

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL MICROBIOLOGY

DISTRIBUTION AND SUSCEPTIBILITY PROFILE OF CANDIDA ISOLATES
FROM HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS



BY

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DECLARATION

I hereby declare that the submission of this compilation is the true findings of my own researched work presented towards an award of a second degree in Medical Microbiology under the supervision of Dr. Japheth A. Opintan and Dr. Nicholas Dzifah Dayie and that, to the best of my knowledge, it contains no material previously published by another person nor submitted to any other University or institution for the award of degree except where due acknowledgement has been made in text. However, references from the work of others have been clearly stated.

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DEDICATION

This work is dedicated to my wife, Mrs. Ophelia Asare Quansah and children for their support and encouragement. It is also dedicated to Mr. Frederick Kumi-Ansah of the Cape Coast Teaching Hospital for his support and guidance.

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My appreciation goes to God for giving me the strength to go through this programme. I am also grateful to my supervisors, Dr. Japheth A. Opintan and Dr. Nicholas Dzifa Dayie for their patience, support and guidance throughout this study. Furthermore, I appreciate the efforts of the entire technical staff of the Cape Coast teaching hospital, especially, Mr. Daniel Edem Azumah, Mr. Francis Arthur and Mr. Philemon Wintiger.

ABSTRACT

Background: Oropharyngeal candidiasis (OPC) has been indicated as the most prevalent fungal opportunistic infection in individuals infected with Human Immunodeficiency Virus (HIV). *Candida albicans* has been the most isolated species in OPC. However, other *Candida* species have been implicated as potential pathogens. Majority of these species have been found to be less susceptible to commonly administered antifungal drugs.

Aim: The aim of this study was to determine the distribution and prevalence of antifungal resistance among *Candida* isolates from HIV-infected patients presenting with OPC.

Methodology: The study participants were recruited from HIV-infected patients with OPC attending anti-retroviral clinics of selected health facilities, using non-probability sampling methods. Clinical data including information on ART therapy were extracted from patient's folder. Oropharyngeal specimens were collected from participants for culture using sterile swabs. Speciation of presumptive *Candida* isolates recovered from Sabouraud dextrose agar (SDA) plates was done using standard identification methods. The E-TEST stable agar gradient minimum inhibitory concentration (MIC) method was used to determine the susceptibility of the *Candida* isolates to selected antifungals. CD4⁺ T-lymphocyte counts were also estimated using the BD FACS count machine.

Results: Of the 286 samples collected, 67.8% ($n=194$) were culture positive for seven (7) different *Candida* species, whose distributions were as follows: *C. albicans* (69.1%, $n=134$), *C. tropicalis* (10.3%, $n=20$), *C. glabrata* (6.7%, $n=13$), *C. parapsilosis* (5.7%, $n=11$), *C. krusei* (4.1%, $n=8$), *C. dubliniensis* (2.6%, $n=5$) and *C. lusitaniae* (1.5%, $n=3$). *C. albicans*-associated OPC was significantly higher among HIV-infected patients on ART compared to ART-naïve

patients [(73.9% vs. 26.1%; OR=2.16, 95% CI, 1.141-4.101) (p=0.018)]. Non-*C. albicans* isolates were significantly more resistant to fluconazole than *C. albicans* identified [Non-*C. albicans* = 45% (n=27) vs. *C. albicans* = 29.1 % (n=39); p= 0.033]. Fluconazole resistance was significantly higher among participants with previous history of fluconazole therapy compared to fluconazole-naïve patients [Fluconazole exposed patients = 70% (14/20) vs. Fluconazole naïve patients = 29.9 % (52/174); p= 0.001]. The *C. albicans* isolates showed a reduced susceptibility to Amphotericin B relative to the non-*Candida albicans* isolates (1.5% vs. 3.3%; p= 0.266). Prevalence of flucytosine resistance were 2.3% (3/134) and 8.3% (5/60) for *C. albicans* and non-*Candida albicans* respectively.

Conclusion: *C. albicans* accounted for majority of OPC in this cohort of HIV patients, with non-*Candida albicans* species showing significantly higher resistance to fluconazole. *C. albicans*-associated OPC was significantly associated with ART, but not with duration of ART, age, gender, and prior antifungal therapy. Previous history of fluconazole therapy and ART were associated with reduced susceptibilities to fluconazole. Without any contraindication, flucytosine and Amphotericin B may be considered for OPC not responding to fluconazole therapy.

