Characterization of *Candida* species isolated from the oral mucosa of HIV-positive African patients

By

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Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Medical Biosciences at the University of the Western Cape

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Keywords

Candidiasis
Candida albicans
HIV infection
Fluconazole
Antifungal drug resistance
TREK Sensititre
Proteomics
SDS-PAGE
HPLC-MS

Abstract

One of the most common HIV-associated opportunistic infections is candidiasis, caused by *Candida albicans* or other *Candida* species. In immune suppressed subjects, this commensal organism can cause an increase in patient morbidity and mortality due to oropharyngeal or systemic dissemination. Limited information exists on the prevalence and antifungal susceptibility of *Candida* species in the African continent, the most HIV-affected region globally and home to new and emerging drug resistant *Candida* species. The mechanisms of *Candida* drug resistance in the African continent have also not been described.

In this study, 255 *Candida* species isolated from the oral mucosa of HIV-positive South African and Cameroonian patients were identified using differential and chromogenic media and their drug susceptibility profiles tested using the disk diffusion method and the TREK Sensititre system, an automated broth microdilution method. *Candida* cell wall fractions were run on SDS-PAGE and HPLC-MS with the aim of identifying peptides specifically expressed by antifungal drug resistant isolates.

Comparisons between the two groups of isolates revealed differences in *Candida* species prevalence and drug susceptibility with interesting associations observed between specific drug resistance and duration of ARV therapy. This study showed that fluconazole, the drug of choice for the treatment of candidiasis in the African continent, is not an effective therapy for most cases of *Candida* infection, and suggests that regional surveillance be implemented in the continent. A multiple-drug resistant *Candida* strain was identified in this study, a finding that has not previously been documented.

The use of proteomics tools allowed for the identification of peptides involved in drug resistance and the elucidation of *Candida* colonization mechanisms in HIV-infected African patients.

Declaration

I declare that this work is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Pedro Miguel dos Santos Abrantes

Signed: …….. Date: ……..

I dedicate this thesis to my parents, Margarida Abrantes and Carlos Abrantes, true role models who gave me the tools to reach for my dreams and never stopped believing in me. For this I am very grateful.

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I would like to thank my supervisors, Prof. Charlene Africa and Prof. Patrick Bouic for all their guidance, patience and support throughout this journey. I am very lucky to have such amazing individuals as my supervisors. I would also like to thank the following persons and institutions for their help during the course of this study: Mr. Norman Coldrey, Mr. Ernest Maboza and Mr. Claude Bayingana from the Medical Microbiology Cluster, Medical Biosciences Department, UWC for their assistance and endless support in the Medical Microbiology laboratory; Dr. Mike Mackenzie, Dr. Ali Shadari, Sr. Dumisa Bizani, nurses and counselors at the ARV clinic, Delft Day Hospital for their assistance during the sample collection process and for their patience while filling in the patient's questionnaires and consent forms; Dr. Leo Ayuk, Dr. Charles Awasom and nursing staff at the Bamenda Regional Hospital, Cameroon, for making sample collection in Bamenda a reality; Prof. Carole McArthur from the University of Missouri, for an incredible research trip to Cameroon and her collaborative work on the TREK Sensititre platform; Mr. Marinus Barnard from the National Health Laboratory Services in Greenpoint for training me on the TREK Sensititre system; Mr. Randall Fisher from the Molecular Virology laboratory, Medical Biosciences Department, UWC for the SDS-PAGE training and assistance and for having a genuine will to teach and help others; Dr. Salome Smit from the CAF – University of Stellenbosch for training me on the FASP protocol and for the HPLC-MS analysis; Prof. Richard Madsen from the University of Missouri for his guidance with the statistical analysis; My sister, Dr. Katya Abrantes, who truly is an inspiration to me; My friends in Cape Town, who make my stay in this beautiful city so pleasant; The National Research Foundation of South Africa and the Division for Postgraduate Studies at UWC for funding this project. And finally, I would like to express my gratitude to all the patients who agreed to participate in this study. Without them none of this would have been possible.

Research Output

The following peer-reviewed manuscripts and conference proceedings published in scientific journals were generated during the course of this study:

Pedro MDS Abrantes, Charlene WJ Africa. "*Candida* species isolated from the oral mucosa of a South African population of HIV-positive women". *South Afr J Epidemiol Infect* 26(3):127-8, 2011.

Ilze Messeir, Pedro MDS Abrantes, Charlene WJ Africa. "Strengths and Limitations of different Chromogenic Media for the Identification of *Candida* species". *J Microbiol Res* 2(5): 133-140, 2012.

Pedro MDS Abrantes, Carole McArthur, Charlene WJ Africa. "A Comparison of Susceptibility Patterns of Oral *Candida* Isolates from South African and Cameroonian HIV- Positive Populations". *J Dent Res* 91(Spec Iss B):35;138, 2012.

Pedro MDS Abrantes, Charlene WJ Africa. "HIV/*Candida* co-infection in Sub-Saharan African women on ART". *South Afr J Epidemiol Infect* 28(3):245. ISSN (Print): 1015-8782, ISSN (Web): 2220-1084, 2013.

Pedro MDS Abrantes, Carole McArthur, Charlene WJ Africa. "Multi-drug resistant (MDR) oral *Candida* species isolated from HIV-positive patients in South Africa and Cameroon". *Diagn Microbiol Infect Dis*. DOI: 10.1016/j.diagmicrobio.2013.09.016.

Peer-Reviewed Conference Presentations

Below is a list of abstracts from conferences in which sections of this study were presented:

PMDS Abrantes, CWJ Africa. "Profiles of fluconazole-resistant *Candida* strains". International Union of Biological Sciences 30th General Assembly; Darwin 200 Scientific Symposium 2009, UWC, Cape Town.

The ability to combat infections with the use of appropriate antimicrobials is being hampered by the emergence of more and more resistant strains of pathogenic bacteria and fungi and an increase in HIV-AIDS. This poses a threat of an increase in untreatable infections with devastating outcomes.

Candida samples (128) were isolated from mouth swabs of HIV-positive patients at community hospitals in the Western Cape, by sample-scraping of the patient's oral mucosa and tongue. The samples were inoculated onto selective (Sabouraud's) and differential agar (modified Fluka chromogenic *Candida* identification agar with selective supplement, and tomato (V8) agar). Antimicrobial susceptibility testing was done on Sabouraud's and Yeast Nitrogen Base Agar plates using fluconazole antimicrobial disks (25μg) and incubated for 24 hours at 37˚C. Resistance or susceptibility was measured from the edge of the disk to the edge of the susceptibility zone. The presence of microcolonies within the susceptibility zone was also scored.

Fifty-seven percent (57%) of the *Candida* samples demonstrated different degrees of resistance to fluconazole. More isolates of *C. albicans* (60%) than *C. dubliniensis* (30%) and *C. krusei* (50%) were resistant to fluconazole. These results clearly show that resistance to fluconazole is becoming widespread throughout clinical samples which could contribute to an increase in patient morbidity and mortality. In order to have a better understanding of this resistance, the samples were analysed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). This investigation is currently underway and specific membrane proteins related to drug resistance are being characterised.

PMDS Abrantes, CWJ Africa. "Fluconazole susceptibility of *Candida* species present in the oral mucosa of HIV-positive South African patients". 11th International Union Against Sexually Transmitted Infections World Congress Africa 2009, PO2.1.8, Nedbank Conference Centre, BOE Building, V&A Waterfront, Cape Town.

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Fifty-three percent (53%) of the *Candida* samples demonstrated different degrees of resistance to fluconazole. More isolates of *C. albicans* (58%) than *C. glabrata* (42%) and *C. dubliniensis* (50%) were resistant to fluconazole. These results clearly show that resistance to fluconazole is becoming widespread throughout clinical samples which could contribute to an increase in patient morbidity and mortality. More detailed studies on the mechanisms of drug resistance in these samples is ongoing.

PMDS Abrantes, R Fisher, CWJ Africa. "Characterization of Fluconazole-Resistant and -Susceptible Oral *Candida* Isolates from HIV-Positive Patients Using SDS-PAGE". Cape Biotechnology Forum 2010, PP01, Lord Charles Hotel, Somerset West.

Candida infections are known contributors to the higher morbidity and mortality rates seen in HIV-positive patients, especially in underdeveloped countries. Localized and systemic *Candida* infections are normally treated with fluconazole in public health facilities in South Africa. It is thought that the widespread and improper use of this drug over the past decade has resulted in an increase in fluconazole-resistant isolates.

Three *Candida* species were identified from one hundred and twenty eight (128) *Candida* strains isolated from the oral mucosa of HIV-positive patients. These were investigated for susceptibility to fluconazole and protein expression was analysed using SDS-PAGE.

Results yielded very characteristic and well expressed protein bands in both susceptible and resistant *Candida* strains. A 24kDa protein was expressed in *C. albicans* fluconazole-resistant and *C. glabrata* samples. Protein bands in the region of 37kDa were found in *C. dubliniensis* fluconazole-susceptible and *C. glabrata* fluconazole-resistant samples while a 44kDa protein consistent with exoglucanase was found to be expressed in fluconazole-susceptible *C. dubliniensis* samples. A 50kDa protein band was present in fluconazole-susceptible *C. glabrata* samples.

This study shows that clinical *Candida* strains seem to express drug-resistance protein patterns in resistant *C. albicans* samples. However, the expression of peptides previously described as related to fluconazole resistance seemed to be present in both fluconazole-resistant and -susceptible non-albicans species. More detailed characterization of these peptides is underway.

PMDS Abrantes, CWJ Africa "*Candida* species isolated from the oral mucosa of a South African population of HIV-positive women". Federation of Infectious Diseases Societies of Southern Africa 4WARD 2011 4th Congress, P18, Elangeni Hotel, Durban.

Candida infections are known contributors to the higher morbidity and mortality rates seen in HIV-positive patients, especially in underdeveloped countries.

Females are more predisposed to *Candida* infections than their male counterparts. In this study, the prevalence and fluconazole susceptibility of *Candida* species in HIV-positive women was investigated.

A significant association between *Candida* species colonization and health status was found, as well as between *Candida* species and drug susceptibility results.

C. albicans was the only species isolated from pregnant/recently pregnant women. Because an association between pregnancy outcomes and *Candida* has previously been reported, this deserves further investigation.

PMDS Abrantes, C McArthur, CWJ Africa "A Comparison of Susceptibility Patterns of Oral *Candida* Isolates from South African and Cameroonian HIV- Positive Populations". International Association for Dental Research 90th General Session 2012, Oral Presentation Seq. 35; S038, Iguaçu Falls, Brazil.

Objectives: *Candida* infections are known contributors to the high morbidity and mortality rates seen in HIV-positive patients, especially in underdeveloped countries. Candidiasis is commonly present in the mouths of these individuals, with *Candida albicans* being the most commonly identified species. The prevalence of drug-resistant Candida species in HIV-positive populations in South African has, to our knowledge, not previously been compared

with HIV-positive populations in other parts of Africa and the possible emergence of drug-resistant species is a cause for concern that deserves to be investigated.

Methods: In this study, *Candida* isolates were collected from the oral mucosa of 128 South African and 126 Cameroonian HIV-positive patients, by scraping the mouths of consenting patients using sterile cotton swabs. Ethics clearance for this project was granted by the University of the Western Cape. Confirmation of *Candida* species was done by growth on differential media, Gram staining and microscopy. The isolates were grown on selective media and differentiated using two commercial chromogenic agars and Tomato (V8) agar. Changes in colony colour, morphology and pseudohyphae/chlamydospore expression could then be observed, allowing for species differentiation. Isolates were also examined for antifungal susceptibility patterns using the TREK system.

Results: The results from this study suggest that the prevalence of *Candida* species varies according to geographical region and HIV-subtype. Discrepancies in antifungal drug susceptibility patterns were also observed in the two populations.

Conclusion: The emerging drug-resistance raises the need for increased species prevalence surveillance, as this information can have clinical implications in the choice of more appropriate and effective patient treatment.

C McArthur, PMDS Abrantes, L Ayuk, C Awasom, CWJ Africa "Widespread Azole Resistance of Oral *Candida* species Isolated from HIV-positive Cameroonian patients". American Society for Microbiology 113th General Meeting 2013, 199/2335, Denver, Colorado, U.S.A.

Background: *Candida* infections are a common cause of death in immunocompromised patients. The prevalence and anti-mycotic drug susceptibility profiles of *Candida* species from Cameroon in Africa are unavailable. This study was prompted by an increasing incidence of treatment failure. Drug susceptibility profiles, necessary to improve treatment outcomes, is particularly important in countries where the sale of antimicrobials and antifungals is uncontrolled and resistance may emerge due to the indiscriminate use.

Objective: The goal of this study was to characterize and determine drug susceptibility of oral *Candida* species in Cameroonian patients with HIV/AIDS.

Materials and Methods: *Candida* species were isolated from the oral cavity of 126 HIV-positive patients attending a local HIV/AIDS clinic in the Cameroon. Drug susceptibility to azoles and echinocandins was determined using the commercial TREK Sensititre® YeastOne™ platform that provides the minimal inhibitory concentration of amphotericin B, 5-flucytosine, anidulafungin, caspofungin, micafungin, fluconazole, itraconazole, posaconazole, and voriconazole.

Results: Ninety two isolates identified were *Candida albicans.* Remaining isolates were *C. glabrata* (24), *C. tropicalis* (4), *C. krusei* (3), *C. parapsilopsis/lusitanreae/keyfr* (2), and one isolate was *C. dubliniensis.* More than 50% of *C. albicans* isolated were resistant to azoles but 115 *Candida* species (87%) were susceptible to amphotericin B. Twenty one of the twenty four *C.glabrata* identified (88%) were resistant to micafungin. The majority of Cameroonian *Candida* species were sensitive to flucytosine (5-FC) (95%) and echinocandins (79%).

Conclusions**:** The report of azole resistance in all *Candida* species isolated from immunocompromised patients in Cameroon is a new and important observation. We found the approach using a broad screening platform an effective means to obtain data rapidly. We propose confirmation of these data and regional surveillance of *Candida* species in other areas in Cameroon and surrounding countries to develop an effective public health management and treatment strategy.

PMDS Abrantes, CWJ Africa. "HIV/*Candida* co-infection in Sub-Saharan African women on ART". Federation of Infectious Diseases Societies of Southern Africa 5th Congress 2013, P77, Champagne Sports Resort, Winterton.

Introduction: Sub-Saharan Africa has 23.5 million cases of HIV and is home to 92% of the world's HIV-positive pregnant women of whom 24% die of pregnancy related complications. Oral candidiasis is a common condition in HIV-AIDS patients, caused by commensal yeasts which may colonise the mucous membranes of the mouth causing morbidity due to several factors including immunosuppression, smoking, poor nutrition and the use of antibiotics.
Methods: One hundred and ninety-four South African and Cameroonian HIV-positive women participated in the study. Only subjects who had white pseudomembranous plaque on the tongue or visible oral candidiasis were included. Samples were collected by scraping the patient's oral mucosa and tongue with a sterile swab. *Candida* species were differentiated using selective and chromogenic media and their susceptibility to antifungal drugs was tested using the TREK Sensititre system.
Results and conclusion: One hundred and ninety-six isolates, representative of six *Candida* species were identified. *C. albicans* was the predominating species, with *C. glabrata* and *C. dubliniensis* being the more frequent of the non-albicans isolates. Azole drug resistance patterns were very high for *C. albicans*, while *C. glabrata* showed high resistance patterns to echinocandins drugs. The duration of ART could be associated with the presence of different *Candida* species but no concrete conclusions could be drawn concerning HIV/*Candida* co-infection when controlling for other risk factors such as HIV stage, pregnancy, age and treatment for tuberculosis. This may be a cause for concern, particularly in the case of pregnancy, where co-infection may pose a risk for maternal morbidity and mortality.

List of abbreviations

AA – Amino acid

AIDS – Acquired immunodeficiency syndrome

APS – Ammonium persulfate

ART – Antiretroviral therapy

ARV – Antiretroviral

ATCC – American type culture collection

AZT – Azidothymidine (zidovudine)

BSA – Bovine serum albumin

$CaCO_3$ – Calcium carbonate

CD4 – Cluster of differentiation 4

CLSI – Clinical laboratory standards institute

Da – Dalton

DDI – Didanosine

DNA – Deoxyribonucleic acid

D4T – Stavudine

EDTA - Ethylenediaminetetraacetic acid

EFV – Efavirenz

FASP – Filter aided sample preparation

g – Grams

g – Relative centrifugal force

GMB – Methylene-blue and glucose-enriched Mueller-Hinton agar

GTE – Glucose-Tris-EDTA

HAART – Highly active antiretroviral treatment

HCl – Hydrogen chloride

HIV – Human immunodeficiency virus

HPLC-MS – High performance liquid chromatography – mass spectrometry

kDa – Kilodalton

KLT – Kaletra (lopinavir)

kV – Kilovolt

LPV/r – Alluvia

mA - Milliamperes

MIC – Minimum inhibitory concentration

mL – Milliliter

mm – Millimeter

mM – Millimolar

MS – Mass spectrometry

MW – Molecular weight

m/z – Mass to charge ratio

NCPF – National collection of pathogenic fungi

NVP – Nevirapine

PAA – Polyacrylamide

PCR – Polymerase chain reaction

PSM – Peptide spectrum matches

PMSF - phenylmethylsulfonyl fluoride

RNA – Ribonucleic acid

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SPSS – Statistical product and service solutions

TEMED – Tetramethylene diamine

TDF – Tenofovir disoproxil fumarate (tenofovir)

Tris - tris(hydroxymethyl)aminomethane

v – Volt

v/v – Volume to volume

YNBG – Yeast nitrogen base agar

YO9 – YeastOne 9

YPD – Yeast peptone dextrose

3TC – Lamivudine

µg - Microgram

µl - Microliter

°C – Degrees centigrade

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Chapter 1: Review of the Literature

1.1 *Candida* in HIV infection

Human Immunodeficiency Virus (HIV) is a retrovirus that infects cells bearing the CD4 cell surface antigen, which include T_h cells, monocytes, dendritic cells and microglia (Mims *et al*, 2004). This results in an impaired host immune response and consequent predisposition to opportunistic infections (Cury *et al*, 2003).

HIV infection was responsible for approximately 1.7 million deaths worldwide in 2011, with an estimated 2.8 million people being newly infected in 2010 (WHO, 2013). An estimated 35.3 million people are infected with the HI virus globally, with most of these (22.9 million) living in Africa (WHO, 2013).

The prevalence of HIV in Africa is not uniform: while South Africa has an estimated 17.3% of its adult population living with HIV, the overall infection rate in the Cameroon is thought to be approximately 4.6% (WHO, 2013). Statistics for 2007 show that the HIV prevalence rate is much higher in Southern Africa than in other African regions: More than a quarter (26.1%) of the adult population of Swaziland was reported to be infected with HIV, followed by Botswana (23.9%) and Lesotho (23.2%). In West Africa the rates were found to be lower: Nigeria had 3.1% of its adult population infected by HIV, followed by Equatorial Guinea (3.4%), Chad and Congo (both with 3.5%), Gabon (5.9%) and the Central African Republic (6.3%) (WHO African Health Observatory, 2010).

In South Africa and Cameroon HIV is the leading cause of disease burden (40.7% in South Africa and 14.4% in Cameroon) accounting for 46% and 5% of the infant mortality rate in South Africa and Cameroon respectively (WHO African Health Observatory, 2010).

Human immunodeficiency virus – type 1 (HIV-1) is the predominant HIV type in most regions, with HIV-type 2 infections being found in about 1 to 2 million people in West Africa (Gottlieb *et al*, 2008). Although AIDS-related symptoms such as tuberculosis and pneumonia appear to be similar in late HIV-1 and -2 infections, the two HIV subtypes are known to have different disease progression patterns (Popper *et al*, 1999, MacNeil *et al*, 2007), which could possibly influence the development of opportunistic infections.

One of the most common HIV-associated opportunistic infections is candidiasis, caused mainly by *Candida albicans* or *Candida dubliniensis* (a novel species first discovered in 1995). Other *Candida* species that can become invasive include *C. tropicalis, C. krusei* and *C. glabrata*. These can cause an increase in patient morbidity and mortality due to oropharyngeal or systemic dissemination. HIV-related *Candida* infections were found to be associated with a higher patient mortality in developing countries than in developed countries (Clark and Hajjeh, 2002).

Candida infection can present as pseudomembranous candidiasis (thrush), characterized by white pseudomembranes in the oral mucosa and/or upper digestive tract; acute atrophic candidiasis, characterized by pain and inflammation of the mouth or tongue; chronic hyperplastic candidiasis, characterized by homogenous white lesions on the oral mucosa or tongue and angular cheilitis, characterised by lesions in the corners of the mouth, usually accompanying intra-oral candidiasis (Akpan and Morgan, 2002). In HIV-positive individuals linear gingival erythema, a characteristic periodontal lesion, is associated with *Candida* infection (Samaranayake, Keung Leung and Jin, 2009). In severely immunocompromised patients, *Candida* species can spread through the bloodstream and gastrointestinal tract. This can lead to systemic candidiasis, which has a mortality rate of up to 57% (Fraser *et al*, 1992). *Candida* is currently the 4th most commonly isolated microorganism in nosocomial bloodstream infections (Budhavari, 2009).

Candida species are pseudohyphae-forming yeasts, which grow as ovoid single cells and multiply by budding and division. It has been shown by proteomic analysis that *Candida* species undergo dimorphic transitions from yeast to hyphae, with this factor being related to a shift between colonization and actual infection. Different proteins were identified as being involved in factors such as metabolism and protein synthesis, creating a possible link between protein expression and *Candida* invasion (Hernandez *et al*, 2004).

The *Candida* cell wall, known to change over time, plays a crucial role in its pathogenicity (Chaffin *et al*, 1998). It is composed of 80-90% carbohydrate, forming a complex extracellular matrix of β-glucans, chitin, mannan and manno-proteins. The latter accounts for around 40% of the total carbohydrate content (Cihlar and Calderone, 2009).

Innate immune defenses against *Candida* are based on the antifungal efficacy of cytokine-mediated tissue macrophages and circulating neutrophils (polymorphonuclear leukocytes). These cytokines include granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF). T-helper cell 1 (T_h1) cytokines interferon γ (IFNγ), interleukin (IL)-12, IL-15 and tumour necrosis factor α (TNFα) have also been implicated in the cytokine-mediated immune response against *Candida* species. T-helper cell 2 (T_h2) mediated cytokines IL-4 and IL-10, however, have been shown to suppress the antifungal action of phagocytes against *Candida* species (Ernst and Rogers, 2005).

Candida species can survive and multiply inside macrophages and neutrophils. Because neutrophils are partly responsible for controlling the establishment of invading fungi, immunosuppression may cause an overgrowth of *Candida* to occur (Mims *et al*, 2004). *C. dubliniensis* was shown to be more vulnerable to the fungicidal activity of leukocytes than *C. albicans* (Vilela *et al*, 2002).

Another study showed that pre-exposure of insect larvae to *Candida albicans* or glucan/laminarin resulted in a protection mechanism against a subsequent *Candida* lethal dose. This was shown to be due to an increased expression of antimicrobial peptides by the larvae, demonstrating that there can also be peptide changes in the host that can lead to different outcomes in *Candida* infection and colonization (Bergin, 2006, Nett *et al*, 2006).

It has been demonstrated by DNA subtyping that *C. albicans* has a minimum of 70 different subtypes, and that patients with multiple *Candida* subtype infections had lower CD4 counts than those with single infections (Redding *et al*, 1997). Another study identified around 85 immunoreactive serum protein species in patients infected by *C. albicans* and allowed for the characterization of 42 enzymes identified as *C. albicans* antigens (Pitarch *et al*, 2004). Cell wall surface proteins of *C. albicans* are known to cause immune responses in the host, and have been used with some success in trials for the development of drugs and vaccines against *Candida* species (Thomas *et al*, 2006b).

The complete genome of *Candida* is known and is available in different databases (Rossignol *et al*, 2008). However, there are challenges that cannot be solved by using a purely genomic approach, and some of these are the mechanisms of drug resistance in fungal species. Proteins

perform essential roles in cellular processes, making their study the next logical step after detailed mapping of genes. Proteomic studies are generally more complex than genomic studies, as in the proteome there is a dynamic response to genetic and environmental factors (leading to changes in protein expression) and the specific proteins being analysed are often present in a very small amount of sample with many other non-relevant proteins being present. Sample processing in proteomics studies can also be more complex, due to protein degradation and other factors (Reinders and Sickmann, 2009). The use of proteomic analysis in drug resistance studies is able to provide important information on the biological complexities and pathogenic behaviour of *Candida* species, and can lead to new approaches in the management of fungal infections (Thomas *et al*, 2006a).

1.2 Susceptibility and resistance to treatment

Various antifungal drugs with different modes of action have been developed over the years. These include polyene antifungals (e.g.: nystatin and amphotericin B), which interfere with ergosterol synthesis, thereby causing cell membrane leakage; the imidazoles (e.g.: miconazole, clotrimazole and ketoconazole), which also interfere with ergosterol and other cell membrane sterol synthesis; the echinocandins (e.g.: anidulafungin, micafungin and caspofungin), which inhibit β 1-3 glucan synthesis, affecting the fungal cell wall and 5-Flucytosine, which interferes with fungal RNA and DNA synthesis. The triazoles (comprising of fluconazole, posaconazole, voriconazole, itraconazole, isavuconazole, pramiconazole and ravuconazole), which interfere with the synthesis of ergosterol, have been shown to have fewer side effects than some of the other antifungal drug classes (Khan and Jain, 2000).

Fluconazole is responsible for the inhibition of the enzyme cytochrome P450 14α-demethylase, which converts lanosterol to ergosterol and is required in fungal cell wall synthesis (Sweetman, 2004). This antifungal is routinely given as treatment for candidiasis in healthcare facilities, as it is less toxic and more effective than imidazole antifungals such as ketoconazole or amphotericin B, a polyene antibiotic which binds to ergosterol in the fungal cell wall, leading to leakage of cellular contents and subsequent cell death (Kshirsagar *et al*, 2005). It is, however, not recommended for pregnant women, as it is a teratogenic drug (Pursley *et al*, 1996, Lopez-Rangel and Allen, 2005). The primary mechanism of fluconazole

resistance in *C. dubliniensis* has been shown to be overexpression of the major facilitator efflux pump Mdr1p (Sullivan and Coleman, 1997). A 2002 study identified two proteins in *Candida glabrata* induced by fluconazole exposure, namely a 169-kDa protein which was later identified using mass spectrometry as the ATP binding cassette-type drug efflux transporter CgCdr1p, and a 61-kDa protein, later identified as lanosterol 14α-demethylase, an enzyme targeted by fluconazole (Niimi *et al*, 2002).

It has also been shown that an increased expression of plasma membrane drug efflux pump Cdr1p and Cdr2p causes a decrease in azole susceptibility in *C. albicans* clinical isolates (Holmes *et al*, 2006). This may explain why patients initially infected with *C. albicans* and treated with fluconazole demonstrate a switch to *C. dubliniensis* colonization (Martinez *et al*, 2002, Sullivan and Coleman, 1997).

Other studies have shown that clinical *Candida* isolates with high resistance often present with multiple resistance mechanisms, including the overexpression of efflux pumps, changes in the expression of lanosterol 14α-demethylase enzyme and other mechanisms that lower the drug concentration in fungal cells, in such a way that ergosterol synthesis is not interrupted (Niimi *et al*, 2005, White *et al*, 2002). This is especially true in the case of *C. glabrata*, which seems to have an innate resistance to fluconazole and whose numbers have increased in patients presenting with candidiasis in recent years (Vermitsky and Edlind, 2004).

Different studies have shown that resistance to available antifungal therapies is widespread, including the case of more recent antifungal drugs such as fluconazole and itraconazole (Luque *et al*, 2009, Manzano-Gayosso *et al*, 2008). It is thought that this rapid resistance occurred due to the widespread and repeated use of these drugs (Jia *et al*, 2008). Another study has also demonstrated that fluconazole has an exposure-response relationship with patient mortality, showing that most patients who died from candidiasis had fluconazole resistant strains and that patient mortality was associated with low fluconazole therapeutic dosages (Baddley *et al*, 2008).

Different *Candida* species have varying resistance patterns, which appear to be geographically determined. Therefore an early identification facilitates the selection of an appropriate antifungal drug. It has been suggested that the use of oral antifungals in oropharyngeal candidiasis must be reserved for cases where there is no response to topical

antifungal treatment, as resistance to azole antifungal agents is increasing (Powderly, Mayer and Perfect, 1999). A 2003 study also stressed the need for fungal species and resistance pattern surveillance to avoid an even higher number of improperly treated, and therefore resistant, fungal infections (Godoy *et al*, 2003). This is a cause of concern in the case of immunocompromised patients, who are at a much higher risk of developing opportunistic complications.

Limited fluconazole susceptibility data from the African continent is available. A previous report of baseline data from South Africa demonstrated 100% susceptibility of *C. albicans* to fluconazole (Blignaut et al, 2002). However, and of importance, is that the study was done before the introduction of fluconazole to patients attending HIV-AIDS clinics. Other more recent African studies have reported a frequent resistance of *C. albicans* and non-albicans species to azoles (Njunda *et al*, 2012; Mulu *et al*, 2013).

1.3 Transmission of drug resistant *Candida* isolates

Studies have shown that oral transmission of *C. albicans* in HIV-positive couples could contribute to the dissemination of fluconazole-resistant isolates (Dromer *et al*, 1997). DNA sampling of oral isolates demonstrated that sexual partners tended to share genetically indistinguishable clones. This could also play a role in the increase in fluconazole resistance that has been seen in recent years.

A demonstration of genetically indistinguishable strains in different hospitalised patients, indicative of person-to-person transmission within the hospital environment, emphasised the need for oral sample collection and initiation of treatment in patients who present with *Candida* in the oral mucosa (Fanello *et al*, 2006).

Earlier studies showed an increased diversity of *C. albicans* strains in HIV-positive patients along a certain period of time, resulting in a change in the *Candida* species genotype, along with changes in fluconazole susceptibility (Lasker *et al*, 2001) and the ability to spread fluconazole-resistant *Candida* strains to other patients and staff (Makarova *et al*, 2003).

The differential expression of several proteins that may contribute to fluconazole resistance in *C. glabrata* has been identified (Rogers *et al*, 2006). These were found to be the up-regulation of the ATP-binding cassette transporter Cdr1p, the ergosterol biosynthesis enzyme Erg11p, proteins involved in glycolysis and glycerol metabolism and proteins involved in the response to oxidative stress and cadmium exposure. Other studies have described this resistance as a result of point mutations in the gene encoding for lanosterol demethylase (ERG11) and an increased expression of ERG11 or the genes encoding for the multidrug efflux pumps CaMDR1, CDR1, and CDR2 (Franz, Ruhnke and Morschhäuser, 1999). It has also been found that membrane-associated multidrug transporter proteins Cdr1p, Cdr2p, and Mdr1p were not found in fluconazole-resistant isolates (Hooshdaran *et al*, 2004). However, no similar proteomics studies have been done on HIV-positive patients, on the African continent or with other *Candida* species.

1.4 Fluconazole susceptibility testing

The process of determining specific values for fluconazole resistance and susceptibility is not straightforward. A recent review addressing this question studied the fluconazole susceptibility results of 13.338 isolates obtained from various studies. Specific fluconazole concentration values for susceptible, resistant and susceptible-dose dependant isolates were determined based on the information collected (Pfaller, Diekema and Sheehan, 2006). However, research on antifungal susceptibility testing remains in its infancy. Factors such as zone of inhibition, time of incubation and appearance of microcolonies within the susceptibility zone when using fluconazole impregnated felt disks are still being used in different ways by different groups. This poses a problem, since unlike bacteria, there is no standardized method for antifungal susceptibility testing (Pfaller, Diekema and Sheehan, 2006). The use of fluconazole impregnated felt disks is a simple, rapid and cost-effective method (Ernst and Rogers, 2005) that produces clear results (Kustimur *et al*, 2003) depending on which disk diffusion test medium is used. Various agars can be used in antifungal susceptibility testing, including Sabouraud Dextrose agar, Yeast Nitrogen Base agar, High Resolution Medium and Casitone agar. It has been found that Yeast Nitrogen Base agar produces the best results with relation to inhibition zone definition and quality of growth when compared with other mycological agars (May, King and Warren, 1997, Yücesoy, Guldas and Yuluq, 2001).

Methylene-blue and glucose-enriched Mueller-Hinton (GMB) agar has also been used, proving to be a reliable and low-cost medium for *Candida* susceptibility testing (Lee *et al*, 2001) and is the the recommended antifungal disk diffusion medium of the Clinical and Laboratory Standards Institute (CLSI, 2009). However, newer drug susceptibility platforms which comply with CLSI standards have been developed, leading to more straightforward ways of testing *Candida* isolates against different antifungal drugs. One of these is the TREK Sensititre drug susceptibility platform (Cat. No. V2020-SYS, Thermo Scientific, USA), a broth microdilution method that provides multiple antifungal drug susceptibility testing.

1.5 Antiretroviral (ARV) therapy and antifungal use

By 2013, approximately 7.53 million Africans were on ARV treatment (WHO, 2013). South Africa has the world's largest ARV therapy programme, with various antiretroviral drugs being used in combination to combat HIV infection in public hospitals. In 2011, it was estimated that 66% of South Africans with advanced HIV infection were on ARV therapy, while the figure for Cameroon in the same year was 41% (WHO Data Repository, 2013).
Azidothymidine (AZT) is a reverse transcriptase inhibitor and the first approved treatment for HIV. It is also given to expectant mothers to prevent mother-to-child transmission. Other ARVs in common use include Stavudine (d4T), Lamivudine (3TC) Nevirapine (NVP) and Efavirenz (EFV). Additional drugs being introduced in the South African Highly Active Antiretroviral Therapy (HAART) list (second-line drugs) include Didanosine (ddl) and Tenofovir Disoproxil Fumarate (TDF), both reverse transcriptase inhibitors and Lopinavir (Kaletra-KLT), a protease inhibitor. A pilot study on resistance to first-line ARV drugs showed low levels of resistance to the different antiviral drugs, but stressed that these would increase over time, mainly due to high infection levels and patient drop-out rates (Morris *et al*, 2009).

Although not much is known about the interactions between fluconazole and antiretroviral drugs, research has shown that the concomitant use of fluconazole and nevirapine resulted in an increased plasma level of nevirapine (Wakeham *et al*, 2010). The use of azoles with nevirapine has been shown to decrease the plasma levels of antifungal drugs (Boehringer Ingelheim Pharmaceuticals, 2008) and it has been reported that the concurrent use of these two medications increases hepatotoxicity (WHO, 2008). *Candida* infections have continued to increase after the introduction of HAART (Traeder, Kowoll and Arasté, 2008).

1.6 Techniques used in the study of *Candida*

Detailed characterization of *Candida* species is essential to the understanding of resistance to antifungals. Recently developed non-culture based techniques to detect *Candida* species include polymerase chain reaction (PCR), *Candida albicans* germ tube (CAGT) antibody detection and 1,3 beta-D (B-D) glucan markers (Budhavari, 2009). However, these techniques are used for patient diagnosis before samples are grown in selective media and do not replace microbiological examination. Furthermore, these techniques have been associated with high numbers of false positive results. Sabouraud's selective media is still the standard in the culturing of fungal isolates, and is widely used for the identification of certain species by direct observation of the shape and colour of the colonies. This selective medium has a low pH and a high sugar concentration, providing ideal growth conditions for fungal species. It is used as a prerequisite growth media for more advanced applications using fungal isolates. Confirmation of presumptive clinical *Candida albicans* or *Candida dubliniensis* isolates is usually done using the germ tube test. This test relies on the ability these two species have to form short lateral filaments (germ tubes) when incubated for 2-3 hours in bovine serum (Haley and Callaway, 1979).

Chromogenic media allows for the differentiation of different *Candida* species based on the colour of the colonies. It is very simple to use and produces results that are accurate and relatively rapid. The synthetic chromogenic substrates present in this media are cleaved by the enzymes hexosaminidase (present in *C. albicans*, *C. dubliniensis* and *C. tropicalis*) and alkaline phosphatase (present in *C. krusei*, *C. glabrata*, *C. kefyr*, *C. parapsilopsis* and *C. lusitaniae*), and result in the production of a specific colour for each species (Rousselle *et al*, 1994) corresponding to specific enzymes present in that organism. An accurate result is normally obtained after 24-48 hours of incubation. The tomato juice (V8) agar (a mixture of tomato juice, $CaCO_3$, dextrose and agar) has been developed to meet the needs of routine clinical laboratories that require a low-cost and rapid technique to distinguish between *C. albicans* and *C. dubliniensis* (Alves *et al*, 2006), as it can be difficult to distinguish these two species in chromogenic agar. In this medium, *C. albicans* presents as smooth, round colonies, while *C. dubliniensis* presents as rough, irregular colonies. Microscopy of *C. dubliniensis* using this medium reveals the presence of pseudohyphae and chlamydospores. Other culturing techniques used in the differentiation of *C. albicans* and *C. dubliniensis* include growing the organisms in Sabouraud agar plates at 45°C (in which case *C. dubliniensis* does

not grow) (Pinjon *et al*, 1998, Gales *et al*, 1999, Us and Cengiz, 2007) and growing the organisms in tobacco agar (a mixture of tobacco and agar) at 28°C for 48-72 hours (Khan *et al*, 2004). In this medium *C. dubliniensis* grows as rough, yellow-brown colonies with pseudohyphae and chlamydospores, while *C. albicans* grows as smooth, white-cream colonies without the presence of pseudohyphae or chlamydospores.

Sabouraud agar, chromogenic and selective agars are used as part of the protocol for the use of other techniques, and remain important microbiological tools in isolating and identifying microorganisms. However, due to improved technology, cultural studies are being replaced by more sophisticated methods of characterization, a few of which are described below.

1.7 Techniques used for protein identification

1.7.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a technique widely used in proteomics research to detect protein modifications, determine relative protein molecular mass and separate proteins according to their electrophoretic ability. Protein separation is done by isolating the organism's protein fractions, standardizing protein concentration, denaturing the protein's secondary and tertiary structures and applying a negative current so that the proteins are separated according to their molecular weight (Schägger and Jagow, 1987).

Comparison of the distance traveled by proteins in the sample relative to those in marker proteins of known molecular weight allows for the determination of the weight of unknown proteins (Abelson, Simon and Deutscher, 1990).

Application of the SDS-PAGE technique to fluconazole-resistant *Candida* isolates showed the expression of proteins Grp2p, Ifd1p, Ifd4p, Ifd5p, Ifd6p Grp2p, and Erg10p, a protein involved in the ergosterol biosynthesis pathway (Hooshdaran *et al*, 2004). Other studies employing SDS-PAGE showed an increase in the expression of a 44kDa protein identified as exoglucanase after *C. albicans* strains were subjected to fluconazole (Angiolella *et al*, 2002), the expression of a 40kDa and 60kDa proteins in *C. albicans* after exposure to fluconazole (Kustos *et al*, 2006), the appearance of a 23.9kDa acid proteinase enzyme in *C. tropicalis* (Okumura, Inoue and Nikai, 2007) and significant differences in the protein band patterns of fluconazole-resistant and susceptible *Candida* strains (Shahid *et al*, 2006), with 23 proteins being more abundant in fluconazole-resistant strains, most of which range from 23kDa to 64 kDa in molecular weight. *Candida glabrata* proteins with a molecular weight of 61kDa and 169kDa have also been implicated in fluconazole resistance, and were identified as 14α-demethylase, a plasma membrane enzyme induced by fluconazole exposure and the drug efflux transporter CgCdr1p, respectively (Niimi *et al*, 2002).

1.7.2 High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

The use of mass spectrometry for the study of proteins has been previously documented (Domon and Aebersold, 2006). This analytical technique relies on the ionization of a sample and the subsequent measurement of the charge to mass ratios of the samples' compounds (Sparkman, 2000). Proteins are typically characterized by mass spectrometry by means of techniques such as time-of-flight MS (TOF-MS) or Fourier transform ion cyclotron resonance MS (FT-ICR). Liquid chromatography mass spectrometry (LC-MS) is a very sensitive mass spectrometry technique that can also be used in protein identification. This technique combines the separation ability of a chromatographic technique and the determination of charge to mass ratios of mass spectrometry, to ultimately identify components within a sample.

High performance liquid chromatography is a chromatographic technique that can be applied in proteomics to separate, identify or quantify different proteins. This method works by passing a pressurized liquid and sample mixture through a sorbent-filled column. The interaction of the sorbent particles and different sample components result in the separation of the latter (Touchstone, 1993). The equipment consists of a sampler, which allows the sample mixture to be carried into a column, different pumps used to pass the pressurized liquid through the column and a detector, which produces a signal proportional to the amount of sample which originates in the column. The detector is connected to a user computer interface, thereby allowing for data analysis.

High performance liquid chromatography is an efficient chromatography technique when compared with other similar platforms, as higher column pressures and smaller sample sizes are used. This ultimately results in better peak resolution.

Previous studies on *Candida* species using this technique have relied on the exposure of *Candida* strains to antifungal drugs (Li *et al*, 2007) and the determination of antifungal drug concentration in plasma (Marchetti *et al*, 2001) or suspension (Amin *et al*, 2009). The approach of exposing a *Candida* type strain to fluconazole and subsequently reading the fluconazole levels in the cultures using HPLC has been reported (Casalinuovo *et al*, 2008). The use of HPLC for *Candida* species detection has also been documented (Goldenberg *et al*, 2005). The large-scale analysis of clinical *Candida* protein fractions using HPLC is, to the best of our knowledge, undocumented.

Objectives

The objective of this study was to characterize *Candida* species isolated from immunocompromised patients, by means of microbiological (for species differentiation and antimicrobial susceptibility testing) and proteomic (for specific drug-resistance protein identification) tools.

Analysis of oral *Candida* specimens from different HIV-positive African patients using selective and differential media and their exposure to various antifungal drugs (with an emphasis on the most widely used drug, fluconazole) is an important approach which will aid in our understanding and knowledge of the current *Candida* prevalence and drug resistance patterns in African populations, details of which are at present very limited. This, in turn, will have beneficial repercursions in patient treatment. This study could also contribute to the management and treatment of *Candida* based on the *Candida* species present in HIV-positive patients in different regions of the African continent. The use of SDS-PAGE and HPLC-MS for characterization would identify specific *Candida* proteins that are related to fluconazole and other antifungal drug resistance, which could be used as the benchmark for new research in this area. This presents as a gap in the research that has already been done, as no study has compared the expression of specific resistance proteins in *Candida* with the much more detailed results that are obtainable from an HPLC-MS reading in a large number of clinical isolates.

Chapter 2: Materials and Methods

2.1 Sample collection

Two hundred and twelve (212) samples were collected between 2006 and 2008 from HIV-positive patients at community hospitals in Khayelitsha (Site B hospital) in and Delft (Delft Day Hospital –ARV clinic), both located in the greater Cape Town metropolitan area, in South Africa. A further 262 samples were collected in 2011 from HIV-positive patients at the Bamenda Regional Hospital in the North West Province of Cameroon, West Africa. This was done by scraping the patient's oral mucosa and tongue using a mouth swab. Only HIV-positive patients presenting with white pseudomembranous plaque on the tongue or other visible oral candidiasis were selected, as these patients had a higher chance of harbouring oral *Candida*. Ethical clearance for this project was given by the Ethics Committee at UWC. Approval from the Ministry of Health Regional Hospital Institutional Review Board (IRB) in Cameroon was obtained for sample collection in Bamenda. Prior to sample collection, the reasons for, and nature of the study were explained to the patients who willingly consented to participate. Data from the patient's hospital folder was collected, where appropriate. The participants were required to sign consent forms agreeing to participate in the study (Appendix 1) and to submit some personal information in a questionnaire, namely, their gender, gender of the sexual partner, age, race, immune status (HIV stage), date diagnosed and duration of ARV treatment (Appendix 2).

2.2 Isolation of *Candida* species

The South African samples were examined in the Medical Microbiology laboratories at UWC while the Cameroonian samples were examined in a private laboratory in Bamenda.
Swabs were initially streaked onto Sabouraud's agar (Cat. no. BO0408T, Oxoid, UK) and incubated at 37˚C for 24 hours. Plates showing no growth were re-incubated for a further 24 hours before being discarded as negative.

All isolates were stored at -80°C in Pro-Lab Microbank microbial preservation vials (Cat. no. PL.170/M, Pro-Lab, Canada), allowing them to be re-grown as and when needed. Cameroonian isolates were transported in these frozen preservation vials to UWC for characterisation which included the confirmation of *Candida* species using chromogenic media, Gram staining and the germ tube test.

2.3. Characterisation of isolates.

2.3.1. *Candida* species identification using chromogenic media

Selective agar and chromogenic media included Sabouraud's agar modified Fluka chromogenic *Candida* identification agar, (Cat. no. 94382, Sigma-Aldrich, USA) with respective selective supplement (Cat. no. 68067, Sigma-Aldrich, USA), Oxoid chromogenic *Candida* agar (Cat. no. CM1002A, Oxoid, UK) with respective selective supplement (Cat. no. SR0231E, Oxoid, UK), tomato (V8) agar (Alves *et al*, 2006) and tobacco agar (Khan *et al*, 2004).

Type strains of *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512) and *C. krusei* (ATCC 2159) served as positive controls for the chromogenic species differentiation.

