.4	.1	17	No
51	No	No	No	No	No	No	No	No	Yes	No	No	No	No	No		No	.3	.3	23	Yes
52	No	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	2	Yes	.3	.3	21	No
53	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No	No	0	No			92	No
54	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1.1	1.1	62	Yes

55	No	Yes	No	Yes	No	No	No	Yes	No	No	No	No	No	No	1	No	1.1	.6	35	Yes
56	No	No	No	No	No	No	No	No	No	Yes	No	No	No	No		No	1.2	.6	14	No
57	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1.5	.5	21	No
58	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.6	.5	5	Yes
59	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.7	.6	12	Yes
60	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No	No		No	1	1.5	25	No
61	No	No	No	No	No	No	No	Yes	No	No	Yes	No	No	Yes	2	No	.8	.7	8	Yes
62	No	Yes	No	No	No	No	No	No	No	No	Yes	No	No	No	1	Yes	1	.6	34	Yes
63	No	No	No	No	No	No	No	Yes	Yes	No partner	Yes	Yes	No	No		No			93	No
64	Yes	Yes	No	Yes	No	No	No	No	No	No	Yes	No	No	Yes		No	1	.67	14	Yes
65	No	No	No	No	No	No	No	No	No	No	No	No	No	No	6	Yes	.58	.33	10	No
66	No	No	No	No	No	No	No	No	No	No	No	No	No	No	8	Yes	1.66	1.66	20	No
67	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1.17	.5	20	No
68	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	4	No	.83	.67	23	Yes
69	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No		Yes	.83	.17	20	Yes
70	No	No	No	No	No	No	No	No	No	No	No	No	No	No	2	No	.83	.5	45	No
71	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.92	.67	4	Yes
72	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.3	.42	11	No
73	No	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	0	No	1	.33	28	No
74	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	0	No	1	.9	12	Yes
75	No	Yes	No	No	No	No	No	Yes	No	No	Yes	No	No	No	4	No	1	.67	20	No
76	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1	.67	23	No
77	No	Yes	No	No	No	No	No	No	No	No	Yes	No	No	No		No	1.3	.8	64	Yes
78	No	No	No	No	No	No	No	No	No	No	Yes	Don't know	No	No	2	No	.8	.6	14	Yes
79	No	Yes	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	.5	.1	31	No
80	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		Yes	.5	0	58	Yes
81	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.8	.5	26	Yes
82	No	No	No	No	No	No	No	No	No	No	Yes	No	No	Yes	1	No	1	.6	0	Yes

83	No	No	No	No	No	No	No	Yes	Don't know	No	Yes	No	No	No	5	No	.6	.8	11	No
84	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.1	.5	24	Yes
85	No	No	No	No	No	No	No	Yes	Yes	Yes	No	Yes	Yes	No		Yes	.1	.4	28	No
86	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No	Yes		Yes	1.6	2	61	Yes
87	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.5	.3	5	Yes
88	No	No	No	No	No	No	No	Yes	Yes	No	No	Yes	Yes	No		No	.3	1	67	No
89	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.5	.5	18	Yes
90	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.9	1	8	Yes
91	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes	No	0	No	.5	.3	3	Yes
92	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.9	.5	0	No
93	No	No	No	No	Yes	No	No	Yes	No	No	No	No	No	No	2	No	1.1	.6	30	No
94	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.5	.5	12	No
95	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	0	No	.7	1.33	5	No
96	No	No	No	No	No	No	No	No	No	No	No	No	No	No	4	No	.5	.6	4	No
97	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.7	.5	0	No
98	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	1	.6	64	Yes
99	No	Yes	No	No	Yes	No	No	Yes	No	No	Yes	No	No	No		No	2	2.3	58	Yes
100	No	No	No	No	No	No	No	Yes	No	No	No	Yes	No	No		No	.1	.6	4	Yes
101	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.08	.08	2	Yes
102	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	1.4	.8	0	No
103	No	No	No	No	No	No	No	No	No	No	No	Yes	No	No		No	.2	.5	47	No
104	Yes	Yes	No	No	No	No	No	Yes	No	No	No	No	No	Yes	1	No	.3	.5	78	Yes
105	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1.3	1.5	15	Yes
106	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.9	.5	23	No
107	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No		No	.9	.6	11	Yes
108	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.4	.4	27	Yes
109	No	No	No	Yes	No	No	No	No	No	No	No	No	No	No	0	Yes	.3	.6	18	Yes
110	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	1	1	8	Yes
111	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.4	.6	0	No