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LIST OF ABBREVIATIONS

5FC	5-Fluorocytosine
AFST	Antifungal susceptibility testing
AIDS	Acquired immunodeficiency syndrome
ALS	Agglutinin-like sequence
ART	Antiretro therapy
BMD	Broth microdilution method
CCTH	Cape Coast Teaching Hospital
CLSI	Clinical and Laboratory Standards Institute
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee for Antimicrobial Susceptibility Testing
GMB	Glucose Methylene Blue
ART	Anti-retroviral Therapy
HIV	Human immunodeficiency virus
IC	Invasive candidiasis
ITS	Internally transcribed spacer
MDR	Multiple drug resistance
MIC	Minimum inhibitory concentration

MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NCCLS	National Committee for Clinical Laboratory Standards
OIs	Opportunistic infections
OPC	Oropharyngeal candidiasis
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose broth
PNA	Peptide nucleic acid
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal RNA
SBAHS	School of Biomedical and Allied Health Sciences
SDA	Sabouraud Dextrose Agar
S-DD	Susceptible dose dependent
VVC	Vulvo-vaginal candidiasis
WHO	World health organization
YNB	Yeast Nitrogen Base

YPD

Yeast

Potato

Dextrose

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Human immunodeficiency virus (HIV) infection still remains a public health problem claiming more than 35 million lives. The world health organization (WHO) reported that the number of people living with HIV at the end of 2016 was 36.7 million. Africa remains the most affected region, accounting for almost two thirds of the world's total HIV infection. In Ghana, an estimated 290,000 people lived with HIV at the end of 2016 (WHO, 2016).

HIV primarily impairs the functions of the immune system making infected persons highly susceptible to opportunistic infections (Chu *et al.*, 2011). These opportunistic infections (OIs) are the leading cause of morbidity and mortality during the natural course of the disease (Lawn *et al.*, 2001; Walensky *et al.*, 2006). By far, oropharyngeal candidiasis (OPC) dominates opportunistic fungal infections associated with HIV/AIDS. An estimated 90% or more of patients diagnosed with HIV/AIDS develop OPC with 60% having at least an episode of infection in a year and 50% - 60% recurrent infections during the course of their illness (Samaranayake *et al.*, 2002, Li *et al.*, 2012).

Candida albicans in many studies have been reported as the most isolated species in OPC, accounting for up to 81% of cases (Sangeorzan *et al.*, 1994). The distribution pattern has however changed over time, with some non-*albicans* species implicated as significant opportunistic pathogens in 20–40% of all cases (Shang *et al.*, 2010; Mushi *et al.*, 2016).

OPC is an indicator of immune suppression, and can be employed as a tool for early detection, testing and management of patients infected with HIV/AIDS (Klein *et al.*, 1984). OPC is also a

marker for poor prognosis (Adurogbangba *et al.*, 2004), reported during the middle and late stages of HIV/AIDS infection, when the CD4+ cell count is low (Dull *et al.*, 1991).

1.2 BURDEN OF OPC INFECTION

Patients who are immunocompromised frequently develop both superficial and life-threatening *Candida* infections which gradually develop into severe complications (Diro *et al.*, 2008). Associated with these complications are local discomfort, changes in taste, poor food intake leading to malnutrition and wasting and early death among HIV infected patients (Diro *et al.*, 2008). Invasive infections can also develop following the spread of oral infection into the bloodstream causing significant morbidity and mortality (Akpan and Morgan, 2002; Fanello *et al.*, 2006). Economic burden can be substantial as a result of slow recovery and prolonged hospital stay. Considerable number of people (1.2 million) still die due to HIV related infections including OPC every year (USAID, 2015) despite the introduction of ART. These deaths occur during the working age of victims posing a major economic impact (Collins *et al.*, 2007, Herrmann *et al.*, 2016). Cost of implementing programmes relating to HIV/AIDS is another heavy burden.

Patient's outcome is directly impacted when the identity of the causative fungi is known and the right antifungal therapy is timely initiated (Patel *et al.*, 2009). Available drugs for treatment of *Candida* infections include the polyene (Amphotericin B and its different formulations), Azoles and its derivatives (itraconazole, fluconazole, isavuconazole, and voriconazole), Echinocandins (micafungin, caspofungin and anidulafungin) and 5-flucytosine. Extensive use of these drugs

induces selective pressure on fungal strains, leading to emergence of resistance (Lass-Florl, 2009).