On Fluka chromogenic agar, *C. albicans* grows as light green colonies, *C. dubliniensis* as dark green colonies, *C. glabrata* as smooth cream/white colonies and *C. tropicalis* as metallic blue colonies, while *C. krusei* grows as pink/purple, fuzzy colonies. The identification of *C. kefyr/parapsilopsis/lusitaneae* is not described in the Fluka catalogue. In Oxoid chromogenic media, *C. albicans* grows as light green colonies, *C. dubliniensis* as dark green colonies, *C. glabrata* as smooth beige/yellow/brown colonies, *C. kefyr/parapsilopsis/lusitaneae* as variable, natural pigment colonies and *C. tropicalis* as metallic blue colonies, while *C. krusei* grows as pink/brown, fuzzy colonies.

2.3.2. Microscopy

The Gram stain was used for the staining of *Candida* isolates prior to light microscopy observation. Pure colonies of the isolates were heat fixed in a glass slide and flooded with crystal violet (primary stain), iodine (which acts as a mordant), alcohol (used for decolorization) and carbol fuchsin, which acts as a counterstain.

2.3.3. Germ tube test

Presumptive *C. albicans* and *C. dubliniensis* cultures were incubated at 37˚C for 2-3 hours in fetal bovine serum (Cat. no. A15-101, PAA Laboratories, Austria) for the stimulation of germ tube production. Germ tube formation was observed microscopically in a wet mount preparation.

Type strains of *C. albicans* (ATCC 90028 and NCPF 3281) and *C. dubliniensis* (NCPF 3949a) served as positive controls for the germ tube test, while *C. tropicalis* (ATCC 950) (which forms pseudohyphae with constriction at the point of attachment when grown in serum) served as a negative control.

2.3.4 *Candida albicans* and *C. dubliniensis* species differentiation

Presumptive *C. albicans* and *C. dubliniensis* cultures were incubated at 37˚C for 48 hours in Tomato (V8) agar; at 28˚C for 48-72 hours in Tobacco agar and at 45˚C for 24-48 hours in Sabouraud dextrose agar. Differences in growth, colony morphology and pseudohyphae/chlamydospore expression allowed for species differentiation.

2.4 Antimicrobial susceptibility testing

Fluconazole antimicrobial susceptibility test disks 25µg (Cat. no. X7148, Oxoid, UK) were used for the antifungal susceptibility test, and three selective media were used, namely Sabouraud's (Cat. no.CM0041, Oxoid, UK), Difco Yeast Nitrogen Base with glucose

(YNBG) (Cat. no. 239210, Beckton, Dickinson and Company, UK) and Methylene-blue and glucose-enriched (GMB) Mueller-Hinton (Cat. No. CM0337, Oxoid, UK) agar.

2.4.1. Preparation of agar plates

2.4.1.1. Yeast Nitrogen Base agar with Glucose

A 10X solution of Yeast Nitrogen Base in powder form (6.7 g/100 mL) and dextrose (5 g/100 mL) was filtered using a 0.45 µm disposable filter (Ref. no. 25NS, MSI filters, USA) attached to a 50mL sterile plastic syringe into a sterile bottle containing Difco granulated bacteriological agar (1.3 g/100 mL) (Cat. no. 214530, Difco, USA), according to the manufacturer's instructions.

2.4.1.2 Methylene-blue and glucose-enriched Mueller-Hinton agar

Methylene-blue and glucose-enriched Mueller-Hinton (GMB) agar was prepared by adding 2% glucose and 5 µg of methylene blue dye/ml to Mueller-Hinton agar (Cat. no. CM0337, Oxoid, UK).

2.4.2. Disk diffusion susceptibility testing

Clinical strains were incubated for 24 hours at 37˚C in Sabouraud's, yeast nitrogen base agar with glucose (YNBG) and Methylene-blue and glucose-enriched Mueller-Hinton (GMB) agar. A sweep of colonies was added to sterile plastic Greiner tubes containing 5mL of sterile distilled water and the inoculum was adjusted according to McFarland standards, a technique using known densities of microorganism suspensions for standardization (McFarland, 1907). The dilution ratio used was of approximately $3x10^8$ microorganisms per ml in Sabouraud and YNBG. In the case of GMB agar, further dilutions were used to yield a final concentration of $2x10^4$ to $4x10^4$ microorganisms per ml, as described in the CLSI protocol. Three random samples were also analyzed in triplicate using a Bausch&Lomb Spectronic 20 spectrophotometer (Cat. no. 33-31-72 Bausch&Lomb, USA) at 450nm, and showed an absorbance in the range of 0.13 to 0.18.

A sterile cotton swab was dipped in the solution and inoculated on Sabouraud's, YNBG and GMB plates. Fluconazole-impregnated felt disks were then placed on the inoculated plates and incubated for 24 hours at 37˚C. The resistance patterns of the samples were seen as

inhibition areas around the fluconazole disks, which were then measured, from the edge of the disk to the edge of the susceptibility area. The presence of microcolonies within the susceptibility zone was also noted and given a score (0= a clear zone with no microcolonies, 1=a few microcolonies present, 2= moderate growth of microcolonies and 3= many microcolonies in the susceptibility area). This was done in duplicate. When differences in susceptibility/microcolony growth were seen, the susceptibility test was repeated. In random samples, microcolony and outer growth areas were stained and observed microscopically for *Candida* species confirmation. These were subsequently grown in chromogenic media and were shown to be the same species. Samples with susceptibility areas less than 7 mm in Sabouraud and YNBG (14 mm or less in GMB) and with the presence of microcolonies were regarded as resistant, as well as samples with more than a microcolony score of 2, while samples with susceptibility areas higher than 12 mm in Sabouraud and YNBG (19 mm or more in GMB) and with a microcolony score of 2 or less were regarded as susceptible to fluconazole. Strains with a susceptibility area ranging from 7 to 12 mm in Sabouraud and YNBG and from 15 to 18 mm in GMB agar were regarded as intermediate (or dose-dependent) strains, as described in studies employing these media (May, King and Warren, 1997, Lee *et al*, 2001). For the purpose of the proteomics section of this study, however, intermediate strains were considered resistant, as these would still express drug resistance proteins.

2.4.3. TREK Sensititre susceptibility testing

The TREK Sensititre (Cat. No. V2020-SYS, Thermo Scientific, USA) YeastOne 9 (YO9) system, a CLSI approved broth microdilution system, consists of microtiter plates embedded with nine different drugs (anidulafungin, micafungin, caspofungin, 5-flucytosine, posaconazole, voriconazole, itraconazole, fluconazole and amphotericin B) in ascending concentrations. The drug concentration ranges on the YO9 wells are 0.008-8 µg/ml for micafungin, caspofungin, posaconazole and voriconazole; 0.015-8 µg/ml for anidulafungin; 0.12-256 µg/ml for fluconazole; 0.015-16 µg/ml for itraconazole; 0.06-64 µg/ml for 5-flucytosine and 0.12-8 µg/ml for amphotericin B. The wells are also coated with a colorimetric agent, allowing for the minimum inhibitory concentration (MIC) of each drug to be easily detected both with the naked eye and with the supplied Vizion computer-assisted plate reading system.

Running of the samples on the TREK Sensititre system was done by diluting second-generation *Candida* strains onto sterile phosphate buffered saline tubes to a 0.5 McFarland standard, using the supplied TREK nephelometer. This was followed by vortexing the suspension according to protocol, dispensing 100 µl of the solution into the YeastOne broth, dispensing the inoculated broth onto the YO9 plate wells using an Ovation 25-1250 µl automated multichannel pipette (VistaLab, NY, USA, cat. no. 1160-1250), sealing the plates with the supplied plate film and incubating for 24 hours at 37°C.
The plates were then read using the supplied Vizion plate reader and the TREK SWIN software.

Newly developed species-specific clinical breakpoints were used for the determination of echinocandin drug resistance (anidulafungin, caspofungin and micafungin) for *C. albicans*, *C. tropicalis* and *C. krusei* (Pfaller *et al*, 2012), while CLSI breakpoint categories were used for 5-flucytosine, itraconazole, fluconazole and amphotericin B (Eraso *et al*, 2008) and proposed breakpoints were used for voriconazole (Pfaller *et al*, 2006). In the case of posaconazole, for which no clinical breakpoints have been established, wild-type MIC values were used (Pfaller *et al*, 2011). MICs were defined as the lowest concentrations that inhibited growth at 100%.

2.5 Protein identification using SDS-PAGE

SDS-PAGE protein identification was done on South African samples using a Hoefer MightySmall II system (Ref. no. SE 250-10A-.75, Hoefer, USA), a Labnet Enduro 250V power supply (Ref. no. E-0203, Labnet, USA) and a Fluka SDS gel preparation kit (which includes the separation gel buffer, 30% acrylamide/0.8% bisacrylamide solution, running buffer 10x concentrate, Tetramethylene diamine (TEMED) 10% solution, sample incubation buffer concentrate and concentrated ammonium persulfate (APS) solution) (Ref. no. 08091, Sigma-Aldrich, USA). Type strains of *C. albicans* (NCPF 3281) and *C. dubliniensis* (NCPF 3949a) served as positive controls for the electrophoresis analysis.

Clinical specimens were grown in Sabouraud agar plates for 24 hours, after which individual colonies were incubated in 15ml screw cap plastic centrifuge tubes containing 10mL of YPD broth (peptone 10 g/L and dextrose 40 g/L distilled water) at 37˚C for 24 hours in a GFL agitator (Ref. no. 3015, GFL, Germany). The optical densities of the type strains and six random clinical broth cultures were read at 600 nm using a Bausch&Lomb Spectronic 20 spectrophotometer (Cat. no. 33-31-72 Bausch&Lomb, USA) with the samples having an approximate absorbance reading of 5.5. The broth tubes were centrifuged at 3000 *g* using a MSE Super Minor centrifuge (Ref. no. 12-79, MSE, UK). The broth was subsequently removed from the tubes using sterile plastic Pasteur pipettes. Two milliliters (2 mL) of sterile distilled water was then added to the fungal cells, followed by centrifugation at 3000 *g* for 10 minutes using a MSE Super Minor centrifuge (Ref. no. 12-79, MSE, UK) to wash the cells. The isolated pellet was then suspended in 2 mL homogenizing buffer (50 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride), using a protein isolation method first described by Niimi *et al* (2002). One gram (1 g) of borosilicate 1 mm solid glass beads (Cat. no. Z273619, Sigma-Aldrich, USA) was added to the centrifuge tubes containing the fungal pellets and homogenizing buffer. The fungal cells were then disrupted by placing the tubes in a vortex for 6 minutes. This was followed by transferring the solution to 2 mL plastic microcentrifuge tubes and centrifugation at 5000 *g* at 4˚C for 10 minutes to remove cell debris and unbroken cells, using an Eppendorf benchtop centrifuge (Ref. no. 5415C, Eppendorf, Germany). The obtained lysate solutions (now containing only fungal cell membrane components) were then centrifuged at 20 000 *g* at 4˚C for 60 minutes using an

Eppendorf microcentrifuge (Ref. no. 5424, Eppendorf, Germany), resulting in the isolation of a crude membrane fraction.

Protein concentration was determined using a Bio-Rad Bovine Serum Albumin (BSA) Standard Set (Cat. no. 500-0207, Bio-Rad, USA), a protein assay kit based on the Bradford protein assay (Bradford, 1976). Sixteen microliters (16 µL) of a 10 m*M* Tris-HCl, pH 7.0, 5 m*M* EDTA solution were added to the isolated pellets and vortexed. Five microliters (5µL) of concentrated BSA solutions (0.125 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 0.75 µg/ml, 1 µg/ml, 1.5 µg/ml and 2 µg/ml) and 5 µL of each sample were then transferred to 96-well plastic microplates in duplicate (two wells per sample) and 250 µL of Bradford dye reagent was added to each well with the aid of a Labnet multichannel pipette (Ref. no. P4508-300, Labnet, USA). The microplates were then placed in a Thermo Scientific Multiskan 355 EX microplate reader with computer-assisted user interface (Ref. no. 5118170, Thermo-Scientific, USA) for absorbance reading at 620 nm. Sample concentration was standardized using a plot of the seven prediluted BSA solutions' concentrations and absorbance readings. Samples which expressed a protein concentration higher than 0.8 mg/ml were diluted in GTE buffer (10 m*M* Tris-HCl, pH 7.0, 5 m*M* EDTA, 20% v/v glycerol) to an approximate protein concentration of 0.65 mg/ml. Four microliters (4 µL) of glycerol and 20 µL of SDS loading buffer (incubation buffer) were then added to the samples. This loading solution was then vortexed to solubilise the protein samples in the buffers and subsequently incubated at 37˚C for 30 minutes.

A Bio-Rad Precision Plus Dual Colour Protein marker (Cat. no. 161-0374, Bio-Rad, USA) was used for protein weight determination. Protein samples were separated in a 15% SDS polyacrylamide gel, prepared by adding 7.5 mL PAA stock solution, 5 mL separation gel buffer, 2.25 mL distilled water and 0.25 mL TEMED 10% solution to 0,1 mL APS 10% and subsequently loaded between glass and alumina plates supplied with the Hoefer SDS-PAGE gel caster system. One hundred percent (100%) ethanol was then added to the top of the gel to smoothen its surface and the separating gel was subsequently left to dry. A 3% stacking gel solution (0.5 ml PAA stock solution/1 ml stacking gel buffer/3.4 ml distilled water/0.1 ml TEMED 10% solution and 0.03 mL APS 10% solution) was then added to the top of the separating gel. Ten wells were created in the stacking gel by inserting a plastic comb in the top section of the stacking gel solution before it solidified. The glass plate-polyacrylamide

gel-alumina plate assemblies were then transferred to the Hoefer electrophoresis unit after the stacking gel solidified. The unit's lower chamber and upper buffer chamber were filled with the diluted Fluka SDS kit running buffer. Ten microliters (10 μL) of protein marker and 30 μL of protein samples were added to their respective wells in the gel using a P20 Gilson pipette (Ref. no. F123600, Gilson, France). 20 μL of GTE buffer (10 m*M* Tris-HCl, pH 7.0, 5 m*M* EDTA, 20% v/v glycerol) was added. Twenty microliters (20 μL) of incubation buffer solution was used as a negative control.

A 250V/30mA electric current was applied to the gels after which they were stained with Coomassie Brilliant Blue R250 (0.05-0.1% brilliant blue R250 in 10% acetic acid, 40% distilled water, 50% methanol) overnight in a GFL agitator (Ref. no. 3015, GFL, Germany).

Protein gels were subsequently soaked in Coomassie de-stain solution (10% methanol, 30% acetic acid, 60% distilled water) for approximately 4 hours (until the gels were clear and protein bands could be seen) and soaked in gel drying solution (7.5% acetic acid, 10% glycerol, 40% methanol, 50% distilled water) for 3-5 minutes. Gels were then dried using a Promega gel drying kit (Cat. no. V7120, Promega, USA). Gel drying films were used to dry the protein gels, which were moistened in gel drying solution, secured in a gel drying frame using the supplied metal clamps and left to dry overnight.

2.6 Protein identification using HPLC-MS

High performance liquid chromatography-mass spectrometry analysis was performed on 40 isolate fractions from both populations (10 fluconazole-susceptible fractions and 10 fluconazole-resistant fractions from *C. albicans* isolates and 20 fractions from the non-albicans isolates found in this study), by using the same *Candida* cell surface fraction isolation protocol used for the SDS-PAGE analysis. The cell fractions were taken from UWC to the Central Analytical Facility (CAF) Proteomics laboratory at Tygerberg Medical Campus for analysis.

Filter-aided sample preparation (FASP) was used on the cell fractions, in accordance with the protocol set by the University of Stellenbosch's Central Analytical Facility Proteomics laboratory: samples were mixed 1:1 with lysis buffer (50 µl sample with 50 µl SDT lysis buffer (4% SDS, 100 m*M* Tris-HCl pH 7.6, 0.1 M DTT that was added freshly just before use)). The 100 µl sample was then mixed with 100 µl UA buffer (8 *M* urea, 100 m*M* Tris-HCl, pH 8.5) and placed on an Amicon ultra 0.5 centrifugal 10 kDa filter (EMD Millipore, USA) and subsequently centrifuged for 40 minutes at 14000 *g*. This was followed by the addition of 200 µl UA buffer and centrifugation at 14000 *g* for 40 minutes. The proteins were then alkylated by the addition of 100 µl of 0.05 *M* iodoacetamide in UA buffer. This was then mixed and incubated for 5 minutes before centrifugation at 14 000 *g* for 30 minutes, followed by the addition of 100 µl of UB buffer (8 *M* urea, 0.1 *M* Tris-HCl pH 8.0), centrifuged for 30 minutes at 14 000 *g* and repeated once more. After centrifugation, 100 µl of a 50 m*M* ammonium bicarbonate solution was added, centrifuged at 14 000 *g* for 30 minutes and repeated once more. This was followed by the addition of 40µl trypsin and incubation at 37°C for 17 hours in a wet chamber. The following morning the filter was placed in a clean Eppendorf tube and centrifuged for 40 minutes at 14 000 *g*, followed by the addition of 40 µl of a 0.5 *M* sodium chloride solution and centrifuged for 20 minutes at 14 000 *g*. Finally, the solution was acidified by the addition of 2.4 µl FA solution. The filtrate was then desalted using C18 StageTips (Thermo-Fisher Scientific, USA) according to the manufacturer's instructions. The desalted solution was dried *in vacuo* and stored at -20^{0}C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10 µl injections were made for nano-LC chromatography.

All mass spectrometry experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electropsray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100 µm, 5 µm, C18) pre-column followed by XBridge BEH130 NanoEase column (15 cm, ID 75 µm, 3.5 µm, C18) column with a flow rate of 300 nl/min. The gradient used was from 5-17 % B in 5 min, 17-25% B in 90 min, 25-60% B in 10 min, 60-80% B in 5 min and kept at 80% B for 10 min. Solvent A was 100% water in 0.1 % formic acid, and solvent B was 100 % acetonitrile in 0.1% formic acid.

The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 x 10^6. The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 1.5 x 10^4) using collision induced dissociation. The lock mass option (polydimethylcyclosiloxane; *m/z* 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250°C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms were also applied for MS/MS.

2.7 Statistical analysis

Statistical analysis was done using the SPSS 21.0 statistical software. Descriptive statistics and chi-square tests were used for the comparison of different *Candida* colonization patterns and patient data. Analysis of fluconazole and other antifungal drug susceptibility results was also done by means of chi-square tests ($p<0.05$).

Chapter 3: Results

3.1 *Candida* species growth patterns

Table 1 shows the colony growth patterns of *Candida* species isolates 24 hours after sample collection and incubation and the respective percentage of isolates for each species (more detailed information can be seen in appendix 3). Growth was defined as scanty when ≤ 5 colonies were seen after the first sample incubation, while a higher number (> 6 colonies) and a more uniform appearance of colonies was categorized as light growth. In the South African samples, *Candida albicans* was the most prevalent species, followed by *C. glabrata* and *C. dubliniensis*.

Table 1: Growth pattern results of the first inoculation of all South African *Candida* isolates.

***Candida* spp/Growth**	**Scanty (%)**	**Light (%)**	**Moderate (%)**	**Heavy (%)**
C. albicans	52 (49%)	40 (38%)	12 (11%)	2 (1.9%)
C. dubliniensis	4 (40%)	5 (50%)	1 (10%)	0 (0%)
C. glabrata	4 (33%)	7 (58%)	1 (8.3%	0 (0%)

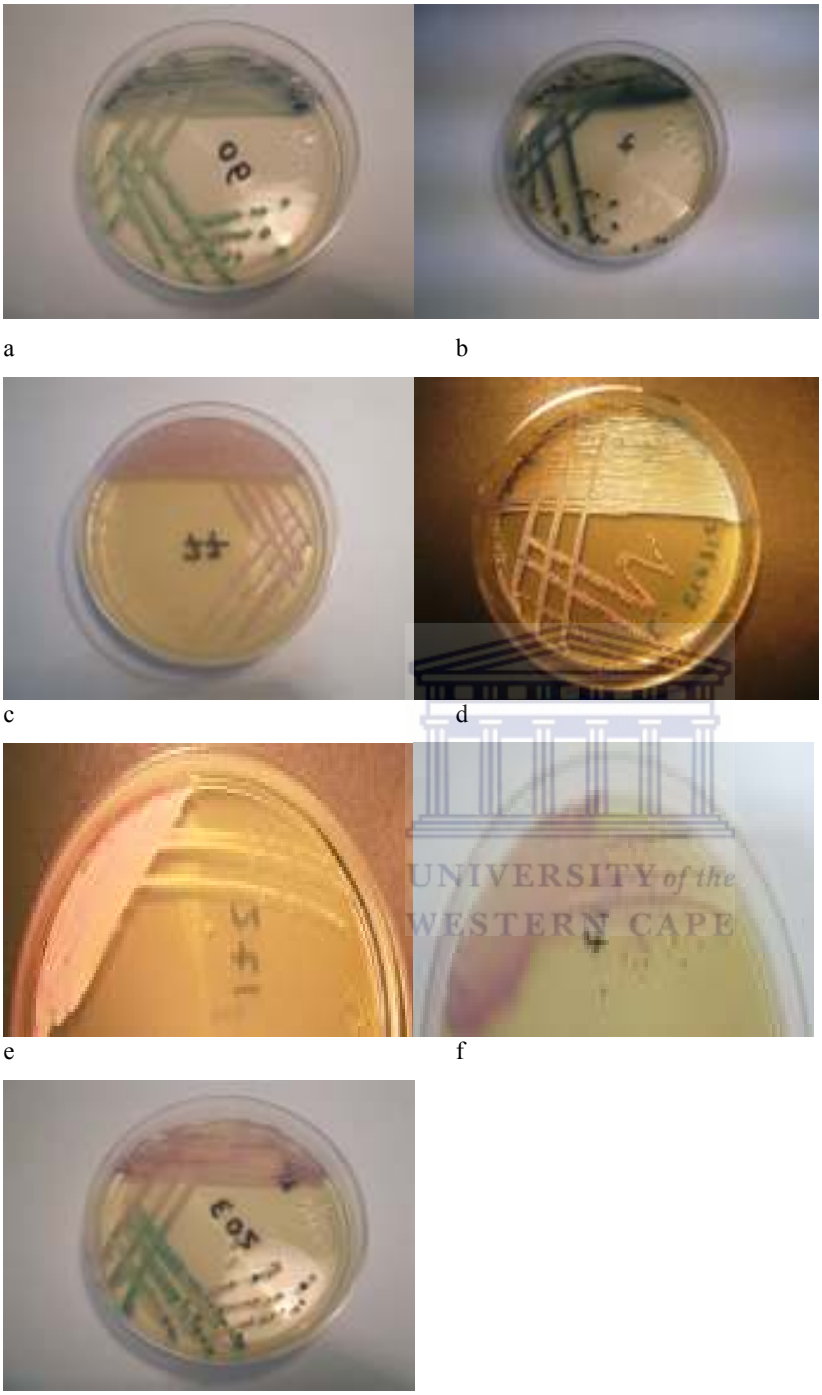
C. albicans was also the predominant isolate from the Cameroonian samples, followed by *C. glabrata* and other *Candida* species (Table 2).

Table 2: Growth pattern results of the first inoculation of all Cameroonian *Candida* isolates.

***Candida* spp/Growth**	**Scanty (%)**	**Light (%)**	**Moderate (%)**	**Heavy (%)**
C. albicans	61 (66.3%)	21 (22.8%)	9 (9.8%)	1 (1.1%)
C. dubliniensis	0 (0%)	1 (100%)	0 (0%)	0 (0%)
C. glabrata	24 (100%)	0 (0%)	0 (0%)	0 (0%)
C. krusei	4 (100%)	0 (0%)	0 (0%)	0 (0%)
C. tropicalis	2 (50%)	1 (25%)	0 (0%)	1 (25%)
C. kefyr/ parapsilopsis/lusitaneae	2 (100%)	0 (0%)	0 (0%)	0 (0%)

3.2 Colonial morphology on selective/differential media

Isolates were inoculated onto chromogenic agar and incubated at 30˚C for 24-48 hours. The different colours and textures that distinguish the various species are clearly demonstrated in Figures 1 and 2 using Fluka and Oxoid chromogenic media, respectively. The *Candida* species differentiation results are shown in accordance with the colours/textures expressed by the *Candida* type strains when grown on both chromogenic media, as no individual chromogenic medium is able to accurately distinguish between all *Candida* species found in this study (*C. glabrata*, for example, shows a slight pink coloration when grown on Fluka chromogenic agar, instead of the cream white colour described in the product catalogue). A mixed culture of different species on Fluka chromogenic medium can be observed in Figure 1(g).



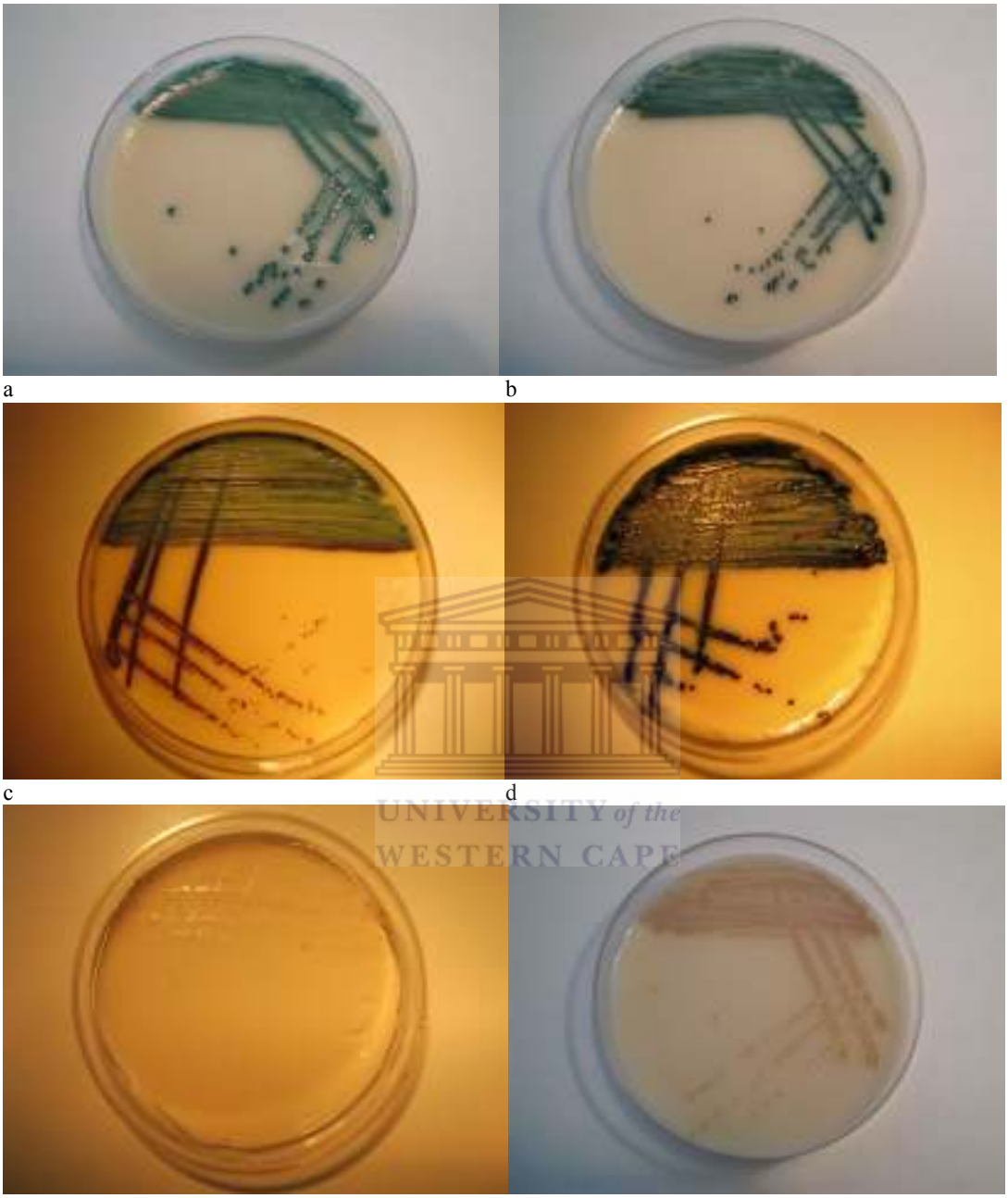
a b

c d

e f

g

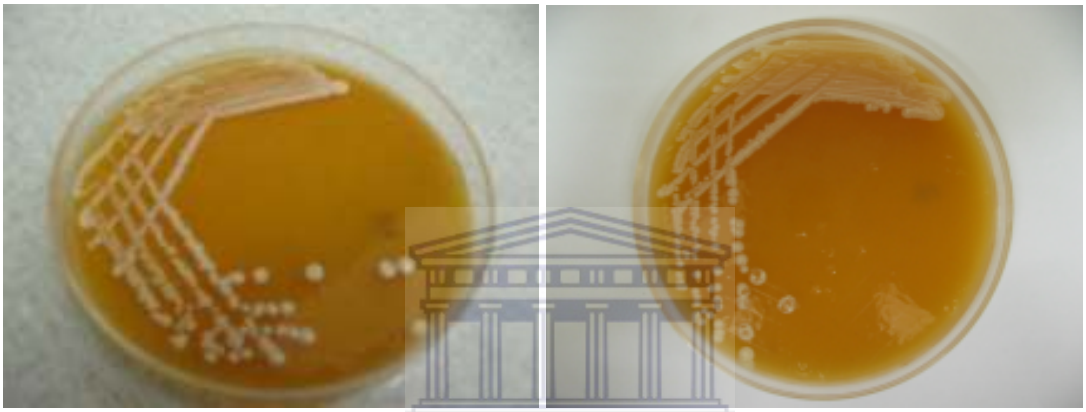
Fig. 1: Growth of *Candida* species on Fluka chromogenic media:
a: *C. albicans*
b: *C. dubliniensis*
c: *C. glabrata*
d: *C. tropicalis*
e: *C. krusei*
f: C. *kefyr*/*C. parapsilopsis*/*C. lusitaneae*
g: Mixed growth



a b c d e f

Fig. 2: Growth of *Candida* species on Oxoid chromogenic media:
a: *C. albicans*
b: *C. dubliniensis*
c: *C. glabrata*
d: *C. tropicalis*
e: *C. krusei*
f: *C. kefyr*/*C. parapsilopsis*/*C. lusitaneae*

Tomato juice agar was used for the further differentiation between *C. albicans* and *C. dubliniensis*. Figure 3(a) shows a *C. albicans* culture after inoculation onto tomato juice agar and subsequent incubation at 37˚C for 48 hours (detailed results on chromogenic and tomato juice agars can be seen in appendix 4). Figure 3(b) represents a *C. dubliniensis* culture on tomato juice agar under the same growth conditions. *C. albicans* grew as a smooth, shiny colony, while *C. dubliniensis* yielded a characteristic rough, dry colony. Similar results were observed for growth on Tobacco agar incubated at 28˚C for 48 hours (Figure 4).

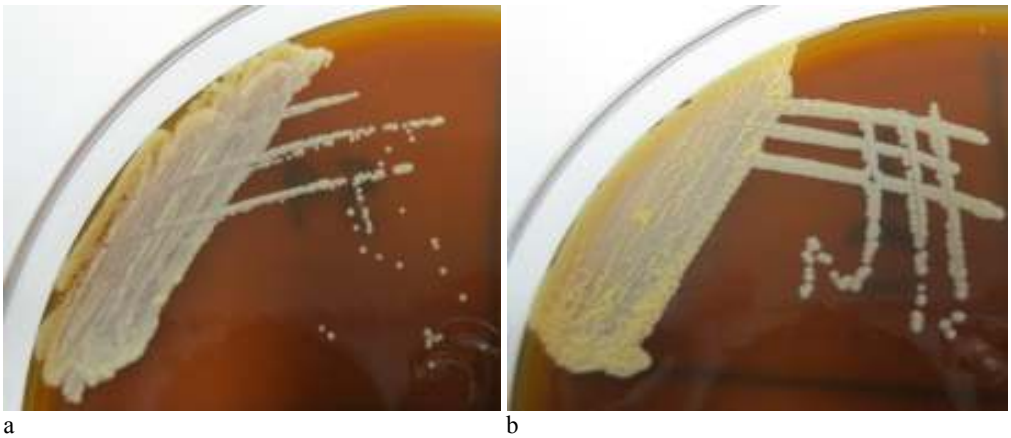


a b

Fig. 3: Growth of *Candida* species on tomato juice agar:

a: *C. albicans*

b: *C. dubliniensis*



a b

Fig. 4: Growth of *Candida* species on tobacco agar:

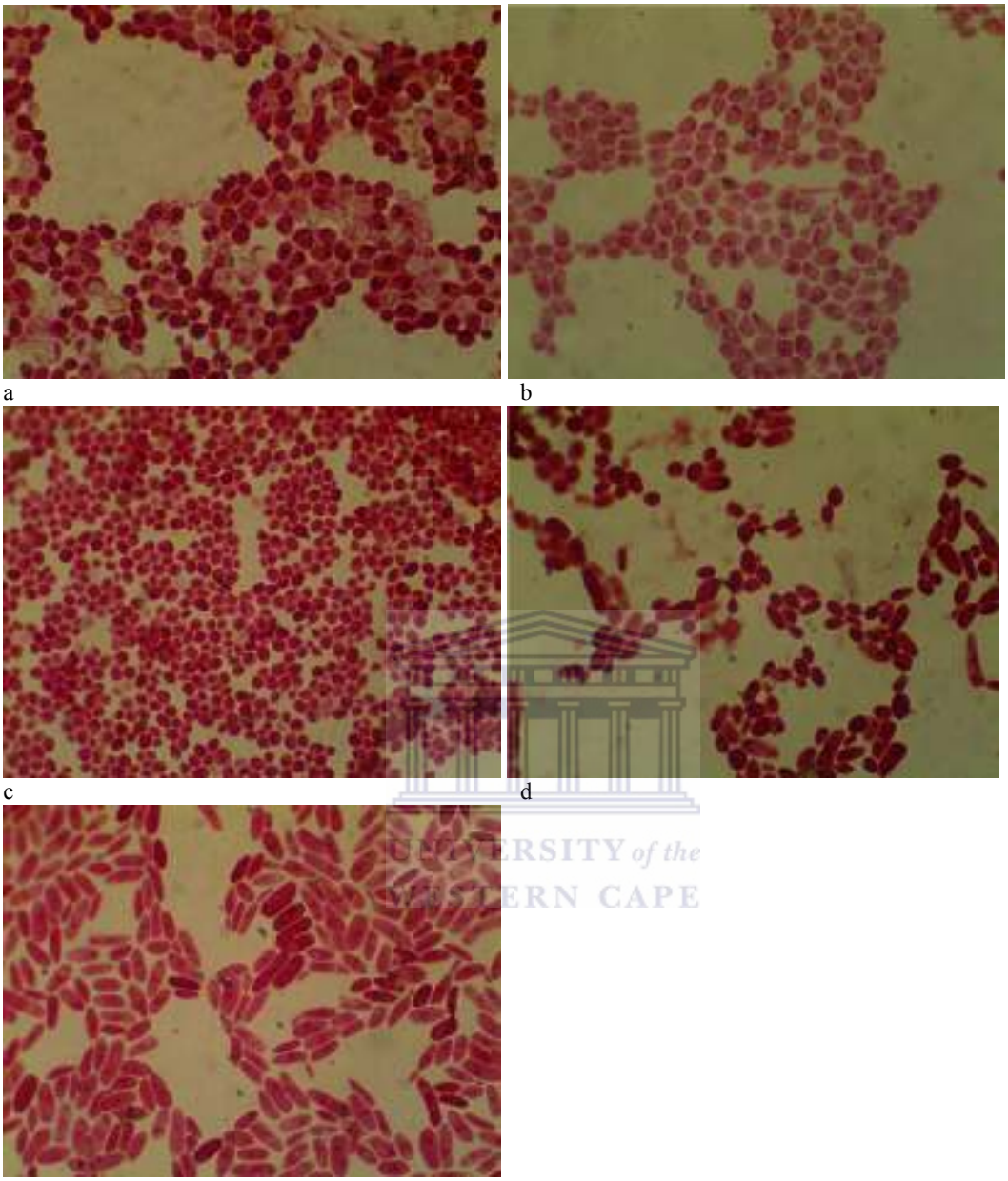
a: *C. albicans*

b: *C. dubliniensis*

3.3 *Candida* species microscopical morphology

Figure 5 shows the different *Candida* type strains stained with carbol fuschin and examined by oil immersion microscopy using an Optikam B3 camera (Optika Microscopes, Italy) attached to an optical microscope. Figure 6 shows some of the different *Candida* cell morphologies isolated from clinical samples in this study.

Microscopy played an important role in the initial identification of *Candida* species. *Candida krusei* was the only *Candida* species that could be presumptively identified by microscopy, due to its noticeably larger cells.



a b c d e

Fig. 5: *Candida* type strain cell morphologies (1000X).

a: *C. albicans* ATCC 90028
b: *C. dubliniensis* NCPF 3949a
c: *C. glabrata* ATCC 26512
d: *C. tropicalis* ATCC 950
e: *C. krusei* ATCC 2159

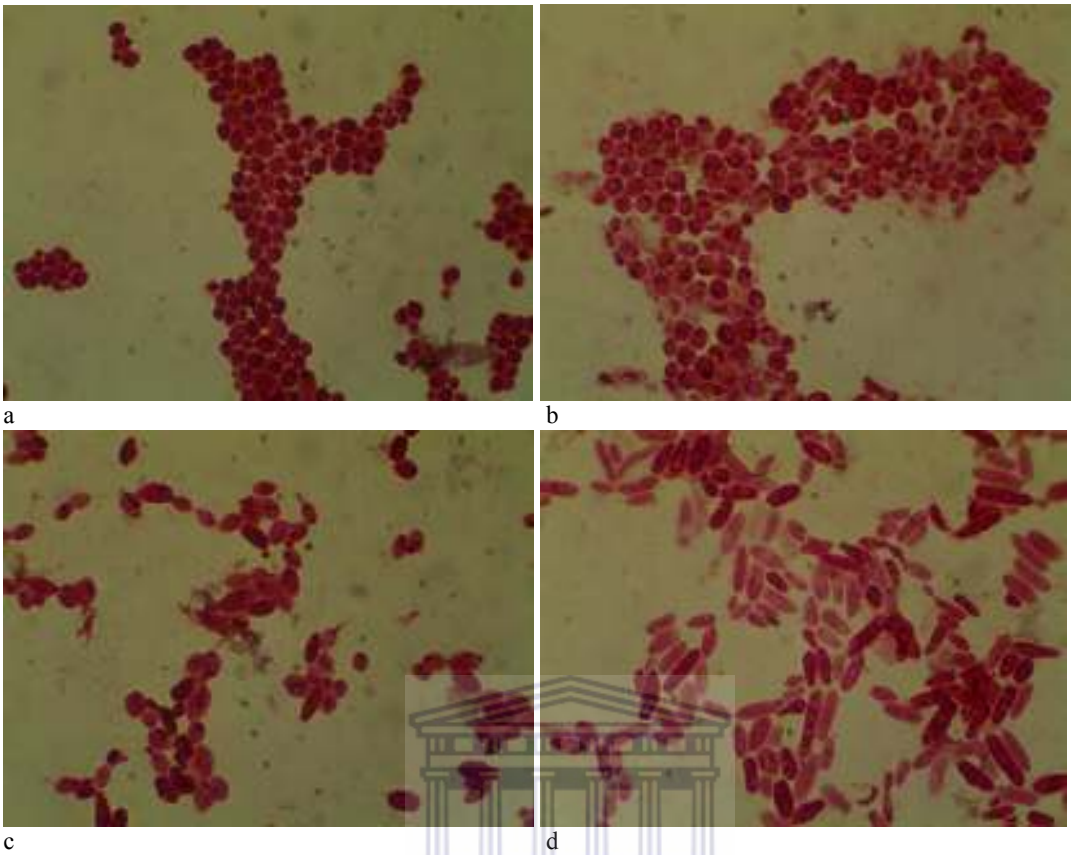


Fig. 6: *Candida* clinical strain cell morphologies (1000X).

a: *C. albicans*/*C. dubliniensis*/*C. kefyr*/*C. parapsilopsis*/*C. lusitaneae*
b: *C. glabrata*
c: *C. tropicalis*
d: *C. krusei*

3.4 Frequency distribution of *Candida* species identified from clinical isolates

One hundred and twenty six (126) of the swabs collected from the South African population resulted in positive *Candida* growth, with two patients harbouring two *Candida* species. This equates to 59.4% of the total number of patients testing positive for the presence of *Candida* in their oral mucosa. Eighty three percent (83%) of the patient's isolates were identified as *C. albicans* (106 isolates), 9.4% as *C. glabrata* (12 isolates) and 7.8% as *C. dubliniensis* (10 isolates).

In the case of the Cameroonian population, 127 (48.5%) of the swabs collected resulted in positive *Candida* growth. Seventy one point three percent (71.3%) of the patient's isolates were identified as *C. albicans* (92 isolates), 18.9% as *C. glabrata* (24 isolates), 3.1% as *C. krusei* (4 isolates), 3.1% as *C. tropicalis* (4 isolates), 0.77% as *C. dubliniensis* (1 isolate) and 1.55% as either either *C. kefyr*, *C. parapsilopsis* or *C. lusitaneae* (2 isolates) (Figure 7).

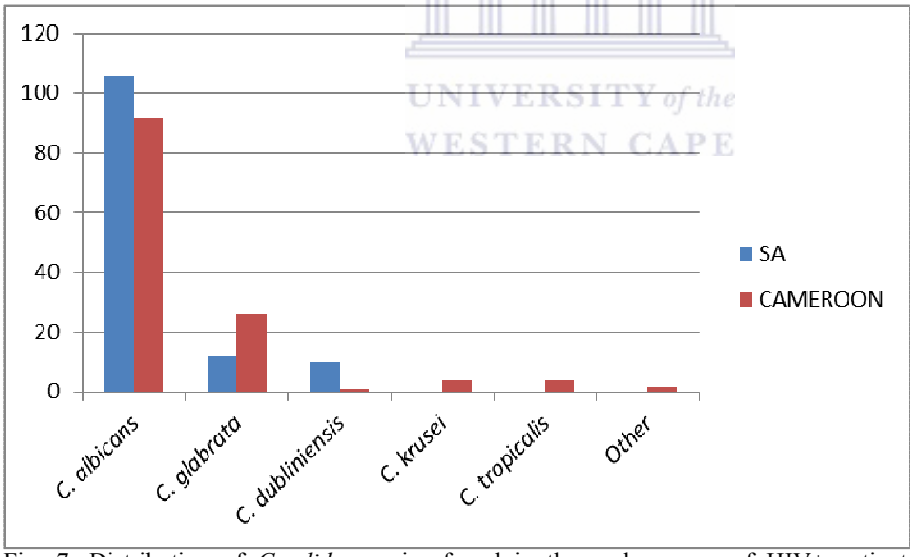


Fig. 7: Distribution of *Candida* species found in the oral mucosa of HIV+ patients (number of isolates represented on the y-axis).

3.5 Antifungal susceptibility testing

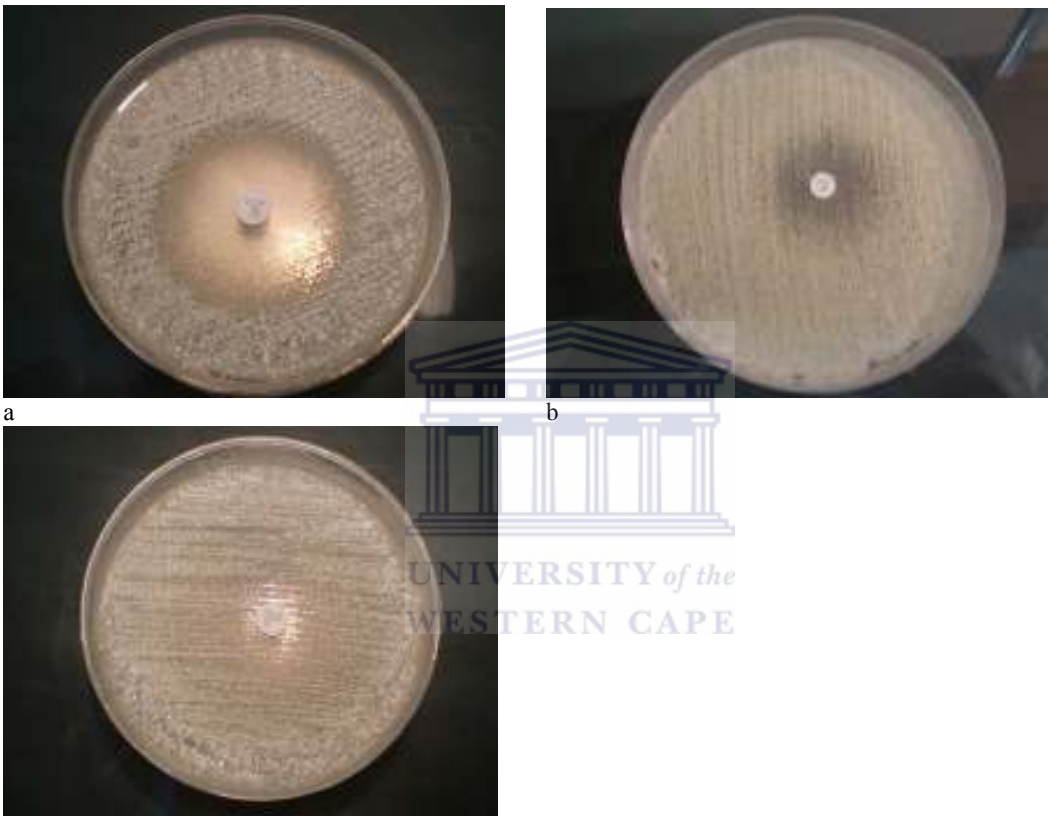
3.5.1 Fluconazole disc diffusion susceptibility testing

Fluconazole susceptibility testing was optimized by inoculating three random clinical strains in triplicate following the same methodology as previously described for clinical isolates (subheading 2.4), with a McFarland standard of 1, 2 and 3. The same susceptibility patterns were observed for all dilutions (Table 3). This was done for the case of a possible dilution mistake while visually adjusting the clinical fungal cell dilutions, to confirm if the susceptibility results would remain the same in a more concentrated *Candida* dilution.