112	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	1	No	.2	0	0	No
113	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No		No	1.1	1	0	No
114	No	No	No	No	Yes	No	No	No	No	No	No	No	No	Yes		Yes			76	No
115	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	2	No	1.1	1	68	Yes
116	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	10	No	.3	.2	18	Yes
117	No	No	No	No	Yes	No	No	No	No	No	No	No	No	No		No	1.5	1.8	35	Yes
118	No	No	No	No	No	No	No	Yes	Yes	No	Yes	No	No	No	1	Yes	.3	.5	50	No
119	No	No	No	No	No	No	No	No	No	No	Yes	No	No	Yes		Yes	0	0	5	Yes
120	No	No	No	No	No	No	No	Yes	No	No	No	No	No	Yes		Yes	.6	.8	38	Yes
121	No	No	No	No	No	No	No	No	No	Yes	No	No	No	Yes	2	No	.2	.4	34	Yes
122	No	No	No	No	No	No	No	No	No	No	No	No	No	No	2	Yes	1	1.3	15	No
123	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.83	1.33	16	No
124	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No		Yes	1	.3	74	Yes
125	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	1	No	1.5	1.1	22	Yes
126	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No	1	Yes	1	1	19	Yes
127	No	No	No	No	Yes	No	No	No	No	No	Yes	No	No	No	1	No	.5	.5	1	Yes
128	No	Yes	No	No	No	Yes	No	No	No	No	No	No	No	Yes		No	1.2	1.3	57	Yes
129	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	4	No	.5	.5	1	Yes
130	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No		No	.6	1	39	Yes
131	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	3	No	.4	0	0	No
132	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes	No	2	Yes	.4	.5	27	Yes
133	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	Yes	.5	.8	27	Yes
134	Yes	Yes	No	No	No	No	No	No	No	Yes	No	No	No	No	1	Yes	.6	.6	61	Yes
135	Yes	No	No	No	No	No	No	Yes	No	No	No	No	No	No	1	Yes	.7	1	13	No
136	No	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes	10	Yes	.7	.2	34	Yes
137	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.5	.3	26	No
138	Yes	Yes	No	Yes	No	No	No	No	No	No	No	No	No	No		Yes	.8	1	10	Yes
139	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes	No		Yes	.5	.8	5	No

140	No	Yes	No	No	No	No	No	No	No	Yes	No	No	No	No		Yes	1.33	1.08	32	Yes
141	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	.1	.1	13	Yes
142	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.6	.6	4	No
143	No	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.2	.5	2	Yes
144	No	No	No	No	No	No	No	No	No	No	No	No	No	No	1	No	.9	.5	51	Yes
145	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.8	.3	12	No
146	Yes	No	No	No	Yes	No	No	No	No	No	No	No	No	No		Yes	1.3	1	8	Yes
147	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No		No	1.1	.3	45	No
148	No	Yes	No	No	No	No	No	Yes	No	No	No	No	Yes	No	1	Yes	1.08	1	0	No
149	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		Yes	.5	.3	39	Yes
150	No	No	No	No	No	No	No	Yes	Yes	No	No	Yes	No	No		No	1.1	.5	16	No
152	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.6	.3	6	No
153	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.7	.3	20	No
154	No	No	No	No	No	No	No	No	No	No	No	No	No	No	2	No	.6	.1	8	No
155	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.4	.3	17	Yes
156	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	7	Yes	.2	0	0	Yes
157	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No				Yes
158	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	5	Yes				Yes
159	No	No	No	No	No	No	No	No	No	No	No	No	No	No	5	No	.167	.33	0	No
160	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.67	.58	17	No
161	No	No	No	No		No	No	No	No	No	No	No	No	No	1	No	1	1.08	29	No
162	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.92	2.2	22	Yes
163	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No	1	No	.25	.25	0	No
164	No	Yes	No	No	No	No	No	No	No	No	No	No	No	Yes		Yes	.25	.083	82	Yes
165	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No		Yes	.167	.417	38	Yes
166	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes	2	No	.167	.083	0	Yes
167	No	No	No	No		No	No	No	No	No	No	No	No	Yes		Yes	.417	.083	0	Yes
168	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	1.17	1	39	Yes

169	No	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No		No	.67	.5	23	Yes
170	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	1	.67	23	Yes
172	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.5	.167	17	No
173	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	.75	1	18	Yes
174	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.67	.75	16	No
175	No	No	No	No	No	No	No	No	No	No	Yes	No	No	Yes		No	.583	.417	0	Yes
176	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.583	.5	17	Yes
177	No	No	No	No	No	No	No	Yes	No	No	No	No	No	Yes		Yes	.83	1.75	30	No
178	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes		No	.5	.5	45	Yes
179	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.67	.75	0	Yes
180	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.167	.33	12	Yes
181	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.25	.583	49	Yes
182	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.83	1	6	Yes
183	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.5	.417	0	Yes
184	No	No	No	No	No	No	No	No	No	No	Yes	No	No	No		No	.75	.66	10	No
185	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No	1	No	.33	.67	4	Yes
186	No	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes		No	.75	.75	40	Yes
187	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.25	.167	13	Yes
188	No	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No		No	.417	.83	35	Yes
189	Yes	No	No	No	No	No	No	No	No	No	No		No	No		No	.92	.5	12	No
190	No	No	No	No	No	No	No	Yes	No	No	No	No	No	No		No	.5	.5	17	Yes
191	No	Yes	No	No	No	No	No	No	Yes	No	No	No	No	No		No	.83	.583	25	Yes
192	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.2	.5	0	No
193	Yes	No	No	No	No	No	No	No	No	No	Yes	No	No	No		Yes	1	.6	20	Yes
194	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.6	.7	32	No
195	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes	No		No	1.1	.9	5	No
196	No	No	No	No	No	No	No	No	No	No	No	No	No	No		No	.1	.3	28	Yes
197	No	No	No	No	No	No	No	Yes	Yes	No	No	No	No	No		No	.583	.083	28	Yes