Differential diagnosis of OPC is done using a set of clinical criteria. Non-responding cases are however diagnosed in the laboratory using microscopy and culture procedures. Antifungal susceptibility testing (AFST) procedures are applied to determine suitable antifungal agents for treating non-responding cases and to monitor antifungal resistance locally and globally (Borman and Johnson, 2014).

Standardized methods for performing AFST have been described elsewhere (Espinel-Ingroff, 2003; Orosky-Zeichner *et al.*, 2003; Pfaller *et al.*, 2005; Pfaller *et al.*, 2006). Availability of these methods provide the means for conducting large-scale surveillance studies and documentation of the potency of antifungal drugs against clinical fungal isolates.

1.3 PROBLEM STATEMENT

Several research findings point to a growing problem of antifungal resistance among *Candida* species especially the non- *Candida albicans* species (Diro *et al.*, 2008, Pfaller *et al.*, 2010, Pfaller *et al.*, 2011; Yapar *et al.*, 2011, Lortholary *et al.*; 2012, Arendrup *et al.*, 2013). Also a concern, is the epidemiological shift in the trend of candida infections from *C. albicans* to non-*Candida albicans* species. This shift differ geographically and even among health settings and cohorts of patients within a country (Nweze *et al.*, 2011).

Global prevalence of antifungal resistance among *Candida* species is estimated at 9.3% to 56.7% (Manzano-Gayosso *et al.*, 2008; Falagas *et al.*, 2010; Rosana *et al.*; 2015; Salari *et al.*; 2016; Mulu

et al., 2013; Terças *et al.*, 2017). In Africa, prevalence in excess of 50% have been reported (Dos Santos *et al.*, 2014, Owotade *et al.* 2016). In Ghana, a study by Feglo *et al.* (2012) in Kumasi revealed that 4.5% to 22.2% of yeast isolates tested against flucytosine, amphotericin B, fluconazole and itraconazole were resistant.

Outside Africa, prevalence of antifungal resistance ranging between 1% and 14% have been reported in some parts of Europe (Bagg *et al.*, 2005, Arendrup *et al.*, 2014). The ARTEMIS antifungal surveillance programme conducted between 1997 and 2007 in 41 countries reported a high fluconazole-resistance among non-*Candida albicans* isolates (*C. guilliermondii* 11.4%, *C. inconspicua* 53.2%, *C. rugosa* 41.8%, and *C. norvegensis* 40.7%). The rate of isolation of these resistant species also increased 5 to 10 fold over the 10year study period (Pfaller *et al.*, 2010).

These epidemiological changes in antifungal resistance and *Candida* species underscore the need for constant monitoring especially in the immunocompromised patients to determine the burden of fungal infections due to antifungal resistance and recommend possible prevention and control measures.

In Ghana, particularly the Central Region however, little is known about the etiologic agents of OPC and their antifungal susceptibility pattern in HIV-patients due to the fact that routine speciation and susceptibility testing of yeast isolates are not done by most laboratories before treatment is given. This study was therefore conducted to help fill the knowledge gaps on the efficacy of antifungal agents, emergence of resistance, and further knowledge that could impact treatment guidelines.

1.4 JUSTIFICATION

Although *C. albicans* are the predominant isolated *Candida* species in OPC (Horn *et al.*, 2009), non-*C. albicans* species, such as *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. dubliniensis*, *C. guilliermondii*, *C. parapsilosis*, *C. kefyr*, and *C. pelliculosa* have also been implicated as important causes of infections in patients diagnosed with HIV/AIDS (Gugnani *et al.*, 2003, Nweze *et al.*, 2011). The clinical significance of isolating these non-*albicans* species is that some have been found to be less susceptible to the most commonly used antifungal drugs, a situation that presents significant compounding challenges for chemotherapy (Mane *et al.*, 2010). Hence it is important to determine the distribution of *Candida* species and their antifungal susceptibility patterns to help provide further knowledge that could impact treatment of OPC.

1.5 AIM

The aim of the study was to determine the distribution and prevalence of antifungal resistance among *Candida* isolates from HIV/AIDS patients with OPC.

1.6 SPECIFIC OBJECTIVES:

1. To determine the distribution of *Candida* species responsible for OPC among HIV-infected patients.
2. To determine the susceptibility of the *Candida* isolates to fluconazole, Amphotericin B and flucytosine.