Table 3: McFarland dilution optimization test, showing inhibition areas and microcolony formation after fluconazole susceptibility testing of three different samples.

No.	McFarland dilution	Inhibition area (mm)	Microcolonies
1	McFarland1	4	0
	McFarland2	4	0
	McFarland3	4	0
2	McFarland1	2	1
	McFarland2	2	1
	McFarland3	2	1
3	McFarland1	resistant	resistant
	McFarland2	resistant	resistant
	McFarland3	resistant	resistant

Figure 8 demonstrates susceptibility or resistance to fluconazole. Susceptibility is demonstrated by a zone of inhibition around the fluconazole-impregnated disc (Fig. 7a), intermediate resistance is indicated by the growth of fungal microcolonies in the susceptibility area (Fig.7b) and resistance is indicated where the fungal growth grew over the impregnated disc (Fig. 7c), when cultures were incubated in YNBG at 37˚C for 24 hours.



a b

c

Fig. 8: Inhibition of *Candida* growth in YNBG media in the presence of a fluconazole disk (a clear inhibition area can be seen around the disk) (a), presence of microcolonies in the susceptibility area (b) and resistance to fluconazole (no inhibition area can be seen on the plate) (c).

Susceptibility and resistance were also clearly demonstrated when cultures were incubated in GMB at 37˚C for 24 hours.

a b

Fig. 9: Inhibition of *Candida* growth in GMB media in the presence of a fluconazole disk (a clear inhibition area can be seen around the disks) (a) and resistance to fluconazole (there is no distinct inhibition area around the disks) (b).

Figures 10 and 11 show the susceptibility results obtained after growing the South African and Cameroonian *Candida* strains in the presence of fluconazole impregnated disks in YNBG agar. More than half of the isolates showed resistance in both the South African and Cameroonian populations, with the Cameroonian group showing higher numbers of intermediate, or dose-dependent, isolates.

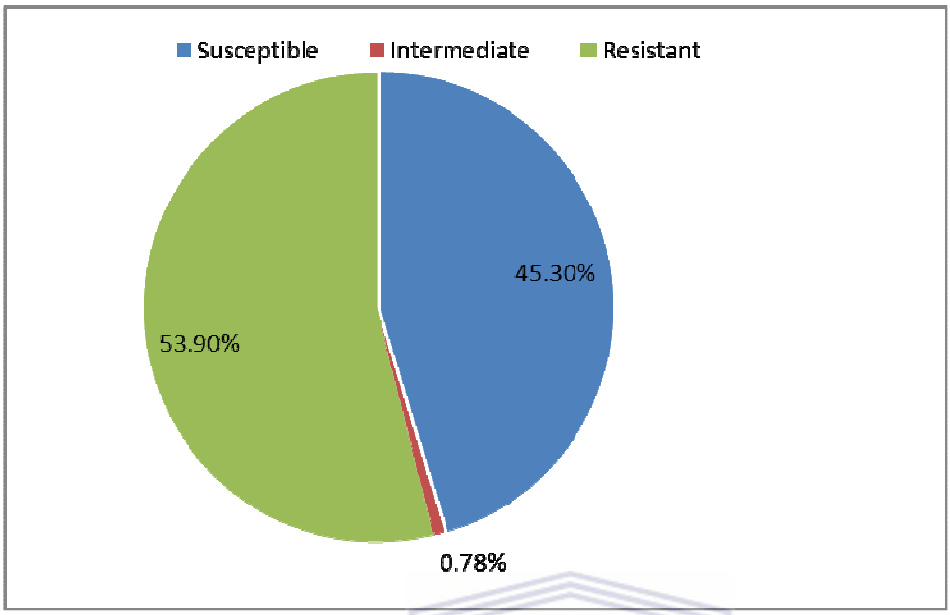


Fig. 10: South African fluconazole susceptibility results in Yeast Nitrogen Base agar.

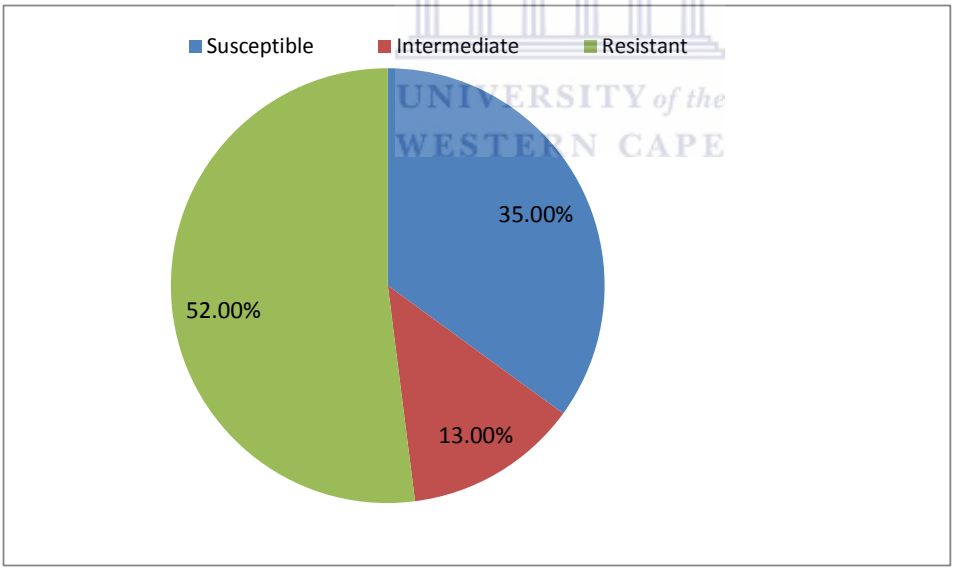


Fig. 11: Cameroonian fluconazole susceptibility results in Yeast Nitrogen Base agar.

Fifty four point seven percent (54.7%) of the South African *Candida* samples demonstrated some degree of resistance to fluconazole in YNBG agar (resistant and intermediate samples). The value for the Cameroonian group was higher, at 65%. All YNBG resistant South African samples showed resistance on Sabouraud's and GMB, apart from two *C. glabrata* samples which showed intermediate resistance in GMB. In the case of intermediate resistance samples grown on YNBG, all these showed up as resistant in the other two media. Tables 4 and 5 show the fluconazole susceptibility values on YNBG agar for the South African and Cameroonian populations, respectively.

Table 4: Fluconazole susceptibility results of South African *Candida* species grown on YNBG agar.

SA *Candida* spp/Fca susceptibility	**n=128**	**Susceptible (%)**	**Intermediate (%)**	**Resistant (%)**
C. albicans	106 (83%)	46 (43.4%)	0 (0%)	60 (56.6%)
C. dubliniensis	10 (7.8%)	9 (90%)	0 (0%)	1 (10%)
C.glabrata	12 (9.4%)	3 (25%)	1 (8.3%)	8 (66.7%)

Table 5: Fluconazole susceptibility results of Cameroonian *Candida* species grown on YNBG agar.

Cam *Candida* spp/ Fca susceptibility	**n=127**	**Susceptible (%)**	**Intermediate (%)**	**Resistant (%)**
C. albicans	92 (72.4%)	44 (47.8%)	9 (9.8%)	39 (42.4%)
C. glabrata	24 (18.9%)	1 (4.2%)	7 (29.2%)	18 (75%)
C. krusei	4 (3.1%)	0 (0%)	0 (0%)	4 (100%)
C. tropicalis	4 (3.1%)	0 (0%)	0 (0%)	4 (100%)
C. dubliniensis	1 (0.8%)	0 (0%)	1 (100%)	0 (0%)
Other	2 (1.6%)	0 (0%)	1 (50%)	1 (50%)

Tables 6 and 7 show the chi-square results of the susceptibility test done on *Candida* species grown on YNBG agar. The significance values demonstrate the statistical association between different *Candida* species and their susceptibility to fluconazole on YNBG and confirm the reliability of this medium in fluconazole susceptibility testing. No statistically significant associations were seen in the other agars used in this study. The note in (a.) is generated by the SPSS statistics programme and shows which p-value should be read based on the percentage value obtained.

Table 6: Chi-square susceptibility results of South African *Candida* spp. grown on YNBG agar.

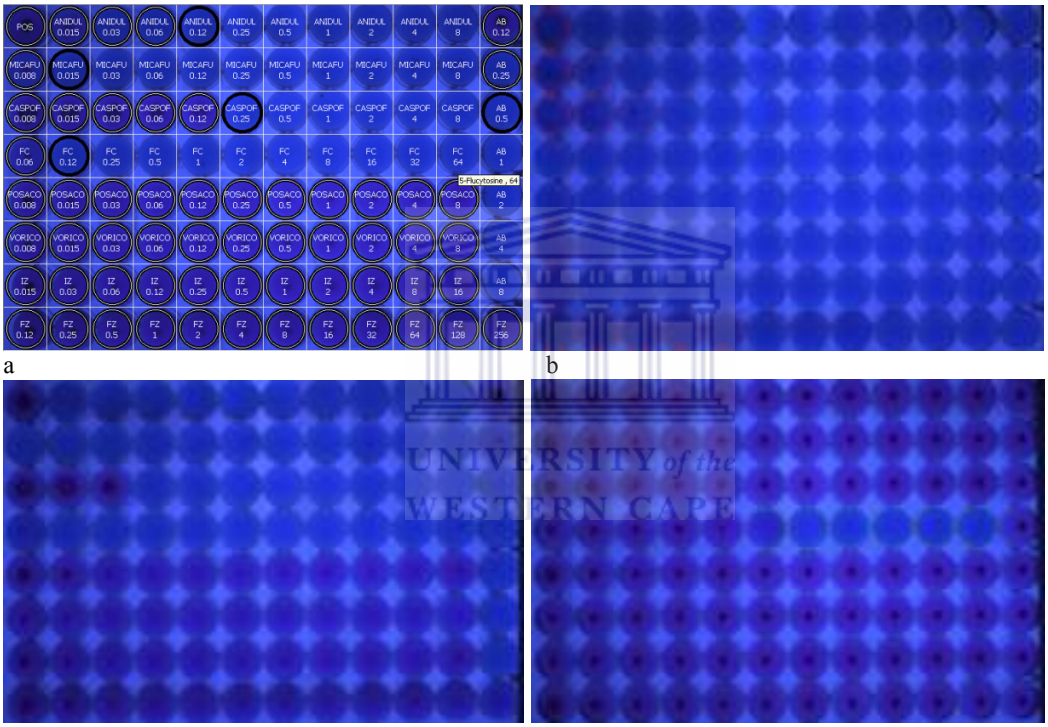
Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	27.564[a]	2	p=0.000
Likelihood Ratio	19.640	2	p=0.000
Linear-by-Linear Association	21.454	1	p=0.000
N of Valid Cases	128		
a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 1.65.			

Table 7: Chi-square susceptibility results of Cameroonian *Candida* spp. grown on YNBG agar.

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	26.856[a]	5	p= 0.000
Likelihood Ratio	38.207	5	p= 0.000
N of Valid Cases	127		
a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .34.			

3.5.2. Susceptibility testing using the TREK system

Figure 9 shows the TREK Sensititre YO9 drug panel and the different results seen on the TREK Sensititre plates. The different drugs and their concentrations are demonstrated in Fig.12(a), susceptible strains are indicated by the absence of growth on wells without a red ring around them (b), azole drug resistance showing growth within the wells (c), while multiple drug resistance is indicated by growth in most of the wells (d).



a b

c d

Fig. 12: Drug panel and different results seen on the TREK Sensititre plates.
a: Different drugs and concentrations of the TREK panel
b: Susceptible strain (growth only in red circled wells)
c: Azole drug resistance
d: Multiple drug resistance (only 5-Flucytosine >2μg/ml inhibits the growth of the organism)

As described by Eraso *et al* (2008) and Pfaller *et al* (2006, 2012), Table 8 demonstrates the different antifungal drug susceptibility breakpoints used for the different *Candida* species using the TREK system. A blank cell indicates that to our knowledge, no break points have been established for this drug.

Table 8: Different drug susceptibility clinical breakpoints used in this study.

	C. albicans			*C. glabrata*		
	Susceptible	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Anidulafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25 µg/mL	≥0.5 µg/mL
Caspofungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25 µg/mL	≥0.5 µg/mL
Micafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.06 µg/mL	0.12 µg/mL	≥0.25 µg/mL
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	<0.016µg/mL	_	≥0.016µg/mL	<0.5µg/mL	_	≥0.5µg/mL
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL
	C. tropicalis			*C. krusei*		
	Susceptible	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Anidulafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
Caspofungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
Micafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	<0.03µg/mL	_	≥0.03µg/mL	<0.25µg/mL	_	≥0.25µg/mL
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL
	C. dubliniensis			*C. kefyr/para/lusi*		
	Susceptible	**Intermediate**	**Resistant**	**Susceptible**	**Intermediate**	**Resistant**
Anidulafungin	_	_	_	_	_	_
Caspofungin	_	_	_	_	_	_
Micafungin	_	_	_	_	_	_
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	_	_	_	_	_	_
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL

(- : no breakpoint available for the organism/drug)

The TREK drug susceptibilities of *Candida* species isolated from the South African population are summarised in Table 9. These results demonstrate the differences in susceptibility patterns of different *Candida* species when exposed to the different drugs present on the YO9 panel. The significance values show the statistical associations between different *Candida* species and their susceptibility to the various antifungal drugs. Statistical associations were seen for all drugs with the exception of 5-Flucytosine.

Table 9: TREK susceptibility results of *Candida* species obtained from the South African population.

		C. albicans n=106	*C. glabrata* n=12	*C. dubliniensis* n=10	Spp/resistance associations
Amphotericin B	Susceptible	97	7	9	p=0.01
	Intermediate	0	0	0	
	Resistant	9	5	1	
5-Flucytosine	Susceptible	101	11	10	
	Intermediate	0	0	0	
	Resistant	5	1	0	
Anidulafungin	Susceptible	101	11	_	p=0.000
	Intermediate	3	0	_	
	Resistant	2	1	_	
Caspofungin	Susceptible	98	9	_	p=0.000
	Intermediate	8	3	_	
	Resistant	0	0	_	
Micafungin	Susceptible	106	12	_	p=0.000
	Intermediate	0	0	_	
	Resistant	0	0	_	
Fluconazole	Susceptible	53	8	9	p=0.032
	Intermediate	1	4	0	
	Resistant	52	0	1	
Itraconazole	Susceptible	43	4	9	p=0.008
	Intermediate	1	6	0	
	Resistant	62	2	1	
Voriconazole	Susceptible	49	12	9	p=0.000
	Intermediate	0	0	0	
	Resistant	57	0	1	

(- : no breakpoint available for the organism/drug).

Isolates from the Cameroonian population yielded the susceptibility patterns shown in Table 10. Cameroonian sample 174 did not grow in the TREK broth and was therefore not included in this section. These results demonstrate the differences in susceptibility patterns of different *Candida* species when exposed to the different drugs present on the YO9 panel. The significance values show the statistical associations between different *Candida* species and their susceptibility to the various antifungal drugs. Statistical associations were seen for all drugs with the exception of 5-Flucytosine.

Table 10: TREK drug susceptibility results of *Candida* species obtained from the Cameroonian population.

		C. albicans n=92	*C. glabrata* n=24	*C. tropicalis* n=4	*C. krusei* n=3	*C. para/lusi/kefyr* n=2	*C. dubliniensis* n=1	Spp/ resistance associations
Amphotericin B	Susceptible	88	23	2	1	1	0	p=0.001
	Intermediate	0	0	0	0	0	0	
	Resistant	4	1	2	2	1	1	
5-Flucytosine	Susceptible	86	24	4	2	1	1	
	Intermediate	0	0	0	1	0	0	
	Resistant	6	0	0	0	0	0	
Anidulafungin	Susceptible	92	16	4	3	-	-	p=0.000
	Intermediate	0	5	0	0	-	-	
	Resistant	0	3	0	0	-	-	
Caspofungin	Susceptible	92	16	4	3	-	-	p=0.000
	Intermediate	0	7	0	0	-	-	
	Resistant	0	1	0	0	-	-	
Micafungin	Susceptible	92	3	4	3	-	-	p=0.000
	Intermediate	0	5	0	0	-	-	
	Resistant	0	16	0	0	-	-	
Fluconazole	Susceptible	45	16	4	1	2	1	p=0.041
	Intermediate	1	7	0	0	0	0	
	Resistant	46	1	0	2	0	0	
Itraconazole	Susceptible	44	5	1	1	2	1	p=0.044
	Intermediate	1	15	3	2	0	0	
	Resistant	47	4	0	0	0	0	
Voriconazole	Susceptible	46	23	4	2	2	1	p=0.000
	Intermediate	0	0	0	0	0	0	
	Resistant	46	1	0	1	0	0	

(- : no breakpoint available for the organism/drug)

Isolates from South Africa and Cameroon tested with posaconazole (for which there are no established breakpoints) showed high resistance to this drug across all tested *Candida* species. The results for this drug are shown in Table 11.

Table 11: Posaconazole susceptibility results.

	C. albicans		***C. glabrata***		***C. tropicalis***		***C. krusei***	
	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)
South Africa	17 (16%)	89(83.9%)	7 (58.3%)	5 (41.7%)	_	_	_	_
Cameroon	18 (19.6%)	74 (80.4%)	9 (37.5%)	15 (62.5%)	0 (0%)	4 (100%)	1 (33.3%)	2 (66.6%)

(- : no breakpoint available for the organism/drug)

Tables 12 and 13 show the chi-square statistical association results and symmetric measures of the susceptibility test done on South African and Cameroonian *Candida* species run on the TREK Sensititre system. The significance values are seen for the individual drugs on the YO9 panel and elucidate the statistical associations between different *Candida* species and their drug susceptibility results. In both populations, 5-Flucytosine was the only drug on the YO9 panel for which no statistically significant association was seen when comparing the prevalence of *Candida* species and drug susceptibility. The symmetric measures show how strong the different associations were. Detailed results of these statistical associations can be seen in appendix 8.

Table 12: Chi-square and symmetric measure results of South African species vs drug susceptibility.

South Africa	**Chi-square test results**	**Symmetric measures**
Amphotericin B	p=0.01	Strong association
5-Flucytosine	p=0.685	Weak association
Anidulafungin	p=0.000	Strong association
Caspofungin	p=0.000	Strong association
Micafungin	p=0.000	Strong association
Fluconazole	p=0.032	Moderate association
Itraconazole	p=0.008	Moderate association
Voriconazole	p=0.000	Strong association

Table 13: Chi-square and symmetric measure results of Cameroonian species vs drug susceptibility.

Cameroon	Chi-square test results	Symmetric measures
Amphotericin B	p=0.001	Strong association
5-Flucytosine	p=0.265	Moderate association
Anidulafungin	p=0.000	Strong association
Caspofungin	p=0.000	Strong association
Micafungin	p=0.000	Strong association
Fluconazole	p=0.041	Moderate association
Itraconazole	p=0.044	Moderate association
Voriconazole	p=0.000	Strong association

Antifungal drug susceptibility testing was done on all type strains (Table 14) for comparative reasons, using the fluconazole disk diffusion method and the three media used in the clinical strains, namely Sabourauds, GMB and YNBG, and the TREK Sensititre micro dilution broth method.

Table 14: Comparison of fluconazole drug susceptibility results using disk diffusion and the TREK system.

	Sab			GMB			YNBG			TREK	
Type strain	**mm**	**Mc**	**Interpretation**	**mm**	**Mc**	**Interpretation**	**mm**	**Mc**	**Interpretation**	**Result**	**Interpretation**
C. albicans NCPF3281	0	Res.	Resistant	22	0	Susceptible	0	Res.	Resistant	>256	Resistant
C. dubliniensis NCPF3949a	5	1	Resistant	20	0	Susceptible	>20	0	Susceptible	=0.5	Susceptible
C. albicans ATCC90028	17	2	Susceptible	15	0	Intermediate	0	Res.	Resistant	>256	Resistant
C. tropicalis ATCC950	12	0	Intermediate	10	0	Resistant	16	0	Susceptible	=2	Susceptible
C. krusei ATCC2159	3	0	Resistant	4	0	Resistant	0	Res.	Resistant	=64	Resistant
C. glabrata ATCC26512	3	0	Resistant	10	0	Resistant	0	Res.	Resistant	=32	Intermediate

3.5.3. Distribution and susceptibility patterns of *Candida* species by gender

The distribution and susceptibility patterns of *Candida* species with relation to factors such as gender, ethnicity and HIV status did not originally form part of the objectives of this study. However, the analysis of the data deemed these comparisons worth mentioning.

Candida species prevalence according to patient gender is summarised in Table 15. Differences in *Candida* species colonization between the two genders can be seen, with females harbouring more species than males. Out of the total 196 *Candida* strains isolated from the oral mucosa of 194 HIV-positive women, 152 were identified as *C. albicans*, 27 as *C. glabrata*, 8 as *C. dubliniensis*, 4 as *C. krusei*, 3 as *C. tropicalis* and 2 as either *C. parapsilopsis*, *C. lusitaneae* or *C. kefyr*. Two women were colonized by both *C. albicans* and *C. glabrata*. The number of isolates may differ from the number of patients, as multiple species were isolated in two South African patients. Table 15 examines the prevalence of *Candida* according to gender within population groups.

Table 15: *Candida* species prevalence according to patient gender.

Spp. According to gender	**South African n=128**		**Cameroonian n=127**	
	Males (%)	Females (%)	Males (%)	Females (%)
C. albicans	31 (88.6%)	75 (81.5%)	15 (65.2%)	77 (74%)
C. dubliniensis	3 (8.6%)	7 (9.3%)	0 (0%)	1 (0.96%)
C. glabrata	2 (5.7%)	10 (10.9%)	7 (30.4%)	17 (16.3%)
C. tropicalis	0 (0%)	0 (0%)	1 (4.3%)	3 (2.9%)
C. krusei	0 (0%)	0 (0%)	0 (0%)	4 (3.8%)
C. kefyr/ parapsilopsis/ lusitaneae	0 (0%)	0 (0%)	0 (0%)	2 (1.9%)

Tables 16 and 17 show the associations between *Candida* species and fluconazole susceptibility in relation to gender distribution for both populations, using the YNBG disk diffusion method and the TREK Sensititre system. As with the previous table and for future tables, the number of cases may differ from the number of patients, as multiple species were isolated in two South African patients.

Table 16: South African and Cameroonian *Candida* species prevalence and fluconazole susceptibility according to patient gender using the YNGB disk diffusion method (percentages are per gender/per species).

Spp. and susceptibility according to gender		**Susceptible (%)**		**Intermediate (%)**		**Resistant (%)**	
		SA (%)	**Cam (%)**	**SA (%)**	**Cam (%)**	**SA (%)**	**Cam (%)**
C. albicans	Females	35 (46.7%)	37 (46.8%)	0 (0%)	8 (10.4%)	40 (53.3%)	32 (41.6%)
	Males	11 (35.5%)	7 (46.7%)	0 (0%)	1 (66.7%)	20 (64.5%)	7 (46.7%)
C. dubliniensis	Females	6 (85.7%)	0 (0%)	0 (0%)	1 (100%)	1 (14.3%)	0 (0%)
	Males	3 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C. glabrata	Females	3 (30%)	1 (5.9%)	1 (10%)	4 (23.5%)	6 (60%)	12 (70.6%)
	Males	0 (0%)	0 (0%)	0 (0%)	2 (28.6%)	2 (100%)	5 (71.4%)
C. tropicalis	Females	-	0 (0%)	-	0 (0%)	-	4 (100%)
	Males	-	0 (0%)	-	0 (0%)	-	0 (0%)
C. krusei	Females	-	0 (0%)	-	0 (0%)	-	4 (100%)
	Males	-	0 (0%)	-	0 (0%)	-	0 (0%)
C. kefyr/ parapsilopsis/ lusitaneae	Females	-	0 (0%)	-	1 (50%)	-	1 (50%)
	Males	-	0 (0%)	-	0 (0%)	-	0 (0%)

Table 17: South African and Cameroonian *Candida* species prevalence and fluconazole susceptibility according to patient gender using the TREK Sensititre system (percentages are per gender/per species).

Spp. and susceptibility according to gender		**Susceptible (%)**		**Intermediate (%)**		**Resistant (%)**	
		SA (%)	**Cam (%)**	**SA (%)**	**Cam (%)**	**SA (%)**	**Cam (%)**
C. albicans	Females	40 (53.3%)	41 (53.2%)	1 (1.3%)	0 (0%)	34 (45.3%)	36 (46.8%)
	Males	13 (41.9%)	4 (26.7%)	0 (0%)	1 (6.7%)	18 (58.1%)	10 (66.7%)
C. dubliniensis	Females	6 (85.7%)	1 (100%)	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)
	Males	3 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C. glabrata	Females	7 (70%)	11 (64.7%)	3 (30%)	5 (29.4%)	0 (0%)	1 (5.9%)
	Males	1 (50%)	5 (71.4%)	1 (50%)	2 (28.6%)	0 (0%)	0 (0%)
C. tropicalis	Females	-	3 (100%)	-	0 (0%)	-	0 (0%)
	Males	-	1 (100%)	-	0 (0%)	-	0 (0%)
C. krusei	Females	-	1 (25%)	-	0 (0%)	-	3 (75%)
	Males	-	0 (0%)	-	0 (0%)	-	0 (0%)
C. kefyr/ parapsilopsis/ lusitaneae	Females	-	2 (100%)	-	0 (0%)	-	0 (0%)
	Males	-	0 (0%)	-	0 (0%)	-	0 (0%)

3.5.4 Distribution, associations and susceptibility patterns of *Candida* species in women

Since women are known to be more predisposed to *Candida* than their male counterparts, the colonization patterns, antifungal drug susceptibility results and different parameters that are unique to women, including their pregnancy status, were investigated. For this section, antifungal drugs were grouped into the azoles and echinocandins, as these are drug classes which would most probably be dispensed for treating these patients and both are well represented on the TREK panel. In the combined female population, resistance to azole drugs was very high in the case of *C. albicans* (54%) and *C. krusei* (100%). The echinocandin drugs showed high levels of resistance the case of *C. glabrata* (40.7%) (Table 18).
No associations were seen between *Candida* species prevalence and age, health status or presence of tuberculosis. It was noted that *C. albicans* was the only species isolated from the oral mucosa of patients who were either pregnant or had recently given birth (Table 18).

Table 18 shows the *Candida* species associations and drug susceptibility results in females, when combining the female patient results from both populations. For the susceptibility testing results an isolate was considered to be resistant if it fell within the established "resistant" breakpoint category for one or more of the tested drugs in that class, as detailed in the breakpoint studies described in Table 8.

Table 18: *Candida* species associations and drug susceptibility in females.

	C. albicans		*C. dubliniensis*		*C. glabrata*		*C. tropicalis*		*C. krusei* *		*C. kefyr/para/lusi*		p-values
	(n=152)		(n=8)		(n=27)		(n=3)		(n=4)		(n=2)		
Age distribution													
10-20yrs (n=3)	3(100%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		
21-50yrs (n=163)	127(77.9%)		8(4.9%)		21(12.9%)		2(1.2%)		4(2.4%)		1(0.6%)		
>50yrs (n=30)	22(73.3%)		0(0%)		6(20%)		1(3.3%)		0(0%)		1(3.3%)		
Health status													
AIDS - (n=147)	112(76.2%)		8(5.4%)		20(13.6%)		2(1.4%)		3(2%)		2(1.4%)		
AIDS + (n=49)	40(81.6%)		0(0%)		7(14.3%)		1(2%)		1(2%)		0(0%)		
Pregnancy													
Not pregnant (n=182)	138(75.8%)		8(4.4%)		27(14.8%)		3(1.6%)		4(2.2%)		2(1.1%)		
Pregnant/recent birth (n=14)	14(100%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		
Patients on TB treatment													
No (n=172)	133(77.3%)		8(4.7%)		24(14%)		2(1.2%)		3(1.7%)		2(1.2%)		
Yes (n=24)	19(79.2%)		0(0%)		3(12.5%)		1(8.3%)		1(8.3%)		0(0%)		
ARV therapy													
No ARV therapy (n=35)	30(85.7%)		2(5.7%)		2(5.7%)		1(2.9%)		0(0%)		0(0%)		
AZT/NVP/ 3TC (n=73)	57(78.1%)		0(0%)		13(17.9%)		0(0%)		3(4.1%)		0(0%)		
d4T/NVP/ 3TC (n=31)	21(67.7%)		3(9.7%)		5(16.1%)		0(0%)		1(3.2%)		1(3.2%)		
d4T/EFV/ 3TC (n=24)	22(91.7%)		1(4.2%)		1(4.2%)		0(0%)		0(0%)		0(0%)		
AZT/EFV/ 3TC (n=17)	13(76.5%)		0(0%)		3(17.6%)		1(5.9%)		0(0%)		0(0%)		
LPV/r combinations (n=5)	3(60%)		1(20%)		1(20%)		0(0%)		0(0%)		0(0%)		
AZT/DDI/ KLT (n=3)	1(33.3%)		1(33.3%)		1(33.3)		0(0%)		0(0%)		0(0%)		
TDF/3TC (n=2)	2(100%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		
AZT/3TC/ lopinavir/ritonavir (n=1)	0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		1(100%)		
AZT/3TC/ KLT (n=1)	1(100%)		0(0%)		0(0%)		0(0%)		0(0%)		0(0%)		
Not known (n=4)	2(50%)		0(0%)		1(25%)		1(25%)		0(0%)		0(0%)		
Duration of ARV therapy													
No ARV therapy (n=35)	30(85.7%)		2(5.7%)		2(5.7%)		1(2.9%)		0(0%)		0(0%)		p=0.008
<1yr (n=50)	43(86%)		1(2%)		6(12%)		0(0%)		0(0%)		0(0%)		
≥1-<3yrs (n=49)	35(71.4%)		5(10.2%)		7(14%)		1(2%)		1(2%)		0(0%)		
≥3yrs (n=60)	44(73.3%)		0(0%)		11(18.3%)		0(0%)		3(5%)		2(3.3%)		
Unknown (n=2)	0(0%)		0(0%)		1(50%)		1(50%)		0(0%)		0(0%)		
Susceptibility patterns	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant	
Azoles	70(46%)	82(54%)	7(87.5%)	1(12.5%)	22(81.5%)	5(18.5%)	3(100%	0(0%)	0(0%)	3(100%)	2(100%)	0(0%)	p=0.000
Echinocandins	150(98.7%)	2(1.3%)	-	-	16(59.3%)	11(40.7%)	3(100%)	0(0%)	3(100%)	0(0%)	-	-	p=0.000

*One of the *C. krusei* isolates did not grow on the TREK plate, and was therefore not included in the susceptibility section of this table.

"-" = no established breakpoint for the organism/drug.

3.6 Distribution of *Candida* species related to age and ethnicity.

Age and race were examined for frequency of species distribution. Frequency was calculated for each age group as a percentage of each individual species isolated. In the South African population, *C.albicans* was most prevalent in the 31-40 year age group as was the case for the Cameroonian group (Table 19). *C. glabrata* and *C.dubliniensis* were also detected in this age group more than in any of the other age groups in South Africa. As female patients approached or passed menopause, *C. glabrata* became the dominant species in the Cameroonian population.

The effect of ethnicity or race was also examined within the South African population, which was composed of patients from different racial groups (Table 20). Species distribution and prevalence was markedly increased in the black population, compared to the other race groups.

Table 19: *Candida* species associated with age.

Age	***C. albicans***		***C. dubliniensis***		***C. glabrata***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
≤20 years	3 (2.80%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
21-30 years	33 (31%)	14 (15.2%)	2 (20%)	0 (0%)	3 (25%)	0 (0%)
31-40 years	44 (41.5%)	34 (37%)	4 (40%)	0 (0%)	9 (75%)	8 (33.3%)
41-50 years	19 (18%)	22 (23.9%)	2 (20%)	1 (100%)	0 (0%)	6 (25%)
51-60 years	5 (4.7%)	16 (17.4%)	2 (20%)	0 (0%)	0 (0%)	9 (37.5%)
≥61 years	2 (1.9%)	3 (3.3%)	0 (0%)	0 (0%)	0 (0%)	1 (4.2%)
Age	***C. tropicalis***		***C. krusei***		***C. kefyr/para/lusi***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
≤20 years	_	0 (0%)	_	0 (0%)	_	0 (0%)
21-30 years	_	1 (25%)	_	2 (50%)	_	0 (0%)
31-40 years	_	1 (25%)	_	2 (50%)	_	1 (50%)
41-50 years	_	1 (25%)	_	0 (0%)	_	0 (0%)
51-60 years	_	1 (25%)	_	0 (0%)	_	1 (50%)
≥61 years	_	0 (0%)	_	0 (0%)	_	0 (0%)

Table 20: South African *Candida* species associated with race.

***Candida* species and race**	**Black (%)**	**Coloured (%)**	**Indian (%)**	**White (%)**
C. albicans	98 (83%)	6 (75%)	1 (100%)	1 (100%)
C. dubliniensis	9 (7.6%)	1 (12.5%)	0 (0%)	0 (0%)
C. glabrata	11 (9.3%)	1 (12.5%)	0 (0%)	0 (0%)

3.7 Distribution of *Candida* species related to HIV status

HIV infection is associated with opportunistic co-infections such as *Candida*. This study examined the prevalence of *Candida* in HIV-positive subjects (Table 21, where AIDS refers to individuals with late-stage HIV-related opportunistic infections) and attempted to associate the findings with the treatment (Tables 22 and 23) and duration of ARV therapy (Table 24). Detailed information on these tables can be found in appendices 5, 6 and 7. Species prevalence appeared to decrease in AIDS patients possibly because most of them were on ARVs (only patients with lower CD4 counts are routinely enrolled for ART in both countries' state hospitals). This is confirmed in Table 21 where patients not on ARV showed a higher prevalence of *Candida* than those being treated with ARVs even though the prevalence altered with different treatment regimes in the South African group. In the Cameroonian group, species prevalence seemed to increase in those being treated with the AZT, NVP, 3TC cocktail (Table 23).

Table 21: *Candida* species associated with HIV status.

HIV Status	***C. albicans***		***C. dubliniensis***		***C. glabrata***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
HIV+	65 (61%)	68 (73.9%)	10 (100%)	1 (100%)	9 (75%)	17 (70.8%)
AIDS	41 (39%)	24 (26.1%)	0 (0%)	0 (0%)	3 (25%)	7 (29.2%)
HIV Status	***C. tropicalis***		***C. krusei***		***C. kefyr/para/lusi***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
HIV+	-	3 (75%)	-	3 (75%)	-	2 (100%)
AIDS	-	1 (25%)	__-	1 (25%)	-	0 (0%)

Table 22: *Candida* species associated with antiretroviral therapy in the South African group.

***Candida* species and ARV therapy**	**No ARVs**	**d4T, EFV, 3TC**	**d4T, NVP, 3TC**	**AZT, NVP, 3TC**
C. albicans	40 (38%)	33 (31%)	24 (23%)	7 (6.6%)
C. dubliniensis	2 (20%)	3 (30%)	4 (40%)	0 (0%)
C. glabrata	2 (17%)	2 (17%)	5 (42%)	1 (8.3%)
	AZT, EFV, T3C	**AZT, DDI, KLT**	**AZT, 3TC, KLT**	**TDF, EFV, 3TC**
C. albicans	0 (0%)	1 (0.9%)	1 (0.9%)	1 (0.9%)
C. dubliniensis	0 (0%)	1 (10%)	0 (0%)	0 (0%)
C. glabrata	1 (8.3%)	1 (8.3%)	0 (0%)	0 (0%)

AZT: Azidothymidine (zidovudine), DDI: Didanosine, D4T: Stavudine, EFV: Efavirenz, KLT: Kaletra (lopinavir), NVP: Nevirapine, TDF: Tenofovir disoproxil fumarate (tenofovir), 3TC: Lamivudine.

Table 23: *Candida* species associated with antiretroviral therapy in the Cameroonian group.

***Candida* species and ARV therapy**	**No ARVs (%)**	**d4T, 3TC (%)**	**d4T, NVP, 3TC (%)**	**AZT, NVP, 3TC (%)**
C. albicans	4 (4.3%)	1 (1.1%)	3 (3.3%)	57 (62%)
C. dubliniensis	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C. glabrata	(0%)	0 (0%)	2 (8.3%)	14 (58.3%)
C. tropicalis	1 (25%)	0 (0%)	0 (0%)	0 (0%)
C. krusei	0 (0%)	0 (0%)	1 (25%)	3 (75%)
C. kefyr/ parapsilopsis/ lusitaneae	0 (0%)	0 (0%)	1 (50%)	0 (0%)
	AZT, EFV, T3C (%)	**TDF, T3C (%)**	**AZT, 3TC, KLT (%)**	**LPV/r + (%)**
C. albicans	19 (20.7%)	1 (1.1%)	0 (0%)	4 (4.3%)
C. dubliniensis	0 (0%)	0 (0%)	0 (0%)	1 (100%)
C. glabrata	5 (20.1%)	1 (4.2%)	0 (0%)	1 (4.2%)
C. tropicalis	2 (50%)	0 (0%)	0 (0%)	0 (0%)
C. krusei	0 (0%)	0 (0%)	0 (0%)	0 (0%)
C. kefyr/ parapsilopsis/ lusitaneae	0 (0%)	0 (0%)	1 (50%)	0 (0%)

AZT: Azidothymidine (zidovudine), DDI: Didanosine, D4T: Stavudine, EFV: Efavirenz, KLT: Kaletra (lopinavir), LPV/r: Alluvia, NVP: Nevirapine, TDF: Tenofovir disoproxil fumarate (tenofovir), 3TC: Lamivudine.

Patients on ARVs for less than 1 year showed a greater prevalence of *Candida* than those who had not received treatment. The longer the duration above one year, the lower the prevalence in both groups (Table 24).

Table 24: *Candida* species associated with duration of antiretroviral therapy.

ARV Duration	***C. albicans***		***C. dubliniensis***		***C. glabrata***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
No ARVs	39 (36.8%)	5 (5.2%)	2 (20%)	0 (0%)	2 (16.7%)	0 (0%)
<1year	41 (38.7%)	17 (17.7%)	2 (20%)	0 (0%)	7 (58.3%)	0 (0%)
1-2years	13 (12.3%)	12 (12.5%)	5 (50%)	0 (0%)	1 (8.3%)	5 (20.8%)
2-3years	9 (8.5%)	9 (9.4%)	1 (10%)	0 (0%)	1 (8.3%)	2 (8.3%)
≥3years	4 (3.8%)	53 (55.2%)	0 (0%)	1 (100%)	1 (8.3%)	17 (70.8%)
ARV Duration	***C. tropicalis***		***C. krusei***		***C. kefyr/para/lusi***	
	SA (%)	Cam (%)	SA (%)	Cam (%)	SA (%)	Cam (%)
No ARVs	-	1 (25%)	-	0 (0%)	-	0 (0%)
<1year	-	0 (0%)	-	0 (0%)	-	0 (0%)
1-2years	-	1 (25%)	-	1 (25%)	-	0 (0%)
2-3years	-	1 (25%)	-	0 (0%)	-	0 (0%)
≥3years	_-	1 (25%)	-	3 (75%)	-	2 (100%)

Antiretroviral therapy duration and *Candida* species prevalence statistical association for Cameroonian results: p=0.034.

3.8 Protein Identification

3.8.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Table 25 places together the molecular mass and function of fluconazole-resistance associated proteins identified in the different studies explained in chapter 1.

Table 25: *Candida* proteins expressed in the presence of fluconazole, their approximate molecular mass and function.

Protein	Molecular Mass	Function
Pdc11p	5.4kDa	Carbohydrate metabolism
Ifd5p	5.4kDa	Alcohol dehydrogenase-oxidoreductase
Ifd1p	5.6kDa	Alcohol dehydrogenase-oxidoreductase
Ifd6p	5.9kDa	Alcohol dehydrogenase-oxidoreductase
Ifd4p	6kDa	Alcohol dehydrogenase-oxidoreductase
Cdc19p	6.5kDa	Carbohydrate metabolism
Gap1p	6.6kDa	Carbohydrate metabolism
Tropiase	23.9kDa	Acid proteinase
Grp2p	37.62kDa	Reductase
Unknown	40kDa	Unknown
Erg10p	41.7kDa	Ergosterol biosynthesis pathway
Exoglucanase	44kDa	
Unknown	60kDa	Unknown
14a-demethylase	61kDa	Ergosterol biosynthesis
Cdr2p	96kDa	Membrane multidrug transporter
Mdr1p	109.258kDa	Membrane multidrug transporter
CgCdr1p	169kDa	Membrane multidrug transporter
23 others	23-64kDa	Unknown

Figure 13 shows a Coomassie blue stained protein gel obtained using SDS-PAGE, already dried and sandwiched between two drying films. The second protein row corresponds to the Bio-Rad broad range protein marker used to determine the approximate molecular weights of the sample proteins. Figures 14 to 17 reveal the individual protein gel bands for the *Candida* species identified as *C. albicans* (susceptible and resistant strains are shown, based on TREK results), *C. dubliniensis* and *C. glabrata*, respectively, with the patient's number shown

below and the colours denoting resistance patterns. A protein marker band is shown on the left, as well as a type strain for each species, where available.

Fig. 13: A Coomassie-stained protein gel with nine sample protein lanes and a protein marker lane with specific and easily identifiable protein standards (values shown in kiloDaltons).

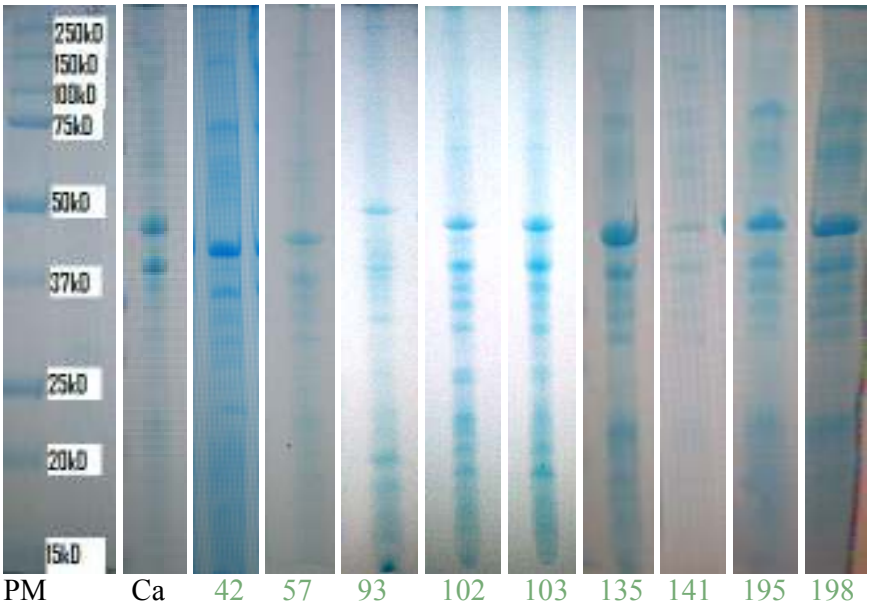
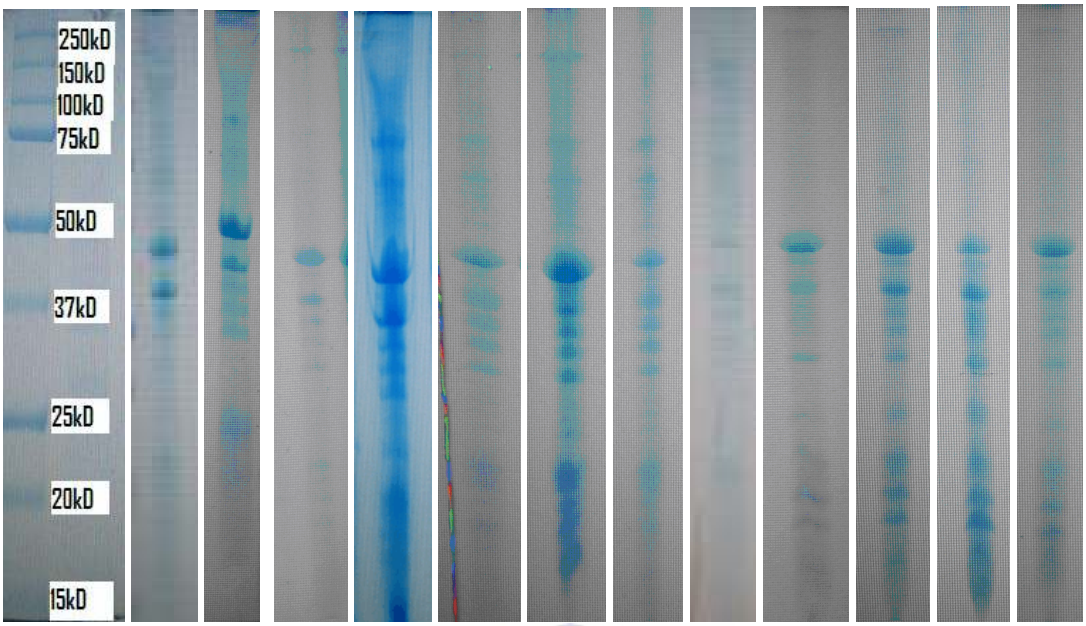
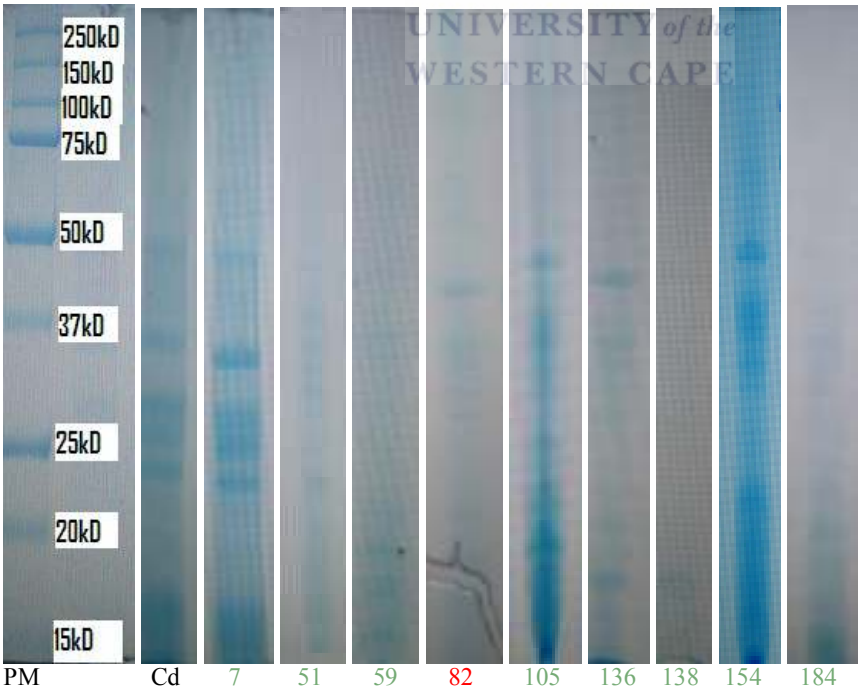


Figure 14: Fluconazole-susceptible *C. albicans* SDS-PAGE protein gel results.