198	No	No	No	No	No	No	No	No	No	Yes	Yes	No	Yes	No	3	No	.33	.167	6	Yes
199	No	No	No	No	No	No	No	No	No	No	No	No	No	No		Yes	.167	.083	11	Yes

Table 9.3.3 Clinical factors and laboratory investigations in HIV positive women colonised with *Candida*

ID	On ARV	Time since on ARV (months)	Current ARV	CD4 cell/mm3	Viral Load (copies/ml)	Colonised	Yeast Count (cfu/ml)	YeastID	YeastID2
1	Yes	24	3TC,TDF,EFV	405	1698	Yes	20	*C. famata*	
2	Yes	84	3TC,TEN,Alluvia	546	40	Yes	560	*C. albicans*	
3	Yes	60	3TC,TDF,EFV	347	40	Yes	30	*C. albicans*	
4	Yes	72	DDI,AZT,Alluvia	580	40	No			
5	Yes	24	3TC,TDF,EFV	250		Yes	220	*C. albicans*	*C. tropicalis*
6	Yes	48	3TC,TDF,NVP	196	5	Yes	40	*C. albicans*	
7	Yes	36	TDF,AZT,Alluvia	346	351	Yes	1040	*C. dubliniensis*	
8	Yes	24		511	1191	No			
9	Yes	6	3TC,TDF,EFV	83		No			
10	Yes	36	3TC,d4t,EFV	418	13314	Yes	720	*C. albicans*	
11	Yes	24	3TC,TDF,EFV	567		Yes	20	*C. albicans*	
12	No		3TC,TDF,EFV	571		Yes	170	*C. albicans*	
13	Yes	60	3TC,d4t,EFV	444	40	No			
14	Yes	45				Yes	10	*C. dubliniensis*	
15	Yes	24	3TC,TDF,NVP	274	5	No			
16	Yes	5	3TC,TDF,EFV	104		Yes	470	*C. albicans*	
17	Yes	72	3TC,EFV,TEN	842	5	No			
18	Yes	5	3TC,TDF,NVP	101		No			
19	Yes	36	3TC,EFV,TEN			No			
20	Yes	84				No			
21	Yes	6		266		No			
22	Yes	28	3TC,TDF,EFV	419	40	No			
23	Yes	24	3TC,d4t,EFV	526	5	No			
24	Yes	96		1200	5	Yes	10	*C. albicans*	
25	No	0	HAART NAIVE	595		Yes	530	*C. albicans*	
26	No	0	HAART NAIVE	398		Yes	90	*C. albicans*	
27	Yes	60	Alluvia,EFV,TEN	370		Yes	10	*C. albicans*	
28	Yes	84		393	40	Yes	30	*C. albicans*	
29	Yes	12	3TC,TDF,EFV	650		No			
30	No		HAART NAIVE	646	40	Yes	200	*C. dubliniensis*	
31	Yes	24	3TC,TEN,Alluvia	409		No			
32	Yes	8	3TC,TDF,EFV	156	5	Yes	30	*C. albicans*	
33	Yes	84	3TC,TDF,EFV	324	2312	No			
34	Yes	12	3TC,d4t,EFV	27	183298	Yes	380	*C. albicans*	
35	Yes	36	3TC,TDF,EFV	821	5	Yes	130	*C. albicans*	
36	Yes	26	3TC,d4T,Alluvia	904	26	Yes	10	*C. tropicalis*	
37	Yes	84	3TC,TDF,NVP	626	5	Yes	130	*C. albicans*	
38	Yes	6	3TC,TDF,NVP	246	223	Yes	60	*C. albicans*	
39	Yes	6	3TC,TDF,EFV	178	451	No			
40	Yes	6	3TC,TDF,EFV	40		No			