CHAPTER TWO

LITERATURE REVIEW

2.1 CLINICAL SPECTRUM AND EPIDEMIOLOGY OF CANDIDA INFECTIONS

Candida species remain important human pathogens especially in patients who have critical underlying health conditions and compromised immune system. Infections caused by *candida* species are diverse and may involve any organ or system of the body. Clinically, these infections may be localized to the vagina, mouth, fingers, nails, gastrointestinal, lungs and nails or develop into systemic infections such as septicemia, endocarditis and meningitis (Luna and Tortoledo, 1993).

Candida infections in healthy individuals are usually presented superficially and are seen in all age groups but very common in children and the elderly (Fanello *et al.*, 2006). Systemic candidiasis such as myocarditis, hepatosplenic abscess, pulmonary infection, central nervous system infection are commonly seen in individuals who have compromised immune system with reported mortality rate of 71 – 79% (Akpan and Morgan 2002). The greater number of *candida* infections are seen on mucosal surfaces and comprise of vulvovaginal and oropharyngeal candidiasis.

2.1.1 Vulvo-vaginal candidiasis (VVC)

Vulvo-vaginal candidiasis (VVC) remains one of the most common infections caused by *Candida* species. Approximately 75% of women experience VVC at least once in life with 8% experiencing repeated infections (Sobel, 2007). *Candida albicans* account for approximately

90% of Vulvo-vaginal candidiasis cases, while *Candida glabrata* remains the second most common cause of infection (Sobel, 1998). In Ghana, studies have shown that 53.4% of vulvovaginal candidiasis are caused by *Candida albicans* (Adjapong et al; 2014).

Clinical symptoms of VVC include itching, irritation, pain and redness of the vulva. Risk factors of VVC include antibiotic use, contraception, pregnancy and diabetes.

2.1.2 Systemic Candidiasis

Introduction of *Candida* cells into the bloodstream can be disseminated throughout the body to infect almost every organ leading to death if not diagnosed early and treated.

Invasive candidiasis has been reported as the fourth most common cause of bloodstream infections (Wisplinghoff *et. al.*, 2004). Mortality rate of invasive candidiasis for adults has been reported as 49% (Gudlaugsson *et. al.*, 2003) and 14.5% (Zaoutis *et. al.*, 2005). Crude mortality rates has been reported in the range of 0.4 deaths per 100,000 from the period 1997 to 2003 (Pfaller and Diekema 2007).

In patients with cancer, invasive fungal infection is associated with high morbidity and mortality. It is estimated that 5%-40% of invasive fungal disease occur among patients with hematological malignancies with close to 95% of these infections caused by *Aspergillus* and *Candida* species (Halpern *et al*; 2015). A comparative study to find out the changing prevalence of different *Candida* species among Ghanaian HIV/AIDS patients with candidaemia reported an increase in isolation of non-*Candida albicans* from 2003-2005 to 2010-2014 (Siakwa *et al.*, 2008; Siakwa *et al*; 2014).

Candida albicans as in the case of other candidiasis has been identified as the most common cause of invasive candidiasis accounting for approximately 50% to 60% of all cases. More than

90% of cases of invasive candidiasis are as a result of *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* with the remaining species not often seen in bloodstream infections (Pfaller and Diekema 2007, Perlroth *et al.*, 2007).

2.1.3 Oropharyngeal Candidiasis

The risk of oropharyngeal Candidiasis varies depending on the existence of some underlying medical conditions. For instance, oral or throat candidiasis is not commonly found in healthy adults. However, they frequently occur in people who have HIV/AIDS and cancers such as leukemia and lymphoma. Denture wearers, diabetics, the elderly and neonates are also susceptible to OPC infections.

It is estimated that patients with diabetes have yeast carriage of up to 54% with *C. albicans* accounting for 25- 69% of the isolates (Aly *et al.*; 1995). Incidence of oral infections due to *Candida albicans* among patients with acute leukemia undergoing chemotherapy and radiation therapy is estimated at 90 to 95% (Leung *et al.*, 2000). Impaired salivary function due glandular tissue damage by radiation is thought to be a major factor leading to *Candida* infection in these patients (Haveman & Redding 1998).

Despite the introduction of ART, OPC is still considered a major problem for HIV/AIDS patients in countries where resources are limited (Thompson *et al.*; 2010). Prevalence of OPC among HIV/AIDS patients is estimated at 0.9% to 83% globally (Fabian *et al.*, 2009; Thompson *et al.*, 2010; Maury *et al.*, 2011; Patel *et al.*, 2012; Kwamin *et al.*, 2013; Maurya *et al.*, 2013; Mulu *et al.*, 2013; Kirti *et al.*, 2015; Das *et al.*, 2016; Konaté *et al.*, 2017; Tamí- Terças *et al.*, 2017). In Africa, prevalence ranging from 0.9 to 81% have been reported among HIV/AIDS patients (Fabian *et al.*, 2009; Mulu *et al.*, 2013; Kwamin *et al.*, 2013; Konaté *et al.*, 2017).