PM Ca 14 40 44 46 47 48 60 95 100 101 112

Figure 15: Fluconazole-resistant *C. albicans* SDS-PAGE protein gel results.



PM Cd 7 51 59 82 105 136 138 154 184

Figure 16: *C. dubliniensis* SDS-PAGE protein gel results.

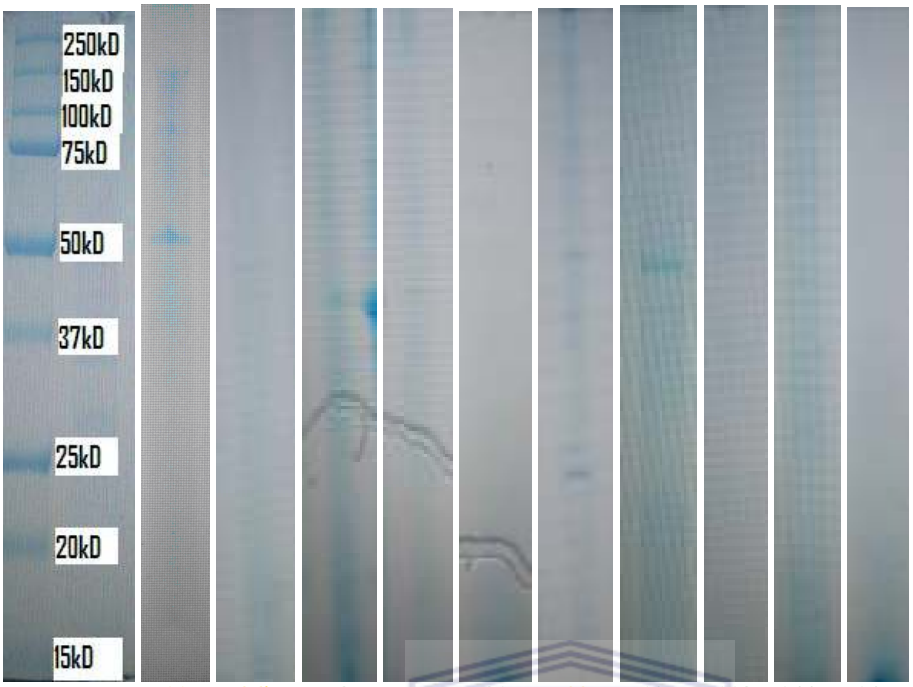


Figure 17: *C. glabrata* SDS-PAGE protein gel results.

Table 26 shows the molecular weights of the unique bands identified by SDS-PAGE in this study and the fluconazole susceptibility of the respective isolates. Proteins with a molecular weight of 24kDa were only seen in intermediate (dose-dependent) or drug-resistant cell fractions.

Table 26: Molecular weight and fluconazole susceptibility of proteins of interest identified by SDS-PAGE.

Molecular weight	Fluconazole susceptibility
24kDa	I,R
23-27kDa	S
36kDa	S
37kDa	S
44kDa	S,R
49kDa	S
50kDa	S,I

S=susceptible; I=intermediate; R=resistant.

3.8.2 High Performance Liquid Chromatography-Mass Spectrometry

Thermo Proteome Discoverer 1.3 software (Thermo Scientific, Bremen, Germany) was used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK, and Sequest) of all tandem mass spectra against the Uniprot *Candida* database. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine, N-acetylation and deamidation (NQ) was used as variable modifications. The precursor mass tolerance was set to 10 ppm, and fragment mass tolerance set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 1 tryptic peptides per proteins, a Mascot score threshold of 20 and Sequest score threshold of 1.5. Percolator was used for peptide validation with a maximum delta Cn of 0.5, and decoy database searches with a FDR of 0.02 and 0.05 with validation based on the q-value.

The individual peptides' information was then further searched on the Uniprot *Candida* database (Uniprot, 2013) to identify specific peptide functions related to antifungal drug resistance.

Tables 27 and 28 show the detailed information of drug-resistance related proteins identified using HPLC-MS for *C. albicans* fluconazole-susceptible and intermediate/resistant isolate fractions, respectively, and their function as described on the UniProt database. Tables 29 and 30 show the HPLC-MS results for the non-albicans species which were found to be susceptible and intermediate/resistant to fluconazole, respectively. Only proteins above a mascot score of 24 were considered significant, and with a protein sequence coverage above 5%.

Table 27: Fluconazole-susceptible *C. albicans* cell fraction HPLC-MS results.

Isolate number	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Function
C50	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	2130.39	33.69	3	10	19	66	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C80			2342.92	46.19	2	11	24	84	656	70.3	5.17	
C88			2868.32	44.05	4	11	26	129	656	70.3	5.17	
C132			1956.02	44.66	3	11	26	110	656	70.3	5.17	
C182			1858.36	47.56	4	21	28	122	656	70.3	5.17	
SA3			704.61	24.85	4	6	14	42	656	70.3	5.17	
SA70			111.74	17.38	4	2	10	18	656	70.3	5.17	
SA126			910.50	26.52	5	4	17	50	656	70.3	5.17	
SA163			1756.18	45.43	3	10	27	97	656	70.3	5.17	
SA201			692.88	22.87	4	5	13	27	656	70.3	5.17	
C50	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	1334.50	22.79	1	3	11	50	645	70.0	5.06	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity
C80			1565.69	29.61	1	2	14	61	645	70.0	5.06	
C88			1816.01	27.44	2	1	17	86	645	70.0	5.06	
C132			1415.21	28.53	1	2	14	75	645	70.0	5.06	
SA3			455.98	14.73	1	1	8	29	645	70.0	5.06	
SA70			86.30	13.80	1	1	8	13	645	70.0	5.06	
SA126			573.48	19.53	2	1	12	30	645	70.0	5.06	
SA163			1233.05	39.07	1	5	22	71	645	70.0	5.06	
SA201			488.06	19.53	1	2	10	19	645	70.0	5.06	
C88	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	620.44	27.02	1	11	19	35	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C132			377.90	14.14	1	3	9	21	707	80.8	4.88	
C182			424.47	20.08	1	7	11	16	707	80.8	4.88	
C80	P43084	Probable NADPH dehydrogenase OS=Candida albicans GN=EBP1 PE=1 SV=2 - [EBP1_CANAX]	280.05	32.43	1	9	9	20	407	46.0	6.39	Oxidoreductase that binds mammalian estrogens with high affinity.
SA163			52.80	9.83	1	1	4	4	407	46.0	6.39	
SA201			37.74	8.11	1	3	3	4	407	46.0	6.39	
C80	O13318	pH-responsive protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=PHR2 PE=2 SV=2 -	390.41	11.58	2	5	5	18	544	58.7	4.64	Required for apical cell growth and plays an essential role in morphogenesis. May be integral to the
C88			335.93	13.42	2	5	5	20	544	58.7	4.64	
C132			422.77	10.85	2	4	5	22	544	58.7	4.64	
C182			328.88	4.96	1	2	2	12	544	58.7	4.64	
SA3			53.31	4.96	1	1	2	2	544	58.7	4.64	

SA70		[PHR2_CANAL]	67.03	7.54	1	3	3	4	544	58.7	4.64	pathogenic ability of the organism
SA126			370.51	6.80	1	3	3	10	544	58.7	4.64	
SA163			192.31	10.48	1	3	4	5	544	58.7	4.64	
SA201			369.62	6.80	2	3	3	12	544	58.7	4.64	
C80	Q06099	S-(hydroxymethyl) glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	192.83	5.77	1	2	2	13	381	40.0	6.70	Confers resistance to formaldehyde.
C88			61.62	5.77	1	2	2	4	381	40.0	6.70	
C132			170.76	5.77	1	2	2	10	381	40.0	6.70	
C182			130.24	9.71	1	1	4	11	381	40.0	6.70	
SA3			122.16	7.87	1	3	3	12	381	40.0	6.70	
SA70			46.10	6.82	1	2	3	8	381	40.0	6.70	
SA126			229.23	7.87	1	3	3	18	381	40.0	6.70	
SA201			208.85	5.77	1	2	2	9	381	40.0	6.70	
C50	P43071	Multidrug resistance protein CDR1 OS=Candida albicans GN=CDR1 PE=3 SV=1 - [CDR1_CANAX]	148.36	6.46	2	5	6	8	1501	169.8	6.98	Transporter, whose physiological function is not yet established. Confers resistance to the chemical cycloheximide.
SA163			34.99	2.66	2	2	4	4	1501	169.8	6.98	

Legend: Accession: UniProt database query number; Score: mascot score; Coverage: the % sequence coverage of the protein as detected by the MS; # of proteins: total nr of proteins detected; # unique peptides: total nr of peptides detected for the protein that is unique to the protein; #PSM: peptides spectrum matches, estimation of the false positive protein identifications using decoy database containing reversed sequences. The PSM shown are all within the parameters due to the use of percolater that calculates statistically meaningful q-values. #AA: nr of amino acids present within the protein as given in the database. MW: molecular weight of the protein as given in database; pI: pI of protein as given in database.

Table 28: Fluconazole-intermediate and -resistant *C. albicans* cell fraction HPLC-MS results.

Isolate number	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Function
C35	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	1246.81	38.57	3	10	21	55	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C42			1626.76	37.35	3	11	21	107	656	70.3	5.17	
C73			1905.13	44.66	2	22	24	101	656	70.3	5.17	
C76			331.14	17.07	2	2	10	26	656	70.3	5.17	
C199			3521.43	48.32	1	12	30	174	656	70.3	5.17	
C255			521.37	24.70	4	11	16	59	656	70.3	5.17	
SA28			1369.57	31.71	3	6	19	73	656	70.3	5.17	
SA40			320.86	10.52	3	1	7	38	656	70.3	5.17	
SA50			1121.96	25.30	5	10	15	90	656	70.3	5.17	
SA78			316.91	20.73	5	8	13	56	656	70.3	5.17	
SA100			704.10	26.83	4	5	16	47	656	70.3	5.17	
C35	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	916.33	28.22	1	4	14	39	645	70.0	5.06	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity
C199			1812.37	35.50	1	5	18	104	645	70.0	5.06	
SA28			1019.96	27.91	1	3	15	54	645	70.0	5.06	
SA40			320.62	11.16	3	1	7	36	645	70.0	5.06	
SA100			477.11	22.02	1	1	13	36	645	70.0	5.06	
C42	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	372.85	16.12	1	7	11	24	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C76			154.89	10.18	1	3	10	17	707	80.8	4.88	
C199			960.76	24.33	1	12	14	32	707	80.8	4.88	
SA50			207.22	16.27	1	7	12	19	707	80.8	4.88	
SA78			283.68	9.90	1	4	7	15	707	80.8	4.88	
C42	P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	651.81	19.16	1	1	12	45	642	69.6	5.11	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C76			479.69	26.32	1	2	15	43	642	69.6	5.11	
C199			1098.03	23.36	1	3	15	61	642	69.6	5.11	
C76	P10592	Heat shock protein SSA2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA2 PE=1 SV=3 - [HSP72_YEAST]	518.71	28.01	1	2	16	50	639	69.4	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C35	O13318	pH-responsive	188.95	9.38	2	4	4	8	544	58.7	4.64	May be

C42		protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=PHR2 PE=2 SV=2 - [PHR2_CANAL]	703.02	11.40	2	4	5	28	544	58.7	4.64	integral to the pathogenic ability of the organism
C73			1074.28	9.56	2	5	5	38	544	58.7	4.64	
C199			178.31	9.56	1	4	4	11	544	58.7	4.64	
SA28			57.02	7.54	1	3	3	4	544	58.7	4.64	
SA40			118.12	6.99	1	2	4	7	544	58.7	4.64	
SA50			76.33	9.38	1	3	4	6	544	58.7	4.64	
SA78			205.11	6.80	2	3	3	8	544	58.7	4.64	
SA100			140.40	12.68	1	3	5	9	544	58.7	4.64	
C35	P43084	Probable NADPH dehydrogenase OS=Candida albicans GN=EBP1 PE=1 SV=2 - [EBP1_CANAX]	154.00	17.20	1	5	5	10	407	46.0	6.39	Oxidoreductase that binds mammalian estrogens with high affinity.
C50			243.37	32.92	1	10	10	13	407	46.0	6.39	
C73			166.13	19.90	1	5	7	15	407	46.0	6.39	
C199			66.64	12.53	1	4	6	6	407	46.0	6.39	
C42	Q06099	S-(hydroxymethyl) glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	152.77	5.25	1	2	2	8	381	40.0	6.70	Confers resistance to formaldehyde.
C73			244.05	9.97	1	3	4	20	381	40.0	6.70	
C199			39.40	3.15	1	1	1	1	381	40.0	6.70	
C255			147.09	7.87	1	2	3	13	381	40.0	6.70	
SA28			97.46	9.71	1	1	4	9	381	40.0	6.70	
SA40			88.42	7.87	1	2	3	7	381	40.0	6.70	
SA50			121.47	9.97	1	3	4	8	381	40.0	6.70	
SA78			67.40	7.35	1	2	3	8	381	40.0	6.70	
SA100			75.32	8.14	1	1	3	5	381	40.0	6.70	
C73	P78595	Multidrug resistance protein CDR2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=CDR2 PE=3 SV=2 - [CDR2_CANAL]	152.73	2.87	2	1	4	6	1499	168.9	6.98	Multidrug efflux transporter. Confers resistance to azole antifungal agents, to other antifungals (terbinafine, amorolfine) and to a variety of metabolic inhibitors.
C42	P43071	Multidrug	34.79	2.40	2	1	5	9	1501	169.8	6.98	Transporter,

C199		resistance protein CDR1 OS=Candida albicans GN=CDR1 PE=3 SV=1 - [CDR1_CANAX]	37.92	4.06	2	1	5	5	1501	169.8	6.98	whose physiological function is not yet established. Confers resistance to the chemical cycloheximide.

Legend: Accession: UniProt database query number; Score: mascot score; Coverage: the % sequence coverage of the protein as detected by the MS; # of proteins: total nr of proteins detected; # unique peptides: total nr of peptides detected for the protein that is unique to the protein; #PSM: peptides spectrum matches, estimation of the false positive protein identifications using decoy database containing reversed sequences. The PSM shown are all within the parameters due to the use of percolator that calculates statistically meaningful q-values. #AA: nr of amino acids present within the protein as given in the database. MW: molecular weight of the protein as given in database; pI: pI of protein as given in database.

Table 29: Fluconazole-susceptible non-albicans cell fraction HPLC-MS results.

Isolate number	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Function
C. dubliniensis												
SA7	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	511.08	24.24	3	4	14	42	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
SA138			343.94	20.58	4	3	13	39	656	70.3	5.17	
SA184			475.62	23.63	5	6	14	43	656	70.3	5.17	
SA7	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	403.67	16.43	1	1	9	26	645	70.0	5.06	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity
SA138			296.68	21.55	1	1	12	30	645	70.0	5.06	
SA7	Q06099	S-(hydroxymethyl)glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	135.77	9.97	1	4	4	12	381	40.0	6.70	Confers resistance to formaldehyde.
SA138			45.23	12.60	1	2	5	9	381	40.0	6.70	
SA184			90.61	7.87	1	2	3	8	381	40.0	6.70	
SA138	O13318	pH-responsive protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=PHR2 PE=2 SV=2 - [PHR2_CANAL]	55.96	2.21	1	1	1	2	544	58.7	4.64	May be integral to the pathogenic ability of the organism
SA184			46.28	4.96	1	1	2	2	544	58.7	4.64	
C. glabrata												
SA72	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	99.87	13.80	4	3	8	19	645	70.0	5.06	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity
C219			212.42	14.73	2	1	10	39	645	70.0	5.06	
SA107	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	309.00	16.92	4	5	9	20	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C219	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	440.46	18.25	1	7	13	38	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C219	P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	385.11	22.27	1	2	13	36	642	69.6	5.11	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C219	P10592	Heat shock protein SSA2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA2 PE=1 SV=3 - [HSP72_YEAST]	265.40	23.94	1	1	14	47	639	69.4	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
SA107	Q06099	S-(hydroxymethyl) glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	55.84	6.82	1	1	3	7	381	40.0	6.70	Confers resistance to formaldehyde.
SA107	O13318	pH-responsive protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=PHR2 PE=2 SV=2 - [PHR2_CANAL]	76.23	7.54	1	3	3	6	544	58.7	4.64	May be integral to the pathogenic ability of the organism

C. parapsilopsis/lusitaneae/kefyr												
C17	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	1214.22	27.60	6	2	18	96	645	70.0	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C21			1296.94	23.72	2	1	14	101	645	70.0	5.06	
C17	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	579.20	19.38	1	7	14	39	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C21			662.48	21.78	1	9	17	56	707	80.8	4.88	
C17	P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	627.09	21.50	4	1	17	71	642	69.6	5.11	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C21	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	1299.31	27.90	2	2	17	103	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C21	P10592	Heat shock protein SSA2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA2 PE=1 SV=3 - [HSP72_YEAST]	694.17	18.15	2	1	11	71	639	69.4	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C21	O13318	pH-responsive protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=PHR2 PE=2 SV=2 - [PHR2_CANAL]	94.18	6.25	1	2	3	5	544	58.7	4.64	May be integral to the pathogenic ability of the organism
C. krusei												
C258	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	99.76	4.24	1	2	3	8	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C. tropicalis												
C245	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	678.38	30.18	2	3	16	81	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C250			711.49	28.05	2	2	16	73	656	70.3	5.17	
C245	P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4 - [HSP71_YEAST]	652.16	16.51	1	1	10	57	642	69.6	5.11	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C250			576.74	17.76	1	1	12	58	642	69.6	5.11	
C245	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 -	585.05	31.47	2	2	17	74	645	70.0	5.06	Binds human HTN3/histatin-5, a peptide
C250			586.43	27.60	2	1	16	68	645	70.0	5.06	

		[HSP72_CANAL]										from saliva, and mediates its fungicidal activity.
C250	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	117.31	10.75	1	5	8	12	707	80.8	4.88	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.

Legend: Accession: UniProt database query number; Score: mascot score; Coverage: the % sequence coverage of the protein as detected by the MS; # of proteins: total nr of proteins detected; # unique peptides: total nr of peptides detected for the protein that is unique to the protein; #PSM: peptides spectrum matches, estimation of the false positive protein identifications using decoy database containing reversed sequences. The PSM shown are all within the parameters due to the use of percolator that calculates statistically meaningful q-values. #AA: nr of amino acids present within the protein as given in the database. MW: molecular weight of the protein as given in database; pI: pI of protein as given in database.

Table 30: Fluconazole-intermediate and -resistant non-albicans cell fraction HPLC-MS results.

Isolate number	Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI	Function
C. glabrata												
SA11	P10592	Heat shock protein SSA2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA2 PE=1 SV=3 - [HSP72_YEAST]	167.98	8.14	4	1	6	15	639	69.4	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C237			1471.64	49.92	1	5	25	108	639	69.4	5.06	
SA11	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	167.95	11.13	3	1	8	12	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
SA36b			127.92	17.07	4	5	10	15	656	70.3	5.17	
SA77			46.84	8.99	4	2	6	9	656	70.3	5.17	
SA92			71.82	6.10	4	2	4	4	656	70.3	5.17	
C237	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	242.16	8.06	1	4	6	17	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
SA11	Q06099	S-(hydroxymethyl)glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	33.20	2.62	1	1	1	2	381	40.0	6.70	Confers resistance to formaldehyde.
SA36b			0.00	5.77	1	2	2	2	381	40.0	6.70	
C. dubliniensis												
SA82	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	521.81	27.44	3	8	15	49	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
SA82	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	212.78	9.19	1	4	6	10	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
SA82	P10591	Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=4 SV=4 - [HSP71_YEAST]	206.84	12.77	1	1	7	28	642	69.6	5.11	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
SA82	Q06099	S-(hydroxymethyl)glutathione dehydrogenase OS=Candida maltosa GN=FDH1 PE=3 SV=1 - [FADH_CANMA]	102.72	9.97	1	2	4	12	381	40.0	6.70	Confers resistance to formaldehyde.
SA82	O13318	pH-responsive protein 2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876)	89.27	4.78	1	2	2	5	544	58.7	4.64	May be integral to the pathogenic

		GN=PHR2 PE=2 SV=2 - [PHR2_CANAL]										ability of the organism.
C. krusei												
C144	P41797	Heat shock protein SSA1 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA1 PE=1 SV=2 - [HSP71_CANAL]	117.63	11.74	4	2	8	14	656	70.3	5.17	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C172	P10592	Heat shock protein SSA2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA2 PE=1 SV=3 - [HSP72_YEAST]	38.12	5.48	2	1	4	8	639	69.4	5.06	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C172	P46587	Heat shock protein SSA2 OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=SSA2 PE=1 SV=3 - [HSP72_CANAL]	58.03	5.12	2	1	3	12	645	70.0	5.06	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.
C172	P46598	Heat shock protein 90 homolog OS=Candida albicans (strain SC5314 / ATCC MYA-2876) GN=HSP90 PE=1 SV=1 - [HSP90_CANAL]	171.58	2.97	1	1	2	4	707	80.8	4.88	Binds HTN3/histatin-5, a peptide from human saliva, and mediates its fungicidal activity.

Legend: Accession: UniProt database query number; Score: mascot score; Coverage: the % sequence coverage of the protein as detected by the MS; # of proteins: total nr of proteins detected; # unique peptides: total nr of peptides detected for the protein that is unique to the protein; #PSM: peptides spectrum matches, estimation of the false positive protein identifications using decoy database containing reversed sequences. The PSM shown are all within the parameters due to the use of percolator that calculates statistically meaningful q-values. #AA: nr of amino acids present within the protein as given in the database. MW: molecular weight of the protein as given in database; pI: pI of protein as given in database.

Table 31 summarizes the drug resistance-related proteins identified by HPLC-MS, showing which *Candida* species expressed the proteins, their resistance to fluconazole, the protein description and function.

Table 31: Summary of drug resistance-related *Candida* proteins identified by HPLC-MS.

***Candida* spp.**	**Fluconazole resistance**	**Description of protein**	**Function**
C. albicans, *C. dubliniensis*, *C. glabrata*, *C. parapsilopsis/kefyr/lusitaneae*, *C. tropicalis*	S, R	Heat shock protein SSA1	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C. albicans, *C. dubliniensis, C. glabrata*, *C. parapsilopsis/kefyr/lusitaneae*, *C. tropicalis*	S, R	Heat shock protein SSA2	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C. albicans, *C. glabrata*, *C. parapsilopsis/kefyr/lusitaneae*, *C. krusei*, *C. tropicalis*	S,R	Heat shock protein 90	Binds human HTN3/histatin-5, a peptide from saliva, and mediates its fungicidal activity.
C. albicans	S, R	Probable NADPH dehydrogenase	Oxidoreductase that binds mammalian estrogens with high affinity.
C. albicans, *C. dubliniensis*, *C. glabrata*, *C. parapsilopsis/kefyr/lusitaneae*	S, R	pH-responsive protein 2	May be integral to the pathogenic ability of the organism
C. albicans, *C. dubliniensis*, *C. glabrata*	S, R	S-(hydroxymethyl) glutathione dehydrogenase	Confers resistance to formaldehyde
C. albicans	S, R	Multidrug resistance protein CDR1	Confers resistance to the chemical cycloheximide
C. albicans	R	Multidrug resistance protein CDR2	Multidrug efflux transporter. Confers resistance to azole antifungal agents, to other antifungals (terbinafine, amorolfine) and to a variety of metabolic inhibitors.

S=susceptible; R=resistant.

Figures 18 and 19 show the chromatograms obtained after HPLC-MS analysis of the *Candida* cell wall fraction using the FASP method on a *C. albicans* drug-susceptible isolate and an azole-resistant isolate, respectively. Figures 20 and 21 show the HPLC-MS analysis chromatograms for a *C. glabrata* isolate which demonstrated intermediate susceptibility to azole drugs and a *C. krusei* isolate which was found to be resistant to fluconazole.

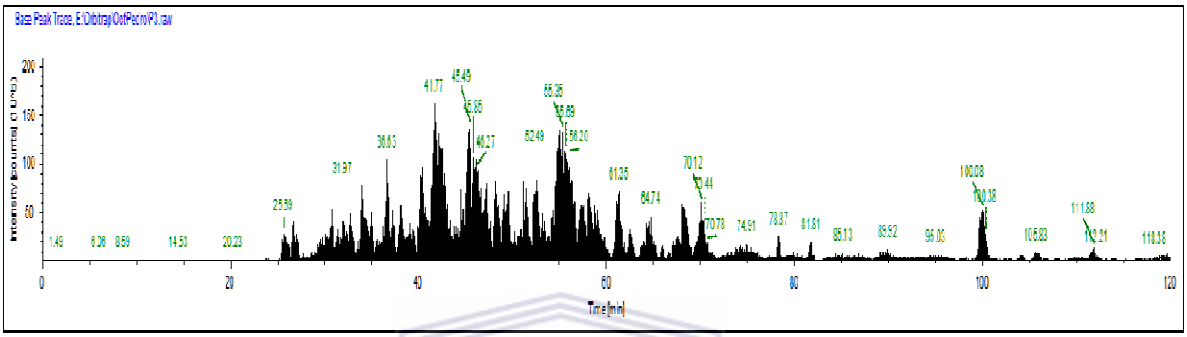


Fig. 18: Chromatogram for drug-susceptible *C. albicans* isolate SA163.

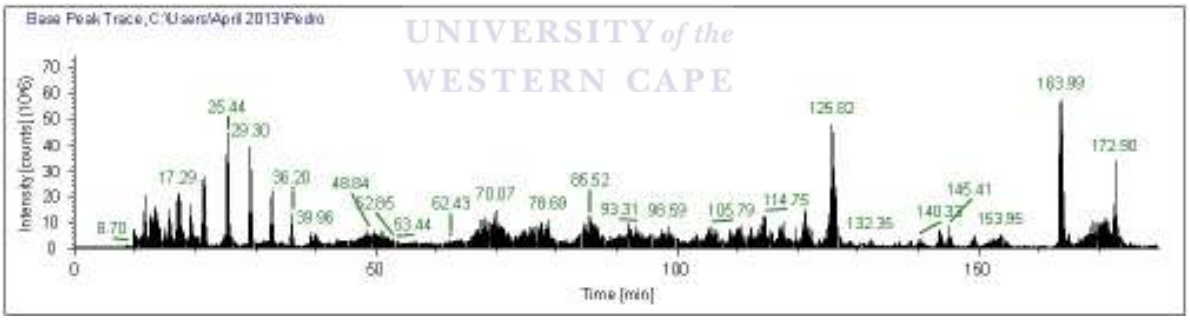


Fig. 19: Chromatogram for azole-resistant *C. albicans* isolate C73.

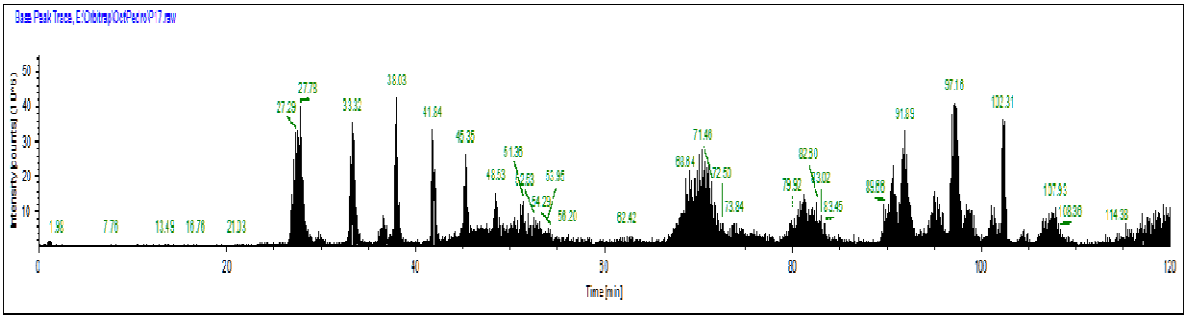


Fig. 20: Chromatogram for azole-intermediate *C. glabrata* isolate SA92.

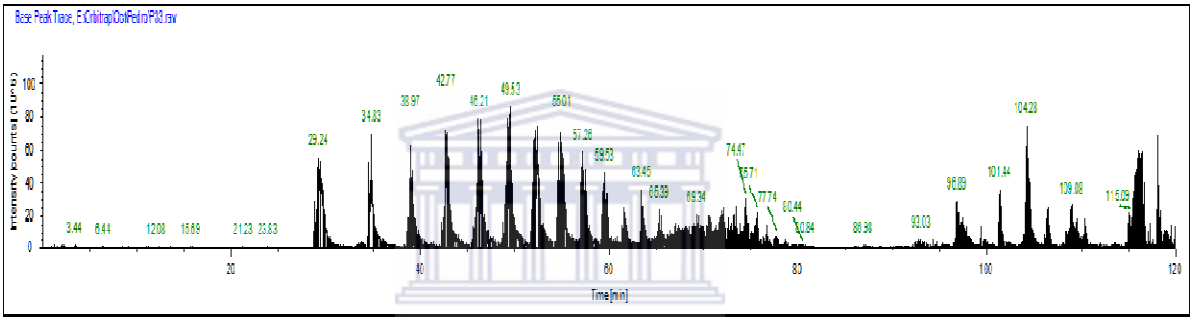


Fig. 21: Chromatogram for fluconazole-resistant *C. krusei* isolate C144.

Chapter 4: Discussion and Conclusion

Oral candidiasis is a common opportunistic infection in the course of HIV disease progression. Changes in the clinical severity of oral candidiasis and type of *Candida* species profile may be a reflection of immunological changes in patients. Although *C. albicans* is the most commonly reported species in HIV literature, there appears to be a gradual trend globally toward change in the *Candida* species prevalence with non-albicans *Candida* being more frequently isolated from patients with HIV/AIDS along with an associated intrinsic or acquired antifungal resistance becoming apparent in several *Candida* species. The emergence of resistant non-albicans species might pose a problem in treatment strategies and requires further investigation. Studies related to changes in the distribution of *Candida* species during the progression of HIV infection and the development of resistance to antimycotics are rare in Africa.

The aim of this study was therefore to isolate and characterize *Candida* species from two different HIV-positive African populations namely South Africa and Cameroon from which very high rates of HIV infection have been reported. This is important, as no similar study has previously been done. Also, to our knowledge, no study had combined the use of SDS-PAGE and HPLC-MS for a broad analysis of clinical yeast isolates from HIV-positive patients in South Africa and Cameroon. *Candida* species, as opportunistic organisms, are a cause of concern in HIV-positive patients. Of even greater concern is the emergence of antifungal drug resistance. Fluconazole is very often the only oral antifungal available to HIV-positive patients in African public hospitals, and the emerging resistance renders it ineffective against *Candida* infections.

The characterization of *Candida* species employed the use of chromogenic/selective media for species differentiation and drug susceptibility testing. Other factors, such as patient gender, immune status and age were also taken into account.

4.1 Species identification

Different microbiological media have been developed in recent years to rapidly identify *Candida* species. The use of different media in this study for species isolation and identification produced clear, reproducible results in all species. The only exception was the identification of *Candida parapsilopsis*/*lusitaneae*/*kefyr*, for which there was no concrete differentiation agar based on chromogenic substrates.

It is important to note that no individual solid culture media can be relied upon for *Candida* species differentiation. The results obtained from the different chromogenic media had to be compared to correctly identify the individual species, as in some cases two or three different species presented with the same colour in certain media. In the case of *C. albicans* and *C. dubliniensis* differentiation, although a slight colour difference could be seen in both chromogenic agars, no medium was able to give clear results. Therefore, suspected samples (which demonstrated a darker tonality) were subsequently inoculated and grown on tomato juice agar and tobacco agar and incubated at 45˚C followed by microscopy. The use of these techniques greatly aided in the correct differentiation of these two species.

Species differentiation in chromogenic and differential culture media was a crucial step for further microbiological and proteomic analysis of the isolates. Correct identification of clinical specimens cannot be done exclusively using more detailed techniques such as HPLC-MS, for example, which is extremely sensitive to protein expression and is only able to accurately identify cultured type strains in the computer's database to species level if a database is present. The use of chromogenic and differential agars in the microbiological identification of the clinical species eliminated this problem.

Chromogenic culture media also allowed for the identification and isolation of multiple species present in swab samples.

4.2 *Candida* species prevalence in South African and Cameroonian patients

One hundred and twenty-eight (128) of the swab samples collected from South African patients yielded *Candida* growth, with 127 *Candida* isolates from the swabs collected in the Cameroon. The results of the first inoculation of the swabs into Sabouraud's agar showed that most specimens showed scanty to light growth after incubation at 37˚C for 24 hours, with no significant differences observed for the different *Candida* species. *Candida* species were identified and differentiated using chromogenic agar. Confirmatory tests were done to differentiate between *C. albicans* and *C. dubliniensis*.

The prevalence of *Candida* in the oral mucosa of healthy individuals is approximately 40-60% (Odds, 1987). However, a study done on healthy and HIV-positive patients (Sánchez-Vargas *et al*, 2005) showed that the prevalence of *Candida* in HIV infection was higher than in healthy individuals. The overall *Candida* prevalence in the present study was found to be similar to that of healthy individuals, although in the higher percentage values, especially in the case of the South African group.

The prevalence of *Candida albicans* in South African HIV-positive patients was found to be much higher than that of other *Candida* species with *C. glabrata* more frequently isolated than *C. dubliniensis*. Another South African study (Blignaut, 2007) also demonstrated *C. albicans* to be the most predominant species, followed by *C. dubliniensis*. Further studies from this region would confirm the shift in species prevalence of *C. glabrata*.

Species such as *C. krusei, C. parapsilopsis* and *C. tropicalis*, which have been described as invasive *Candida* species in other studies (Arredondo-García *et al*, 2009, Chen *et al*, 2009, Prakoeswa *et al*, 2009) were not observed in the South African population. However, these three species were observed in the Cameroonian samples where the most prevalent species was also found to be *C. albicans*. Other species isolated from the Cameroonian samples were a large number of *C. glabrata* and two other species characterized as *C. kefyr*, *C. lusitaneae* or *C. parapsilopsis*. An association between diet and *Candida* carriage, especially non-albicans species, has been previously documented (Coleman *et al*, 1995, Jabra-Rizk *et al*, 2001, Kadir, Uygun and Akyüz, 2005, Kwamin *et al*, 2013). We can speculate that this may

well have been the case with the Cameroonian patients from whom non-albicans species were isolated.

The frequency of *C. albicans* as the predominant species, followed by *C. glabrata*, has been reported by Mousavi *et al* (2012), Badiee *et al* (2010), Fidel, Vazquez and Sobel (1999) and Hamza *et al* (2008). However, the distribution of non-albicans species differed in each of these studies with *C. tropicalis*, *C. parapsilopsis* and *C. krusei* reported by Hamza *et al* (2008) in Tanzania and *C. dubliniensis*, *C. krusei*, *C. kefyr*, *C. parapsilopsis* and *C. tropicalis* reported from Iran (Badiee *et al*, 2010, Mousavi *et al*, 2012). The distribution of *Candida* species appears to vary according to geographic region and sometimes within the same region (Mares *et al*, 2008, Basma *et al*, 2009).

A study from Venezuela reported distribution in the order of *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilopsis* and *C. krusei* (Magaldi *et al*, 2001). *Candida albicans*, followed by *C. tropicalis*, has also been reported in a study from Cameroon with *C. dubliniensis* reported less frequently with an absence of *C. glabrata* (Njunda *et al*, 2013). A study from Ghana reported a distribution of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilopsis*, C. sake, *C. dubliniensis*, *C. globosa*, *C. formata*, *C. glabrata* and *C. lusitaneae* (Kwamin *et al*, 2013).

A study done in Indonesia (Prakoeswa *et al*, 2009) found that only 35.29% of *Candida* infections in HIV-positive patients were due to *C. albicans*, with the remainder (64.71%) being *C. tropicalis*, *C. dubliniensis*, *C. glabrata* and *C. guilliermondi* collectively.

Only one case of *C. dubliniensis* was seen in the Cameroonian group in the present study. *C. dubliniensis* has previously been reported in the Cameroon but also in very low numbers (Njunda 2013). This species has been associated with HIV/AIDS in Caucasian and well-resourced populations (Blignaut *et al*, 2003). In the South African group, which represents a more genetically mixed population, *C. dubliniensis* numbers were slightly higher confirming this species as a contributor to *Candida* carriage in HIV-positive South African patients.

C. dubliniensis was first identified in Africa in HIV-positive patients in 2001 (Fisher, Basson and van Zyl, 2001) and is considered to have a colonial morphology phenotypically similar to *C. albicans* with a proposed genotypical differentiation using molecular techniques only. This study has shown a clear differentiation between *C. albicans* and *C. dubliniensis* by growth on

tomato juice agar and tobacco agar, a method which may be employed in a resource-poor setting where the reagents required for molecular methods may not be affordable.

4.3 Antifungal susceptibility testing

The comparison of fluconazole drug susceptibility tests used on the type strains showed similar results for YNBG agar and the TREK system, which further confirms the reliability of these two techniques. The other media used (Sabouraud and GMB agars) only showed similar results as the first two in the case of the *C. krusei* and *C. glabrata* type strains and individually in the case of the *C. albicans* (Sabouraud) and *C. dubliniensis* (GMB) NCPF type strains.

Seventy-seven percent (77%) of South African *Candida* isolates were found to express resistance to fluconazole when grown in Sabouraud agar, while 18% of isolates were classified as intermediate, or dose-dependent. The number of resistant (78%) *C. albicans* strains in this medium was much higher than the intermediate (19.8%) and susceptible ones (1.9%), with 60% of *C. dubliniensis* and 83% of *C. glabrata* isolates showing resistance to fluconazole on Sabouraud agar.

Although Sabouraud agar is the ideal medium to grow *Candida* isolates, it performed poorly in antimicrobial susceptibility testing. This has been previously reported (May, King and Warren, 1997). The GMB quality control organism susceptibility test showed values outside the range specified by the CLSI. The clinical strain results and South African population results for fluconazole susceptibility using Sabouraud and GMB agars were presented for comparative reasons, but because of discrepancies YNBG agar was preferred, as this agar has shown the best performance when compared with other media due to its increased sensitivity in the susceptibility testing of *Candida* species (May, King and Warren, 1997, Yücesoy, Guldas and Yuluq, 2001). Although this medium was more difficult to prepare, the dilution protocols were more straightforward than in the case of GMB, leading to faster processing of samples, reduced use of consumables and less chance of dilution errors.

South African antimicrobial susceptibility results in YNBG showed very high resistance levels in *C. glabrata* (66.7%), followed by *C. albicans* (56.6%) and *C. dubliniensis* (10%).

The intermediate (dose-dependent) result for *C. glabrata* was 8.3%. In theory, patients harbouring these species could be treated with fluconazole if higher doses are used and/or if treatment is extended.

In the Cameroonian population, the YNBG results showed the highest resistance levels in *C. krusei* and *C. tropicalis* (100%), followed by *C. glabrata* (75%), *C. kefyr/parapsilopsis/lusitaneae* (50%) and *C. albicans* (42.4%). The only *C. dubliniensis* isolate collected from this population showed intermediate (dose-dependent) resistance. Other intermediate (dose-dependent) results were seen in *C. kefyr/parapsilopsis/lusitaneae* (50%), *C. glabrata* (29.2%) and *C. albicans* (9.8%).

The present study showed that resistance to fluconazole is present in *Candida* species isolated from clinical samples. This is a cause for concern, as this is one of the few and in some cases the only oral antifungal drug available at public hospitals on the continent. The high levels of resistance seen in *Candida* species could contribute to an increase in patient morbidity and mortality. Different studies consider *C. dubliniensis* to be more resistant to fluconazole than *C. albicans* (Fisher, Basson and van Zyl, 2001, Powderly, Mayer and Perfect, 1999). However, a high difference in resistance levels was not seen in this study. Studies in other parts of the world have shown similar azole-resistant *Candida* species in HIV-positive patients (Fidel, Vazquez and Sobel, 1999, Hamza *et al*, 2008, Badiee *et al*, 2010).

The high fluconazole resistance levels seen in the present study are disturbing, but can be partly blamed on the prolonged prescription of this drug in clinical candidiasis cases. *Candida* resistance to antifungal drugs in Cameroon, where the sale of medicines is not regulated, can also be blamed on the unregulated sale of medications, which leads to the distribution of antifungal drugs by untrained persons and/or self-medication by the patients.

Candida albicans isolated from patients who had not previously been treated with fluconazole showed resistance to fluconazole (9.8%) and itraconazole (4.9%) and this resistance was found to increase to 44.7% and 44.15% after treatment with these drugs (Magaldi *et al*, 2001).

Prophylactic administration of fluconazole in patients with low CD4 counts may result in clinical treatment failure which is significantly correlated with reduced susceptibility to fluconazole and other azoles (Müller *et al*, 2000).

Although *C. albicans* was the most prevalent species in the present study, drug resistance in both populations was much more prominent in non-albicans species. The representation of five non-albicans *Candida* species isolated from the Cameroon could be the cause of the higher fluconazole intermediate/resistant results in this population, due to non-albicans species being inherently more resistant than *C. albicans*.

The co-existence of two *Candida* species (*C. albicans* and *C. glabrata*) in patients SA36 and SA203, with both species demonstrating the same resistance patterns to fluconazole, could signal the exchange of genetic information related to drug resistance and predisposed the host to a higher degree of fungal colonization and infection. It is possible that these two species formed an important part of a microbial biofilm in the oral mucosa of these patients, which might thus have resulted in increased pathogenicity.

Cross-resistance to azoles has been reported for *Candida* species (Magaldi *et al*, 2001) with all colonies from the same swabs developing the same resistance patterns. Horizontal transmission where the same resistance patterns in genetically identical species isolated from partners was observed, even when one partner had never received previous treatment (Dromer *et al*, 1997). Cross-resistance to a range of clinically used antifungals may also be attributed to the antifungal agents used (Sojakova *et al*, 2004).

Non-albicans *Candida* species such as *C. glabrata* have been implicated as the causative agents of 46% of systemic *Candida* infections (Wingard, 1995). Since very high levels of *C. glabrata* isolates from both populations were found to be either resistant or intermediate to fluconazole in immunocompromised patients, this poses a cause for concern, as these species are able to cause a very high level of systemic infections even though they occur in much lower numbers than *C. albicans*. *Candida glabrata* and *C. krusei* have been reported to be less susceptible/intrinsically resistant to fluconazole (Bodey *et al*, 2002, Badiee *et al*, 2010) and this occurrence in HIV-positive patients is increasing.

There is a possibility that different susceptibility results would have been obtained in some of the clinical samples tested if a higher fluconazole dosage had been used, even though it has been shown that 25 μg fluconazole impregnated felt disks give similar results to 50 μg felt disks (Kustimur *et al*, 2003). In the same line of thought, it is possible and probable that, in some cases, clinical *Candida* strains may demonstrate a higher susceptibility to fluconazole if the antifungal therapeutic dose is increased. For the purpose of this study, however, these fluconazole dose-dependent strains were still considered resistant, as they showed particular molecular patterns related to fluconazole resistance.

Recently developed echinocandins and third generation azole compounds have shown a better efficacy in combating certain *Candida* infections (Pemán and Almirante, 2008) and could be considered as second-line drugs to deal with fluconazole-resistant fungal infections.

The results from the TREK Sensititre system showed a 45.3% overall fluconazole resistance (including intermediate drug resistance isolates) of *Candida* species in HIV-infected patients in the Western Cape of South Africa. In Cameroon the value was 45.2%. Although the values were very similar in these two regions, the numbers of resistant isolates were much higher than those previously documented in other studies.

The TREK Sensititre system proved to be a very useful tool in determining the resistance patterns of all clinical isolates against various antifungal drugs. This CLSI-approved method substituted the GMB agar diffusion technique (the standard CLSI method) in this study, as it provided repeatable and consistent results. The fact that it includes newer generation drugs, including the echinocandins, was also very valuable.