41	Yes	48	3TC,TDF,EFV	1023	5	Yes	70	*C. albicans*	
42	Yes	18	3TC,TDF,NVP	462	337	Yes	20	*C. albicans*	
43	Yes	72	3TC,d4t,EFV	239	400	No			
44	Yes	94	3TC,TDF,EFV	1172	5	No			
45	Yes	60	3TC,TDF,NVP	35	5	Yes	165	*C. albicans*	
46	Yes	72	3TC,TDF,NVP	166	40	Yes	60	*C. albicans*	
47	Yes	72	3TC,TDF,EFV	194	5	Yes	10	*C. albicans*	
48	Yes	12	3TC,TDF,EFV	180	40	No			
49	Yes	36	3TC,TDF,EFV	576	5	No			
50	Yes	6	3TC,TDF,NVP	267		No			
51	Yes	72	3TC,TDF,AZT	555	5	Yes	10	*C. albicans*	
52	Yes	84	3TC,TDF,EFV	145	14411	No			
53	Yes	96	3TC,d4T,Alluvia	482	5	No			
54	Yes	6	3TC,TDF,EFV	86		Yes	690	*C. albicans*	
55	Yes	12	3TC,TDF,EFV	302	5	Yes	1475	*C. albicans*	
56	Yes	36	3TC,AZT,Alluvia	134	5	No			
57	Yes	48	TDF,AZT,Alluvia	655	189	No			
58	Yes	6	3TC,TDF,NVP	292		Yes	570	*C. tropicalis*	
59	Yes	6	3TC,TDF,EFV	19		Yes	70	*C. albicans*	
60	Yes	24	3TC,TDF,EFV	684	1631	No			
61	Yes	36	3TC,TDF,EFV	386	40	Yes	230	*C. tropicalis*	*C. parapsilosis*
62	Yes	6	3TC,TDF,EFV	202		Yes	50	*C. albicans*	
63	Yes	48	3TC,TDF,EFV	716	5	No			
64	Yes	6	3TC,TDF,EFV	58		Yes	210	*C. albicans*	
65	Yes	6	3TC,TDF,EFV	40		No			
66	Yes	36	3TC,TEN,Alluvia	400	5	No			
67	Yes	12	3TC,TDF,EFV	418	5	No			
68	Yes	12	3TC,TEN,Alluvia	640	315002	Yes	370	*C. albicans*	
69	Yes	4	3TC,TDF,EFV	92		Yes	10	*C. tropicalis*	
70	Yes	24	3TC,TDF,NVP	778	40	No			
71	Yes	48	3TC,TDF,EFV	505	5	Yes	10	*C. albicans*	
72	Yes	120	3TC,TDF,EFV	621	5	No			
73	Yes	12	3TC,TDF,EFV	364	40	No			
74	Yes		3TC,TDF,EFV	699	5	Yes	10	*C. albicans*	
75	Yes	72	3TC,TDF,EFV	574	5	No			
76	Yes	60	3TC,TDF,EFV	141		No			
77	Yes	48	3TC,TDF,EFV	564	5	Yes	10	*C. albicans*	
78	Yes	108	3TC,TDF,EFV	307	5	Yes	240	*C. albicans*	
79	Yes	70	3TC,TDF,EFV	706	5	No			
80	Yes	60	3TC,TDF,EFV	321	110	Yes	80	*C. albicans*	
81	Yes	24	3TC,TDF,EFV	295		Yes	20	*C. albicans*	
82	Yes	12	3TC,TDF,EFV	34	46	Yes	180	*C. albicans*	
83	Yes	12	3TC,TDF,NVP	868		No			
84	Yes	60	3TC,TDF,EFV	701	5	Yes	20	*C. albicans*	
85	Yes	48	3TC,TDF,EFV	690		No			