The Indian sub-continent have also reported prevalence of 5.0 to 38.8% OPC cases among HIV/AIDS patients (Nadagir *et al.*, 2008; Maurya *et al.*, 2013; Kirti *et al.*, 2015; Das *et al.*, 2016). In Ghana, OPC has been reported as the third commonest clinical oral infection among HIV/AIDS patients (Kwamin *et al.*, 2010). A baseline study on distribution of *Candida* species among HIV patients attending clinic at the Korle-bu Teaching hospital in Accra, reported a prevalence of 75.3% *Candida* infection among HIV-patients (Kwamin *et al.*, 2013).

Candida albicans have been implicated as the main causative species of OPC accounting for 37.2–95.2% of cases (Sharifzadeh *et al.*; 2013; Maurya *et al.*, 2013, Berberi *et al.*; 2015, Pappas *et al.*; 2016)). Nonetheless, a trend of rising *Candida* infections due to *non-Candida albicans* have been reported in HIV- patients (Pfaller, 2012). In one study carried out in Chile, *C. parapsilosis* constituted majority of isolated species (Ajenjo *et al.*, 2011). Again, prevalence of *C. dubliniensis* infections in HIV-patients is reported to have risen globally after it was first discovered (Khan *et al.*, 2012). Infections due to *Candida guilliermondii* and *Candida rugosa*, a previously uncommon agents is also reported to have risen (Pfaller *et al.*, 2009).

Positive selection pressure as a result of exposure to antifungal agents has been cited as the reason for the increase in these *non-albicans* species (Pfaller, 2012). The rise in isolation of the *non-candida albicans* species has significant challenge for chemotherapy because of the innate resistance shown by some of the *non-albicans* species to antifungals agents particularly the azoles (Mane *et al.*, 2010; Lai *et al.*, 2012).

2.2 EPIDEMIOLOGY OF ANTIFUNGAL RESISTANCE

Antifungal resistance has become a global problem since the introduction and widespread use of triazoles in the early 1990s (Jabra-Rizk *et al.*, 2004). This emerging antifungal resistance varies from samples, patients and countries in terms of incidence and prevalence rates (Toure *et al.*; 2016). Multiple resistance to commonly used antifungal agents have also been reported in some species of fungi such as the *C. rugosa* (Pfaller *et al.*, 2010).

In one of the antifungal resistance surveillance programmes conducted by the ARTEMIS in 41 countries between 1997 and 2007, high fluconazole-resistance ranging from 11.4% to 53.2% was reported among some species of *Candida* (Pfaller *et al.*, 2010). The China Hospital Invasive Fungal Surveillance Net (CHIF-NET) in a recent surveillance study involving 8,829 *Candida* isolates collected from Sixty-five tertiary hospitals also found high azole resistance among some non- *C.albicans* species (*C. tropicalis*: 13.3%, *C. glabrata* complex:18.7% and uncommon *Candida* species 44.1%) (Xiao *et al.*, 2018). Again, a nationwide cohort study in Dutch involving 144 patients with influenza pneumonia also showed 29% triazoles-resistance among patients diagnosed of influenza-associated invasive aspergillosis (Van de Veerdonk *et al.*, 2017). A study conducted in Kumasi, Ghana to determine the prevalence of yeast species and their antifungal susceptibility profiles in different samples found 4.5% to 22.2% resistance to flucytosine, amphotericin B, fluconazole and itraconazole with all the isolated cases of *Candida krusei* being resistant to fluconazole (Feglo *et al.*; 2012).

Among HIV/AIDS patients, prevalence of azole resistance have been estimated in a range of 9.3 to 56.7% globally (Manzano-Gayosso *et al.*, 2008; Falagas *et al.*, 2010; Mulu *et al.*, 2013; Rosana *et al.*; 2015; Salari *et al.*; 2016; Terças *et al.*, 2017).