In the South African population, both *C. albicans* and *C. glabrata* showed low resistance levels to echinocandin drugs. Very high azole resistance levels were noted in *C. albicans* and high azole resistance in the case of *C. glabrata* isolates. *C. dubliniensis* isolates responded well to azole drugs, with the exception of one isolate.

Four point seven percent (4.7%) of *C. albicans* and 8.3% of *C. glabrata* species were found to be resistant to 5-flucytosine. All *C. dubliniensis* isolates were susceptible to this drug.

An extreme drug resistance *Candida* isolate was identified using the TREK system. Isolate SA82, a *Candida dubliniensis* strain, was found to be resistant to eight drugs on the TREK panel. The only exception was 5-flucytosine, which inhibited growth of this isolate in concentrations above 2µg/ml. This is, to our knowledge, the first time that such high resistance levels have been documented. This could signal the emergence of multiple drug resistant *Candida* species.

In the Cameroonian population, *C. albicans*, *C. tropicalis* and *C. krusei* strains showed no resistance to echinocandin drugs, while *C. glabrata* strains showed high resistance levels against micafungin. In the case of the azole drugs, the reverse was seen: *C. albicans* strains were more resistant to azoles (greater than or equal to 50% resistance in all azoles for *C. albicans*), with *C. glabrata* responding better to this class of drugs. The *C. dubliniensis* strain and two species identified as *C. parapsilopsis/lusitaneae/kefyr* showed no resistance to azole drugs, with *C. tropicalis* strains showing susceptibility to both fluconazole and voriconazole. Most species tested with 5-flucytosine showed very promising results. The exceptions were *C. krusei*, with only one isolate (33.3% of total) showing intermediate resistance, and *C. albicans*, where 5.4% of isolates were totally resistant.

Resistance to amphotericin B was seen with respect to all *Candida* species, with non-albicans species demonstrating especially high resistance levels, particularly *C. glabrata* obtained from the South African population.

There is a possibility that the repeated exposure of *Candida* species to antifungal drugs, particularly in the Cameroon where more non-albicans species are present, might have led to changes in the distribution of *Candida* species. Species-specific azole resistance has been documented in Brazil (Colombo *et al*, 2002) and resistance to a specific antifungal drug has been shown to result in cross-resistance to other drugs of the same class (Müller *et al*, 2000).

Statistical tests done between *Candida* species and resistance patterns showed moderate to strong associations between the former, with the exception of 5-flucytosine. These results further demonstrate that the resistance patterns of specific antifungal drugs are related to the specific *Candida* species they target.

4.4 The role of gender

In South Africa, female patients were found to have a higher incidence of *Candida* when compared to their male counterparts, as 62% of females tested positive for *Candida*, as opposed to 55% of males. In Cameroon, however, these values were 48.4% and 48.9% respectively. As in the South African group in the present study, candidiasis was reported to be higher in females (76.8%) than males (23.2%) in the study by Njunda *et al* (2013), which associated CD4+ T cell counts <200 cells/µl with frequency of candidiasis.

No significant association was seen to exist between patient's gender and *C. albicans* and *C. dubliniensis* colonization. South African female patients had a much higher prevalence of *C. glabrata* in the oral mucosa when compared with the male patients, with double the percentage. In the Cameroonian patients, the reverse was observed, as the percentage of males with *C. glabrata* present in their oral mucosa was higher than that of females, with females showing a greater diversity of species.

The fact that *Candida albicans* was the only species isolated from the oral mucosa of patients who were either pregnant or had recently given birth could indicate a link between pregnancy and *Candida* species colonization. It would be interesting to increase the sample size and do a comparison of the *Candida* present in other body sites of these patients (Nowakowska, Kurnatowska and Wilkzynsky, 2001) especially since *Candida* species have been implicated in pre-term delivery (Hay and Czeizel, 2007). *Candida krusei* (an intrinsically resistant species) and *C. parapsilopsis/lusitaneae/kefyr* were only seen in the female population, with fewer representatives of other *Candida* species being present in males. These results support the literature on the estrogen target of *Candida* species, which predisposes females to a higher burden of *Candida* colonization.

4.5 Effect of age and ethnicity

A higher percentage of *Candida* species was isolated from South African patients ranging between 21 to 30 and 31 to 40 year old age groups. This seemed to shift to older age groups in the Cameroonian population. Similar results within the same age group were reported in a

recent study of candidiasis in HIV-positive patients in Cameroon (Njunda *et al*, 2013) and an earlier study in Iran (Mousavi *et al*, 2012). A high percentage (75%) of *C. glabrata* isolates was obtained from South African patients in the 31 to 40 year old age group (Table 19). This species was more uniformly distributed in the Cameroonian population.

No association was seen between *Candida* species and race. The majority of the patients who participated in the study were black, with few representatives of other racial groups. A study on a larger sample size done on different geographical areas would be needed to ascertain whether species prevalence can be related to race.

4.6 Effect of HIV stage

Thirty-four percent (34%) of South African patients and 25.6% of Cameroonian patients who tested positive for *Candida* carriage in their oral mucosa had symptoms of late-stage immunosuppression and opportunistic infections (AIDS) which included tuberculosis, lymph node enlargement and to a lesser degree systemic and localized fungal infections elsewhere in the body. Thirty-nine percent (39%) of South African patients with *C. albicans* and 25% of South African patients with *C. glabrata* were severely immunosuppressed (AIDS patients). However, none of the patients from South Africa or Cameroon with *C. dubliniensis* carriage were found to have late stage immunosuppression. In Cameroon the percentages of severely immunocompromised patients were more uniform for *C. albicans* (26.1%), *C. glabrata* (29.2%), *C. krusei* (25%) and *C. tropicalis* (25%), with the few *C. dubliniensis* and *C. kefyr*/*parapsilopsis*/*lusitaneae* isolates only being present in HIV-positive patients with no symptoms of late-stage disease.

4.7 Effect of ARV therapy

Overall, 52.8% of South African patients on ARV medication had *Candida* in their oral mucosa. Thirty-three percent (33%) of South African patients presenting with *Candida* in the oral mucosa were not on antiretroviral therapy at the time of sample collection. The highest number of *C. albicans* isolates was collected from this group (38%). Most patients on ARV therapy were either on d4T/Efavirenz/3TC or d4T/Nevirapine/3TC triple therapy, which

belong to the first antiretroviral regimen. A reduced number of patients were on Zidovudine (AZT), which is used during pregnancy or KLT/DDL/TDF therapies, which at the time was being introduced in public hospitals.

Of the Cameroonian patients 46.4% on ARV medication had *Candida* in their oral mucosa. In this group, only 3.9% of patients with *Candida* in their oral mucosa were not on antiretroviral therapy at the time of sample collection. In this group, patients on the AZT/NVP/3TC cocktail had the highest *C. albicans* (62%) and *C. glabrata* (58.3%) colonization, probably because this was the most commonly dispensed ARV drug cocktail at the site. In Cameroon, it was noted that AZT was part of most drug combinations, while d4T was the drug of choice for the South African group.

A high number of both *C. dubliniensis* (40%) and *C. glabrata* (42%) colonization was seen in South African patients taking the d4T/Nevirapine/3TC ARV cocktail. A future study with a higher sample number of these species would confirm if this drug combination renders the patient more susceptible to these non-albicans species.

It can also be postulated that, because this is an ARV combination that has been in use for a longer period of time in this part of the continent, HIV might have acquired resistance to this cocktail over the years. This has been suggested by another study from Ghana (Kwamin *et al*, 2013). Much lower carriage values were found with the more recent ARV combinations such as AZT/DDL/Kaletra, AZT/3TC/Kaletra and TDF/Efavirenz/3TC in the present study. No similar associations were seen in the Cameroonian group.

The number of *C. albicans* isolates steadily declined in South African patients on prolonged ARV therapy. This makes sense, as the lower viral count caused by the antiretroviral therapy over a longer period would confer better immunity. These immune responses would, in turn, reduce the burden of opportunistic infections and prevent their spread to other anatomical sites. However, the reverse is true for the Cameroonian population, as most *Candida* isolates were obtained from patients on antiretroviral drugs for longer than 3 years.

The number of *C. dubliniensis* South African isolates increased in patients who were on ARV therapy between 12 and 23 months. This number declined after more than 24 months of therapy, while *C. glabrata* isolates were increased in patients who were on ARV therapy for

less than 12 months. A higher prevalence of candidiasis among patients who were not on ARV treatment has been previously reported (Njunda *et al*, 2013).

The shift from *C. albicans* to the more drug-resistant non-albicans species seen after continued ART is therefore a cause for concern. A future study employing a larger sample size and focusing on ARV combinations and CD4 counts, for example, would provide more information on the colonization behaviour of these species on immunocompromised patients.

4.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Limited studies have examined the protein profile in fluconazole-resistant *Candida* species. The generation of protein profiles by SDS-PAGE has been accepted as an effective typing method for the characterization of *Candida* (Rodrigues *et al*, 2004).

The Fluka SDS gel preparation kit allowed for quicker, easier, safer and less expensive preparation of polyacrylamide gels, when compared to conventional SDS-PAGE reagents. The Hoefer system allowed for the simultaneous running of two 8X8cm gels in approximately 1 hour and proved to be a simple and effective SDS-PAGE system for this type of application, while use of the gel drying kit allowed for a quick drying process and better gel consistency for handling and storage of cast gels.

The protein assay kit used in this study allowed for protein quantification in all samples that were above the desired concentration, as these were simply diluted in buffer solution. However, samples with very low cell densities analyzed using SDS-PAGE resulted in blank or very faint protein lanes. It was noted that some non-albicans species took longer to grow in solid and liquid media and that after surface protein isolation and Bradford protein quantification the protein bands in some cases were very faint or could not be seen. In these cases the process was repeated with a longer incubation time (48 hours) and a new gel was run. It must be noted that even when a sample had a certain cell density, after dilution with glycerol and running buffer the sample would be much more diluted. This did not occur in the case of *C. albicans* species, as in most cases *C. albicans* samples with lower concentrations than other *Candida* species would appear in the SDS-PAGE gel results as very

clear protein bands, while the non-albicans species often appeared as blank lanes or as very faint protein bands, even though protein concentration was standardized.

Polyacrylamide gel electrophoresis analysis showed well expressed protein bands in both susceptible and resistant *C. albicans* samples. The most defined one was present at approximately 46kDa, followed by four protein bands between 30 and 36kDa. These were very similar to the protein bands found in the *C. albicans* NCPF control.

A 24kDa protein related to the tropiase acid proteinase was found to be expressed in *C. albicans* isolates SA90, SA94 and SA109 (all resistant to fluconazole).

In the case of *C. dubliniensis* a protein band in the region of 49 kDa was found in the *C. dubliniensis* NCPF control (a drug-susceptible type strain), as well as a band in the region of 36 kDa, consistent with Grp2p reductase, followed by 3 protein bands ranging from 23 kDa, consistent with acid proteinase, to 27 kDa. It was interesting to note that isolate fraction 7 (which was susceptible to all drugs on the TREK panel) had very similar protein patterns to those of the *C. dubliniensis* NCPF type strain. A ~44 kDa protein consistent with exoglucanase was found to be expressed in isolate fractions SA82, an extreme drug resistant strain, and SA136, a drug-susceptible strain.

In the case of *C. glabrata*, a protein band in the region of 37 kDa was found in isolate fraction SA144, a fluconazole-susceptible sample, consistent with Grp2p reductase. A 24 kDa protein consistent with the tropiase acid proteinase was found to be expressed in isolate fraction SA92 (a fluconazole-intermediate (dose-dependant) isolate). A 50 kDa protein band was also present in isolate fraction SA92 and in fraction SA107 (a fluconazole susceptible isolate).

These results show that clinical *Candida* strains seem to express drug-resistance protein patterns in resistant *C. albicans* isolates. However, the expression of proteins previously described as related to fluconazole resistance seemed to be present in both resistant and susceptible non-albicans species.

It must be noted that the proteins described above, previously found to be related to fluconazole resistance, often expressed resistance patterns to other azoles or different drug

classes. Isolate SA144, a fluconazole –susceptible isolate which expressed a 37 kDa protein, was resistant to 5-flucytosine, while isolate SA107, also susceptible to fluconazole, demonstrated resistance to amphotericin B. The 24 kDa protein found in the fluconazole-intermediate isolate SA92 was resistant to amphotericin B, itraconazole and posaconazole. This molecular weight is consistent with an acid proteinase, an enzyme found in *Candida* which is known to destroy important host immune proteins, destroy host cells and induce changes in the organism's pathogenicity (Staib, 1969).

Previous studies have exposed *Candida* species to fluconazole, allowing for the overexpression of resistance proteins before the cellular components were run on the acrylamide gels. This study suggests that while the expression of probable fluconazole-resistance proteins in fluconazole-susceptible strains could be due to a similar response mechanism to other azole drugs (such as in the case of SA92), the appearance of resistance proteins in isolates resistant to other groups of antifungals (with different modes of action) needs to be further investigated.

A more sensitive stain, such as a silver stain, could have been used in the SDS-PAGE protein analysis. However, the use of such a stain would have resulted in the appearance of many protein bands in the gels, which would have made it difficult to identify bands of interest. Therefore, the stain used in this study remains the best option in reading protein bands, especially as cell fractions with a relatively limited array of individual proteins were used.
This study analysed the South African isolates' cell fractions by SDS-PAGE, looking for the expression of proteins of interest in relation to drug resistance. This provided a different perspective into the *Candida* proteome *in vivo*, as *Candida* SDS-PAGE proteomics studies usually rely on the analysis of type strains or few clinical strains, often with prior exposure to fluconazole and then analysed by gel electrophoresis.

4.9 High Performance Liquid Chromatography – Mass Spectrometry

Fluconazole resistance in *C. albicans* is thought to occur by reduced fluconazole accumulation (Sanglard *et al*, 1995, Venkasteswarlu *et al*, 1995) as a result of reduced cellular uptake or due to increased efflux. Another mechanism of resistance could be via an

altered drug target, namely 14α-sterol demethylase, encoded by ERG16 (Casalinuovo *et al*, 2004).

With the elucidation of the full proteome of various organisms, the use of modern techniques such as high performance liquid chromatography and updated online databases, it is now possible to identify proteins accurately and with extreme sensitivity. Overall, this platform was by far superior to SDS-PAGE, as the results were very specific and detailed and the proteins identified by mass spectrometry could easily be searched using a protein online database.

The sample preparation used for the SDS-PAGE and HPLC-MS analysis allowed for the selection of proteins present in the *Candida* cell wall, although some proteins that do not form part of the cell wall were identified by liquid chromatography-mass spectrometry. This seems to show that the cell wall fraction isolation technique used in this study (originally developed for the less sensitive gel electrophoresis analysis), which involves lysing the cells, allows for some intracellular components to remain in the final cell fraction, and these can still be read by the very sensitive mass spectrometer. This did not interfere with the results, as the description and origin of the individual proteins identified by HPLC-MS could be determined when matching the results to the UniProt database, allowing for these readings to be excluded.

Up to 206 individual proteins were identified per cell fraction analysis using the HPLC FASP method. This number was not uniform across different cell fractions: *C. parapsilopsis/lusitaneae/kefyr* yielded fewer proteins than other species, but proteins of interest were still detected.

Different proteins, some of which could be involved in mechanisms of drug resistance, were identified using HPLC-MS. These included membrane transporter proteins; proteins described as being related to cellular response to heat, osmotic stress, oxidative stress, starvation, pH changes and toxic substances; molecular chaperones (heat shock proteins) that regulate target proteins; proteins that cause allergic reactions in humans and proteins that elicit immune responses in patients with malignant haematological disorders. However, due to the nature of this study, only proteins that were confirmed as being responsible for/related to drug resistance were included. Cell membrane proteins that affected the organism's

pathogenicity and virulence were also considered to be of interest and were included in the results.

Due to the limited amount of proteins identified by SDS-PAGE and the fact that only 40 of the *Candida* cell fractions were analysed by HPLC-MS, no meaningful statistical analyses could be performed on these sections.

Different colonization tactics used by oral *Candida* species could be elucidated using HPLC-MS: *C. albicans* drug-susceptible isolates seemed to bind to the oral mucosa by expressing proteins that bind to HTN3/histatin-5 found in saliva, affecting the fungicidal activity of these human antimicrobial proteins. Salivary histatins have a potent antifungal activity and the mediation of fungicidal activity by this mechanism was first described in 2003 (Li *et al*). The presence of histatin-binding proteins in all *Candida* isolates by HPLC-MS demonstrated that this is a crucial mechanism in oral colonization across all *Candida* species. Other colonization mechanisms seen in these isolates included the expression of oxidoreductases that bind strongly to estrogen (which might explain the higher female *Candida* colonization seen in this study) and the expression of pH responsive proteins, which affect the pathogenicity of these *Candida* isolates. Changes in the expression of pH-regulated genes have been shown to induce changes in the virulence of *Candida* species (Mühlschlegel and Fonzi, 1997).

The expression of a multidrug resistance transporter protein CDR1 of unknown physiological function found in four isolates in this group, which confers resistance to cycloheximide, chloramphenicol and miconazole (Prasad *et al*, 1995), was a finding that has previously been documented in the development of *Candida* drug resistance. Due to this, two of these proteins, which had a protein sequence coverage below 5%, were included in the results, as the presence of this protein showed that the same mechanism was visibly present in both populations.

It has been demonstrated that CDR1 is present in greater amounts than the CDR2 multidrug efflux transporter protein in *Candida albicans* (thereby explaining the 4-fold presence of this protein when compared to CDR2) and that it may play a role in fluconazole drug resistance (Holmes *et al*, 2008). It has been shown that when this protein is overexpressed it leads to resistance to different chemicals as well as to azole antifungal drugs (Niimi *et al*, 2004). It

may be speculated that in the presence of specific physiological stimuli, the overexpression of CDR1 might confer these isolates with resistance against certain antifungal drugs.

Salivary HTN3/histatin-5 –binding proteins, estrogen-binding oxidoreductases and pH responsive proteins were also seen in fluconazole-resistant *C. albicans* isolates. In this group a drug-resistance related protein was identified in a Cameroonian cell fraction: multidrug efflux transporter protein CDR2, which is responsible for resistance to azole antifungal agents, to other antifungals (terbinafine, amorolfine) and to a variety of metabolic inhibitors (Sanglard *et al*, 1997), were found on isolate C73. Although this multidrug efflux protein has also been implicated in echinocandin resistance (Schuetzer-Muehlbauer *et al*, 2003), this was not seen in this isolate, which was susceptible to all drug classes except the azoles. Salivary HTN3/histatin-5 –binding proteins were also identified in drug-susceptible and -resistant non-albicans species.

The comparison of SDS-PAGE gels and HPLC-MS analysis yielded some interesting results: isolate SA82, a multi-drug resistant *C. dubliniensis* strain, presented with five different proteins related to its pathogenicity and drug resistance: three heat shock proteins that bind to HTN3/histatin, a pH-responsive protein and a protein that confers resistance to formaldehyde were found in this isolate's cell fraction. A protein of approximately 40kDa seen on the SDS-PAGE gel of this and other isolates was consistent with S-(hydroxymethyl) glutathione dehydrogenase, the protein found by HPLC that confers resistance to formaldehyde. The same 40kDa identified by HPLC-MS that confers resistance to formaldehyde could have been described in 2006 in a study employing SDS-PAGE analysis of *Candida* species (Kustos *et al*), as an unknown protein expressed in the presence of fluconazole. This protein was found across different *Candida* species, both susceptible and resistant to fluconazole.

A 169kDa drug efflux transporter protein has been reported to be expressed by *C. glabrata* in the presence of fluconazole (Niimi *et al*, 2002). This was not the case in the present study. However, proteins with very similar molecular weights (168.9kDa and 169.8kDa) and with the same function (multidrug transporter proteins), which confer resistance to cycloheximide and to azoles, other drugs and metabolic inhibitors were seen in three *C. albicans* fluconazole-resistant isolates.

These results further demonstrate the specificity of HPLC-MS results in elucidating the drug resistance mechanisms of *Candida* species present in these HIV-infected individuals. It was noted, however, that the use of SDS-PAGE allowed for the identification of proteins of low molecular weight that were not seen in the HPLC-MS analysis. Gel electrophoresis analysis allowed for the identification of proteins between 23 kDa and 36kDa that are suspected of being related to drug resistance. The proteins of interest seen by liquid chromatography had a molecular weight of 40kDa or more.

4.10. Summary and Conclusion

Although a single species of *Candida* was isolated from the majority of samples cultured, two patients in this group were colonized by both *C. albicans* and *C. glabrata*. This may signal the existence of a symbiotic relationship between these two species, within a *Candida* biofilm in the oral mucosa. The increased pathogenicity of *C. glabrata* when forming part of a biofilm has been previously documented (Sereviratne, 2010).

The prevalence and drug susceptibility of *Candida* species in HIV-positive patients varied across these two regions of the African continent (South Africa and Cameroon). Therefore, regional surveillance is recommended in different regions of the continent, as this knowledge would ultimately lead to better patient care.

In the South African group, a severely immunocompromised patient was seldomly seen. This could be attributed to better access to healthcare facilities and modern medications as well as the fact that these patients are tracked by healthcare providers.

In HIV-positive patients, *C. albicans* infections have the best prognosis compared to infections caused by non-albicans species. The resistance to fluconazole of these non-albicans species plays a very important role in the management of HIV-positive patients. It can therefore be speculated that if the patients had not been receiving treatment (which results in a CD4+ increase), a higher number of non-albicans invasive (and potentially more resistant) species could have been seen in this study's results. However, studies looking at the interaction between fluconazole and tipranavir/ritonavir combination (Vourvahis and Kashuba, 2007) and etravirine (Kakuda, Schöller-Gyüre and Hoetelmans, 2011) and the

increased plasma half-life of zidovudine when administered with fluconazole (Sahai *et al*, 1994) have indicated that certain ARV drugs may interact with azole compounds, rendering them less effective (Boehringer Ingelheim Pharmaceuticals, 2008). Similar studies involving ARV regimes used in Africa interacting with fluconazole would contribute greatly to our understanding of the emerging drug resistance in Africa.

The *Candida* isolates used in this study were collected either at baseline or while the patient was already on ARV treatment. It is estimated that, by December 2008, only 44% of patients in Sub-Saharan Africa in need of ARV therapy were taking antiretroviral drugs (WHO, 2013). This means that the remaining 66% of immunocompromised patients would be more susceptible to HIV opportunistic infections, including *Candida*.

It was noted that a much higher number of females attended the ARV clinics when compared to their male counterparts. This could be because of the higher predisposition that females have to HIV when compared to males, and/or the presence of HIV/Sexually transmitted infections screening programs available to pregnant women in healthcare facilities, which would result in more women finding out about their seropositivity. Another important factor to consider is the difference in the ARV regimen compliance of males and females, as social and cultural factors present in different geographical regions may influence the decision of patients to comply with ARV therapy.

The use of traditional disk diffusion antifungal drug susceptibility testing using YNBG agar produced very similar results to the TREK Sensititre CLSI-approved modern microdilution system, and is therefore the susceptibility testing medium of choice. Both these techniques can be used in resource-limited laboratories, with the TREK system being the best system due to its simplicity and ability to test organisms against different drugs simultaneously.

Programmes on species prevalence and antifungal use and resistance pattern surveillance have been successfully developed and introduced in Europe, Asia-Pacific, Latin America and North America (Adriaenssens *et al*, 2010, Cuenca-Estrella *et al*, 2008, Pfaller *et al*, 2011). The high HIV prevalence and accompanying immunodeficiency in sub-Saharan Africa and the presence of different HIV subtypes in the Continent are strong driving factors emphasizing the need for regional *Candida* surveillance programmes. Changes in drug

susceptibility over time serve as a reminder for the need to test clinical *Candida* isolates for sensitivity to antifungal drugs in the effort to improve patient care and reduce patient morbidity and mortality. The use of the TREK Sensititre platform for drug susceptibility testing can be done rapidly and with minimal training and reagents and is therefore a promising method for use in resource-limited laboratories in Africa. This type of technology should be widely available in public hospitals, as the emergence of drug-resistant species due to incorrect empirical treatment is a cause of concern.

Human immunodeficiency virus-infected groups that were found to be the most vulnerable to *Candida* infections included women, who are the most predisposed to *Candida* in general, and patients who were on antiretroviral treatment for longer periods of time, who were more predisposed to non-albicans drug resistant isolates.

When comparing drug resistance patterns of *Candida* in females, strong associations were seen between specific *Candida* species and their susceptibility to azoles and echinocandins (high azole resistance patterns were seen in *C. albicans* isolates and *C. krusei*, while *C. glabrata* was the most resistant species against the echinocandins). These findings emphasize the need for regional surveillance of *Candida* species, as the different species prevalence seen in different geographical regions and their distinct susceptibility to different drug classes means that empirical treatment of these patients might not be working effectively in treating *Candida* infections.

It is known that *Candida* species express estrogen-binding proteins, which result in a higher predisposition of females to *Candida* infection (Tarry *et al*, 2005 and Cheng, Yeater and Hoyer, 2006). High performance liquid chromatography-mass spectrometry analysis of cell surface fractions obtained from African *Candida* isolates in this study revealed the presence of oxireductase proteins that bind to mammalian estrogen with high affinity. These findings further elucidate the predisposition of females to *Candida* colonization.

Drug resistance-related proteins were identified in *Candida* species using both SDS-PAGE and HPLC-MS. The use of gel electrophoresis was found to be a valuable tool in the proteomic study of drug resistance in clinical *Candida* isolates, especially in the identification

of low molecular weight proteins. However, its usefulness is surpassed by the more sensitive high performance liquid chromatography platform, which provided much more detailed results.

The expression of *Candida* proteins that are related to colonization and pathogenicity mechanisms were found in different *Candida* species, in both drug-susceptible and –resistant isolates. The combination of different drug resistance mechanisms and binding abilities to salivary histatins and estrogen found in *Candida* species through HPLC-MS analysis seem to be instrumental in the ability of these organisms to colonize immunocompromised patients and resist the action of different chemicals and antifungal drugs.

The protein band of 24kDa identified in fluconazole-resistant cell fractions analysed by SDS-PAGE and the presence of a multidrug resistance protein known to confer resistance to azole drugs, which was seen only on a fluconazole-resistant *C. albicans* cell fraction, demonstrates differences in the protein expression of fluconazole-susceptible and –resistant isolates.

The results from this study demonstrated a need for regional surveillance of *Candida* species in African countries and improved control over the sale of medications. Results also showed that the prevalent *Candida albicans* does not respond to specific antifungal drugs that might be dispensed empirically. *Candida glabrata* from Cameroon demonstrated resistance to micafungin while South African isolates were susceptible, which shows significant regional differences. The reverse of this pattern was seen in the case of 5-flucytosine, thereby re-emphasizing the need for more epidemiological studies on the African continent.

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Appendix 1A

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of Project: Characterization of fluconazole-resistant *Candida* species from the oral cavity of HIV-positive patients in the Western Cape (SA)/ Bamenda (Cameroon).

Names of Researchers: Pedro Santos Abrantes, Prof P Bouic, Prof Charlene WJ Africa

If you would like to participate in this study please tick the relevant boxes:

1. Have you read the attached information sheet and has the purpose of the research project been explained to you?

YES	NO

2. Do you understand the method of sample collection and any risks involved?

YES	NO

3. Do you grant permission for information from your medical records to be disclosed to the research team as and when necessary?

YES	NO

4. Do you agree that samples collected for research or diagnostic testing can be stored for possible use in future research projects conducted by the above named researchers and /or other research collaborators?

YES	NO

I declare that my participation in this research project is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that any information contained in my file will remain confidential and that I (or my doctor) will be informed if any of the results of the medical tests done (as part of the research) have implications for my health. I know how to contact members of the research team should I change my mind about participating in this study.

…….	…….	…….
Name of patient (BLOCK CAPITALS)	**Date**	**Signature**
…….	…….	…….
Name of person taking consent	**Date**	**Signature**
…….	…….	…….
Name of researcher	**Date**	**Signature**

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

Appendix 1B

INFORMATION SHEET

Prospective participants are requested to read this information sheet carefully and to ask questions where necessary, before signing the attached consent form. This sheet must be detached and retained by the participant and the consent form filed for record.

Candida is a yeast found as part of the normal flora of the gut and mouth of some individuals. Normally, it does not cause a problem but in the case of persons infected with HIV, it may cause infection, particularly in the oral cavity. Most patients respond to treatment with fluconazole, but cases showing no improvement following treatment with fluconazole have been reported. This study will improve our knowledge of why this resistance happens.

The clinical procedure will entail the collection of samples from the mouth using cotton swabs. The sample collection procedure is non-invasive and safe and will be carried out with the utmost care to ensure the comfort of the patient.

Patients will be required to sign the attached form granting consent for the collection of swab samples and for the use of the samples donated and clinical parameters recorded. The patient will also be required to grant permission for his/her HIV status and other medical history to be disclosed if necessary. Participants will not be recorded by name, but samples and information will be coded to protect the identity of the individual. However, the coding will be used by the clinic to trace the individual if relevant information (as a result of the study) should be passed to him/her or his/her doctor. Permission will also be sought for the use of additional biological material collected in the clinic, which is usually discarded but which the researchers may find useful for future research.

Participation in this study is voluntary and refusal to participate will not prejudice the treatment of the patient in any way. Consent to participate will be recorded by completing the attached form. Should individuals agree to participate and later change their minds, they may withdraw by calling the following persons:
Prof C. Africa, University of the Western Cape, Department of Medical Biosciences, Tel: 021 9592342, or Prof P Bouic, Synexa, University of Stellenbosch, Tel: 021 9339582.

Appendix 2

University of the Western Cape

Department of Medical Biosciences

Patient Questionnaire

Gender	M	F		
Partner's gender	M	F		
Age:				
Race	Black	Coloured	White	Other
HIV sub-type	HIV-1	HIV-2		
Date diagnosed:				
Immune status	HIV +ve	AIDS		
ARV	Yes (specify)	No		
Duration of ARV therapy:				
Clinical presentation:				

Appendix 3

Tables 1 and 2 show the colony growth patterns of all *Candida* species 24 hours after sample collection and incubation (1=scanty growth, 2=light growth, 3=moderate growth, 4=heavy growth).

Table 1: Growth patterns of the first inoculation of all South African *Candida* samples.

Patient No.	Growth pattern	Patient No.	Growth pattern	Patient No.	Growth pattern
3	2	72	2	135	1
7	3	73	1	136	1
10	2	77	1	138	2
11	3	78	2	141	1
12	3	80	1	142	2
13	2	81	1	144	2
14	2	82	2	145	1
16	3	83	2	146	2
19	2	85	2	147	2
21	1	88	4	154	2
23	3	89	1	155	1
24	1	90	3	156	1
25	3	92	2	159	1
26	2	93	1	161	2
27	2	94	1	163	1
28	2	95	1	167	2
30	2	96	1	168	1
36	2	97	2	169	1
37	1	99	1	174	1
38	2	100	1	175	1
40	3	101	1	176	1
41	2	102	2	180	1
42	1	103	1	181	1
44	3	105	2	182	1
46	2	107	2	183	1
47	2	109	3	184	1
48	2	110	2	185	2
50	1	111	2	186	1
51	1	112	2	188	2
57	2	115	1	191	1
58	1	116	1	192	1
59	2	117	2	194	1
60	2	118	1	195	1
61	2	119	1	196	2
62	1	120	2	197	1
65	1	122	1	198	1
66	2	123	1	199	2
67	2	126	1	201	1
68	3	127	2	203	1
69	3	131	1	205	1
70	3	132	2	206	2
71	3	134	4	207	1

Table 2: Growth patterns of the first inoculation of all Cameroonian *Candida* samples.

Patient No.	Growth pattern	Patient No.	Growth pattern	Patient No.	Growth pattern
7	2	85	4	180	1
10	1	88	3	181	2
11	1	90	1	182	1
12	2	92	1	183	3
13	2	93	1	184	1
14	1	98	1	186	1
15	1	99	1	196	1
17	1	100	1	197	1
19	1	103	2	198	2
21	1	106	1	199	1
24	1	108	1	200	1
26	1	109	1	201	1
28	1	111	1	202	1
32	4	114	3	206	2
33	1	115	1	207	1
35	2	116	1	216	1
36	2	117	1	219	1
39	1	118	1	220	1
40	3	119	1	221	2
41	2	121	3	222	1
42	2	124	1	226	1
44	1	127	1	228	1
48	1	132	1	229	3
50	1	134	1	233	2
51	1	135	1	234	1
55	1	136	2	236	1
56	1	137	2	237	1
59	1	141	1	238	1
60	1	143	1	241	1
64	2	144	1	244	2
69	1	146	2	245	2
70	3	148	1	246	1
72	1	154	1	247	1
73	1	156	1	248	1
74	2	158	1	249	1
76	1	159	1	250	1
79	1	164	1	253	3
80	2	172	1	255	1
81	2	174	1	256	1
82	1	175	1	257	1
83	2	177	1	258	1
84	1	178	1	260	3
				261	1

Appendix 4

Tables 3 and 4 show the results obtained using Sabouraud's agar, which allows for *Candida* growth, chromogenic agar which differentiates different *Candida* species and the tomato juice agar, which allows for *C. albicans* and *C. dubliniensis* differentiation.

Table 3: South African results obtained from the different selective media.

Patient no.	Sabouraud's agar	Chromogenic agar	Tomato juice agar
3	*Candida*	*C. albicans*	*C. albicans*
7	*Candida*	*C. dubliniensis*	*C. dubliniensis*
10	*Candida*	*C. albicans*	*C. albicans*
11	*Candida*	*C. glabrata*	
12	*Candida*	*C. albicans*	*C. albicans*
13	*Candida*	*C. albicans*	*C. albicans*
14	*Candida*	*C. albicans*	*C. albicans*
16	*Candida*	*C. albicans*	*C. albicans*
19	Candida	*C.albicans*	*C.albicans*
21	Candida	*C. albicans*	*C. albicans*
23	*Candida*	*C. albicans*	*C. albicans*
24	*Candida*	*C. albicans*	*C. albicans*
25	*Candida*	*C. albicans*	*C. albicans*
26	*Candida*	*C. albicans*	*C. albicans*
27	*Candida*	*C. albicans*	*C. albicans*
28	*Candida*	*C. albicans*	*C. albicans*
30	*Candida*	*C. albicans*	*C. albicans*
36	*Candida*	*C. albicans and C. glabrata*	*C. albicans*
37	*Candida*	*C. albicans*	*C. albicans*
38	*Candida*	*C. albicans*	*C. albicans*
40	*Candida*	*C. albicans*	*C. albicans*
41	*Candida*	*C. albicans*	*C. albicans*
42	*Candida*	*C. albicans*	*C. albicans*
44	*Candida*	*C. albicans*	*C. albicans*
46	*Candida*	*C. albicans*	*C. albicans*
47	*Candida*	*C. albicans*	*C. albicans*
48	*Candida*	*C. albicans*	*C. albicans*
50	*Candida*	*C. albicans*	*C. albicans*
51	*Candida*	*C. dubliniensis*	*C. dubliniensis*
57	*Candida*	*C. albicans*	*C. albicans*
58	*Candida*	*C. albicans*	*C. albicans*
59	*Candida*	*C. dubliniensis*	*C. dubliniensis*
60	*Candida*	*C. albicans*	*C. albicans*
61	*Candida*	*C. albicans*	*C. albicans*
62	*Candida*	*C. albicans*	*C. albicans*
65	*Candida*	*C. albicans*	*C. albicans*
66	*Candida*	*C. albicans*	*C. albicans*
67	*Candida*	*C. albicans*	*C. albicans*
68	*Candida*	*C. albicans*	*C. albicans*

69	*Candida*	*C. albicans*	*C. albicans*
70	*Candida*	*C. albicans*	*C. albicans*
71	*Candida*	*C. albicans*	*C. albicans*
72	*Candida*	*C. glabrata*	
73	*Candida*	*C. albicans*	*C. albicans*
77	*Candida*	*C. glabrata*	
78	*Candida*	*C. albicans*	*C. albicans*
80	*Candida*	*C. albicans*	*C. albicans*
81	*Candida*	*C. glabrata*	
82	*Candida*	*C. dubliniensis*	*C. dubliniensis*
83	*Candida*	*C. albicans*	*C. albicans*
85	*Candida*	*C. albicans*	*C. albicans*
88	*Candida*	*C. albicans*	*C. albicans*
89	*Candida*	*C. albicans*	*C. albicans*
90	*Candida*	*C. albicans*	*C. albicans*
92	*Candida*	*C. glabrata*	
93	*Candida*	*C. albicans*	*C. albicans*
94	*Candida*	*C. albicans*	*C. albicans*
95	*Candida*	*C. albicans*	*C. albicans*
96	*Candida*	*C. albicans*	*C. albicans*
97	*Candida*	*C. albicans*	*C. albicans*
99	*Candida*	*C. albicans*	*C. albicans*
100	*Candida*	*C. albicans*	*C. albicans*
101	*Candida*	*C. albicans*	*C. albicans*
102	*Candida*	*C. albicans*	*C. albicans*
103	*Candida*	*C. albicans*	*C. albicans*
105	*Candida*	*C. dubliniensis*	*C. dubliniensis*
107	*Candida*	*C. glabrata*	
109	*Candida*	*C. albicans*	*C. albicans*
110	*Candida*	*C. albicans*	*C. albicans*
111	*Candida*	*C. albicans*	*C. albicans*
112	*Candida*	*C. albicans*	*C. albicans*
115	*Candida*	*C. albicans*	*C. albicans*
116	*Candida*	*C. albicans*	*C. albicans*
117	*Candida*	*C. albicans*	*C. albicans*
118	*Candida*	*C. glabrata*	
119	*Candida*	*C. albicans*	*C. albicans*
120	*Candida*	*C. albicans*	*C. albicans*
122	*Candida*	*C. albicans*	*C. albicans*
123	*Candida*	*C. albicans*	*C. albicans*
126	*Candida*	*C. albicans*	*C. albicans*
127	*Candida*	*C. albicans*	*C. albicans*
131	*Candida*	*C. albicans*	*C. albicans*
132	*Candida*	*C. albicans*	*C. albicans*
134	*Candida*	*C. albicans*	*C. albicans*
135	*Candida*	*C. albicans*	*C. albicans*
136	*Candida*	*C. dubliniensis*	*C. dubliniensis*
138	*Candida*	*C. dubliniensis*	*C. dubliniensis*
141	*Candida*	*C. albicans*	*C. albicans*
142	*Candida*	*C. albicans*	*C. albicans*
144	*Candida*	*C. glabrata*	
145	*Candida*	*C. albicans*	*C. albicans*

146	*Candida*	*C. albicans*	*C. albicans*
147	*Candida*	*C. glabrata*	
154	*Candida*	*C. dubliniensis*	*C. dubliniensis*
155	*Candida*	*C. albicans*	*C. albicans*
156	*Candida*	*C. albicans*	*C. albicans*
159	*Candida*	*C. albicans*	*C. albicans*
161	*Candida*	*C. albicans*	*C. albicans*
163	*Candida*	*C. albicans*	*C. albicans*
167	*Candida*	*C. albicans*	*C. albicans*
168	*Candida*	*C. albicans*	*C. albicans*
169	*Candida*	*C. albicans*	*C. albicans*
174	*Candida*	*C. albicans*	*C. albicans*
175	*Candida*	*C. albicans*	*C. albicans*
176	*Candida*	*C. albicans*	*C. albicans*
180	*Candida*	*C. albicans*	*C. albicans*
181	*Candida*	*C. albicans*	*C. albicans*
182	*Candida*	*C. albicans*	*C. albicans*
183	*Candida*	*C. albicans*	*C. albicans*
184	*Candida*	*C. dubliniensis*	*C. dubliniensis*
185	*Candida*	*C. albicans*	*C. albicans*
186	*Candida*	*C. albicans*	*C. albicans*
188	*Candida*	*C. albicans*	*C. albicans*
191	*Candida*	*C. albicans*	*C. albicans*
192	*Candida*	*C. albicans*	*C. albicans*
194	*Candida*	*C. albicans*	*C. albicans*
195	*Candida*	*C. albicans*	*C. albicans*
196	*Candida*	*C. glabrata*	
197	*Candida*	*C. albicans*	*C. albicans*
198	*Candida*	*C. albicans*	*C. albicans*
199	*Candida*	*C. albicans*	*C. albicans*
201	*Candida*	*C. albicans*	*C. albicans*
203	*Candida*	*C. albicans and C. glabrata*	*C. albicans*
205	*Candida*	*C. albicans*	*C. albicans*
206	*Candida*	*C. albicans*	*C. albicans*
207	*Candida*	*C. dubliniensis*	*C. dubliniensis*

Table 4: Cameroonian results obtained from the different selective media.

Patient No.	Sabouraud agar	Chromogenic agar	Tomato juice agar
7	*Candida*	*Candida albicans*	*Candida albicans*
10	*Candida*	*Candida albicans*	*Candida albicans*
11	*Candida*	*Candida albicans*	*Candida albicans*
12	*Candida*	*Candida albicans*	*Candida albicans*
13	*Candida*	*Candida albicans*	*Candida albicans*
14	*Candida*	*Candida albicans*	*Candida albicans*
15	*Candida*	*Candida albicans*	*Candida albicans*
17	*Candida*	*Candida kefyr/parapsilopsis/lusitaneae*	
19	*Candida*	*Candida glabrata*	
21	*Candida*	*Candida kefyr/parapsilopsis/lusitaneae*	
24	*Candida*	*Candida albicans*	*Candida albicans*
26	*Candida*	*Candida albicans*	*Candida albicans*
28	*Candida*	*Candida albicans*	*Candida albicans*
32	*Candida*	*Candida albicans*	*Candida albicans*
33	*Candida*	*Candida albicans*	*Candida albicans*
35	*Candida*	*Candida albicans*	*Candida albicans*
36	*Candida*	*Candida albicans*	*Candida albicans*
39	*Candida*	*Candida glabrata*	
40	*Candida*	*Candida albicans*	*Candida albicans*
41	*Candida*	*Candida albicans*	*Candida albicans*
42	*Candida*	*Candida albicans*	*Candida albicans*
44	*Candida*	*Candida glabrata*	
48	*Candida*	*Candida albicans*	*Candida albicans*
50	*Candida*	*Candida albicans*	*Candida albicans*
51	*Candida*	*Candida albicans*	*Candida albicans*
55	*Candida*	*Candida albicans*	*Candida albicans*
56	*Candida*	*Candida albicans*	*Candida albicans*
59	*Candida*	*Candida albicans*	*Candida albicans*
60	*Candida*	*Candida albicans*	*Candida albicans*
64	*Candida*	*Candida albicans*	*Candida albicans*
69	*Candida*	*Candida albicans*	*Candida albicans*
70	*Candida*	*Candida albicans*	*Candida albicans*
72	*Candida*	*Candida albicans*	*Candida albicans*
73	*Candida*	*Candida albicans*	*Candida albicans*
74	*Candida*	*Candida albicans*	*Candida albicans*
76	*Candida*	*Candida glabrata*	
79	*Candida*	*Candida albicans*	*Candida albicans*
80	*Candida*	*Candida albicans*	*Candida albicans*
81	*Candida*	*Candida albicans*	*Candida albicans*
82	*Candida*	*Candida albicans*	*Candida albicans*
83	*Candida*	*Candida albicans*	*Candida albicans*
84	*Candida*	*Candida albicans*	*Candida albicans*
85	*Candida*	*Candida tropicalis*	
88	*Candida*	*Candida albicans*	*Candida albicans*
90	*Candida*	*Candida albicans*	*Candida albicans*
92	*Candida*	*Candida glabrata*	
93	*Candida*	*Candida glabrata*	
98	*Candida*	*Candida albicans*	*Candida albicans*

99	*Candida*	*Candida albicans*	*Candida albicans*
100	*Candida*	*Candida albicans*	*Candida albicans*
103	*Candida*	*Candida albicans*	*Candida albicans*
106	*Candida*	*Candida albicans*	*Candida albicans*
108	*Candida*	*Candida glabrata*	
109	*Candida*	*Candida glabrata*	
111	*Candida*	*Candida albicans*	*Candida albicans*
114	*Candida*	*Candida albicans*	*Candida albicans*
115	*Candida*	*Candida albicans*	*Candida albicans*
116	*Candida*	*Candida glabrata*	
117	*Candida*	*Candida albicans*	*Candida albicans*
118	*Candida*	*Candida albicans*	*Candida albicans*
119	*Candida*	*Candida albicans*	*Candida albicans*
121	*Candida*	*Candida albicans*	*Candida albicans*
124	*Candida*	*Candida glabrata*	
127	*Candida*	*Candida albicans*	*Candida albicans*
132	*Candida*	*Candida albicans*	*Candida albicans*
134	*Candida*	*Candida albicans*	*Candida albicans*
135	*Candida*	*Candida albicans*	*Candida albicans*
136	*Candida*	*Candida dubliniensis*	*Candida dubliniensis*
137	*Candida*	*Candida albicans*	*Candida albicans*
141	*Candida*	*Candida tropicalis*	
143	*Candida*	*Candida albicans*	*Candida albicans*
144	*Candida*	*Candida krusei*	
146	*Candida*	*Candida albicans*	*Candida albicans*
148	*Candida*	*Candida glabrata*	
154	*Candida*	*Candida albicans*	*Candida albicans*
156	*Candida*	*Candida glabrata*	
158	*Candida*	*Candida albicans*	*Candida albicans*
159	*Candida*	*Candida glabrata*	
164	*Candida*	*Candida albicans*	*Candida albicans*
172	*Candida*	*Candida krusei*	
174	*Candida*	*Candida krusei*	
175	*Candida*	*Candida albicans*	*Candida albicans*
177	*Candida*	*Candida albicans*	*Candida albicans*
178	*Candida*	*Candida glabrata*	
180	*Candida*	*Candida albicans*	*Candida albicans*
181	*Candida*	*Candida albicans*	*Candida albicans*
182	*Candida*	*Candida albicans*	*Candida albicans*
183	*Candida*	*Candida albicans*	*Candida albicans*
184	*Candida*	*Candida albicans with pseudohyphae*	*Candida albicans with pseudohyphae*
186	*Candida*	*Candida glabrata*	
196	*Candida*	*Candida glabrata*	
197	*Candida*	*Candida albicans*	*Candida albicans*
198	*Candida*	*Candida albicans*	*Candida albicans*
199	*Candida*	*Candida albicans*	*Candida albicans*
200	*Candida*	*Candida glabrata*	
201	*Candida*	*Candida albicans*	*Candida albicans*
202	*Candida*	*Candida albicans*	*Candida albicans*
206	*Candida*	*Candida albicans with pseudohyphae*	*Candida albicans with pseudohyphae*
207	*Candida*	*Candida albicans*	*Candida albicans*

216	*Candida*	*Candida glabrata*	
219	*Candida*	*Candida glabrata*	
220	*Candida*	*Candida glabrata*	
221	*Candida*	*Candida albicans*	*Candida albicans*
222	*Candida*	*Candida albicans*	*Candida albicans*
226	*Candida*	*Candida albicans with pseudohyphae*	*Candida albicans with pseudohyphae*
228	*Candida*	*Candida glabrata*	
229	*Candida*	*Candida albicans*	*Candida albicans*
233	*Candida*	*Candida albicans*	*Candida albicans*
234	*Candida*	*Candida glabrata*	*Candida glabrata*
236	*Candida*	*Candida albicans*	*Candida albicans*
237	*Candida*	*Candida glabrata*	
238	*Candida*	*Candida albicans with pseudohyphae*	*Candida albicans with pseudohyphae*
241	*Candida*	*Candida albicans*	*Candida albicans*
244	*Candida*	*Candida albicans*	*Candida albicans*
245	*Candida*	*Candida tropicalis*	
246	*Candida*	*Candida glabrata*	
247	*Candida*	*Candida albicans*	*Candida albicans*
248	*Candida*	*Candida albicans*	*Candida albicans*
249	*Candida*	*Candida albicans*	*Candida albicans*
250	*Candida*	*Candida tropicalis*	
253	*Candida*	*Candida albicans*	*Candida albicans*
255	*Candida*	*Candida albicans*	*Candida albicans*
256	*Candida*	*Candida albicans*	*Candida albicans*
257	*Candida*	*Candida albicans*	*Candida albicans*
258	*Candida*	*Candida krusei*	
260	*Candida*	*Candida albicans*	*Candida albicans*
261	*Candida*	*Candida albicans*	*Candida albicans*

Appendix 5

Table 5 shows the cumulative results obtained from all South African patients. Data from the questionnaire and patient's folder are included, as well as the results obtained by the different culturing methods.