86	Yes	30	3TC,TDF,EFV	301	5	Yes	580	*C. albicans*	
87	Yes	24	3TC,AZT, Alluvia	194	63855	Yes	3750	*C. famata*	
88	Yes	60	3TC,TDF, EFV	151	40	No			
89	Yes	24	3TC,d4t,EFV	108	40	Yes	30	*C. albicans*	
90	Yes	30	3TC,AZT,Alluvia	508	5	Yes	100	*C. dubliniensis*	
91	Yes	15	3TC,TDF,EFV	273	5	Yes	350	*C. albicans*	
92	Yes	1	3TC,TDF,EFV	59		No			
93	Yes	60	3TC,TEN,Alluvia	277	1463	No			
94	Yes	84	TDF,AZT,Alluvia	264	40	No			
95	Yes	72	3TC,TDF,NVP			No			
96	Yes	24	3TC,TDF,EFV	467	5	No			
97	Yes	12	3TC,TDF,EFV	59	55	No			
98	Yes	24	3TC,TDF,EFV	167	20	Yes	20	*C. albicans*	
99	Yes	6	3TC,d4t,EFV	242	64000	Yes	110	*C. albicans*	*S. cerevisiae*
100	Yes	48	3TC,TEN,Alluvia	400	43	Yes	20	*C. albicans*	
101	Yes	36	3TC,TDF,AZT	102	5	Yes	40	*C. dubliniensis*	
102	Yes	13	3TC,d4t,EFV	619	5	No			
103	Yes	133	3TC,TDF,EFV	1000	20	No			
104	Yes	84		485		Yes	10	*C. albicans*	
105	Yes	48	3TC,TEN,Alluvia	400	2200	Yes	10	*C. albicans*	
106	Yes	48	3TC,TDF,EFV	508		No			
107	Yes	3	3TC,TDF,EFV	260		Yes	90	*C. albicans*	
108	Yes	36	AZT,EFV,TEN	255	5	Yes	30	*C. dubliniensis*	
109	No		HAART NAIVE			Yes	30	*C. dubliniensis*	
110	Yes	36	3TC,TDF,EFV	280	5	Yes	320	*C. albicans*	
111	Yes	16	3TC,d4t,EFV	500		No			
112	Yes	36	3TC,TEN,Alluvia	895	5	No			
113	Yes	56	3TC,d4t,EFV	279	40	No			
114	Yes	70	3TC,TDF,AZT	704		No			
115	No		HAART NAIVE	123		Yes	955	*C. tropicalis*	
116	Yes	1	3TC,TDF,EFV	114		Yes	275	*C. krusei*	
117	Yes	25	3TC,TDF,EFV	410	40	Yes	10	*C. albicans*	
118	Yes	36	3TC,TDF,EFV	389	40	No			
119	Yes	16	3TC,d4t,EFV	187	55	Yes	60	*C. albicans*	
120	Yes	22	3TC,TDF,EFV	355	40	Yes	20	*C. albicans*	
121	Yes	28	3TC,TEN,Alluvia	228	5	Yes	10	*C. albicans*	
122	Yes	36	3TC,TDF,EFV	573	5	No			
123	Yes	48		704		No			
124	Yes	84	3TC,TDF,EFV	1057	25	Yes	75	*C. dubliniensis*	
125	Yes	12	3TC,TEN,Alluvia	225	40	Yes	100	*C. albicans*	
126	Yes	48	AZT,EFV,TEN	750	5	Yes	565	*C. tropicalis*	
127	Yes	3	3TC,TDF,EFV	163	251345	Yes	265	*C. dubliniensis*	
128	Yes	48	3TC,TEN,Alluvia	298	40	Yes	1210	*C. dubliniensis*	
129	Yes	12	3TC,TDF,NVP	321	56	Yes	670	*C. famata*	
130	Yes	33	3TC,TDF,NVP	456	568	Yes	100	*C. famata*	

131	Yes	1				No			
132	Yes	3	3TC,TDF,EFV	78		Yes	380	*C. albicans*	
133	Yes	12	3TC,TDF,EFV	562	5	Yes	30	*C. dubliniensis*	
134	Yes	48	AZT,EFV,TEN	748	5	Yes	10	*C. albicans*	
135	Yes	36	3TC,TDF,NVP	357	5	No			
136	Yes	3	3TC,TEN,Alluvia	7	131117	Yes	320	*C. parapsilosis*	
137	Yes	72	3TC,TDF,NVP	686	5	No			
138	No	0	HAART NAIVE	19		Yes	2470	*C. albicans*	
139	Yes	48	3TC,TDF,EFV	529	5	No			
140	Yes	72	3TC,TDF,NVP	584	25	Yes	50	*C. albicans*	
141	Yes	4	3TC,TDF,EFV	146		Yes	40	*C. albicans*	
142	Yes	3	3TC,TDF,EFV	286		No			
143	Yes	1	3TC,TDF,EFV	109	111000	Yes	230	*C. albicans*	
144	Yes	72	3TC,TDF,NVP	581	5	Yes	160	*C. albicans*	
145	Yes	24	3TC,TDF,NVP	332	5	No			
146	Yes	1	3TC,TDF,EFV	200		Yes	100	*C. famata*	
147	Yes	0	HAART NAIVE	158		No			
148	Yes	3	3TC,TDF,EFV	600	5	No			
149	Yes	60	3TC,TDF,EFV	388	5	Yes	10	*C. albicans*	
150	Yes	60	3TC,TDF,EFV	733		No			
152	Yes	48				No			
153	Yes	12				No			
154	Yes	3				No			
155	Yes	72	3TC,TDF,EFV	392		Yes	40	*C. famata*	
156	No	0	HAART NAIVE	398		Yes	570	*C. albicans*	
157	Yes	24	3TC,TDF,EFV	620	5	Yes	20	*C glabrata*	
158	Yes	72	3TC,TEN,Alluvia	513	5	Yes	490	*C glabrata*	
159	Yes	72	3TC,TDF,EFV	386	5	No			
160	Yes	48	3TC,TDF,NVP	418	5	No			
161	Yes	36	3TC,TDF,EFV	448		No			
162	Yes	1	3TC,TDF,EFV	349		Yes	20	*S. cerevisiae*	
163	Yes	72	3TC,TDF,NVP	835	5	No			
164	Yes	60	3TC,TEN,Alluvia	464	175	Yes	20	*C. albicans*	
165	Yes	2	3TC,TDF,EFV	163	139008	Yes	850	*C. albicans*	
166	Yes	12	3TC,TDF,EFV	350	40	Yes	290	*C. albicans*	
167	Yes	9	3TC,TDF,EFV	588	255	Yes	10	*C. albicans*	
168	Yes	84	3TC,AZT,Alluvia	926	664	Yes	330	*C. albicans*	
169	Yes	1	3TC,TDF,EFV	236	5	Yes	450	*C. albicans*	
170	Yes	120	3TC,TDF,EFV	400	5	Yes	150	*C. albicans*	
172	Yes	45	3TC,TDF,NVP	300	5	No			
173	Yes	12	3TC,TEN,Alluvia	469		Yes	50	*C. albicans*	
174	Yes	72	3TC,d4t,EFV	429	40	No			
175	Yes	15	3TC,TDF,EFV	357	5	Yes	10	*C. dubliniensis*	
176	No		HAART NAIVE	377		Yes	380	*C. albicans*	
177	Yes	72	TDF,AZT,Alluvia	262	261	No			