In Africa, antifungal resistance in excess of 50% has been reported among HIV/AIDS patients in South Africa (Owotade *et al* 2016, Dos Santos *et al*, 2014). This high resistance reported in South Africa contradict findings of Blignaut *et al*, (1999) and (2002) which showed high rate of antifungal susceptibility among *Candida* species. Variation in these findings could not be clearly explained but it was noted that the earlier studies were conducted prior to the introduction of fluconazole in 2002 as prophylaxis to patients attending HIV-AIDS clinics in South Africa (Wertheimer *et al*, 2004). Cross-resistance to fluconazole in patients receiving itraconazole prophylaxis and other previously administered azole therapies, such as ketoconazole and miconazole have also been reported (Rautemaa *et al* 2008).

Findings from a study to determine the distribution and susceptibility pattern of yeasts isolates from oropharyngeal swab of HIV patients in Tanzania indicated 5% resistance to fluconazole and 8.4% resistance to itraconazole (Hamza *et al.*, 2008). In Nigeria, Enwuru *et al.* (2008) also reported that 9.5% of yeasts isolates from oro-pharynx of HIV positive patients were resistant to fluconazole. High resistance of *C. albicans* to azoles (50% and 70%) has also been reported among HIV/AIDS patients in two different studies in Cameroun (Dos Santos *et al.*, 2014, Njunda *et al.*, 2012).

Outside Africa, resistance of 14% to fluconazole and 18% to itraconazole have been reported among *Candida* isolates from the oral cavity of HIV patients in the United Kingdom (Bagg *et al*; 2005). In Texas, Taiwan and Turkey, antifungal resistance less than 4% have been reported among HIV/AIDS patients (Satana *et al.* 2010; Wu *et al.* 2012; Patel *et al.* 2012). Extensive use of antifungal agents for empirical treatments and prophylaxis has been found to contribute to these high resistance (Jia *et al*, 2008).

2.3 CLINICAL PRESENTATION AND PATHOGENESIS OF OPC

Candida infections can occur in many forms ranging from simple mucocutaneous to severe invasive infections that can affect virtually any organ (Arora *et al.*, 2011).

Clinical presentations of OPC include the pseudomembranous or thrush, erythematous, hyperplastic and denture-induced stomatitis. The pseudomembranous and erythematous are the forms that are usually seen in HIV-infection (Abi-Said *et al.*, 1997). The pseudomembranous form is characterized by white, curd-like, discrete plaques seen on the buccal mucosa, throat, tongue, or gingivae. The erythematous form is recognized as smooth red patches on the hard or soft palate, dorsum of tongue, or buccal mucosa.

Pathogenesis and onset of OPC is noticed when there is imbalance between *Candida* virulence and defects in host mucosal defenses during HIV infection (Hamza *et al.*, 2008). The precise mechanism explaining OPC pathogenesis is uncertain (Schuman *et al.*, 1998). OPC in HIV/AIDS infection was previously linked to malfunctioning of T cells of the host (Challacombe *et al.*, 1994). Later studies however attributed OPC to a combination of anti-*Candida* host defense mechanisms impairment (Ashman, *et al.*, 1995, Balish *et al.*, 2001, Farah, *et al.*, 2001). In HIV-positive patients, selective decline in the Th17 cells within the CD4+ T cell during infection has been linked to the ability of *C. albicans* to overcome epithelial defenses and cause disease (Cassone and Cauda, 2012).

Candida species have many virulence factors that make them able to cause a wide range of infections. Among these factors are: ability to change from one morphological form to another under different environmental conditions, secretion of adhesions and invasions (glycoproteins glycosylphosphatidylinositol) which helps them to attach to the surfaces of their host, formation

of biofilm and secretion of hydrolases (proteases, phospholipases and lipases) which play important roles in active penetration of the pathogen into host cells and absorption of nutrients from the host environment (Wächtler *et al.*, 2012; Nicholls *et al.*, 2011; Fanning *et al.*, 2012, Murciano *et. al.*, 2012 ; Brock, 2009).

Pathogenesis begins when the yeast cells adhere to surfaces of the host cells through adhesins. This trigger transition from yeast to hypha and direct growth through thiomotropism. Endocytosis is induced through mediation of invasins produced by the fungal cells. A second mechanism of invasion is thought to be facilitated by hydrolases and active penetration of the fungal cell into the host cell. Biofilms are formed when yeast cells attached to the host cells or catheters (Francois *et al.*, 2013). Low absolute CD4 count (<200 cells/ μ L) is considered a high risk factor for developing OPC during the course of HIV/AIDS infection (Calderone, 2001).