Table 5: Cumulative results from all HIV+ South African patients.

Patient No.	Gender	Partner's gender	Age	Race	Date diagnosed	HIV status	ARVs	Duration	Clinical presentation	Other	Results
1	Male	Female	29	Black	Sep-05	HIV +ve	d4T, EFV, 3TC	10 months	NAD		Negative
2	Female	Male	31	Black	Aug-05	HIV +ve	d4T, EFV, 3TC	11 months	NAD	TB patient	Negative
3	Female	Male	30	Black	2003	HIV +ve	AZT, NVP, 3TC	3 years	NAD	8 months pregnant	*C. albicans*
4	Female	Male	?	Black	2000	HIV +ve	d4T, 3TC	2 months	NAD	NVP->rash->d4T	Negative
5	Male	Female	27	Black	Jan-06	HIV +ve	**NO**		NAD		Negative
6	Male	Female	33	Black	Jun-05	HIV +ve	d4T, 3TC	2 weeks	White plaque on mucosa and side of tongue	CD4+: 97	Negative
7	Female	Male	24	Black	Jun-03	HIV +ve	d4T, NVP, 3TC	7 months	NAD		*C. dubliniensis*
8	Female	Male	37	Black	Sep-05	HIV +ve	AZT, EFV, 3TC	9 months	NAD		Negative
9	Female	Male	42	Black	2005	HIV +ve	AZT, NVP, 3TC	1 year	NAD	Diagnosed when pregnant	Negative
10	Female	Male	26	Black	May-05	HIV +ve	AZT, NVP, 3TC	14 months	NAD		*C. albicans*
11	Male	Female	49	Black	Sep-04	HIV +ve	d4T, EFV, 3TC	2 years	NAD		*C. glabrata*
12	Female	Male	31	Black	Nov-02	HIV +ve	**NO**		White plaque in tongue	TB patient	*C. albicans*
13	Female	Male	19	Black	Mar-05	HIV +ve	**NO**		NAD		*C. albicans*
14	Female	Male	28	Black	May-03	HIV +ve	d4T, NVP, 3TC	16 months	NAD		*C. albicans*
15	Male	Female	38	Black	2003	HIV +ve	d4T, EFV, 3TC	2 years	NAD		Negative
16	Female	Male	29	Black	Nov-02	HIV +ve	AZT, NVP, 3TC	2 years	NAD		*C. albicans*
17	Female	Male	35	Black	Nov-04	HIV +ve	d4T, EFV, 3TC	19 months	NAD		Negative
18	Female	Male	44	Black	Mar-02	HIV +ve	d4T, NVP, 3TC	14 months	NAD		Negative
19	Male	Female	53	Coloured	2006	AIDS	d4T,NVP, 3TC	1 month	Candida in tongue		*C.albicans*
20	Male	Female	39	Black	2002	HIV +ve	**NO**		NAD	CD4+: 47	Negative
21	Female	Male	35	Black	?	HIV +ve	d4T, EFV, 3TC	24 months	White plaque in tongue		*C. albicans*

22	Female	Male	24	Black	Jul-08	HIV +ve	AZT, NVP, 3TC	2 weeks	NAD	CD4+: 144, 6 months pregnant, STI	Negative
23	Female	Male	24	Black	2002	AIDS	d4T, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
24	Female	Male	29	Black	Sep-08	HIV +ve	**NO**		NAD	CD4+: 79, 19 weeks pregnant	*C. albicans*
25	Male	Female	37	Black	2005	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
26	Female	Male	25	Black	Jun-08	HIV +ve	**NO**			1 week old child	*C. albicans*
27	Male	Female	34	Black	Aug-08	AIDS	**NO**		Oropharyngeal thrush	Patient took fluconazole	*C. albicans*
28	Female	Male	28	Black	2007	AIDS	d4T, EFV, 3TC	2 weeks	Candida in tongue		*C. albicans*
29	Male	Female	38	Black	Mar-06	HIV +ve	d4T, EFV, 3TC	29 months	White plaque in tongue		Negative
30	Female	Male	28	Black	2006	AIDS	d4T, EFV, 3TC	22 months	White plaque in tongue	TB patient	*C. albicans*
31	Male	Female	25	Black	Jul-08	HIV +ve	**NO**		White plaque in tongue		Negative
32	Male	Female	24	Black	2007	HIV +ve	d4T, EFV, 3TC	16 months	White plaque in tongue		Negative
33	Female	Male	34	Black	Dec-03	HIV +ve	d4T, EFV, 3TC	24 months	NAD		Negative
34	Female	Male	30	Black	2002	HIV +ve	d4T, NVP, 3TC	22 months	NAD		Negative
35	Female	Male	36	Black	Aug-08	AIDS	d4T, EFV, 3TC	Starting Oct-2008	NAD	CD4+: 64, TB patient, Rash, Headaches	Negative
36	Female	Male	38	Coloured	Sep-08	HIV +ve	**NO**		NAD		*C. albicans* and *C. glabrata*
37	Female	Male	43	Black	2008	AIDS	d4T, EFV, 3TC	8 months	NAD	TB patient	*C. albicans*
38	Male	Female	39	Black	Sep-08	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
39	Female	Male	22	Black	May-08	HIV +ve	AZT, NVP, 3TC	2 months	NAD	31 weeks pregnant	Negative
40	Female	Male	38	Black	2005	AIDS	**NO**	Starting Oct-2008	NAD	CD4+: 34, TB patient	*C. albicans*
41	Male	Female	36	Coloured	Aug-08	AIDS	**NO**		White plaque in tongue		*C. albicans*
42	Female	Male	39	Black	2001	AIDS	d4T, EFV, 3TC	24 months	White plaque in tongue		*C. albicans*
43	Female	Male	34	Black	2000	HIV +ve	d4T, EFV, 3TC	26 months	White plaque in tongue		Negative
44	Male	Female	43	White	Sep-08	AIDS	NO		*Candida* lesion in lower lip, Tb patient		*C. albicans*
45	Female	Male	32	Black	2004	HIV +ve	AZT, NVP, 3TC	3 months	NAD	30 weeks pregnant	Negative
46	Male	Female	35	Black	2008	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
47	Female	Male	22	Coloured	2006	HIV +ve	d4T, EFV, 3TC	1 month	White plaque in tongue	ARV treatment defaulted in 2007	*C. albicans*
48	Female	Male	30	Black	2002	AIDS	d4T, NVP, 3TC	12 months	NAD		*C. albicans*
49	Female	Male	30	Black	2005	AIDS	AZT, DDI, KLT	41 months	NAD	TB patient, ARV regimen 2	Negative

50	Male	Female	34	Black	2003	HIV +ve	d4T, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
51	Female	Male	33	Black	2006	HIV +ve	d4T, EFV, 3TC	23 months	White plaque in tongue		*C. dubliniensis*
52	Female	Male	34	Black	2003	HIV +ve	d4T, NVP, 3TC	20 months	White plaque in tongue		Negative
53	Female	Male	50	Black	?	HIV +ve	d4T, EFV, 3TC	15 months	White plaque in tongue		Negative
54	Female	Male	51	Black	2008	HIV +ve	d4T, EFV, 3TC	28 months	White plaque in tongue		Negative
55	Female	Male	27	Black	2006	HIV +ve	d4T, EFV, 3TC	22 months	White plaque in tongue		Negative
56	Female	Male	27	Coloured	2007	HIV +ve	d4T, NVP, 3TC	12 months	White plaque in tongue		Negative
57	Female	Male	46	Black	Jul-08	AIDS	**NO**		Oral candidiasis in tongue	Amphotericin B lozenges taken days before sample collection	*C. albicans*
58	Female	Male	44	Coloured	2007	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
59	Male	Female	39	Black	2007	HIV +ve	d4T, EFV, 3TC	13 months	NAD		*C. dubliniensis*
60	Male	Female	43	Black	Jun-08	HIV +ve	d4T, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
61	Male	Female	36	Black	May-08	HIV +ve	d4T, NVP, 3TC	2 months	White plaque in tongue		*C. albicans*
62	Male	Female	45	Black	Mar-07	AIDS	d4T, EFV, 3TC	12 months	Systemic fungal infection		*C. albicans*
63	Female	Male	31	Black	2005	HIV +ve	d4T, EFV, 3TC	25 months	NAD		Negative
64	Female	Male	37	Black	2005	HIV +ve	d4T, NVP, 3TC	18 months	White plaque in tongue		Negative
65	Male	Female	40	Black	2007	HIV +ve	d4T, EFV, 3TC	19 months	White plaque in tongue		*C. albicans*
66	Female	Male	33	Black	2001	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
67	Female	Male	29	Black	Sep-08	AIDS	**NO**		White plaque in tongue	TB patient	*C. albicans*
68	Female	Male	31	Black	Sep-08	AIDS	**NO**		White plaque in tongue	TB patient	*C. albicans*
69	Female	Male	23	Black	Jul-08	HIV +ve	**NO**		White plaque in tongue	5 months pregnant	*C. albicans*
70	Female	Male	25	Black	2006	AIDS	**NO**	Starting Oct-2008	Oral candidiasis	Fluconazole prescribed for 2 weeks, TB patient	*C. albicans*
71	Female	Male	30	Black	Apr-08	AIDS	**NO**		White plaque in tongue	CD4+: 122	*C. albicans*
72	Female	Male	25	Black	Dec-07	AIDS	AZT, NVP, 3TC	8 months	White plaque in tongue		*C. glabrata*
73	Female	Male	28	Black	Oct-07	HIV +ve	d4T, EFV, 3TC	12 months	White plaque in tongue		*C. albicans*
74	Female	Male	46	Black	2003	HIV +ve	**NO**		White plaque in tongue		Negative
75	Female	Male	34	Black	2004	HIV +ve	d4T, NVP, 3TC	35 months	White plaque in tongue		Negative

76	Female	Male	30	Black	2002	HIV +ve	AZT, NVP, 3TC	47 months	White plaque in tongue		Negative
77	Female	Male	29	Black	2007	HIV +ve	d4T, EFV, 3TC	7 months	White plaque in tongue		*C. glabrata*
78	Male	Female	44	Black	Aug-08	AIDS	**NO**	Starting Oct-2008	Oral candidiasis		*C. albicans*
79	Male	Female	38	Black	2004	HIV +ve	d4T, EFV, 3TC	46 months	White plaque in tongue		Negative
80	Female	Male	24	Black	Apr-07	HIV +ve	d4T, NVP, 3TC	19 months	White plaque in tongue		*C. albicans*
81	Female	Male	35	Black	May-07	HIV +ve	d4T, NVP, 3TC	5 months	White plaque in tongue		*C. glabrata*
82	Female	Male	36	Black	2004	HIV +ve	**NO**	Starting Oct-2008	White plaque in tongue		*C. dubliniensis*
83	Female	Male	50	Coloured	2007	AIDS	**NO**		White plaque in tongue		*C. albicans*
84	Female	Male	40	Black	May-07	HIV +ve	d4T, EFV, 3TC	12 months	White plaque in tongue		Negative
85	Female	Male	30	Black	Mar-07	HIV +ve	d4T, EFV, 3TC	12 months	White plaque in tongue		*C. albicans*
86	Female	Male	33	Black	2007	HIV +ve	d4T, EFV, 3TC	7 months	White plaque in tongue		Negative
87	Male	Female	48	Black	Mar-08	AIDS	d4T, NVP, 3TC	6 months	White plaque in tongue	TB patient	Negative
88	Male	Female	36	Black	Sep-08	AIDS	**NO**		Oral candidiasis	TB patient	*C. albicans*
89	Female	Male	30	Black	Mar-08	HIV +ve	d4T, EFV, 3TC	18 months	White plaque in tongue		*C. albicans*
90	Female	Male	38	Black	2001	HIV +ve	d4T, NVP, 3TC	48 months	White plaque in tongue		*C. albicans*
91	Male	Female	50	Coloured	2007	HIV +ve	d4T, NVP, 3TC	14 months	White plaque in tongue		Negative
92	Female	Male	31	Black	2007	HIV +ve	d4T, NVP, 3TC	18 months	White plaque in tongue		*C. glabrata*
93	Female	Male	40	Black	2005	HIV +ve	d4T, NVP, 3TC	9 months	White plaque in tongue		*C. albicans*
94	Female	Male	42	Black	2007	HIV +ve	d4T, EFV, 3TC	4 months	White plaque in tongue		*C. albicans*
95	Female	Male	38	Black	2006	HIV +ve	AZT, KLT, 3TC	34 months	White plaque in tongue		*C. albicans*
96	Female	Male	50	Black	Oct-07	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
97	Male	Female	49	Black	2006	AIDS	**NO**	Starting Oct-2008	White plaque in tongue	CD4+: 9	*C. albicans*
98	Female	Male	34	Black	2002	HIV +ve	d4T, NVP, 3TC	2 weeks	White plaque in tongue		Negative
99	Male	Female	35	Black	2006	HIV +ve	d4T, NVP, 3TC	12 months	White plaque in tongue		*C. albicans*
100	Female	Male	38	Black	2007	HIV +ve	d4T, EFV, 3TC	10 months	White plaque in tongue		*C. albicans*
101	Female	Male	31	Black	2002	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
102	Male	Female	53	Indian	1997	AIDS	d4T, NVP, 3TC	11 months	Oral candidiasis		*C. albicans*

103	Male	Female	45	Black	2002	AIDS	d4T, EFV, 3TC	56 months	White plaque in tongue		*C. albicans*
104	Male	Female	42	Coloured	2005	HIV +ve	d4T, NVP, 3TC	24 months	White plaque in tongue		Negative
105	Female	Male	25	Black	2006	HIV +ve	AZT, DDI, KLT	20 months	White plaque in tongue		*C. dubliniensis*
106	Female	Male	32	Black	Jul-07	HIV +ve	d4T, NVP, 3TC	12 months	White plaque in tongue		Negative
107	Female	Male	33	Black	2002	HIV +ve	d4T, NVP, 3TC	4 months	White plaque in tongue		*C. glabrata*
108	Female	Male	36	Black	2005	HIV +ve	AZT, DDI, KLT	35 months	White plaque in tongue		Negative
109	Male	Female	28	Black	Apr-08	AIDS	d4T, EFV, 3TC	6 months	Oral candidiasis	TB patient	*C. albicans*
110	Female	Male	36	Black	2003	HIV +ve	AZT, NVP, 3TC	35 months	White plaque in tongue	1 week old child	*C. albicans*
111	Male	Female	45	Black	Apr-08	AIDS	d4T, NVP, 3TC	4 months	White plaque in tongue	TB patient	*C. albicans*
112	Male	Female	31	Black	Sep-07	AIDS	d4T, EFV, 3TC	2 months	White plaque in tongue	TB patient	*C. albicans*
113	Female	Male	37	Black	Jan-07	HIV +ve	AZT, EFV, 3TC	18 months	White plaque in tongue		Negative
114	Female	Male	31	Black	2005	HIV +ve	d4T, EFV, 3TC	33 months	White plaque in tongue		Negative
115	Female	Male	43	Black	Oct-08	HIV +ve	**NO**		White plaque in tongue	12 weeks pregnant	*C. albicans*
116	Female	Male	32	Black	2005	HIV +ve	d4T, EFV, 3TC	28 months	White plaque in tongue		C. albicans
117	Female	Male	64	Black	Oct-08	AIDS	**NO**		White plaque in tongue	TB patient	*C. albicans*
118	Female	Male	31	Black	2002	HIV +ve	AZT, EFV, 3TC	44 months	White plaque in tongue		*C. glabrata*
119	Female	Male	35	Black	Oct-07	HIV +ve	d4T, NVP, 3TC	8 months	White plaque in tongue		*C. albicans*
120	Female	Male	22	Black	Jul-08	AIDS	**NO**	Starting Oct-2008	Oral candidiasis		*C. albicans*
121	Female	Male	37	Black	Jan-08	HIV +ve	d4T, EFV, 3TC	8 months	White plaque in tongue		Negative
122	Female	Male	34	Black	Jun-08	HIV +ve	d4T, EFV, 3TC	2 months	White plaque in tongue		*C. albicans*
123	Male	Female	39	Black	Apr-08	HIV +ve	d4T, NVP, 3TC	5 months	White plaque in tongue		*C. albicans*
124	Female	Male	24	Black	Jul-08	HIV +ve	d4T, NVP, 3TC	1 month	White plaque in tongue		Negative
125	Male	Female	42	Black	1997	HIV +ve	d4T, EFV, 3TC	33 months	White plaque in tongue		Negative
126	Female	Male	30	Black	Sep-07	HIV +ve	d4T, NVP, 3TC	11 months	White plaque in tongue		*C. albicans*
127	Female	Male	24	Black	2006	AIDS	**NO**	Starting Oct-2008	White plaque in tongue		*C. albicans*
128	Female	Male	27	Black	Aug-08	HIV +ve	**NO**	Starting Oct-2008	White plaque in tongue	19 weeks pregnant	Negative
129	Female	Male	28	Black	2005	HIV +ve	AZT, NVP, 3TC	3 weeks	White plaque in tongue		Negative

130	Male	Female	43	Coloured	2005	HIV +ve	**NO**	Defaulted 2005-2007	White plaque in tongue		Negative
131	Female	Male	35	Black	2002	HIV +ve	d4T, EFV, 3TC	6 months	White plaque in tongue		*C. albicans*
132	Female	Male	32	Black	2003	HIV +ve	**NO**	Starting Oct-2008	White plaque in tongue		*C. albicans*
133	Male	Female	32	Coloured	Oct-04	AIDS	**NO**	Starting Oct-2008	White plaque in tongue		Negative
134	Male	Female	38	Black	Aug-08	AIDS	**NO**		Extensive oral candidiasis		*C. albicans*
135	Male	Female	41	Black	Feb-08	HIV +ve	**NO**		White plaque in tongue		*C. albicans*
136	Female	Male	48	Black	2006	HIV +ve	**NO**		White plaque in tongue		*C. dubliniensis*
137	Male	Female	32	Black	2006	HIV +ve	d4T, EFV, 3TC	24 months	White plaque in tongue		Negative
138	Male	Female	51	Black	Nov-07	HIV +ve	d4T, EFV, 3TC	2 weeks	White plaque in tongue		*C. dubliniensis*
139	Female	Male	47	Black	2001	HIV +ve	d4T, EFV, 3TC	41 months	White plaque in tongue		Negative
140	Male	Female	40	Black	2006	HIV +ve	d4T, EFV, 3TC	26 months	White plaque in tongue		Negative
141	Male	Female	33	Black	2006	AIDS	d4T, EFV, 3TC	21 months	White plaque in tongue		*C. albicans*
142	Female	Male	63	Black	Dec-07	HIV +ve	d4T, EFV, 3TC	2 months	White plaque in sides of tongue		*C. albicans*
143	Female	Male	39	Black	2006	HIV +ve	d4T, EFV, 3TC	21 months	White plaque in tongue		Negative
144	Female	Male	28	Black	Jan-04	AIDS	NO	Starting Oct-2008	Oral candidiasis (tongue)		*C. glabrata*
145	Male	Female	43	Black	2005	HIV +ve	d4T, EFV, 3TC	9 months	Oral candidiasis (tongue)		*C. albicans*
146	Female	Male	30	Black	2005	HIV +ve	NO		White plaque in tongue	17 weeks pregnant	*C. albicans*
147	Male	Female	36	Black	2007	HIV +ve	d4T, NVP, 3TC	4 months	White plaque in tongue		*C. glabrata*
148	Female	Male	33	Black	Jun-08	HIV +ve	d4T, NVP, 3TC	3 months	White plaque in tongue		Negative
149	Male	Female	48	Black	Jul-08	HIV +ve	d4T, NVP, 3TC	2 months	White plaque in tongue		Negative
150	Male	Female	45	Black	2005	HIV +ve	d4T, NVP, 3TC	27 months	White plaque in tongue		Negative
151	Male	Female	51	Coloured	May-07	HIV +ve	d4T, EFV, 3TC	14 months	White plaque in tongue		Negative
152	Female	Male	37	Black	2006	HIV +ve	d4T, NVP, 3TC	31 months	White plaque in tongue		Negative
153	Female	Male	41	Coloured	Oct-03	HIV +ve	d4T, NVP, 3TC	17 months	White plaque in tongue		Negative
154	Male	Female	60	Black	2006	HIV +ve	d4T, NVP, 3TC	30 months	White plaque in tongue		*C. dubliniensis*
155	Female	Male	23	Black	Mar-08	HIV +ve	AZT, NVP, 3TC	6 months	White plaque in tongue		*C. albicans*
156	Female	Male	38	Black	Jan-07	HIV +ve	d4T, NVP,	6 months	White plaque in tongue		*C. albicans*

							3TC				
157	Female	Male	57	Coloured	2000	HIV +ve	d4T, EFV, 3TC	32 months	White plaque in tongue		Negative
158	Male	Female	37	Black	2006	HIV +ve	d4T, EFV, 3TC	30 months	White plaque in tongue		G+ cocci
159	Male	Female	33	Black	2007	HIV +ve	d4T, EFV, 3TC	10 months	White plaque in tongue		*C. albicans*
160	Male	Female	39	Black	2002	HIV +ve	d4T, EFV, 3TC	34 months	White plaque in tongue		Negative
161	Male	Female	51	Black	Sep-08	AIDS	NO		White plaque in tongue	TB patient	*C. albicans*
162	Female	Male	51	Coloured	2006	HIV +ve	d4T, NVP, 3TC	7 months	White plaque in tongue		Negative
163	Male	Female	27	Black	Sep-08	AIDS	NO		White plaque in tongue	TB patient	*C. albicans*
164	Male	Female	42	Black	May-07	HIV +ve	d4T, EFV, 3TC	14 months	White plaque in tongue		Negative
165	Male	Female	36	Black	2001	HIV +ve	d4T, EFV, 3TC	36 months	White plaque in tongue		Negative
166	Female	Male	35	Black	2003	HIV +ve	AZT, EFV, 3TC	7 months	White plaque in tongue		Negative
167	Female	Male	29	Black	Sep-01	HIV +ve	NO		White plaque in tongue		*C. albicans*
168	Male	Female	36	Black	2007	HIV +ve	d4T, EFV, 3TC	9 months	White plaque in tongue		*C. albicans*
169	Female	Male	50	Black	2006	HIV +ve	d4T, NVP, 3TC	26 months	White plaque in tongue		*C. albicans*
170	Male	Female	42	Black	May-08	HIV +ve	d4T, NVP, 3TC	2 months	White plaque in tongue		Negative
171	Male	Female	43	Black	Oct-07	HIV +ve	d4T, NVP, 3TC	10 months	White plaque in tongue		Negative
172	Male	Female	31	Black	Mar-08	HIV +ve	d4T, NVP, 3TC	6 months	White plaque in tongue		Negative
173	Male	Female	43	Black	2007	HIV +ve	d4T, EFV, 3TC	8 months	White plaque in tongue		Negative
174	Female	Male	22	Black	2006	AIDS	d4T, EFV, 3TC	4 months	White plaque in tongue		*C. albicans*
175	Female	Male	26	Black	Oct-07	HIV +ve	d4T, EFV, 3TC	7 months	White plaque in tongue		*C. albicans*
176	Female	Male	19	Black	Jun-08	AIDS	d4T, EFV, 3TC	5 months	White plaque in tongue	TB patient	*C. albicans*
177	Female	Male	38	Black	Oct-06	HIV +ve	d4T, NVP, 3TC	25 months	White plaque in tongue		Negative
178	Female	Male	46	Coloured	2005	HIV +ve	d4T, EFV, 3TC	6 months	White plaque in tongue		Negative
179	Female	Male	22	Black	2006	HIV +ve	NO		White plaque in tongue	ARV treatment defaulted	Negative
180	Female	Male	40	Black	2004	HIV +ve	d4T, NVP, 3TC	29 months	White plaque in tongue		*C. albicans*
181	Female	Male	52	Coloured	Apr-08	HIV +ve	d4T, NVP, 3TC	4 months	White plaque in tongue		*C. albicans*
182	Female	Male	31	Black	Sep-07	HIV +ve	d4T,	11	White plaque		*C. albicans*

							NVP, 3TC	months	in tongue		
183	Female	Male	34	Black	2005	AIDS	d4T, EFV, 3TC	32 months	White plaque in tongue	TB patient, ARV treatment defaulted in 2007	*C. albicans*
184	Female	Male	31	Black	1994	HIV +ve	d4T, NVP, 3TC	12 months	White plaque in tongue		*C. dubliniensis*
185	Female	Male	43	Black	2006	AIDS	d4T, EFV, 3TC	8 months	White plaque in tongue	TB patient	*C. albicans*
186	Female	Male	31	Black	2003	HIV +ve	d4T, EFV, 3TC	10 months	White plaque in tongue		*C. albicans*
187	Female	Male	32	Black	2003	HIV +ve	d4T, NVP, 3TC	1 month	White plaque in tongue	37 weeks pregnant	Negative
188	Female	Male	28	Black	1999	AIDS	d4T, NVP, 3TC	5 months	White plaque in tongue	TB patient	*C. albicans*
189	Female	Male	29	Black	2005	HIV +ve	d4T, NVP, 3TC	24 months	White plaque in tongue		Negative
190	Female	Male	32	Black	Aug-07	HIV +ve	d4T, NVP, 3TC	12 months	White plaque in tongue	20 weeks pregnant	Negative
191	Female	Male	33	Black	2001	AIDS	AZT, NVP, 3TC	36 months	White plaque in tongue	6 weeks old child	*C. albicans*
192	Male	Female	56	Black	2002	AIDS	TDF, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
193	Male	Female	42	Coloured	Sep-08	HIV +ve	d4T, NVP, 3TC	2 weeks	White plaque in tongue		Negative
194	Female	Male	32	Black	Nov-07	HIV +ve	d4T, NVP, 3TC	3 months	White plaque in tongue		*C. albicans*
195	Female	Male	35	Black	2006	HIV +ve	d4T, NVP, 3TC	7 months	White plaque in tongue		*C. albicans*
196	Female	Male	38	Black	2007	HIV +ve	d4T, NVP, 3TC	2 weeks	White plaque in tongue		*C. glabrata*
197	Female	Male	32	Black	2006	HIV +ve	NO	Starting Nov-2008	White plaque in tongue	31 weeks pregnant	*C. albicans*
198	Female	Male	25	Black	2003	HIV +ve	d4T, NVP, 3TC	3 months	White plaque in tongue		*C. albicans*
199	Female	Male	19	Black	Oct-08	HIV +ve	NO		White plaque in tongue	23 weeks pregnant	*C. albicans*
200	Female	Male	29	Black	Jun-07	HIV +ve	d4T, EFV, 3TC	14 months	White plaque in tongue		Negative
201	Female	Male	26	Black	Jul-08	HIV +ve	d4T, NVP, 3TC	2 months	NAD	24 weeks pregnant	*C. albicans*
202	Female	Male	57	Black	2004	AIDS	d4T, NVP, 3TC	8 months	White plaque in tongue	Patient complains of difficulty swallowing (oropharyngeal?)	Negative
203	Female	Male	37	Black	Feb-04	AIDS	AZT, KLT, DDI	9 months	White plaque in tongue		*C. albicans* and *C. glabrata*
204	Female	Male	40	Black	2001	HIV +ve	AZT, NVP, 3TC	38 months	White plaque in tongue		Negative
205	Female	Male	39	Black	Jan-08	HIV +ve	AZT, NVP, 3TC	10 months	White plaque in tongue		*C. albicans*

206	Female	Male	35	Black	Nov-06	HIV +ve	d4T, NVP, 3TC	20 months	White plaque in tongue		*C. albicans*
207	Female	Male	42	Coloured	2006	HIV +ve	d4T, NVP, 3TC	22 months	White plaque in tongue		*C. dubliniensis*
208	Female	Male	23	Black	2005	HIV +ve	d4T, EFV, 3TC	33 months	White plaque in tongue		Negative
209	Female	Male	25	Black	1999	HIV +ve	d4T, EFV, 3TC	7 months	White plaque in tongue		Negative
210	Female	Male	22	Black	2003	AIDS	d4T, NVP, 3TC	14 months	White plaque in tongue	10 month old child	Negative
211	Male	Female	36	Black	Jan-07	HIV +ve	d4T, NVP, 3TC	3 months	White plaque in sides and back of tongue		Negative
212	Female	Male	35	Black	2006	HIV +ve	d4T, NVP, 3TC	8 months	White plaque in tongue		Negative

Table 6 shows the cumulative results obtained from all Cameroonian patients. Data from the questionnaire and patient's folder are included, as well as the results obtained by the different culturing methods.

Table 6: Cumulative results from all HIV+ Cameroonian patients.

Patient No.	Gender	Partner's gender	Age	Race	Date diagnosed	HIV status	ARVs	Duration	Clinical presentation	Other	Results
1	Male	Female	35	Black	?	AIDS	d4T, NVP, 3TC	5 years	Oral candidiasis		Negative
2	Male	Female	49	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		Negative
3	Female	Male	51	Black	2005	HIV+	EFV, 3TC	6 years	White plaque in tongue		Negative
4	Female	Male	20	Black	2008	AIDS	d4T, EFV, 3TC	45 months	White lesion in oral mucosa	Tb patient	Negative
5	Male	Female	62	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
6	Female	Male	45	Black	2005	AIDS	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
7	Female	Male	57	Black	2002	HIV+	AZT, NVP, 3TC	9 years	White plaque in tongue		*C. albicans*
8	Female	Male	34	Black	2005	HIV+	d4T, NVP, 3TC	6 years	White plaque in tongue		Negative
9	Female	Male	48	Black	2007	AIDS	AZT, EFV, 3TC	4 years	White plaque in tongue	Tb patient	Negative
10	Female	Male	44	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White lesions in oral mucosa		*C. albicans*
11	Female	Male	40	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		*C. albicans*
12	Female	Male	40	Black	2003	AIDS	AZT, NVP, 3TC	8 years	White plaque in tongue		*C. albicans*
13	Female	Male	38	Black	2003	HIV+	AZT, NVP, 3TC	8 years	White plaque in tongue		*C. albicans*
14	Female	Male	38	Black	?	HIV+	No	n/a	White plaque in tongue		*C. albicans*
15	Female	Male	55	Black	Jul-11	HIV+	AZT, NVP, 3TC	3 months	White plaque in tongue		*C. albicans*
16	Male	Female	48	Black	2009	HIV+	d4T, NVP, 3TC	2 years	White plaque in tongue		Negative
17	Female	Male	56	Black	1996	HIV+	AZT, 3TC, lopinavir, ritonavir	6 years	White plaque in tongue		*Candida kefyr/parapsilopsis/lusitaneae*
18	Female	Male	55	Black	2006	AIDS	AZT, EFV, 3TC	5 years	White plaque in tongue	Tb patient	Negative
19	Female	Male	56	Black	90's	HIV+	AZT, NVP, 3TC	?	NAD		*C. glabrata*
20	Female	Male	36	Black	2003	HIV+	AZT, NVP, 3TC	8 years	White plaque in tongue		*Candida kefyr/parapsilopsis/l*

											usitaneae
21	Female	Male	33	Black	2007	HIV+	d4T, NVP, 3TC	4 years	White plaque in tongue		Negative
22	Male	Female	51	Black	2008	HIV+	AZT, EFV, 3TC	3 years	White plaque in tongue and oral mucosa		Negative
23	Male	Female	62	Black	2003	HIV+	AZT, NVP, 3TC	8 years	NAD		Negative
24	Male	Female	50	Black	2006	AIDS	AZT, EFV, 3TC	5 years	Oral candidiasis		*C. albicans*
25	Female	Male	59	Black	2009	AIDS	AZT, NVP, 3TC	2 years	NAD		Negative
26	Male	Female	39	Black	2007	HIV+	AZT, NVP, 3TC	4 years	Oral candidiasis		*C. albicans*
27	Female	Male	45	Black	2007	AIDS	d4T, NVP, 3TC	4 years	Oral candidiasis		Negative
28	Female	Male	46	Black	2005	AIDS	AZT, NVP, 3TC	6 years	Oral candidiasis		*C. albicans*
29	Female	Male	40	Black	Sep-10	AIDS	TDF, EFV, 3TC	1 year	White plaque in tongue	Tb patient	Negative
30	Female	Male	42	Black	2005	AIDS	d4T, NVP, 3TC	2005	Oral candidiasis		Negative
31	Female	Male	41	Black	May-09	AIDS	AZT, NVP, 3TC	30 months	Oral candidiasis		Negative
32	Male	Female	53	Black	2006	AIDS	AZT, EFV, 3TC	5 years	Oral candidiasis	Tb patient	*C. albicans*
33	Female	Male	40	Black	Oct-10	HIV+	AZT, NVP, 3TC	13 months	White plaque in tongue		*C. albicans*
34	Female	Male	46	Black	1997	AIDS	AZT, NVP, 3TC	4 years	Oral candidiasis		Negative
35	Female	Male	40	Black	Sep-11	HIV+	AZT, NVP, 3TC	1 month	White plaque in tongue		*C. albicans*
36	Male	Female	29	Black	2008	AIDS	AZT, NVP, 3TC	1 year	Oral candidiasis		*C. albicans*
37	Female	Male	49	Black	2009	HIV+	d4T, NVP, 3TC	2 years	White plaque on oral mucosa		Negative
38	Female	Male	36	Black	2007	HIV+	AZT, EFV, 3TC	4 years	White film in oral mucosa		Negative
39	Male	Female	51	Black	2010	AIDS	AZT, EFV, 3TC	1 year	White film in tongue; oral candidiasis	Tb patient	*C. glabrata*
40	Female	Male	52	Black	2009	AIDS	AZT, EFV, 3TC	2 years	Oral candidiasis		*C. albicans*
41	Male	Female	43	Black	2009	HIV+	AZT, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
42	Female	Male	30	Black	Jul-11	HIV+	AZT, EFV, 3TC	4 months	White plaque in tongue	8 months pregnant	*C. albicans*
43	Male	Female	43	Black	2002	AIDS	AZT, NVP, 3TC	7 years	White plaque in tongue; white lesion in oral mucosa	Tb patient	Negative
44	Female	Male	34	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. glabrata*

45	Female	Male	30	Black	2006	HIV+	TDF, NVP, 3TC	20 months	White plaque in tongue and oral mucosa		Negative
46	Female	Male	60	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		Negative
47	Female	Male	51	Black	2001	HIV+	AZT, NVP, 3TC	10 years	White plaque in tongue		Negative
48	Female	Male	50	Black	2010	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		*C. albicans*
49	Female	Male	36	Black	2003	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue and oral mucosa		Negative
50	Female	Male	52	Black	Jan-05	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue and oral mucosa		*C. albicans*
51	Female	Male	40	Black	2005	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
52	Female	Male	49	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
53	Male	Female	35	Black	2010	HIV+	AZT, NVP, 3TC	4 months	White plaque in tongue		Negative
54	Female	Male	39	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White lesion in oral mucosa		Negative
55	Female	Male	37	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. albicans*
56	Female	Male	45	Black	2007	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue, angular cheilitis		*C. albicans*
57	Female	Male	37	Black	2004	HIV+	AZT, EFV, 3TC	6 months	White plaque in tongue		Negative
58	Female	Male	32	Black	2009	HIV+	d4T, EFV, 3TC	2 years	White plaque in tongue, white lesion in oral mucosa		Negative
59	Female	Male	28	Black	Feb-11	AIDS	AZT, EFV, 3TC	9 months	White plaque in tongue	Tb patient	*C. albicans*
60	Female	Male	38	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
61	Female	Male	34	Black	2009	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		Negative
62	Female	Male	50	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue	Tb patient	Negative
63	Female	Male	35	Black	May-11	HIV+	AZT, EFV, 3TC	5 months	White plaque in tongue		Negative
64	Female	Male	40	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
65	Male	Female	48	Black	2004	HIV+	AZT, 3TC, lopinavir, ritonavir	7 years	White plaque in tongue		Negative
66	Female	Male	34	Black	2005	AIDS	d4T, 3TC	6 years	White plaque in tongue	Tb patient	Negative
67	Female	Male	38	Black	1998	HIV+	AZT, NVP, 3TC	4 years	White lesions in gingivae; white plaque in tongue		Negative
68	Female	Male	29	Black	2007	HIV+	AZT,	4 years	White plaque		Negative

							NVP, 3TC		in tongue		
69	Female	Male	52	Black	2005	AIDS	AZT, 3TC	6 years	White plaque in tongue	Tb patient	*C. albicans*
70	Female	Male	46	Black	2005	HIV+	AZT, NVP, 3TC	6 years	Extensive plaque in tongue		*C. albicans*
71	Female	Male	45	Black	2005	AIDS	AZT, NVP, 3TC	6 years	White plaque in tongue	Tb patient	Negative
72	Female	Male	60	Black	Jan-10	HIV+	AZT, NVP, 3TC	22 months	White plaque in tongue		*C. albicans*
73	Male	Female	40	Black	2010	HIV+	AZT, EFV, 3TC	1 year	White plaque in tongue		*C. albicans*
74	Female	Male	45	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
75	Female	Male	48	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		Negative
76	Female	Male	55	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		*C. glabrata*
77	Female	Male	38	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
78	Female	Male	30	Black	2004	HIV+	ABC, LPV/r	7 years	White plaque in tongue		Negative
79	Female	Male	38	Black	2006	AIDS	AZT, NVP, 3TC	5 years	White plaque in tongue	Tb patient	*C. albicans*
80	Male	Female	50	Black	1996	HIV+	AZT, NVP, 3TC	9 years	White plaque in tongue		*C. albicans*
81	Male	Female	47	Black	2001	HIV+	AZT, NVP, 3TC	10 years	White plaque in tongue and oral mucosa		*C. albicans*
82	Female	Male	55	Black	2002	HIV+	AZT, EFV, 3TC	9 years	White plaque in tongue		*C. albicans*
83	Female	Male	29	Black	Feb-11	HIV+	d4T, 3TC	9months	White plaque in tongue		*C. albicans*
84	Female	Male	30	Black	2005	AIDS	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. albicans*
85	Female	Male	30	Black	2010	HIV+	No (highCD 4+, starting ARV treatmen t)	n/a	White plaque in tongue		*C. tropicalis*
86	Female	Male	31	Black	Jun-10	HIV+	AZT, NVP, 3TC	16 months	White plaque in tongue		Negative
87	Female	Male	62	Black	2005	HIV+	d4T, NVP, 3TC	6 years	White plaque in tongue		Negative
88	Female	Male	70	Black	Sep-10	AIDS	TDF, NVP, 3TC	14 months	White plaque in tongue	Patient treated with fluconazol e in the past. Re-current candidiasi s.	*C. albicans*
89	Female	Male	30	Black	Jul-11	HIV+	Yes(?)	4 months	White plaque in tongue, white lesion in gingiva		Negative
90	Female	Male	69	Black	2011	AIDS	AZT,	5 months	White plaque	Tb patient	*C.*

							NVP, 3TC		in tongue and gingivae		*albicans*
91	Female	Male	59	Black	2007	AIDS	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
92	Male	Female	48	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. glabrata*
93	Male	Female	56	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. glabrata*
94	Female	Male	38	Black	2008	AIDS	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
95	Female	Male	35	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
96	Male	Female	35	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
97	Female	Male	29	Black	Sep-10	HIV+	TDF, EFV, 3TC	14 months	White plaque in tongue and oral mucosa		Negative
98	Female	Male	34	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
99	Female	Male	25	Black	2010	HIV+	AZT, EFV, 3TC	1 year	White plaque in tongue		*C. albicans*
100	Female	Male	38	Black	Dec-09	AIDS	AZT, NVP, 3TC	2 years	White plaque in tongue		*C. albicans*
101	Male	Female	42	Black	2008	HIV+	AZT, EFV, 3TC	3 years	White plaque in tongue		Negative
102	Male	Female	42	Black	2001	HIV+	d4T, NVP, 3TC	10 years	White plaque in tongue		Negative
103	Female	Male	32	Black	2006	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. albicans*
104	Female	Male	32	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		Negative
105	Female	Male	39	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
106	Female	Male	39	Black	2006	HIV+	LPV/r (Alluvia, 2nd)	5 years	White plaque in tongue		*C. albicans*
107	Male	Female	40	Black	Aug-11	HIV+	AZT, NVP, 3TC	3 months	White plaque in tongue		Negative
108	Male	Female	51	Black	2007	AIDS	AZT, EFV, 3TC	4 years	White plaque in tongue		*C. glabrata*
109	Female	Male	41	Black	2000	AIDS	AZT, NVP, 3TC	2 years	White plaque in tongue	Tb patient	*C. glabrata*
110	Female	Male	53	Black	Dec-06	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
111	Male	Female	39	Black	Oct-11	AIDS	AZT, EFV, 3TC	1 month	White plaque in tongue and oral mucosa		*C. albicans*
112	Male	Female	40	Black	Aug-10	HIV+	AZT, NVP, 3TC	15 months	White plaque in tongue, white lesion in oral mucosa		Negative
113	Female	Male	45	Black	2004	HIV+	AZT, NVP, 3TC	7 years	NAD		Negative
114	Male	Female	36	Black	2007	HIV+	AZT, NVP,	4 years	White plaque in tongue		*C. albicans*

							3TC				
115	Female	Male	52	Black	2004	HIV+	Yes(?)	5 years	White plaque in tongue		*C. albicans*
116	Female	Male	? (50-60)	Black	2010	HIV+	Yes(?)	1 year	White plaque in tongue		*C. glabrata*
117	Female	Male	39	Black	Aug-10	HIV+	TDF, 3TC	15 months	White plaque in tongue		*C. albicans*
118	Female	Male	30	Black	May-11	AIDS	AZT, NVP, 3TC	6 months	White plaque in tongue	Tb patient	*C. albicans*
119	Female	Male	57	Black	2002	HIV+	AZT, NVP, 3TC	9 years	White plaque in tongue	Patient took fluconazole recently	*Candida albicans*
120	Female	Male	59	Black	2008	HIV+	d4T, NVP, 3TC	3 years	White plaque in tongue		Negative
121	Male	Female	52	Black	2006	HIV+	No (starting now)	n/a	White plaque in tongue		*C. albicans*
122	Female	Male	55	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
123	Female	Male	39	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		Negative
124	Female	Male	41	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. glabrata*
125	Male	Female	50	Black	2005	AIDS	d4T, NVP, 3TC	6 years	White plaque in tongue	Tb patient	Negative
126	Female	Male	36	Black	2007	HIV+	LPV/r, d4T, 3TC	4 years	White plaque in tongue		Negative
127	Female	Male	58	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
128	Female	Male	37	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
129	Female	Male	57	Black	2002	AIDS	AZT, EFV, 3TC	9 years	White plaque in tongue	Tb patient	Negative
130	Female	Male	36	Black	2007	HIV+	AZT, NVP, 3TC	4 years	Extensive white plaque in tongue		Negative
131	Female	Male	30	Black	2004	AIDS	AZT, NVP, 3TC	7 years	White plaque in tongue	Tb patient	Negative
132	Female	Male	33	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
133	Male	Female	49	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		Negative
134	Male	Female	51	Black	2003	AIDS	AZT, EFV	2 years	White plaque in tongue		*C. albicans*
135	Female	Male	34	Black	2009	HIV+	AZT, EFV, 3TC	2 years	White plaque in tongue		*C. albicans*
136	Female	Male	43	Black	1997	HIV+	LPV/r, AZT, 3TC	2 years	White plaque in tongue		*C. dubliniensis*
137	Female	Male	25	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
138	Female	Male	31	Black	Feb-11	AIDS	AZT, EFV, 3TC	5 months	White plaque in tongue	Tb patient	Negative
139	Male	Female	49	Black	2001	HIV+	LPV/r	8 years	White plaque in tongue		Negative