178	Yes	1	3TC,TDF,EFV	396		Yes	20	*C. albicans*	
179	Yes	1	3TC,TDF,EFV	241		Yes	10	*C. albicans*	
180	Yes	4	3TC,TDF,EFV	193		Yes	50	*C. albicans*	
181	Yes	84	3TC,TDF,EFV	471	40	Yes	20	*C. dubliniensis*	
182	Yes	1	3TC,TDF,EFV	270		Yes	60	*C. albicans*	
183	Yes	1	3TC,TDF,EFV	141	119323	Yes	10	*C. albicans*	
184	Yes	60	AZT,d4T,EFV	414		No			
185	No	0	HAART NAIVE	9		Yes	1500	*C. albicans*	
186	Yes	36		770	5	Yes	130	*C. albicans*	
187	Yes	49	3TC,TDF,EFV	300	40	Yes	100	*C. albicans*	
188	Yes	24	3TC,TDF,EFV	190		Yes	50	*C. albicans*	
189	Yes	6	3TC,TDF,EFV	146		No			
190	No	6	3TC,TDF,NVP	253	40	Yes	20	*C. albicans*	
191	Yes	6	3TC,TDF,EFV	363		Yes	20	*C. albicans*	
192	Yes	9	3TC,TDF,NVP			No			
193	Yes	72	3TC,TDF,EFV	600		Yes	110	*C. albicans*	
194	Yes	6	3TC,TDF,EFV	200		No			
195	Yes	12	3TC,TDF,EFV			No			
196	Yes	6	3TC,TDF,EFV	380	5	Yes	20	*C. albicans*	
197	Yes	6	3TC,TDF,NVP	107		Yes	380	*C. albicans*	
198	Yes	14	3TC,TDF,EFV	291		Yes	160	*C. dubliniensis*	
199	Yes	36				Yes	320	*C. tropicalis*	

9.4 Media and reagents

API 20C Aux

Suspension Medium (2 ml)

Demineralized water

NaCl 0.85 % Medium (2 ml)

Sodium chloride 8.5 g

Demineralized water 1000 ml

C Medium (7 ml)

Ammonium sulphate	5 g
Monopotassium phosphate	0.31 g
Dipotassium phosphate	0.45 g
Disodium phosphate	0.92 g
Sodium chloride	0.1 g
Calcium chloride	0.05 g
Magnesium sulphate	0.2 g
Histidine	0.005 g
Tryptophane	0.02 g
Methionine	0.02 g
Agar	0.5 g
Vitamin solution	1 ml
Trace elements	10 ml
Water	qsp 1000 ml
Final pH:	6.4-6.8

CHROMagar Candida

Composition in g/L

Agar	15

Peptone	10.2
Chloramphenicol	0.5
Chromogenic mix	22
pH	6.1 ±0.2

Preparation

Suspend in the proportion of 47.7 g/L in purified water. Heat and bring to boil (100°C) while swirling or stirring regularly until complete fusion of the agar. Cool to 45-50°C and dispense.

Coomassie Blue staining solution

Coomassie Brilliant Blue R-250	2.5 g
Ethanol	450 ml
Acetic acid	100 ml
Distilled water	400 ml

The volume was adjusted to 1 litre with distilled water. The solution was stored at room temperature.

Coomassie Blue destaining solution

Methanol	450 ml
Acetic acid	100 ml
Distilled water	400 ml

The volume was adjusted to 1 litre with distilled water and the solution stored at room temperature.