2.3.1 Host immunity to OPC

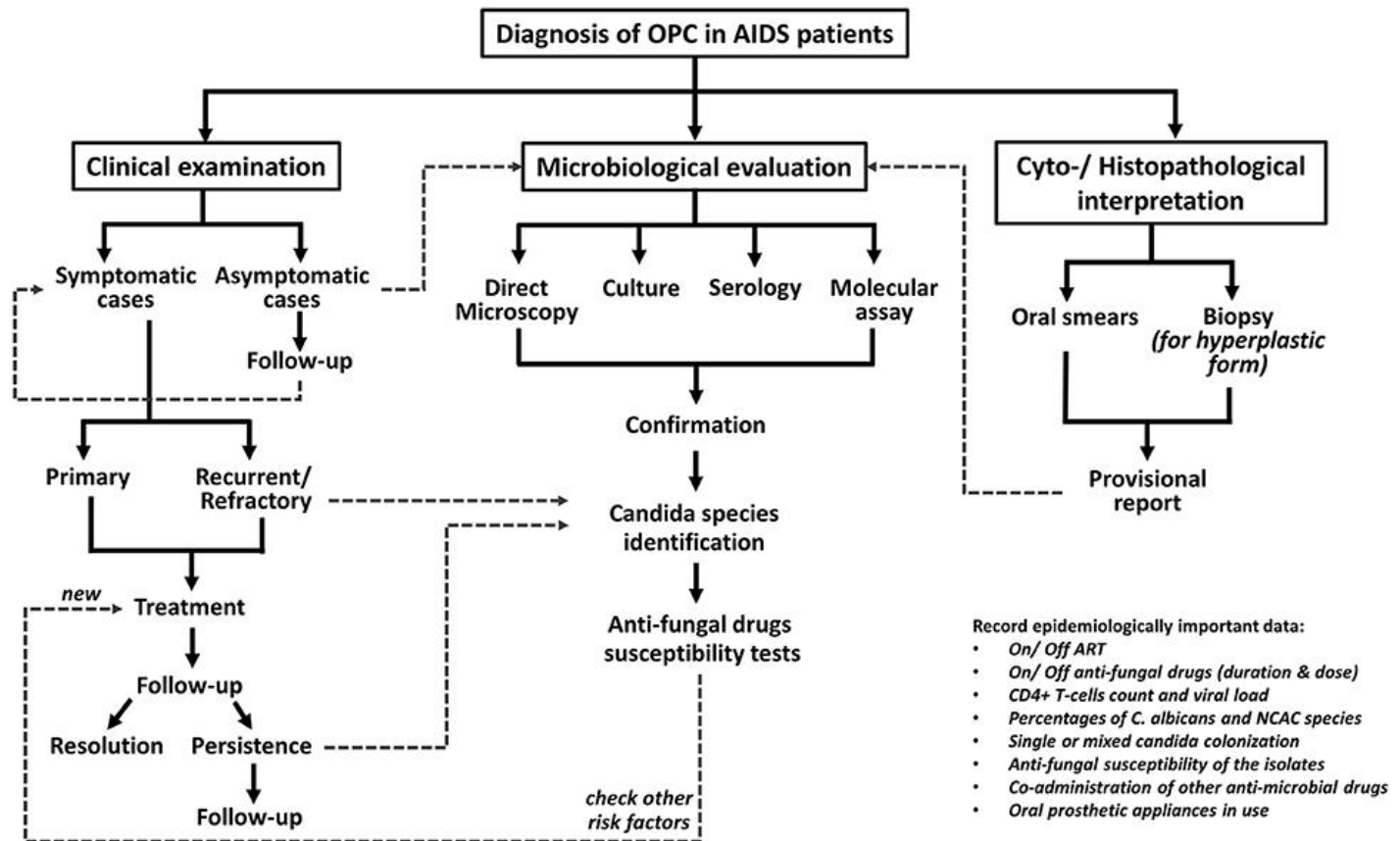
The role of innate and adaptive immunity in the clearing of fungal growth was described by Romani (2000). Mediation of T Helper I cells during infection releases cytokines which activate phagocytes to destroy fungal cells. This is followed by the release of other cytokines by T helper II cells, turning off the fungicidal effector capabilities. Downward release of IL-4 and IL-10 and upward production of IFN- γ and IL-2 have also been found to play a vital role in resistance of infection. The immunoregulatory role by neutrophils in antifungal T cell development have also been described. According to Romani (2000), high risks for fungal infections in neutropenic patients could be explained by this phenomenon.