140	Female	Male	52	Black	2004	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
141	Female	Male	57	Black	?	HIV+	Yes(?)	?	White plaque in tongue		*C. tropicalis*
142	Male	Female	32	Black	2008	HIV+	TDF, EFV, 3TC	3 years	White plaque in tongue		Negative
143	Female	Male	40	Black	Dec-10	AIDS	AZT, EFV, 3TC	1 month	White plaque in tongue		*C. albicans*
144	Female	Male	28	Black	2010	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		*C. krusei*
145	Female	Male	56	Black	2006	HIV+	AZT, EFV, 3TC	3 months	White plaque in tongue		Negative
146	Male	Female	42	Black	2004	HIV+	LPV/r, TDF, 3TC	7 years	White plaque in tongue and white lesion in oral mucosa		*C. albicans*
147	Female	Male	35	Black	2009	AIDS	AZT, NVP, 3TC	2 years	White plaque in tongue	Tb patient	Negative
148	Male	Female	45	Black	2009	HIV+	TDF, EFV, 3TC	2 years	White plaque in tongue		*C. glabrata*
149	Female	Male	60	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
150	Female	Male	50	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
151	Female	Male	50	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
152	Female	Male	32	Black	2004	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
153	Male	Female	36	Black	2010	AIDS	AZT, EFV, 3TC	1 year	White plaque in tongue	Tb patient	Negative
154	Female	Male	71	Black	Now (Nov 2011)	HIV+	No (starting now)		Extensive white plaque in tongue		*C. albicans*
155	Female	Male	42	Black	2007	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
156	Female	Male	72	Black	2006	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		*C. glabrata*
157	Female	Male	53	Black	2007	HIV+	AZT, EFV, 3TC	4 years	White plaque in tongue		Negative
158	Female	Male	39	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
159	Female	Male	38	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		*C. glabrata*
160	Female	Male	26	Black	2009	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		Negative
161	Female	Male	40	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
162	Female	Male	51	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		Negative
163	Female	Male	34	Black	2005	HIV+	LPV/r, TDF, 3TC	2 years	White plaque in tongue		Negative
164	Female	Male	35	Black	Sep-11	HIV+	AZT, NVP,	3 months	White plaque in tongue		*C. albicans*

							3TC				
165	Female	Male	38	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
166	Female	Male	61	Black	2003	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
167	Female	Male	35	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
168	Female	Male	35	Black	2007	HIV+	AZT, EFV, 3TC	4 years	White plaque in tongue		Negative
169	Female	Male	36	Black	2006	HIV+	AZT, EFV, 3TC	3 years	White plaque in tongue		Negative
170	Female	Male	30	Black	Mar-09	HIV+	d4T, NVP, 3TC	30 months	White plaque in tongue		Negative
171	Female	Male	33	Black	2006	AIDS	d4T, NVP, 3TC	5 years	White plaque in tongue	Tb patient	Negative
172	Female	Male	35	Black	2007	HIV+	d4T, NVP, 3TC	4 years	White plaque in tongue		*C. krusei*
173	Female	Male	43	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		
174	Female	Male	27	Black	2007	AIDS	AZT, NVP, 3TC	4 years	White plaque in tongue	Tb patient	*C. krusei*
175	Female	Male	70	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. albicans*
176	Female	Male	32	Black	2003	AIDS	d4T, EFV, 3TC	3 years	White plaque in tongue		Negative
177	Female	Male	53	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
178	Female	Male	38	Black	2004	HIV+	LPV/r, TDF	6 years	White plaque in tongue		*C. glabrata*
179	Female	Male	29	Black	2007	AIDS	AZT, 3TC	4 years	White plaque in tongue	*3 months pregnant*	Negative
180	Female	Male	47	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*C. albicans*
181	Female	Male	30	Black	Oct-11	HIV+	AZT, NVP, 3TC	1 week	White plaque in tongue		*C. albicans*
182	Female	Male	32	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		*C. albicans*
183	Female	Male	27	Black	2009	AIDS	AZT, NVP, 3TC	2 years	White plaque in tongue	Tb patient	*C. albicans*
184	Female	Male	36	Black	Mar-09	HIV+	d4T, NVP, 3TC	21 months	White plaque in tongue		*C. albicans*
185	Male	Female	36	Black	2005	HIV+	d4T, NVP, 3TC	6 years	White plaque in tongue		Negative
186	Male	Female	39	Black	2007	HIV+	d4T, NVP, 3TC	4 years	White plaque in tongue and white lesion in oral mucosa		*C. glabrata*
187	Female	Male	41	Black	2006	AIDS	LPV/r, TDF	5 years	White plaque in tongue	Tb patient	Negative
188	Female	Male	30	Black	2005	AIDS	d4T, NVP, 3TC	6 years	White plaque in tongue	Tb patient	Negative
189	Female	Male	36	Black	Jul-11	HIV+	AZT, NVP, 3TC	1 month	White plaque in tongue		Negative

190	Male	Female	46	Black	2004	HIV+	d4T, NVP, 3TC	7 years	White plaque in tongue		Negative
191	Female	Male	39	Black	Oct-11	HIV+	AZT, NVP, 3TC	11 days	White plaque in tongue		Negative
192	Female	Male	42	Black	2010	HIV+	TDF, EFV, 3TC	1 year	White plaque in tongue		Negative
193	Female	Male	44	Black	2008	HIV+	d4T, NVP, 3TC	3 years	White plaque in tongue		Negative
194	Female	Male	47	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
195	Male	Female	55	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
196	Female	Male	50	Black	2005	HIV+	AZT, NVP, 3TC	6 years	White plaque in tongue		*Candida glabrata*
197	Female	Male	54	Black	2003	HIV+	AZT, NVP, 3TC	8 years	White plaque in tongue		*Candida albicans*
198	Female	Male	49	Black	2002	AIDS	AZT, EFV, 3TC	9 years	White plaque in tongue	Tb patient	*Candida albicans*
199	Female	Male	25	Black	2005	HIV+	d4T, NVP, 3TC	6 years	White plaque in tongue		*Candida albicans*
200	Female	Male	37	Black	Jun-10	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		*Candida glabrata*
201	Female	Male	38	Black	2007	AIDS	AZT, EFV, 3TC	2 months	White plaque in tongue and oral candidiasis	Tb patient	*Candida albicans*
202	Male	Female	37	Black	Aug-11	HIV+	AZT, NVP, 3TC	3 months	White plaque in tongue		*Candida albicans*
203	Female	Male	45	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		Negative
204	Female	Male	42	Black	2004	AIDS	AZT, NVP, 3TC	7 years	White plaque in tongue	Tb patient	Negative
205	Female	Male	33	Black	2007	HIV+	LPV/r, AZT, 3TC	4 years	White plaque in tongue		Negative
206	Female	Male	30	Black	2010	AIDS	AZT, EFV, 3TC	1 year	White plaque in tongue	Tb patient	*C. albicans*
207	Female	Male	31	Black	Jul-11	HIV+	AZT, NVP, 3TC	3 months	White plaque in tongue		*C. albicans*
208	Female	Male	37	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		Negative
209	Female	Male	39	Black	2010	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
210	Female	Male	34	Black	2006	HIV+	AZT, NVP, 3TC	5 years	*White plaque in tongue and white lesion in oral mucosa*		Negative
211	Female	Male	50	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
212	Female	Male	42	Black	2009	AIDS	AZT, EFV, 3TC	2 years	White plaque in tongue	Tb patient	Negative
213	Female	Male	41	Black	2004	AIDS	d4T, NVP, 3TC	7 years	White plaque in tongue	Tb patient	Negative

214	Female	Male	42	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		Negative
215	Female	Male	55	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
216	Female	Male	48	Black	2010	AIDS	AZT, NVP, 3TC	1 year	White plaque in tongue	Tb patient	*C. glabrata*
217	Female	Male	50	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		Negative
218	Female	Male	38	Black	2000	AIDS	d4T, NVP, 3TC	6 years	White plaque in tongue		Negative
219	Female	Male	33	Black	2001	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue		*C. glabrata*
220	Female	Male	39	Black	2006	AIDS	AZT, EFV, 3TC	4 years	White plaque in tongue	Tb patient	*C. glabrata*
221	Female	Male	42	Black	Apr-10	HIV+	AZT, NVP, 3TC	18 months	White plaque in tongue		*C. albicans*
222	Female	Male	40	Black	2004	HIV+	LPV/r, TDF	4 years	White plaque in tongue		*C. albicans*
223	Female	Male	35	Black	2008	HIV+	AZT, EFV, 3TC	3 years	White plaque in tongue		Negative
224				Black					White plaque in tongue		Negative
225	Male	Female	38	Black	2000	AIDS	No (starting now)	n/a	White plaque in tongue		Negative
226	Female	Male	42	Black	2005	HIV+	AZT, EFV, 3TC	3 years	White plaque in tongue		*C. albicans*
227	Female	Male	47	Black	2006	HIV+	d4T, NVP, 3TC	5 years	White plaque in tongue		Negative
228	Female	Male	32	Black	2005	HIV+	d4T, NVP, 3TC	6 years	White plaque in tongue		*C. glabrata*
229	Female	Male	42	Black	Aug-11	HIV+	AZT, NVP, 3TC	2 months	White plaque in tongue		*C. albicans*
230	Female	Male	44	Black	2008	HIV+	d4T, NVP, 3TC	3 years	White plaque in tongue		Negative
231	Male	Female	53	Black	2005	HIV+	AZT, EFV, 3TC	7 years	White plaque in tongue		Negative
232	Female	Male	42	Black	2010	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		Negative
233	Male	Female	43	Black	2005	AIDS	AZT, NVP, 3TC	6 years	White plaque in tongue	Tb patient	*C. albicans*
234	Male	Female	49	Black	2001	AIDS	AZT, EFV, 3TC	10 years	White plaque in tongue	Tb patient	*C. glabrata*
235	Male	Female	62	Black	2003	HIV+	LPV/r, AZT, 3TC	8 years	White plaque in tongue		Negative
236	Female	Male	43	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue and oral mucosa		*C. albicans*
237	Female	Male	60	Black	Oct-10	AIDS	AZT, EFV, 3TC	1 year	White plaque in tongue		*C. glabrata*
238	Female	Male	33	Black	2002	HIV+	AZT, NVP, 3TC	9 years	White plaque in tongue	Patient has an 18 month old child	*C. albicans*

239	Female	Male	43	Black	1997	HIV+	d4T, NVP, 3TC	4 years	White plaque in tongue		Negative
240	Female	Male	52	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		Negative
241	Female	Male	26	Black	Sep-11	HIV+	AZT, EFV, 3TC	2 months	White plaque in tongue	22 weeks pregnancy	*C. albicans*
242	Female	Male	23	Black	Feb-11	HIV+	AZT, NVP, 3TC	4 months	White plaque in tongue	Patient had a miscarriage in Feb 2011	Negative
243	Female	Male	52	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
244	Female	Male	64	Black	2006	HIV+	AZT, NVP, 3TC	5 years	White plaque in tongue		*C. albicans*
245	Male	Female	49	Black	2005	AIDS	AZT, EFV, 3TC	2006	White plaque in tongue	Tb patient	*C. tropicalis*
246	Female	Male	52	Black	2003	HIV+	AZT, NVP, 3TC	9 years	White plaque in tongue		*C. glabrata*
247	Female	Male	50	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		*C. albicans*
248	Female	Male	53	Black	2009	HIV+	LPV/r, AZT, 3TC	2 years	White plaque in tongue		*C. albicans*
249	Female	Male	41	Black	Aug-11	HIV+	AZT, NVP, 3TC	2 months	White plaque in tongue		*C. albicans*
250	Female	Male	40	Black	2009	AIDS	AZT, EFV, 3TC	2 years	White plaque in tongue	Tb patient	*C. tropicalis*
251	Female	Male	55	Black	2008	HIV+	AZT, NVP, 3TC	3 years	White plaque in tongue		Negative
252	Female	Male	29	Black	2005	AIDS	d4T, NVP, 3TC	6 years	White plaque in tongue	Tb patient	Negative
253	Female	Male	?	Black	2004	HIV+	AZT, NVP, 3TC	7 years	White plaque in tongue		*C. albicans*
254	Female	Male	33	Black	2004	HIV+	LPV/r, TDF, 3TC	4 years	White plaque in tongue		Negative
255	Female	Male	33	Black	Nov-11	HIV+	No	n/a	White plaque in tongue		*C. albicans*
256	Female	Male	26	Black	Nov-10	HIV+	AZT, NVP, 3TC	1 year	White plaque in tongue		*C. albicans*
257	Female	Male	42	Black	2009	HIV+	AZT, NVP, 3TC	2 years	White plaque in tongue		*C. albicans*
258	Female	Male	34	Black	2007	HIV+	AZT, NVP, 3TC	4 years	White plaque in tongue and oral mucosa		*C. krusei*
259	Female	Male	30	Black	Apr-11	AIDS	d4T, EFV, 3TC	7 months	White plaque in tongue	Tb patient	
260	Female	Male	28	Black	Nov-11	AIDS	No (starting now)	n/a	White plaque in tongue		*C. albicans*
261	Female	Male	53	Black	2004	HIV+	AZT, EFV, 3TC	7 years	White plaque in tongue		*C. albicans*
262	Female	Male	37	Black	2011	HIV+	No	n/a	White plaque in tongue		Negative

Appendix 6

Tables 7, 8 and 9 show the results obtained from the fluconazole disk diffusion susceptibility testing when samples were grown in triplicate in Sabouraud (SA samples only) and YNBG media, respectively. Microcolony score: 0= a clear zone with no microcolonies, 1=a few microcolonies present, 2= moderate growth of microcolonies and 3= many microcolonies in the susceptibility area.

Table 7.: South African fluconazole susceptibility testing results in Sabouraud agar.

Patient No.	Inhibition area (mm)	Microcolonies	Interpretation	Species
3	4	0	Resistant	*C. albicans*
7	4	1	Resistant	*C. dubliniensis*
10	2	2	Resistant	*C. albicans*
11	4	0	Resistant	*C. glabrata*
12	0	Resistant	Resistant	*C. albicans*
13	0	Resistant	Resistant	*C. albicans*
14	2	3	Resistant	*C. albicans*
16	9	0	Resistant	*C. albicans*
19	7	0	Intermediate	*C. albicans*
21	1	0	Resistant	*C. albicans*
23	5	1	Resistant	*C. albicans*
24	6	1	Resistant	*C. albicans*
25	5	1	Resistant	*C. albicans*
26	0	Resistant	Resistant	*C. albicans*
27	0	Resistant	Resistant	*C. albicans*
28	5	0	Resistant	*C. albicans*
30	5	1	Resistant	*C. albicans*
36a	2	0	Resistant	*C. albicans*
36b	3	2	Resistant	*C. glabrata*
37	2	Resistant	Resistant	*C. albicans*
38	0	0	Resistant	*C. albicans*
40	0	Resistant	Resistant	*C. albicans*
41	0	Resistant	Resistant	*C. albicans*
42	11	1	Intermediate	*C. albicans*
44	8	0	Intermediate	*C. albicans*
46	1	1	Resistant	*C. albicans*
47	5	1	Resistant	*C. albicans*
48	5	1	Resistant	*C. albicans*
50	1	Resistant	Resistant	*C. albicans*
51	2	2	Resistant	*C. dubliniensis*
57	5	1	Resistant	*C. albicans*
58	7	2	Intermediate	*C. albicans*
59	5	1	Resistant	*C. dubliniensis*
60	17	1	Susceptible	*C. albicans*
61	5	1	Resistant	*C. albicans*

62	5	3	Resistant	*C. albicans*
65	2	0	Resistant	*C. albicans*
66	0	Resistant	Resistant	*C. albicans*
67	12	1	Intermediate	*C. albicans*
68	0	Resistant	Resistant	*C. albicans*
69	5	Resistant	Resistant	*C. albicans*
70	5	2	Resistant	*C. albicans*
71	5	Resistant	Resistant	*C. albicans*
72	5	1	Resistant	*C, glabrata*
73	14	1	Susceptible	*C. albicans*
77	5	0	Resistant	*C. glabrata*
78	8	0	Resistant	*C. albicans*
80	6	0	Resistant	*C. albicans*
81	11	0	Intermediate	*C. glabrata*
82	6	1	Resistant	*C. dubliniensis*
83	5	0	Resistant	*C. albicans*
85	0	Resistant	Resistant	*C. albicans*
88	1	Resistant	Resistant	*C. albicans*
89	0	Resistant	Resistant	*C. albicans*
90	0	Resistant	Resistant	*C. albicans*
92	4	0	Resistant	*C. glabrata*
93	7	0	Intermediate	*C. albicans*
94	0	Resistant	Resistant	*C. albicans*
95	0	Resistant	Resistant	*C. albicans*
96	0	Resistant	Resistant	*C. albicans*
97	6	2	Resistant	*C. albicans*
99	10	0	Intermediate	*C. albicans*
100	11	1	Intermediate	*C. albicans*
101	0	Resistant	Resistant	*C. albicans*
102	0	Resistant	Resistant	*C. albicans*
103	5	2	Resistant	*C. albicans*
105	11	0	Intermediate	*C. dubliniensis*
107	13	1	Susceptible	*C. glabrata*
109	0	Resistant	Resistant	*C. albicans*
110	6	1	Resistant	*C. albicans*
111	0	Resistant	Resistant	*C. albicans*
112	5	3	Resistant	*C. albicans*
115	5	3	Resistant	*C. albicans*
116	6	1	Resistant	*C. albicans*
117	10	0	Intermediate	*C. albicans*
118	5	0	Resistant	*C. glabrata*
119	0	Resistant	Resistant	*C. albicans*
120	0	Resistant	Resistant	*C. albicans*
122	5	1	Resistant	*C. albicans*
123	0	Resistant	Resistant	*C. albicans*
126	4	3	Resistant	*C. albicans*
127	0	Resistant	Resistant	*C. albicans*
131	7	0	Intermediate	*C. albicans*
132	0	Resistant	Resistant	*C. albicans*
134	0	Resistant	Resistant	*C. albicans*
135	10	0	Intermediate	*C. albicans*
136	14	0	Susceptible	*C. dubliniensis*

138	13	0	Susceptible	*C. dubliniensis*
141	11	0	Intermediate	*C. albicans*
142	0	Resistant	Resistant	*C. albicans*
144	0	Resistant	Resistant	*C. glabrata*
145	0	Resistant	Resistant	*C. albicans*
146	0	Resistant	Resistant	*C. albicans*
147	2	1	Resistant	*C. glabrata*
154	15	0	Susceptible	*C. dubliniensis*
155	9	1	Resistant	*C. albicans*
156	10	0	Intermediate	*C. albicans*
159	0	Resistant	Resistant	*C. albicans*
161	0	Resistant	Resistant	*C. albicans*
163	1	1	Resistant	*C. albicans*
167	14	3	Resistant	*C. albicans*
168	10	0	Intermediate	*C. albicans*
169	0	Resistant	Resistant	*C. albicans*
174	0	Resistant	Resistant	*C. albicans*
175	0	Resistant	Resistant	*C. albicans*
176	7	2	Intermediate	*C. albicans*
180	8	0	Intermediate	*C. albicans*
181	13	1	Resistant	*C. albicans*
182	0	Resistant	Resistant	*C. albicans*
183	7	3	Resistant	*C. albicans*
184	5	0	Resistant	*C. dubliniensis*
185	10	0	Intermediate	*C. albicans*
186	0	Resistant	Resistant	*C. albicans*
188	0	Resistant	Resistant	*C. albicans*
191	0	Resistant	Resistant	*C. albicans*
192	0	Resistant	Resistant	*C. albicans*
194	6	3	Resistant	*C. albicans*
195	9	0	Intermediate	*C. albicans*
196	0	Resistant	Resistant	*C. glabrata*
197	0	Resistant	Resistant	*C. albicans*
198	8	0	Intermediate	*C. albicans*
199	0	Resistant	Resistant	*C. albicans*
201	5	1	Intermediate	*C. albicans*
203a	0	Resistant	Resistant	*C. albicans*
203b	3	3	Resistant	*C. glabrata*
205	8	0	Intermediate	*C. albicans*
206	6	0	Resistant	*C. albicans*
207	5	0	Resistant	*C. dubliniensis*

Table 8.: South African fluconazole susceptibility testing results in YNBG agar.

Patient No.	Inhibition area (mm)	Microcolonies	Interpretation	Species
3	>20	0	Susceptible	*C. albicans*
7	>20	0	Susceptible	*C. dubliniensis*
10	0	res.	Resistant	*C. albicans*
11	0	res.	Resistant	*C. glabrata*
12	0	res.	Resistant	*C. albicans*
13	0	res.	Resistant	*C. albicans*
14	0	res.	Resistant	*C. albicans*
16	0	res.	Resistant	*C. albicans*
19	0	res.	Resistant	*C. albicans*
21	0	res.	Resistant	*C. albicans*
23	14	1	Susceptible	*C. albicans*
24	14	1	Susceptible	*C. albicans*
25	15	1	Susceptible	*C. albicans*
26	0	res.	Resistant	*C. albicans*
27	18	0	Susceptible	*C. albicans*
28	0	res.	Resistant	*C. albicans*
30	18	0	Susceptible	*C. albicans*
36a	3	0	Resistant	*C. albicans*
36b	9	0	Intermediate	*C. glabrata*
37	18	0	Susceptible	*C. albicans*
38	18	0	Susceptible	*C. albicans*
40	0	res.	Resistant	*C. albicans*
41	18	0	Susceptible	*C. albicans*
42	17	0	Susceptible	*C. albicans*
44	0	res.	Resistant	*C. albicans*
46	0	res.	Resistant	*C. albicans*
47	0	res.	Resistant	*C. albicans*
48	18	0	Susceptible	*C. albicans*
50	0	res.	Resistant	*C. albicans*
51	>20	0	Susceptible	*C. dubliniensis*
57	>20	0	Susceptible	*C. albicans*
58	>20	0	Susceptible	*C. albicans*
59	>20	0	Susceptible	*C. dubliniensis*
60	0	res.	Resistant	*C. albicans*
61	0	res.	Resistant	*C. albicans*
62	0	res.	Resistant	*C. albicans*
65	>20	0	Susceptible	*C. albicans*
66	0	res.	Resistant	*C. albicans*
67	17	0	Susceptible	*C. albicans*
68	0	res.	Resistant	*C. albicans*
69	17	0	Susceptible	*C. albicans*
70	17	0	Susceptible	*C. albicans*
71	0	res.	Resistant	*C. albicans*
72	14	0	Susceptible	*C, glabrata*
73	0	res.	Resistant	*C. albicans*
77	4	0	Resistant	*C. glabrata*
78	>20	0	Susceptible	*C. albicans*
80	0	res.	Resistant	*C. albicans*

81	16	0	Susceptible	*C. glabrata*
82	0	res.	Resistant	*C. dubliniensis*
83	0	res.	Resistant	*C. albicans*
85	14	1	Susceptible	*C. albicans*
88	0	res.	Resistant	*C. albicans*
89	0	res.	Resistant	*C. albicans*
90	0	res.	Resistant	*C. albicans*
92	0	res.	Resistant	*C. glabrata*
93	>20	0	Susceptible	*C. albicans*
94	0	res.	Resistant	*C. albicans*
95	0	res.	Resistant	*C. albicans*
96	0	res.	Resistant	*C. albicans*
97	0	res.	Resistant	*C. albicans*
99	>20	0	Susceptible	*C. albicans*
100	0	res.	Resistant	*C. albicans*
101	0	res.	Resistant	*C. albicans*
102	17	2	Susceptible	*C. albicans*
103	0	res.	Resistant	*C. albicans*
105	>20	0	Susceptible	*C. dubliniensis*
107	16	0	Susceptible	*C. glabrata*
109	0	res.	Resistant	*C. albicans*
110	0	res.	Resistant	*C. albicans*
111	0	res.	Resistant	*C. albicans*
112	0	res.	Resistant	*C. albicans*
115	0	res.	Resistant	*C. albicans*
116	0	res.	Resistant	*C. albicans*
117	0	res.	Resistant	*C. albicans*
118	0	res.	Resistant	*C. glabrata*
119	0	res.	Resistant	*C. albicans*
120	0	res.	Resistant	*C. albicans*
122	19	0	Susceptible	*C. albicans*
123	0	res.	Resistant	*C. albicans*
126	19	0	Susceptible	*C. albicans*
127	19	0	Susceptible	*C. albicans*
131	0	res.	Resistant	*C. albicans*
132	0	res.	Resistant	*C. albicans*
134	0	res.	Resistant	*C. albicans*
135	0	res.	Resistant	*C. albicans*
136	>20	0	Susceptible	*C. dubliniensis*
138	>20	0	Susceptible	*C. dubliniensis*
141	15	0	Susceptible	*C. albicans*
142	0	res.	Resistant	*C. albicans*
144	4	0	Resistant	*C. glabrata*
145	0	res.	Resistant	*C. albicans*
146	0	res.	Resistant	*C. albicans*
147	0	res.	Resistant	*C. glabrata*
154	>20	0	Susceptible	*C. dubliniensis*
155	0	res.	Resistant	*C. albicans*
156	0	res.	Resistant	*C. albicans*
159	0	res.	Resistant	*C. albicans*
161	0	res.	Resistant	*C. albicans*
163	0	res.	Resistant	*C. albicans*

167	>20	0	Susceptible	*C. albicans*
168	19	0	Susceptible	*C. albicans*
169	18	0	Susceptible	*C. albicans*
174	19	0	Susceptible	*C. albicans*
175	>20	1	Susceptible	*C. albicans*
176	17	0	Susceptible	*C. albicans*
180	0t	res.	Resistant	*C. albicans*
181	16	0	Susceptible	*C. albicans*
182	16	0	Susceptible	*C. albicans*
183	19	0	Susceptible	*C. albicans*
184	>20	0	Susceptible	*C. dubliniensis*
185	19	0	Susceptible	*C. albicans*
186	0	res.	Susceptible	*C. albicans*
188	0	res.	Resistant	*C. albicans*
191	17	0	Susceptible	*C. albicans*
192	15	0	Susceptible	*C. albicans*
194	18	0	Susceptible	*C. albicans*
195	>20	0	Susceptible	*C. albicans*
196	0	res.	Resistant	*C. glabrata*
197	14	0	Susceptible	*C. albicans*
198	0	res.	Resistant	*C. albicans*
199	0	res.	Resistant	*C. albicans*
201	>20	0	Susceptible	*C. albicans*
203a	0	res.	Resistant	*C. albicans*
203b	0	res.	Resistant	*C. glabrata*
205	>20	0	Susceptible	*C. albicans*
206	>20	0	Susceptible	*C. albicans*
207	>20	0	Susceptible	*C. dubliniensis*

Table 9.: Cameroonian fluconazole susceptibility testing results in YNBG agar.

Patient No.	Inhibition area (mm)	Microcolonies	Interpretation	Species
7	16	2	Susceptible	*Candida albicans*
10	17	3	Resistant	*Candida albicans*
11	17	3	Resistant	*Candida albicans*
12	17	2	Susceptible	*Candida albicans*
13	15	3	Resistant	*Candida albicans*
14	17	3	Resistant	*Candida albicans*
15	16	3	Resistant	*Candida albicans*
17	9	0	Intermediate	*Candida kefyr/parapsilopsis/lusitaneae*
19	4	0	Resistant	*Candida glabrata*
21	0	res.	Resistant	*Candida kefyr/parapsilopsis/lusitaneae*
24	4	0	Resistant	*Candida albicans*
26	16	3	Resistant	*Candida albicans*
28	0	res.	Resistant	*Candida albicans*
32	17	2	Susceptible	*Candida albicans*
33	17	3	Resistant	*Candida albicans*
35	16	3	Resistant	*Candida albicans*
36	0	res.	Resistant	*Candida albicans*
39	7	2	Intermediate	*Candida glabrata*
40	15	2	Susceptible	*Candida albicans*
41	17	2	Susceptible	*Candida albicans*
42	17	2	Susceptible	*Candida albicans*
44	7	0	Intermediate	*Candida glabrata*
48	7	0	Intermediate	*Candida albicans*
50	18	2	Susceptible	*Candida albicans*
51	>20	2	Susceptible	*Candida albicans*
55	20	2	Susceptible	*Candida albicans*
56	21	2	Susceptible	*Candida albicans*
59	17	3	Resistant	*Candida albicans*
60	19	2	Susceptible	*Candida albicans*
64	17	3	Resistant	*Candida albicans*
69	17	2	Susceptible	*Candida albicans*
70	16	3	Resistant	*Candida albicans*
72	17	2	Susceptible	*Candida albicans*
73	16	2	Susceptible	*Candida albicans*
74	17	3	Resistant	*Candida albicans*
76	5	0	Resistant	*Candida glabrata*
79	17	3	Resistant	*Candida albicans*
80	0	res.	Resistant	*Candida albicans*
81	17	3	Resistant	*Candida albicans*
82	19	0	Susceptible	*Candida albicans*
83	0	res.	Resistant	*Candida albicans*
84	0	res.	Resistant	*Candida albicans*
85	0	res.	Resistant	*Candida tropicalis*
88	0	res.	Resistant	*Candida albicans*
90	16	2	Susceptible	*Candida albicans*
92	4	0	Resistant	*Candida glabrata*

93	5	0	Resistant	*Candida glabrata*
98	17	2	Susceptible	*Candida albicans*
99	17	2	Susceptible	*Candida albicans*
100	0	res.	Resistant	*Candida albicans*
103	15	3	Resistant	*Candida albicans*
106	17	3	Resistant	*Candida albicans*
108	8	0	Intermediate	*Candida glabrata*
109	2	0	Resistant	*Candida glabrata*
111	17	2	Susceptible	*Candida albicans*
114	17	1	Susceptible	*Candida albicans*
115	15	3	Resistant	*Candida albicans*
116	11	0	Intermediate	*Candida glabrata*
117	17	0	Susceptible	*Candida albicans*
118	15	3	Resistant	*Candida albicans*
119	0	res.	Resistant	*Candida albicans*
121	0	res.	Resistant	*Candida albicans*
124	0	res.	Resistant	*Candida glabrata*
127	18	1	Susceptible	*Candida albicans*
132	18	1	Susceptible	*Candida albicans*
134	17	2	Susceptible	*Candida albicans*
135	17	2	Susceptible	*Candida albicans*
136	11	0	Intermediate	*Candida dubliniensis*
137	17	1	Susceptible	*Candida albicans*
141	13	3	Resistant	*Candida tropicalis*
143	17	2	Susceptible	*Candida albicans*
144	0	res.	Resistant	*Candida krusei*
146	18	2	Susceptible	*Candida albicans*
148	0	res.	Resistant	*Candida glabrata*
154	0	res.	Resistant	*Candida albicans*
156	0	res.	Resistant	*Candida glabrata*
158	11	0	Intermediate	*Candida albicans*
159	2	0	Resistant	*Candida glabrata*
164	16	1	Susceptible	*Candida albicans*
172	0	res.	Resistant	*Candida krusei*
174	0	res.	Resistant	*Candida krusei*
175	17	2	Susceptible	*Candida albicans*
177	16	0	Susceptible	*Candida albicans*
178	10	0	Intermediate	*Candida glabrata*
180	17	2	Susceptible	*Candida albicans*
181	17	2	Susceptible	*Candida albicans*
182	0	res.	Resistant	*Candida albicans*
183	17	2	Susceptible	*Candida albicans*
184	6	0	Resistant	*Candida albicans* with pseudohyphae
186	0	res.	Resistant	*Candida glabrata*
196	5	0	Resistant	*Candida glabrata*
197	17	2	Susceptible	*Candida albicans*
198	17	2	Susceptible	*Candida albicans*
199	17	2	Susceptible	*Candida albicans*
200	7	0	Intermediate	*Candida glabrata*
201	11	0	Intermediate	*Candida albicans*
202	8	0	Intermediate	*Candida albicans*

206	0	res.	Resistant	*Candida albicans* with pseudohyphae
207	13	0	Susceptible	*Candida albicans*
216	0	res.	Resistant	*Candida glabrata*
219	5	0	Resistant	*Candida glabrata*
220	0	res.	Resistant	*Candida glabrata*
221	10	0	Intermediate	*Candida albicans*
222	10	0	Intermediate	*Candida albicans*
226	12	0	Intermediate	*Candida albicans* with pseudohyphae
228	>20	1	Susceptible	*Candida glabrata*
229	8	0	Intermediate	*Candida albicans*
233	5	1	Resistant	*Candida albicans*
234	4	1	Resistant	*Candida glabrata*
236	7	0	Intermediate	*Candida albicans*
237	4	0	Resistant	*Candida glabrata*
238	6	0	Resistant	*Candida albicans* with pseudohyphae
241	17	2	Susceptible	*Candida albicans*
244	0	res.	Resistant	*Candida albicans*
245	0	res.	Resistant	*Candida tropicalis*
246	0	res.	Resistant	*Candida glabrata*
247	17	2	Susceptible	*Candida albicans*
248	0	res.	Resistant	*Candida albicans*
249	17	2	Susceptible	*Candida albicans*
250	0	res.	Resistant	*Candida tropicalis*
253	0	res.	Resistant	*Candida albicans*
255	0	res.	Resistant	*Candida albicans*
256	17	2	Susceptible	*Candida albicans*
257	17	2	Susceptible	*Candida albicans*
258	4	0	Resistant	*Candida krusei*
260	0	res.	Resistant	*Candida albicans*
261	18	2	Susceptible	*Candida albicans*

Appendix 7

The following table shows the statistical association between *Candida* species and duration of ARV treatment seen in the Cameroonian population, using the SPSS 21.0 statistics software.

CAM spp vs ART duration Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	33.692[a]	20	.028	.111		
Likelihood Ratio	28.897	20	.090	.016		
Fisher's Exact Test	32.315			**p=0.034**		
Linear-by-Linear Association	2.867[b]	1	.090	.096	.041	.009
N of Valid Cases	126					

a. 25 cells (83.3%) have expected count less than 5. The minimum expected count is .02.

b. The standardized statistic is 1.693.

Appendix 8

The following tables show the chi-square results and symmetric measures of the TREK susceptibility test done on South African and Cameroonian *Candida* species, using the SPSS 21.0 statistics software.

South African results:

Chi-Square Tests for Amphotericin B

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	11.499[a]	2	.003	.009		
Likelihood Ratio	8.081	2	.018	.014		
Fisher's Exact Test	8.724			**p=0.010**		
Linear-by-Linear Association	2.302[b]	1	.129	.156	.107	.056
N of Valid Cases	128					

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 1.17.

b. The standardized statistic is 1.517.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.300	.003	.009
	Cramer's V	.300	.003	.009
N of Valid Cases		128		

Chi-Square Tests for Anidulafungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	128.317[a]	4	.000	.000		
Likelihood Ratio	70.439	4	.000	.000		
Fisher's Exact Test	63.041			**p=0.000**		
Linear-by-Linear Association	84.371[b]	1	.000	.000	.000	.000
N of Valid Cases	128					

a. 5 cells (55.6%) have expected count less than 5. The minimum expected count is .47.

b. The standardized statistic is 9.185.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.001	.000	.000
	Cramer's V	.708	.000	.000
N of Valid Cases		128		

Chi-Square Tests for Caspofungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	132.214[a]	4	.000	.000		
Likelihood Ratio	73.109	4	.000	.000		
Fisher's Exact Test	65.264			**p=0.000**		
Linear-by-Linear Association	82.463[b]	1	.000	.000	.000	.000
N of Valid Cases	128					

a. 4 cells (44.4%) have expected count less than 5. The minimum expected count is .78.

b. The standardized statistic is 9.081.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.016	.000	.000
	Cramer's V	.719	.000	.000
N of Valid Cases		128		

Chi-Square Tests for Micafungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	128.000[a]	2	.000	.000		
Likelihood Ratio	70.186	2	.000	.000		
Fisher's Exact Test	63.098			**p=0.000**		
Linear-by-Linear Association	95.886[b]	1	.000	.000	.000	.000
N of Valid Cases	128					

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is .78.

b. The standardized statistic is 9.792.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.000	.000	.000
	Cramer's V	1.000	.000	.000
N of Valid Cases		128		

Chi-Square Tests for 5-Flucytosine

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	.849[a]	2	.654	1.000		
Likelihood Ratio	1.253	2	.534	.890		
Fisher's Exact Test	.932			**p=0.685**		
Linear-by-Linear Association	.126[b]	1	.722	1.000	.539	.224
N of Valid Cases	128					

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .47.

b. The standardized statistic is -.355.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.081	.654	1.000
	Cramer's V	.081	.654	1.000
N of Valid Cases		128		

Chi-Square Tests for Fluconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	6.667[a]	2	.036	**p=0.032**		
Likelihood Ratio	7.594	2	.022	.028		
Fisher's Exact Test	6.694			.028		
Linear-by-Linear Association	6.575[b]	1	.010	.014	.006	.004
N of Valid Cases	128					

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 4.53.

b. The standardized statistic is -2.564.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.228	.036	.032
	Cramer's V	.228	.036	.032
N of Valid Cases		128		

Chi-Square Tests for Itraconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	9.658[a]	2	**p=0.008**	.006		
Likelihood Ratio	10.512	2	.005	.008		
Fisher's Exact Test	9.597			.007		
Linear-by-Linear Association	5.864[b]	1	.015	.021	.012	.007
N of Valid Cases	128					

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 4.38.

b. The standardized statistic is -2.422.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.275	.008	.006
	Cramer's V	.275	.008	.006
N of Valid Cases		128		

Chi-Square Tests for Voriconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	18.037[a]	2	**p=0.000**	.000		
Likelihood Ratio	23.475	2	.000	.000		
Fisher's Exact Test	19.702			.000		
Linear-by-Linear Association	14.219[b]	1	.000	.000	.000	.000
N of Valid Cases	128					

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 4.53.

b. The standardized statistic is -3.771.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.375	.000	.000
	Cramer's V	.375	.000	.000
N of Valid Cases		128		

Cameroonian results:

Chi-Square Tests for Amphotericin B

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	30.865[a]	5	.000	.002		
Likelihood Ratio	16.499	5	.006	.002		
Fisher's Exact Test	20.455			**p=0.001**		
Linear-by-Linear Association	18.055[b]	1	.000	.001	.001	.000
N of Valid Cases	126					

a. 9 cells (75.0%) have expected count less than 5. The minimum expected count is .08.

b. The standardized statistic is 4.249.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.495	.000	.002
	Cramer's V	.495	.000	.002
N of Valid Cases		126		

Chi-Square Tests for Anidulafungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	153.304[a]	10	.000	.000		
Likelihood Ratio	48.224	10	.000	.000		
Fisher's Exact Test	49.353			**p=0.000**		
Linear-by-Linear Association	30.362[b]	1	.000	.000	.000	.000
N of Valid Cases	126					

a. 15 cells (83.3%) have expected count less than 5. The minimum expected count is .02.

b. The standardized statistic is 5.510.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.103	.000	.000
	Cramer's V	.780	.000	.000
N of Valid Cases		126		

Chi-Square Tests for Caspofungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	153.304[a]	10	.000	.000		
Likelihood Ratio	48.224	10	.000	.000		
Fisher's Exact Test	49.353			**p=0.000**		
Linear-by-Linear Association	30.362[b]	1	.000	.000	.000	.000
N of Valid Cases	126					

a. 15 cells (83.3%) have expected count less than 5. The minimum expected count is .02.

b. The standardized statistic is 5.510.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.103	.000	.000
	Cramer's V	.780	.000	.000
N of Valid Cases		126		

Chi-Square Tests for Micafungin

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	225.968[a]	10	.000	.000		
Likelihood Ratio	111.921	10	.000	.000		
Fisher's Exact Test	107.397			**p=0.000**		
Linear-by-Linear Association	33.862[b]	1	.000	.000	.000	.000
N of Valid Cases	126					

a. 15 cells (83.3%) have expected count less than 5. The minimum expected count is .02.

b. The standardized statistic is 5.819.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	1.339	.000	.000
	Cramer's V	.947	.000	.000
N of Valid Cases		126		

Chi-Square Tests for 5-Flucytosine

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	6.319[a]	5	.276	.267		
Likelihood Ratio	5.755	5	.331	.189		
Fisher's Exact Test	6.756			**p=0.265**		
Linear-by-Linear Association	.074[b]	1	.786	.888	.404	.106
N of Valid Cases	126					

a. 9 cells (75.0%) have expected count less than 5. The minimum expected count is .06.

b. The standardized statistic is .272.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.224	.276	.267
	Cramer's V	.224	.276	.267
N of Valid Cases		126		

Chi-Square Tests for Fluconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	9.899[a]	5	.078	.040		
Likelihood Ratio	12.614	5	.027	.038		
Fisher's Exact Test	9.167			**p=0.041**		
Linear-by-Linear Association	4.103[b]	1	.043	.045	.024	.009
N of Valid Cases	126					

a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .45.

b. The standardized statistic is -2.026.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.280	.078	.040
	Cramer's V	.280	.078	.040
N of Valid Cases		126		

Chi-Square Tests for Itraconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	9.574[a]	5	.088	.051		
Likelihood Ratio	11.034	5	.051	.075		
Fisher's Exact Test	9.170			**p=0.044**		
Linear-by-Linear Association	.050[b]	1	.823	.865	.451	.067
N of Valid Cases	126					

a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .43.

b. The standardized statistic is .224.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.276	.088	.051
	Cramer's V	.276	.088	.051
N of Valid Cases		126		

Chi-Square Tests for Voriconazole

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	20.540[a]	5	.001	.000		
Likelihood Ratio	26.501	5	.000	.000		
Fisher's Exact Test	21.858			**p=0.000**		
Linear-by-Linear Association	9.633[b]	1	.002	.002	.000	.000
N of Valid Cases	126					

a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .38.

b. The standardized statistic is -3.104.

Symmetric Measures

		Value	Approx. Sig.	Exact Sig.
Nominal by Nominal	Phi	.404	.001	.000
	Cramer's V	.404	.001	.000
N of Valid Cases		126		

Appendix 9

The following table shows the statistical association between *Candida* species and duration of ARV treatment, azole and echinocandin susceptibility, respectively, seen in the combined female population, using the SPSS 21.0 statistics software.

Chi-Square Tests for Species vs ARV duration

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	55.070[a]	20	.000	.[b]		
Likelihood Ratio	34.609	20	.022	.007		
Fisher's Exact Test	33.674			**.008**		
Linear-by-Linear Association	5.423[c]	1	.020	.020	.010	.002
N of Valid Cases	195					

a. 23 cells (76.7%) have expected count less than 5. The minimum expected count is .02.

b. Cannot be computed because there is insufficient memory.

c. The standardized statistic is 2.329.

Chi-Square Tests for Species vs Azoles

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	22.378[a]	5	.000	.000		
Likelihood Ratio	26.003	5	.000	.000		
Fisher's Exact Test	22.137			**.000**		
Linear-by-Linear Association	9.972[b]	1	.002	.001	.000	.000
N of Valid Cases	195					

a. 8 cells (66.7%) have expected count less than 5. The minimum expected count is .92.

b. The standardized statistic is -3.158.

Chi-Square Tests for Species vs Echinocandins

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	255.767[a]	10	.000	.000		
Likelihood Ratio	116.243	10	.000	.000		
Fisher's Exact Test	108.772			**.000**		
Linear-by-Linear Association	67.416[b]	1	.000	.000	.000	.000
N of Valid Cases	195					

a. 13 cells (72.2%) have expected count less than 5. The minimum expected count is .10.

b. The standardized statistic is 8.211.

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Strengths and Limitations of different Chromogenic Media for the Identification of *Candida* Species

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Abstract The treatment of invasive candidiasis and other *Candida* infections with the appropriate antifungal agent is assisted by the identification of *Candida* isolates to the species level. Rapid and accurate methods of differentiation are therefore imperative if treatment is to be effective, particularly in HIV-positive patients and in pregnant mothers where intervention may be necessary to reduce the risk for preterm delivery. The time used for isolation, identification and detection of mixed cultures may be reduced with the help of available chromogenic media. In this study, five commercial chromogenic media were evaluated for the differentiation of *Candida* species. Six type-strains of *Candida* species were streaked onto each of five different chromogenic media and incubated for up to 4 days at the different temperatures recommended by the manufacturers. This comparative evaluation demonstrated the strengths and weaknesses of each medium employed and found CHROMagar™ Candida and Chromogenic Candida Agar to be the most effective for distinguishing between different *Candida* species.

Keywords *Candida*, Chromogenic Agar, Rapid Species Differentiation

1. Introduction

There has been a significant increase in the number of *Candida* resistant cases in hospital patients in the last 20 years. Predisposing factors include particularly prolonged and increased use of antifungal agents[1] and patients with compromised immune systems, such as HIV-positive patients[2] and pregnant mothers with asymptomatic vaginal candidiasis who run the risk of preterm delivery[3]. Amongst the species most frequently isolated are *Candida albicans* followed by *Candida glabrata*, *Candida tropicalis* and *Candida krusei*[4].