0.5M EDTA

Dissolve 186.1 g in 700mls distilled water. Adjust pH to 8.0 with 10M NaOH (approx. 50mls)

Add distilled water to 1 liter

Egg Yolk Media

Sabouraud dextrose agar,	13 g
NaCl	11.7 g
$CaCl_2$	0.11 g

10% sterile egg yolk emulsion in 184 ml of distilled water

20ml of egg yolk emulsion was centrifuged at 5000g for 10 minutes. The supernatant was added to cooled sterile medium containing NaCl and $CaCl_2$ before the plates were poured.

Sabouraud Dextrose Agar

Composition

Sabouraud Agar	60 g
Distilled water	1 L

Preparation

The ingredients were boiled whilst stirring until completely dissolved. Thereafter, the solution was autoclaved at 121°C for 15 min. The solution was allowed to cool rapidly to 40-45°C and mixed well. It was aseptically poured into sterile petri dishes.

Sabouraud Dextrose Broth

Sabouraud Broth	20 g
Distilled water	1 L

The ingredients were mixed well and the solution was autoclaved at 121°C for 15 min.

Sensititre YeastOne®

Commercial agent, ingredients known to the manufacturers

10x Tris borate EDTA (TBE) buffer

Tris base	108g
Boric acid	55g

Yeast Carbon Base- Bovine Serum Albumin medium

Agar	1.5 g
Yeast Carbon Base powder	1.17 g
Bovine Serum Albumin	0.2 g
Glucose	0.2 g
Water	100 mls

The ingredients were boiled whilst stirring until completely dissolved. Thereafter, the solution was autoclaved at 121°C for 15 min. The solution was allowed to cool rapidly to 40-45°C and mixed well. It was aseptically poured into sterile petri dishes.

9.5 *C. albicans* isolates from South Africa on the MLST database

mlst.net | View MLST maps

Candida albicans - consensus

All Isolates Key (%total isolates):

- <10
- 11-20
- 21-30
- 31-40
- 41-50
- 51-60
- 61-70
- 71-80
- 81-90
- 91-100

Click on the country marker to view details of isolates.

When displayed you can also view the countries where a particular sequence type is found.

Click to return to all isolate map.

South Africa Epidemiological data - Click to return to Sequence Type Summary for South Africa

id	Sequence Type	strain	Sequencer	Sequencer1	Curator	Region	source
841	90	AM2005/0431	Odds		Frank Odds		blood
2342	95	ML_41.1,	Foluso Owotade		ME Bougnoux		Oral
2338	124	ML_27.2	Foluso Owotade		-1		oral
2352	144	ML_166.1	Foluso Owotade		-1		Oral
914	155	AM2005/0432	Odds		Frank Odds		blood
2339	210	ML_35.1	Foluso Owotade		-1		oral
2359	375	ML_193.3,	Foluso Owotade		ME Bougnoux	Johannesburg	oral
897	392	AM2005/0438	Odds		Frank Odds		blood
2356	392	ML_185.1	Foluso Owotade		-1		Oral
987	610	AM2005/0471	Odds		Frank Odds		blood
827	613	AM2005/0450	Odds		Frank Odds		blood
2351	619	ML_140.3	Foluso Owotade		-1		Oral
982	619	AM2005/0434	Odds		Frank Odds		blood
836	622	AM2005/0479	Odds		Frank Odds		blood
868	622	AM2005/0478	Odds		Frank Odds		blood
837	623	AM2005/0480	Odds		Frank Odds		blood
838	624	AM2005/0481	Odds		Frank Odds		blood
839	625	AM2005/0482	Odds		Frank Odds		blood
840	626	AM2005/0483	Odds		Frank Odds		blood
842	627	AM2005/0433	Odds		Frank Odds		blood
894	627	AM2006/0015	Odds		Frank Odds		blood
866	632	AM2005/0435	Odds		Frank Odds		blood

867	633	AM2005/0449	Odds	Frank Odds		blood
869	634	AM2005/0377	Odds	Frank Odds		blood
870	635	AM2005/0376	Odds	Frank Odds		blood
871	636	AM2005/0362	Odds	Frank Odds		blood
876	640	AM2005/0361	Odds	Frank Odds		blood
882	646	AM2005/0477	Odds	Frank Odds		blood
2347	646	ML_82.2	Foluso Owotade	-1		Oral
895	654	AM2005/0436	Odds	Frank Odds		blood
896	655	AM2005/0437	Odds	Frank Odds		blood
898	656	AM2005/0476	Odds	Frank Odds		blood
2355	656	MI_173.2,	Foluso Owotade	ME Bougnoux		Oral
899	657	AM2005/0473	Odds	Frank Odds		blood
901	659	AM2005/0472	Odds	Frank Odds		blood
2353	661	ML_166.2	Foluso Owotade	-1		oral
908	663	AM2005/0448	Odds	Frank Odds		blood
992	663	AM2005/0447	Odds	Frank Odds		blood
984	724	AM2005/0363	Odds	Frank Odds		blood
985	725	AM2005/0451	Odds	Frank Odds		blood
1027	745	AM2005/0358	Odds	Frank Odds		blood
1028	746	AM2005/0359	Odds	Frank Odds		blood
1045	759	AM2005/0360	Odds	Frank Odds		blood
2350	915	ML_140.1	Foluso Owotade	-1		oral
2349	1019	ML_132.2	Foluso Owotade	-1		Oral
2343	1909	ML_41.2	Foluso Owotade	-1		oral
2319	2164	ML_3.1,	Foluso Owotade	Marie-Elisabeth Bougnoux	Johannesburg	oral
2320	2165	ML_6.3,	Foluso Owotade	Marie-Elisabeth Bougnoux	Johannesburg	oral
2321	2166	ML_10.3	Foluso Owotade	-1	Johannesburg	oral
2336	2167	ML_12.2,	Foluso Owotade	ME Bougnoux		oral
2322	2167	ML_12.1	Foluso Owotade	-1	Johannesburg	oral
2323	2168	ML_12.3	Foluso Owotade	-1	Johannesburg	oral
2324	2169	ML_42.3	Foluso Owotade	-1	Johannesburg	oral
2344	2169	ML_42.1	Foluso Owotade	-1		Oral
2325	2170	ML_45.1,	Foluso Owotade	Marie-Elisabeth Bougnoux	Johannesburg	oral
2326	2171	ML_45.3	Foluso Owotade	-1	Johannesburg	oral
2327	2172	ML_78.1	Foluso Owotade	-1	Johannesburg	oral
2328	2173	ML_78.2	Foluso Owotade	-1	Johannesburg	oral
2329	2174	MI_82.3	Foluso Owotade	-1	Johannesburg	oral
2330	2175	ML_166.3	Foluso Owotade	-1	Johannesburg	oral
2331	2176	ML_193.1,	Foluso Owotade	Marie-Elisabeth Bougnoux	Johannesburg	oral
2332	2177	ML_3.3	Foluso Owotade	-1	Johannesburg	oral
2333	2178	ML_6.1	Foluso Owotade	-1	Johannesburg	oral
2334	2179	ML_6.2,	Foluso Owotade	ME Bougnoux		oral
2345	2181	ML_42.2,	Foluso Owotade	ME Bougnoux	Johannesburg	Oral
2335	2182	ML_10.1	Foluso Owotade	-1		oral
2337	2183	ML_27.1	Foluso Owotade	-1		oral
2357	2184	ML_185.3	Foluso Owotade	-1		Oral
2346	2185	ML_82.1,	Foluso Owotade	ME Bougnoux		Oral
2340	2186	ML_35.2	Foluso Owotade	-1		oral
2341	2187	ML_35.3	Foluso Owotade	-1		oral
2348	2188	ML_132.1	Foluso Owotade	-1		Oral
2354	2189	ML_173.1	Foluso Owotade	-1		
2358	2190	ML_193.2,	Foluso Owotade	ME Bougnoux		Oral

Source: http://calbicans.mlst.net/earth/maps

9.6 Geographic distribution of *C. albicans* strains

STUDYID	DST	Geography
3.1	2164	South Africa
3.2	808	South Africa, Scotland
3.3	2177	South Africa
6.1	2178	South Africa
6.2	2179	South Africa
6.3	2165	South Africa
10.1	2182	South Africa
10.2	2166	South Africa
10.3	2166	South Africa
12.1	2167	South Africa
12.2	2180	South Africa
12.3	2168	South Africa
27.1	2183	South Africa
27.2	124	South Africa, UK, Israel, France, USA, Morocco, Austria
27.3	124	South Africa, UK, Israel, France, USA, Morocco, Austria
35.1	210	South Africa, UK
35.2	2186	South Africa
35.3	2187	South Africa
41.1	95	South Africa, UK, Switzerland, Australia, France, Morocco, Austria, India, Pakistan
41.2	1909	South Africa
41.3	1909	South Africa
42.1	2169	South Africa
42.2	2181	South Africa
42.3	2169	South Africa
45.1	2170	South Africa
45.2	2170	South Africa
45.3	2171	South Africa
78.1	2172	South Africa
78.2	2173	South Africa
78.3	2172	South Africa
82.1	2185	South Africa
82.2	646	South Africa
82.3	2174	South Africa
132.1	2188	South Africa
132.2	1019	South Africa, UK
132.3	1019	South Africa, UK
140.1	915	South Africa, France
140.2	619	South Africa, China
140.3	619	South Africa, China
166.1	144	South Africa, USA, Saudi Arabia
166.2	661	South Africa, USA
166.3	2175	South Africa
173.1	2189	South Africa
173.2	656	South Africa, Mexico, Scotland, UK
173.3	124	UK, Israel, France, USA, Morocco, Austria
185.1	392	South Africa, UK
185.2	392	South Africa, UK
185.3	2184	South Africa
193.1	2176	South Africa
193.2	2190	South Africa
193.3	375	South Africa, Mexico, Scotland, UK