In health, *Candida albicans* is a harmless commensal fungus, while, in immunocompromised patients, it may cause superficial or even life-threatening systemic infections[5]. It is not entirely understood how the mechanisms of change from a non-pathogenic to a pathogenic phenotype occurs. Knowledge of the metabolic activity of *Candida albicans* remains limited even though a great deal of research has been done on aspects of its pathogenicity[5].

Candida dubliniensis is a fairly recently described species of *Candida* with similar characteristics to that of *Candida albicans*. It is clinically important to compare the pathogenesis and management of infection by a newly discovered species, with infection caused by other members of the same genus[6]. *Candida albicans* and *Candida dubliniensis* have the same morphological and physiological characteristics due to the close association in their phylogenetics, e.g. germ-tube and chlamydospore formation[6]. This has caused a problem in differentiating between the two species, with the result that *Candida dubliniensis* strains have been, and will continue to be, identified in the clinical laboratory as *Candida albicans*[6]. To make a precise differentiation between the two species requires PCR-based tests, but due to the high quantities of throughput samples at diagnostic laboratories, this is not feasible and thus PCR-based tests are mostly used in research laboratories[7]. Looking at the phenotypic characteristics is much more inexpensive than that of the genotypic characteristics, and scientists have therefore demonstrated the use of selective and differential media for the presumptive identification of *Candida* species with good sensitivity and specificity[8], thereby reducing the time used for isolation, identification and detection in mixed cultures[9].

The purpose of this study was to perform a comparative evaluation of five different chromogenic media in order to establish which would yield the most reliable differentiation of frequently isolated *Candida* species namely, *Candida albicans*, *Candida dubliniensis*, *Candida tropicalis*, *Candida krusei* and *Candida glabrata*.

2. Materials and Methods

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2.1. Type-strains of *Candida* Used

A total of six type-strains of *Candida* species were used for the evaluation of the five chromogenic media. Of these type-strains, *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 950), *C. krusei* (ATCC 2159), *C. glabrata* (ATCC 26512) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA.) and *C. albicans* (NCPF 3281) and *C. dubliniensis* (NCPF 3949a) from the National Collection of Pathogenic Fungi (NCPF, Bristol, United Kingdom). These type-strains were stored in frozen stocks in cryovials at -70℃ and cultured twice on Sabouraud's dextrose agar (Oxoid, CM 0041) for 24 hours prior to the inoculation of the chromogenic media.

2.2. Inoculation of Chromogenic Media

Chromogenic media used, included commercially prepared CandiSelect™4 Agar (Bio-Rad, 63746) while, Chromogenic Candida Agar (Oxoid, CM1002A), Bismuth Sulphite Glucose Glycine Yeast agar (BiGGY Agar) (Oxoid, CM0589B) also known as Nickerson's medium[10], modified *Candida* Ident Agar, (Fluka, 94382) and CHROMagar™ Candida (CHROMagar, CA220) were purchased in a dehydrated form and prepared according to the manufacturers' instructions. All plates were left to reach room temperature prior to inoculation if previously stored at -4℃. Type-strains of *Candida* species were inoculated onto the different chromogenic media and each incubated for up to 4 days at the different temperatures recommended by the manufacturers. This was done in triplicate. CandiSelect™4 Agar and CHROMagar™ Candida were incubated at 37℃, modified *Candida* Ident Agar, and Chromogenic Candida Agar were incubated at 30℃, and BiGGY Agar was incubated at 28-30℃. The plates were checked after 24, 48, 72 and 96hrs for growth to determine when (according to the manufacturers' claims) the expected colour, morphology or texture of the colonies appeared, and whether prolonged incubation would affect the results.

2.3. Statistical Analysis

Because of the small sample size no meaningful statistical analyses could be performed.

3. Results

All the type-strains grew on the five different chromogenic media. Some type-strains were more distinguishable than others. The appropriate colour, texture and morphology of the colonies were observed after each 24-hour period for a total of 96 hours and compared with the recommended time period of the manufacturers. Some chromogenic media characterized the different type-strains by colour only while others characterized them by colour, texture and morphology.

Both *C. albicans* type-strains (ATCC 90028 and NCPF 3281) appeared as predicted on CHROMagar™ Candida, modified Candida Ident, and Chromogenic Candida Agar (Table 1). They appeared as pink colonies after 24 hours, which darkened to purple after incubation for 48 hours on CandiSelect™4 Agar. On BiGGY Agar, the predicted colour reactions for *C. albicans* were expressed, while the expected mycelial fringe was not observed even after prolonged incubation of 96 hours. (Table 1)

Table 1. Ability of Chromogenic Media to Accurately Differentiate *Candida albicans* From Other *Candida* Species

Agar	Incubation	Predicted	Observed
CHROMagar™ Candida agar @ 37 ℃	48hrs	green	green-turquoise
Candida Ident Agar, (modified) @ 30 ℃	18-24hrs	light green	light green
Chromogenic Candida Agar @ 30 ℃	24hrs	green	green
CandiSelect™4 Agar @ 37 ℃	24hrs	pink-purple	pink
	48hrs	purple	purple
BiGGY Agar @ 28-30 ℃	48-96hrs	smooth, circular brown-black with slight mycelial fringe	smooth, circular brown colonies. No mycelial fringe even after 96hrs

Colonial morphology of *C.dubliniensis* differed from the predicted patterns for all 5 of the chromogenic media used (Table 2). Although the guidelines for CHROMagar™ Candida, modified Candida Ident Agar and BIGGY Agars predicted that *C.dubliniensis* could not be distinguished, results on the CHROMagar™ Candida revealed that *C. albicans* and *C. dubliniensis* could clearly be distinguished with *C. albicans* colonies yielding a green-turquoise colour while *C. dubliniensis* appeared plain green after 48 hours incubation (Table 2). After a longer incubation period (96 hours), no change was observed in *C. albicans* while colonies of *C. dubliniensis* formed a darker centre, a characteristic clearly distinguishing it from *C. albicans* (Fig. 1a,b).

Chromogenic Candida Agar guidelines predicted a green colour, but we observed translucent light-blue colonies after 24 hours which intensified to dark blue on prolonged incubation of 96 hours (Fig. 1c,d). Prolonged incubation was also required for CandiSelect™4 Agar since the pink-purple colonies predicted after 24 hours only appeared after 72 hours of incubation (Table 2).

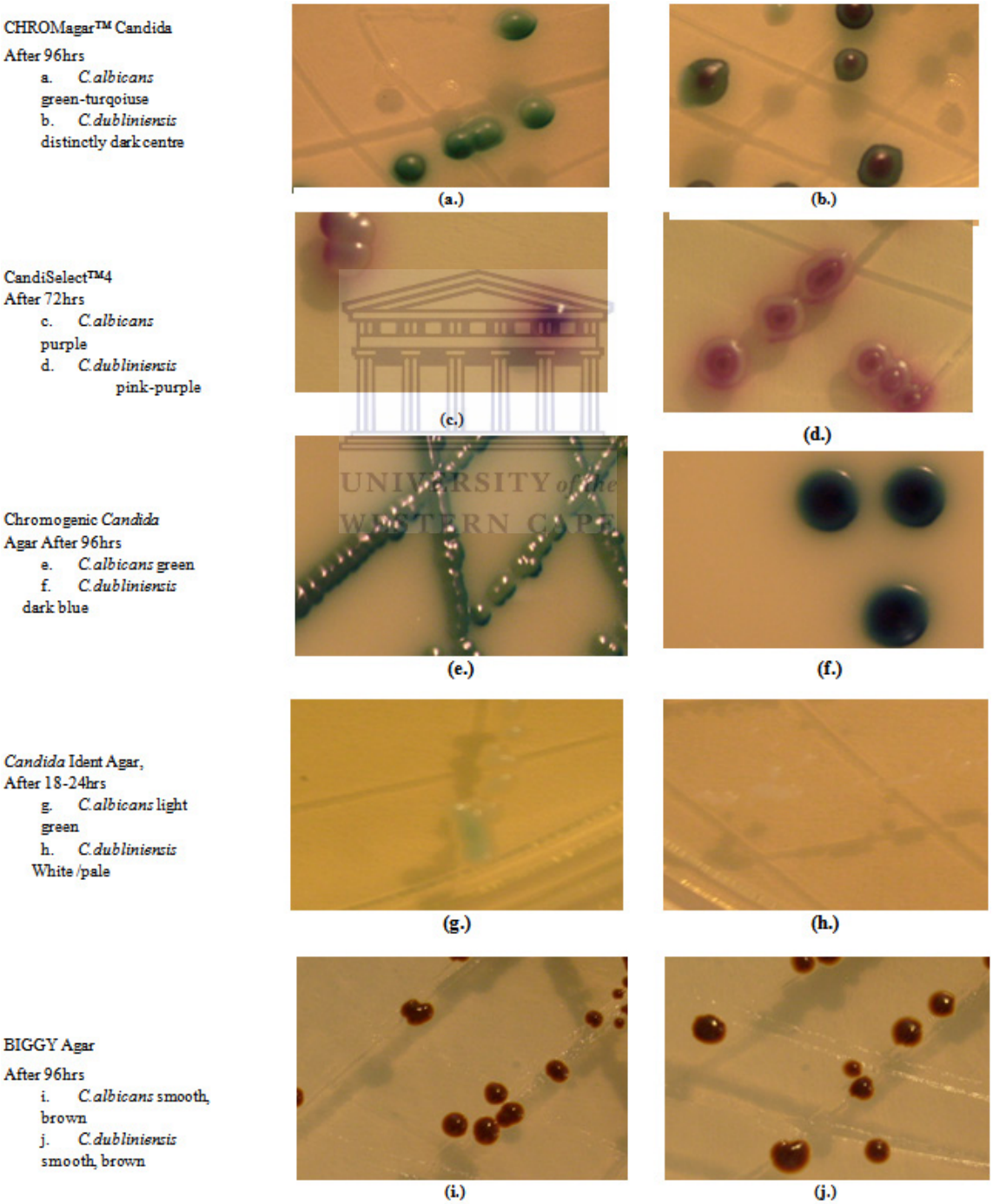

Figure 1. Differentiation of *C.albicans* and *C.dubliniensis* Using Different Chromogenic Media

Table 2. Ability of Chromogenic Media to Accurately Differentiate *Candida dubliniensis* From Other *Candida* Species

Agar	Incubation	Predicted	Observed
CHROMagar™ Candida agar @ 37°C	48hrs	not distinguishable from *C.albicans*	plain green,
	96hrs		green with a dark centre
Candida Ident Agar, (modified) @ 30°C	18-24hrs	ND*	white colonies
	48hrs		metallic green
Chromogenic Candida Agar @ 30°C	24hrs	green	translucent light blue
	25-48hrs		blue
	96hrs		dark-blue
CandiSelect™4 Agar @ 37°C	24-48hrs	Pink to purple	Light pink
	72hrs		pink-purple
BiGGY Agar @ 28-30°C	48hrs	ND*	smooth irregular shaped, light brown
	96hrs		smooth irregular shaped brown

*Not distinguishable from other *Candida* species

Table 3. Ability of Chromogenic Media to Accurately Differentiate *Candida glabrata* From Other *Candida* Species

Agar	Incubation	Predicted	Observed
CHROMagar™ Candida agar @ 37°C	48hrs	not distinguishable (but other species white to mauve)	mauve-colour
Candida Ident Agar, (modified) @ 30°C	18-24hrs	cream white	cream-white to slight pink
Chromogenic Candida Agar @ 30°C	24hrs	variable, natural pigment	beige-cream to light brown
	24-48hrs (max 72hrs)	variable, natural pigment	brown with slight signs of pink
CandiSelect™4 Agar @ 37°C	24hrs	ND*	pale turquoise, flat, shiny, smooth, turquoise center and small white periphery
	48hrs	pale turquoise, flat, shiny, smooth, turquoise center and white periphery	dark turquoise, flat, shiny, smooth, dark turquoise center and white periphery
BiGGY Agar @ 28-30°C	48hrs	not distinguishable	small, cream, opaque

CHROMagar™ Candida, modified Candida Ident Agar, and BIGGY Agar were not able to distinguish *C. glabrata* from other *Candida* species (Table 3), while Candiselect™4 Agar yielded the predicted pale turquoise colonies after 24 hours, which darkened to deep turquoise centred colonies with white peripheries after 48 hours. Chromogenic Candida Agar produced beige-cream to light brown colonies. However, this did not distinguish them from other *Candida* species but when incubated for longer than 72 hours, the colonies started to turn pink.

All of the 5 chromogenic media yielded the predicted results for *C. krusei* (Table 4). Although the guidelines mention silver brown-black, we concede that the reflection of the light in the dark brown colonies could have been interpreted by us as gold rather than silver.

The CHROMagar™ Candida and modified Candida Ident Agar, guidelines predict a metallic blue colony for *C.tropicalis*, but we observed dark-purple blue colonies on the CHROMagar™ Candida (Table 5) and light lilac colonies on modified Candida Ident Agar after 24 hours, which intensified to blue after 48 hours. Neither of the agars grew colonies with a metallic sheen. Growth on CandiSelect™4 Agar appeared to match the overall morphology as described in the guidelines, but the colonies appeared blue and not turquoise in colour. Likewise, the colonies appeared to be similar to the BiGGY Agar guidelines, but no mycelial fringe was evident, nor did the media blacken after 72 hours.

Following the pilot study using only the type-strains, clinical strains from our laboratory collection, previously identified as *C. albicans*, *C. dubliniensis*, *C. krusei*, *C. glabrata* and *C. tropicalis* were also compared for consistency in the evaluation of the chromogenic media. Colony colour and morphology observations from the different clinical strains showed the same results as the type-strains for all chromogenic agars.

Table 4. Ability of Chromogenic Media to Accurately Differentiate *Candida krusei* From Other *Candida* Species

Agar	Incubation	Predicted	Observed
CHROMagar™ Candida agar @ 37°C	48hrs	pink, fuzzy	rough, pink, dry, fuzzy
Candida Ident Agar, (modified) @ 30°C	18-24hrs	purple, fuzzy	light purple, fuzzy
Chromogenic Candida Agar @ 30°C	24-72hrs	brown or pink, dry, irregular	pink with beige to brown periphery, irregular
CandiSelect™4 Agar @ 37°C	24-48hrs	turquoise-blue, rough, dry appearance, irregular	turquoise-blue, rough, dry ,irregular
BiGGY Agar @ 28-30°C	48hrs	large, flat, wrinkled silvery brown-black with brown peripheries; yellow halo diffused into medium	flat, wrinkled, gold glittery dark brown, brown periphery; no halo diffused into medium

Table 5. Ability of Chromogenic Media to Accurately Differentiate *Candida tropicalis* From Other *Candida* Species

Agar	Incubation	Predicted	Observed
CHROMagar™ Candida agar @ 37°C	48hrs	metallic blue	dark purple-blue no metallic appearance
Candida Ident Agar, (modified) @ 30°C	18-24hrs	blue-metallic blue	light lilac
	48hrs		blue
Chromogenic Candida Agar @ 30°C	24-72hrs	blue	blue
CandiSelect™4 Agar @ 37°C	24hrs	ND*	white to light turquoise, mat, uniformly coloured, convex, smooth
	48hrs	intense turquoise, mat, uniformly coloured, convex, smooth	blue, mat, uniformly coloured, convex, smooth
BiGGY Agar @ 28-30°C	48hrs	smooth, dark brown with black centre with mycelial fringe	smooth, dark brown with slightly darker centre, no mycelial fringe
	72hrs	diffuse blackening of media after 72hrs	no diffuse blackening of media, no mycelial fringe

4. Discussion

This study evaluated CHROMagar™ Candida, *Candida* Ident Agar (modified), Chromogenic Candida Agar, CandiSelect™4 Agar and BiGGY Agar for their efficacy in the presumptive identification and differentiation of *Candida* species. An appropriate primary culture medium that assists in the recovery and differentiation of colonies which are phenotypically similar is a vital requirement for the laboratory detection of mixed fungal clinical specimens. Traditional methods for identification of yeast pathogens involves several days and specific mycology media while chromogenic media contains chromogenic substrates which react with enzymes secreted by the organisms to give colour reactions for different species[9] thus complementing traditional methods of identification[11]. CHROMagar™ Candida is the most well-known and widely used chromogenic medium for the identification of different *Candida* species and is the most expensive of the five chromogenic media. Results from mixed cultures are reported to provide results 24 to 48 hours sooner than standard isolation and identification methods. It contains a variety of substrates which interact with the enzymes secreted by the yeast species and has been reported to selectively isolate and identify *Candida* species with a high degree of accuracy[12] sensitivity and specificity[13].

As in our study, previous studies reported green colonies for *C. albicans*[12],[14] dark blue colonies for *C. tropicalis* and pink colonies with a downy appearance for *C.krusei*[8]. Although not clearly distinguishable after 48 hours, prolonged incubation (96 hours) proved useful for differentiating *C.albicans* from *C.dubliniensis*. Modified *Candida* Ident Agar, is a new chromogenic medium on which, we assume, not much research has been done. In this study, modified *Candida* Ident Agar and CHROMagar™ differentiated between the different *Candida* species by colour only. *C. krusei* however, could be differentiated by both colour and texture on both media. With the exception of *C. glabrata*, a more accurate colour expression of the other three species occurred after 48 hours of incubation, which suggested that the colours and texture description of the specific species of *Candida* presented by *Candida* Ident Agar would have been more accurate following an incubation of 48 hours rather than 24 hours. These results confirm that this medium does not reflect the appropriate results suggested by the manufacturer and therefore is not as effective in the differentiation of *Candida* species.

Chromogenic Candida Agar (Oxoid) has been re-named "Oxoid Brilliance Candida Agar" but in this study, we refer to it as "Chromogenic Candida Agar". It is a new commercial ready-to-use chromogenic medium, contains chromogenic substrates which react with the different enzymes of species of *Candida*, such as hexosaminidase and alkaline phosphatase resulting in the expression of a specific colour in the colony. The different colours appear as a result of different enzymes produced by the different species[15]. *C. albicans*, *C. dubliniensis* and *C. tropicalis* produce the enzyme hexosaminidase which results in the colonies being green, but *C. tropicalis* yields dark blue colonies due to other metabolic reactions causing a drop in pH[15]. *C. krusei* yielded brown or pink colonies because it produces alkaline phosphatase and due to a combination of natural pigmentation and some alkaline phosphatase activity, *C. glabrata* yielded a variety of natural colour, such as beige, brown and yellow.

CandiSelect™4 Agar (Bio-Rad) contains two chromogenic substrates which interact with hexosaminidase and phosphatase produced by the different *Candida* species[4], while a combination of antibiotics such as chloramphenicol and gentamicin may suppress bacterial growth. In this study, *C. albicans*, *C. krusei* and *C. glabrata* yielded results described by the manufacturer while the other type-strains did not, thus questioning the reliability of this medium. Each of the different species of *Candida* requires different incubation periods on this medium. Similar results have been reported[4] for *C. krusei*, while identification of *C. tropicalis* and *C. glabrata* were regarded as presumptive only.

In this study, BiGGY Agar was not able to distinguish the different *Candida* species due to the fact that all the type-strains were in the same colour range and that the distinctive characteristics such as the mycelial fringe scarcely occurred and when it did occur, it was never at the recommended incubation period.

We have just touched on the differentiation between *Candida albicans* and *Candida dubliniensis*, since, in addition to looking at the other commonly isolated *Candida* species, we were also interested in establishing whether adj usting incubation times of chromogenic media could adequately differentiate between *Candida albicans* and *Candida dubliniensis*. We believe that we have achieved this.

5. Conclusions

The expression of antifungal susceptibility among different *Candida* species and the misidentification of *C. dubliniensis* as *C. albicans* highlights the potential clinical importance of accurate species differentiation. The use of chromogenic media for the rapid and effective identification of *Candida* species has gained popularity within the clinical laboratory but presents with limitations in that inaccuracies often occur between the reactions described by the manufacturer and the actual results obtained in the laboratory. Differences in colonial morphology may occur as a result of differences in the laboratory conditions under-which the experiments are conducted e.g. the water used for media preparation may be of a different purity, thus affecting the substrate in the medium and thereby producing a different colour expression for specific species. Differences in colour and reflection perceptions by different examiners should also be taken into account. By employing several chromogenic media and optimising the incubation periods for each species,

sometimes deviating from the recommendations of the manufacturers, we were able to establish which media produced the most reliable and consistent results and thus accurately differentiate the *Candida* species commonly infecting HIV-positive individuals and pregnant *Candida*-infected mothers.

This comparative evaluation proved that CHROMagar™ Candida and Chromogenic Candida Agar were the most effective of the chromogenic media evaluated and both yielded the expected colour colonies at the expected time period of incubation as suggested by the manufacturer. Candida Ident Agar (modified) and CandiSelect™4 Agar only yielded results typical of three of the type-strains as suggested by the manufacturer, while BiGGY Agar yielded all of the type-strains in one colour range and none of the differentiating morphological characteristics predicted were ever observed. In order to eliminate inaccuracies in the presumptive identification of *C. dubliniensis*, we strongly support the use of CHROMagar™ Candida since this medium most clearly demonstrated the difference between *Candida albicans* and *Candida dubliniensis* thus reducing error in the identification of the two species.

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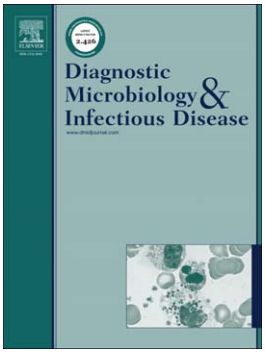

Multi-drug resistant (MDR) oral *Candida* species isolated from HIV-positive patients in South Africa and Cameroon

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Multi-drug resistant (MDR) oral *Candida* species isolated from HIV-positive patients in South Africa and Cameroon.

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Keywords: *Candida*; drug resistance; TREK; antifungal agents

ABSTRACT

Candida species are a common cause of infection in immune-compromised HIV-positive individuals, who are usually treated with the antifungal drug, fluconazole in public hospitals in Africa. However, information about the prevalence of drug resistance to fluconazole and other antifungal agents on *Candida* species is very limited. This study examined 128 *Candida* isolates from South Africa and 126 Cameroonian *Candida* isolates for determination of species prevalence and antifungal drug susceptibility. The isolates were characterized by growth on chromogenic and selective media and by their susceptibility to nine antifungal drugs tested using the TREK™ YeastOne9 drug panel (Thermo Scientific). Eighty three percent (82.8%) of South African isolates were *C. albicans* (106 isolates), 9.4% were *C. glabrata* (12 isolates) and 7.8% were *C. dubliniensis* (10 isolates). Of the Cameroonian isolates, 73.02% were *C. albicans* (92 isolates); 19.05% *C. glabrata* (24 isolates); 3.2% *C. tropicalis* (4 isolates); 2.4% *C. krusei* (3 isolates); 1.59% either *C. kefyr*, *C. parapsilopsis* or *C. lusitaneae* (2 isolates); and 0.79% *C. dubliniensis* (1 isolate). Widespread *C. albicans* resistance to azoles was detected phenotypically in both populations. Differences in drug resistance were seen within *C. glabrata* found in both populations. Echinocandin drugs were more effective on isolates obtained from the Cameroon than in South Africa. A multiple drug resistant (MDR) *C. dubliniensis* strain isolated from the South African samples was inhibited only by 5-flucytosine *in vitro* on the YO9 panel. Drug resistance among oral *Candida* species is common among African HIV patients in these two countries. Regional surveillance of *Candida* species drug susceptibility should be undertaken to ensure effective treatment for HIV-positive patients.

1. Introduction

The chronic nature of HIV infection and the increased incidence of mucosal and disseminated forms of *Candida* infections have necessitated the systemic use of antifungal agents, notably, the azole drugs, fluconazole and itraconazole. Fluconazole is routinely administered for candidiasis in healthcare facilities on the African continent and is also used to treat cases unresponsive to topical antifungal treatment (Powderly et al., 1999).

Widespread and repeated use of azole drugs (Jia et al., 2008) has led to resistance to antifungal therapies; a problem that is apparently spreading widely (Manzano-Gayosso et al., 2008; Luque et al., 2009). Thus, there is an urgent need to determine the extent of this problem on the African continent. High HIV infection rates, the lack of surveillance and the uncontrolled distribution of medications have all contributed to drug resistance that has emerged unchecked. This is especially important in resource-poor countries, where little information and limited resources by which to obtain it, seriously complicates the issue.

Various methods are available for the determination of antifungal drug susceptibility, employing either broth dilution or disk diffusion. These include the use of Yeast Nitrogen Base agar (May et al., 1997) and the methylene-blue and glucose-enriched Mueller-Hinton agar diffusion test, the antifungal disk diffusion medium recommended by the Clinical and Laboratory Standards Institute (2009). However, these time- and resource-consuming methods are being replaced by more modern techniques such as the TREK Vision diagnostic system. The TREK Sensititre YeastOne 9 (YO9) system (Thermo Scientific, USA) is a broth micro-dilution method that provides antifungal drug susceptibility testing for multiple drugs simultaneously and relatively inexpensively. This method has the advantage of being standardized to CLSI standards (Eraso et al., 2008; Pfaller et al., 2012) and consists of

microtiter plates coated with nine different drugs in ascending concentrations which provide for the determination of a minimal inhibitory concentration (MIC). The drugs are as follows: the echinocandins (anidulafungin, micafungin and caspofungin), which inhibit β1-3 glucan synthesis in the fungal cell wall; the fluorinated pyrimidine analogue (5-flucytosine), that inhibits protein and DNA synthesis; triazole drugs (posaconazole, voriconazole, itraconazole and fluconazole), which block ergosterol synthesis thereby affecting the fungal cytoplasmic membrane, and a polyene (amphotericin B), which interferes with ergosterol synthesis, leading to cell membrane leakage. The wells are also coated with a colorimetric agent with the advantage that the MIC of each drug can be easily detected with the naked eye and with the supplied Vizion computer-assisted plate reading system (Thermo Scientific, USA). In resource-limited environments the plates can also be read manually with the aid of a simple inexpensive light box.

The objective of this study was to determine the species prevalence and phenotypic drug susceptibility profiles of *Candida* species isolated from HIV-infected African populations in South Africa and Cameroon, using chromogenic and selective media and the TREK Sensititre diagnostic system. This study was prompted by an increasing number of patients being lost to follow-up or failing conventional therapy.

2. Materials and Methods

Approval from the Ministry of Health Regional Hospital Institutional Review Board (IRB) in Cameroon and from the Ethics Committee at the University of the Western Cape in Cape Town, South Africa, was obtained. A total of 212 HIV-positive patients attending clinics in Khayelitsha (n=18) and Delft (n=204) in the Western Cape, South Africa, and 262 HIV-

positive patients receiving routine care from the HIV clinic at the Bamenda Hospital in Cameroon, participated in the study. Samples were collected over a period of 6 months.

Prior to sample collection, the reasons for, and nature of the study were explained to the patients who willingly consented to participate. Only HIV-positive patients presenting with white pseudomembranous plaque on the tongue or other visible oral candidiasis were selected. Included in the study were two South African patients (male and female) who reported that they had started fluconazole therapy at the time of sample collection. Another South African patient had started taking Amphotericin B lozenges at the time of sample collection and two females from Cameroon reported recent fluconazole therapy. The application of adequate exclusion criteria was limited by the fact that we were unable to collect accurate patient history of previous *Candida* infection or antifungal treatment due to incomplete patient records and patients' lack of knowledge of drug names and usage.

Oral swabs were used to collect samples from the affected areas and swabs were plated onto Sabouraud's agar and incubated for 24 hours at 37 ˚C followed by 24-72 hours of growth at 30 ˚C on Fluka chromogenic *Candida* identification agar, (Cat. no. 94382, Sigma-Aldrich, USA) and Oxoid chromogenic *Candida* agar (Cat. no. CM1002A, Oxoid, UK). Confirmation of *Candida* species was achieved using microscopy, Gram staining and the germ tube test.

Presumptive *C. albicans* and *C. dubliniensis* cultures were incubated at 37 ˚C for 2-3 hours in fetal bovine serum to stimulate germ tube production, and the two species further differentiated by growth at 37 ˚C for 48 hours in Tomato (V8) agar (Alves, Linares et al. 2006) at 28 ˚C for 48-72 hours in Tobacco agar (Khan, Ahmad et al. 2004) and at 45 ˚C for 24-48 hours in Sabouraud dextrose agar (Pinjon, Sullivan et al. 1998). Differences in growth,

colony morphology and pseudohyphae/chlamydospore expression, allowed for species identification (Messeir et al., 2012).

Type strains of *C. albicans* (ATCC 90028 and NCPF 3281) and *C. dubliniensis* (NCPF 3949a) served as positive controls for the germ tube test, while *C. tropicalis* (ATCC 950) served as a negative control. Type strains of *C. albicans* (ATCC 90028 and NCPF 3281), *C. tropicalis* (ATCC 950), *C. dubliniensis* (NCPF 3949a), *C. glabrata* (ATCC 26512) and *C. krusei* (ATCC 2159) served as quality control organisms for the chromogenic species differentiation and drug susceptibility testing.

Second-generation *Candida* strains were diluted with sterile phosphate buffered saline to concentrations of $1x10^6$ to $5x10^6$ cells per ml, corresponding to a 0.5 McFarland standard, measured using the supplied TREK nephelometer. This was followed by vortexing of the suspension and the addition of 100µl of the vortexed solution to YeastOne broth (Product code Y3462, Thermo Scientific). The diluted broth was dispensed into the YO9 plate using an automated 25-1250µl multichannel pipette and incubated for 24 hours at 37 °C.
The plates were then read using the Vizion plate reader and analyzed using the TREK SWIN software (Thermo Scientific, USA).

Recently developed species-specific clinical breakpoints were used (Pfaller et al., 2012) to categorise *C. albicans* and *C. tropicalis* as susceptible, intermediate or resistant to echinocandin drugs (anidulafungin, caspofungin and micafungin). CLSI breakpoint categories were used for 5-flucytosine, itraconazole, fluconazole and amphotericin B (Eraso, et al., 2008) and the breakpoints proposed by Pfaller *et al.* (2006) were used for voriconazole. In the case of posaconazole, for which no clinical breakpoints have been established, wild-

type MIC values were used as previously proposed (Pfaller et al., 2011). MICs were defined as the lowest concentrations that inhibited growth at 100%. The MIC breakpoints of the different drugs for different *Candida* species are listed in Table 1.

Statistical analysis to demonstrate the association between *Candida* species and drug susceptibility was calculated by means of Chi-square tests using the SPSS 21.0 statistics software ($p<0.05$).

3. Results

3.1 Frequency of species

Of the 212 South African samples, 128 (60%) were positive for *Candida* of which 82.8% were identified as *C. albicans* (106 isolates), 9.4% as *C. glabrata* (12 isolates) and 7.8% as *C. dubliniensis* (10 isolates). *Candida albicans* was the most frequently isolated species from both regions. A greater diversity of species was observed among the Cameroonian isolates. One hundred and twenty-six of the 262 Cameroonian samples (48%) were positive for *Candida*, of which 73.02% were *C. albicans* (92 isolates); 19.05% were *C. glabrata* (24 isolates); 3.2% were *C. tropicalis* (4 isolates); 2.4% were *C. krusei* (3 isolates); 1.59% were either *C. kefyr*, *C. parapsilopsis* or *C. lusitaneae* (2 isolates); and 0.79% were *C. dubliniensis* (1 isolate).

3.2. Susceptibility profiles of isolates

All *C. albicans* and *C. glabrata* species isolated from South Africa were susceptible to micafungin and exhibited intermediate resistance to caspofungin and complete resistance to anidulafungin. While more than 50% of the South African *C. albicans* strains were found to demonstrate resistance to all azoles tested (Table 2), *C. glabrata* and *C. dubliniensis* strains demonstrated good overall susceptibility to azoles. A *C. dubliniensis* strain was remarkable in that it was resistant to all the antifungal drugs on the panel except 5-flucytosine in concentrations above 2µg/ml (Table 2).

The results from Cameroonian strains showed that with the exception of *C. glabrata*, other *Candida* species, namely, *C. albicans*, *C. tropicalis* and *C. krusei* strains were susceptible to echinocandin drugs (Table 3). In the case of the azole drugs, the reverse of this pattern was observed: *C. albicans* strains were resistant to azoles (greater than or equal to 50% resistance to all azoles tested). *C. glabrata* was better inhibited by this class of drugs. *Candida dubliniensis* and two species identified as *C. parapsilopsis/lusitaneae/kefyr* were susceptible to all azole drugs tested, while *C. tropicalis* strains were susceptible to both fluconazole and voriconazole.

Six South African isolates, 5(4.7%) *C. albicans* and 1(8.3%) *C. glabrata* were resistant to 5-flucytosine, while most of the species isolated in Cameroon were sensitive to 5-flucytosine. The exceptions among the Cameroonian samples were *C. krusei*, where only one isolate (33.3% of total) showed intermediate non-susceptibility and 6 (6.52%) *C. albicans* were totally resistant.

Notably, although isolates were frequently susceptible to amphotericin B, *Candida* species from both populations contained isolates that were resistant (Tables 2 and 3).

Wild-type MIC determinations with respect to both South African and Cameroonian isolates tested, demonstrated resistance / non-susceptibility to posaconazole (84% for *C. albicans* and 41.7% for *C. glabrata in* South Africa and 80.4%, 62.5%, 100% and 66.7% for *C. albicans, C. glabrata, C. tropicalis* and *C. krusei*, in Cameroon respectively). *C. krusei* is intrinsically resistant to fluconazole and there is a general lack of certainty about the interpretive categories with regard to other azoles. We therefore elected to use the term "non-susceptible" rather than "resistant" to the azoles when referring to *C. krusei* in our results.

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Of the 106 *C. albicans* isolated from the South African population, 13 (12.3%) were resistant to 2 classes of antifungals, while 6/92 (6.5%) *C. albicans* isolates from Cameroon were resistant to 2 classes of antifungals. In Cameroon, the *C. albicans* resistance was either to Amphotericin B and the azoles or to 5-flucytocine and the azoles, while in South Africa, *C. albicans* demonstrated resistance to Amphotericin B, 5-Flucytosine, anidulafungin and the azoles in different combinations.

C. glabrata demonstrated resistance to 2 or more antifungals in 3/12 (25%) of the South African isolates and 10/24 (41.7%) isolates from Cameroon. In Cameroon, the antifungal combinations were anidulafungin, micafungin and azoles, while in South Africa, the antifungal combinations were either Amphotericin B and the azoles or anidulafungin and the azoles.

Statistical analysis showed significant associations between *Candida* species and antifungal drug resistance.

4. Discussion

Oropharyngeal candidiasis (OPC) continues to be a common opportunistic infection in patients infected by HIV. It occurs in up to 90% of HIV/AIDS patients during the prolonged course of HIV disease and greatly reduces their quality of life. They are predisposed to recurrent episodes of OPC that can increase in frequency and severity, resulting in increased morbidity and mortality. This is especially true in Africa, where high incidences of mucosal and deep seated forms of candidiasis have resulted in the use of systemic antifungal agents, especially fluconazole and itraconazole. As in other African countries, the present guidelines for South Africa and Cameroon for the management of HIV-positive patients include fluconazole as a first choice drug for systemic infection (Guidelines, 2009). The widespread use of these antifungal agents have been followed by an increase in antifungal resistance and by a shift from *C. albicans* to non-albicans species prevalence such as those described in this study.

As reported in previous studies from Africa (Blignaut et al., 2002; Hamza et al., 2008), *C. albicans, C. glabrata* and *C. krusei* were the most frequently isolated species from these South African and Cameroonian patients. *Candida dubliniensis* was isolated more frequently from South African patients.

Two South African patients (one male and one female) and two Cameroonian patients (both females) who had recently started fluconazole therapy had visible oropharyngeal thrush at the time of sample collection and their samples yielded the growth of *C. albicans* highly

susceptible to all the drugs tested. Another South African patient who had started taking Amphotericin B lozenges at the time of sample collection, also yielded an isolate of *C. albicans* highly susceptible to all the drugs tested except posaconazole,

However, this study reports that over 50% of *C. albicans* isolated from South Africa and Cameroon have developed resistance to fluconazole. A previous report of baseline data from South Africa demonstrated 100% susceptibility of *C. albicans* to fluconazole indicating a marked change in susceptibility. However, and of importance, is that the study was done before the introduction of fluconazole to patients attending HIV-AIDS clinics (Blignaut et al., 2002) and that the patient population was from another province. High resistance levels were observed to other azole drugs tested. Although lower resistance levels have been previously reported from South Africa (Molepo et al., 2006), compared with the current data, other African studies have reported frequent resistance of *C. albicans* and non-albicans species to azoles (Njunda et al., 2012; Mulu et al., 2013). Cross-resistance to fluconazole has been observed in patients receiving itraconazole prophylaxis (Goldman et al., 2000) and other previously administered azole therapies such as ketoconazole and miconazole (Pelletier et al., 2000; Rautemaa et al., 2008). These observations are a cause for concern as fluconazole is the most widely used antifungal available to treat *Candida* infections in HIV patients in South Africa. The same applies in Cameroon and the rest of the African continent. Second choice is itraconazole. Ketoconazole is also used, but has been superseded by fluconazole and itraconazole.

Resistance to amphotericin B was seen with respect to all *Candida* species, with non-albicans species demonstrating especially high resistance levels, particularly *C. glabrata* isolated from the South African population. The South African *C. dubliniensis* isolate which showed

resistance to all eight of the nine drugs on the YO9 panel may indicate a serious public health problem, since it suggests the emergence of multiple-drug resistant *Candida* species in the HIV population.

Another finding presenting a clinical challenge was the demonstration of *C. albicans* and *C. glabrata* resistance to 2 or more classes of antifungals in this study, with *C. albicans* being the predominant resistant species in the South African population and *C. glabrata* in the Cameroonian population. The dispensing of these antifungal drugs should therefore be carefully monitored and should be based upon established epidemiological data (Blignaut, Pujol et al. 2002). In this way, *in vitro* resistance may be related to treatment failure (Rogers 2006) and aid in the assessment prevalence of multiple drug resistance in the population.

The rate of relapse and clinical response to therapy varies in different populations. Some HIV-positive patients experience recurrent *Candida* infections with shorter disease-free episodes. They are therefore subjected to numerous courses of antifungals which may ultimately result in antifungal resistance. There is a possibility that the repeated exposure of *Candida* species to antifungal drugs, particularly in the Cameroon, might have led to increased variability in the distribution of *Candida* species with more non-albicans species reported. Species-specific azole resistance has been documented in Brazil (Colombo, Da Matta et al. 2002) and resistance to a specific antifungal drug has been shown to result in cross-resistance to other drugs of the same class (Muller, Weig et al. 2000). This was clearly evident in the present study.

As in many other African countries, street access to antifungals and other drugs without prescription is common in Cameroon. This factor, along with a lack of patient knowledge of

antifungal drugs, made it difficult to ascertain whether patients were exposed to these drugs for a long period of time. The possibility exists that specific *Candida* consortia play a role in the acquisition of resistance and with most patients not having access to adequate medical treatment, combined with limited health care resources and the inability of health care workers to monitor the acquisition of antifungals on the street, this problem will escalate leading to an increase in HIV-positive patient morbidity and mortality.

This study demonstrates a need for regional surveillance of *Candida* species on the African continent and improved control over the sale of medications. This study has shown that the prevalent *C. albicans* does not respond to specific antifungal drugs that might be dispensed empirically. *C. glabrata* from Cameroon was resistant to micafungin while South African isolates were susceptible, demonstrating significant regional differences. The reverse of this pattern was seen in the case of 5-flucytosine, thereby re-emphasizing the need for more epidemiological studies.

Limitations of this study include the paucity of patient information (whether early or late presenters of HIV-AIDS), and the lack of patient history of previous episodes of *Candida* infection and treatment. Inconsistencies occur with species differentiation and drug susceptibility techniques due to lack of resources in many African countries, thus complicating a direct comparison of our results with other studies done in Africa.

Finally, the use of the TREK Sensititre platform in this study for drug susceptibility testing proved a rapid and reliable method requiring minimal training and utilizing available reagents. We suggest this approach may be a promising method for use in resource-limited laboratories in Africa. The TREK system avoids the limitations of current drug susceptibility

testing protocols for fungi, as multiple drugs can be tested simultaneously on a single plate using a simple protocol. Furthermore, an inexpensive light box can be used to screen the plates manually.

Standardized methods available from the Clinical and Laboratory Standards Institute (CLSI) are useful for the calculation of clinical breakpoints and epidemiologic cutoff values for reliable in vitro antifungal susceptibility testing. These results indicate that not only does resistance differ from country to country, but also in different regions of the same country. Programmes on species prevalence and antifungal use and resistance pattern surveillance have been successfully developed and introduced in Europe, Asia-Pacific, Latin America and North America (Cuenca-Estrella et al., 2008; Adriaenssens et al., 2010; Pfaller et al., 2011). The high HIV prevalence and accompanying immunodeficiency in sub-Saharan Africa are strong driving factors emphasizing the need for regional *Candida* surveillance programmes. Changes in drug susceptibility over time serve as a reminder for the need to test clinical *Candida* isolates for sensitivity to antifungal drugs in the effort to improve patient care and reduce patient morbidity and mortality.

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Table 1
Drug susceptibility clinical breakpoints used in this study

	C. albicans			*C. glabrata*		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Anidulafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25 µg/mL	≥0.5 µg/mL
Caspofungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25 µg/mL	≥0.5 µg/mL
Micafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.06 µg/mL	0.12 µg/mL	≥0.25 µg/mL
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	<0.016µg/mL	_	≥0.016µg/mL	<0.5µg/mL	_	≥0.5µg/mL
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL

	C. tropicalis			*C. krusei*		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Non-susceptible
Anidulafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
Caspofungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
Micafungin	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL	≤0.25 µg/mL	0.5 µg/mL	≥1 µg/mL
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	<0.03µg/mL	_	≥0.03µg/mL	<0.25µg/mL	_	≥0.25µg/mL
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL

	C. dubliniensis			*C. kefyr/para/lusi*		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Anidulafungin	_	_	_	_	_	_
Caspofungin	_	_	_	_	_	_
Micafungin	_	_	_	_	_	_
5-Flucytosine	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL	≤4 µg/mL	8-16 µg/mL	≥32 µg/mL
Itraconazole	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL	≤0.12 µg/mL	0.25-0.5 µg/mL	≥1 µg/mL
Fluconazole	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL	≤8 µg/mL	16-32 µg/mL	≥64 µg/mL
Amphotericin B	<1 µg/mL	_	≥1 µg/mL	<1 µg/mL	_	≥1 µg/mL
Posaconazole	_	_	_	_	_	_
Voriconazole	≤1 µg/mL	2µg/mL	≥4 µg/mL	≤1 µg/mL	2µg/mL	≥4 µg/mL

"-" No clinical breakpoint available for this drug.

Table 2
Antifungal susceptibility for South African *Candida* isolates

		C. albicans n=106	*C. glabrata* n=12	*C. dubliniensis* n=10	Spp/resistance associations
Amphotericin B	Susceptible	97	7	9	p=0.01
	Intermediate	0	0	0	
	Resistant	9	5	1	
5-Flucytosine	Susceptible	101	11	10	
	Intermediate	0	0	0	
	Resistant	5	1	0	
Anidulafungin	Susceptible	101	11	–	p=0.000
	Intermediate	3	0	–	
	Resistant	2	1	–	
Caspofungin	Susceptible	98	9	–	p=0.000
	Intermediate	8	3	–	
	Resistant	0	0	–	
Micafungin	Susceptible	106	12	–	p=0.000
	Intermediate	0	0	–	
	Resistant	0	0	–	
Fluconazole	Susceptible	53	8	9	p=0.032
	Intermediate	1	4	0	
	Resistant	52	0	1	
Itraconazole	Susceptible	43	4	9	p=0.008
	Intermediate	1	6	0	
	Resistant	62	2	1	
Voriconazole	Susceptible	49	12	9	p=0.000
	Intermediate	0	0	0	
	Resistant	57	0	1	

"-" No clinical breakpoint available for the organism/drug.

Table 3
Antifungal susceptibility for Cameroonian *Candida* isolates

		C. albicans n=92	*C. glabrata* n=24	*C. tropicalis* n=4	*C. krusei* n=3	*C. para/lusi/kefyr* n=2	*C. dubliniensis* n=1	Spp/resistance associations
Amphotericin B	Susceptible	88	23	2	1	1	0	p=0.001
	Intermediate	0	0	0	0	0	0	
	Resistant/non-susceptible	4	1	2	2	1	1	
5-Flucytosine	Susceptible	86	24	4	2	1	1	
	Intermediate	0	0	0	1	0	0	
	Resistant/non-susceptible	6	0	0	0	0	0	
Anidulafungin	Susceptible	92	16	4	3	-	-	p=0.000
	Intermediate	0	5	0	0	-	-	
	Resistant/non-susceptible	0	3	0	0	-	-	
Caspofungin	Susceptible	92	16	4	3	-	-	p=0.000
	Intermediate	0	7	0	0	-	-	
	Resistant/non-susceptible	0	1	0	0	-	-	
Micafungin	Susceptible	92	3	4	3	-	-	p=0.000
	Intermediate	0	5	0	0	-	-	
	Resistant/non-susceptible	0	16	0	0	-	-	
Fluconazole	Susceptible	45	16	4	1	2	1	p=0.041
	Intermediate	1	7	0	0	0	0	
	Resistant/non-susceptible	46	1	0	2	0	0	
Itraconazole	Susceptible	44	5	1	1	2	1	p=0.044
	Intermediate	1	15	3	2	0	0	
	Resistant/non-susceptible	47	4	0	0	0	0	
Voriconazole	Susceptible	46	23	4	2	2	1	p=0.000
	Intermediate	0	0	0	0	0	0	
	Resistant/non-susceptible	46	1	0	1	0	0	

"-" No clinical breakpoint available for the organism/drug.