2.4 CLINICAL AND LABORATORY DIAGNOSIS OF OPC

Differential diagnosis of OPC is done using a set of clinical criteria and symptoms such as sore in the oral cavities, swallowing difficulties, burning sensation, and change in taste. Non-responding cases are however diagnosed in the laboratory using microscopy and culture procedures.

2.4.1 Clinical diagnosis of OPC

Protocol for OPC diagnosis in HIV-positive patients described by (Thompson *et al.*, 2010; Lortholary *et al.*, 2012; Patil *et al.*, 2015) is shown below in figure 2.1.



2.4.1.1 Relationship between WHO clinical staging of HIV and OPC

The World Health Organization (WHO) clinical staging system was developed in 1990. It is a case definition tool used for HIV monitoring and clinical staging of HIV-associated diseases in adults and children. This system employs standardized clinical guidelines that help clinicians to make decisions as far as HIV management is concerned especially in clinical settings where access to laboratory services are limited or lacking (WHO,2005).

The WHO Clinical Staging System has since been revised to monitor when to initiate, change, or stop prophylactic medications, antiretroviral drugs, and other medical interventions. It also helps in the assessment of current clinical status of patients and sequentially group disease from least to most severe (WHO, 2005). The system categorizes patients into one of four clinical stages starting from stage 1 (asymptomatic) to stage 4 (AIDS). Patients are placed in a particular stage when they show at least one clinical symptom associated with that stage's criteria. Patients are moved to a higher stage after they recover from the clinical condition that placed them in that stage (WHO, 2005). OPC is usually seen in stage three with Oesophageal candidiasis seen in stage 4. Clinical staging for adults, adolescents, infants and children are shown in Tables 2.1 and 2.2.

Table 2.1: Clinical staging of HIV/AIDS for adults and adolescents

Clinical stage	
1	Asymptomatic, persistent generalized lymphadenopathy (PGL)
2	Unexplained weight loss (<10% of presumed or measured body weight), recurrent respiratory tract infections, herpes zoster, angular cheilitis, recurrent oral ulcerations, Papular pruritic eruptions, Seborrhoeic dermatitis, fungal nail infections of fingers.
3	Severe weight loss (>10% of presumed or measured body weight), unexplained chronic diarrhoea for longer than one month, unexplained persistent fever, oral candidiasis, oral hairy leukoplakia, pulmonary tuberculosis (TB), severe presumed bacterial infections, acute necrotizing ulcerative stomatitis, gingivitis or periodontitis
4	<p>Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations.</p> <p>HIV wasting syndrome, Pneumocystis pneumonia, Recurrent severe or radiological bacterial pneumonia, Chronic herpes simplex infection (or labial, genital or anorectal of more than one month's duration) Oesophageal candidiasis, Extrapulmonary TB, Kaposi's sarcoma</p> <p>Central nervous system (CNS) toxoplasmosis, HIV encephalopathy</p>

Sourced from the guidelines for antiretroviral therapy in Ghana (3rd edition, 2008)

Table 2.2: WHO clinical staging of HIV and AIDS for infants and children

Clinical stage	
1	Symptomatic, PGL
2	Hepatosplenomegaly, Papular pruritic eruptions, Seborrhoeic dermatitis, extensive human papilloma virus infection, extensive molluscum contagiosum, fungal nail infections, recurrent oral ulcerations, lineal gingival erythema, angular cheilitis, parotid enlargement, herpes zoster, recurrent of chronic RTIs.
3	Moderate unexplained malnutrition not adequately responding to standard therapy, Unexplained persistent diarrhoea (14 days or more), Unexplained persistent fever (intermittent or constant, for longer than one month) Oral candidiasis (outside neonatal period), Oral hairy leukoplakia, Acute necrotizing ulcerative gingivitis/periodontitis, Pulmonary TB, Severe recurrent presumed bacterial pneumonia.
4	Unexplained severe wasting or severe malnutrition not adequately responding to standard therapy. Pneumocystis pneumonia, Recurrent severe presumed bacterial infections (e.g. empyema, pyomyositis, one or joint infection, meningitis, but excluding pneumonia), Chronic herpes simplex infection; (orolabial or cutaneous of more than one month's duration), Extrapulmonary TB, Kaposi's sarcoma, Oesophageal candidiasis, CNS toxoplasmosis (outside the neonatal period)

Source from the guidelines for antiretroviral therapy in Ghana (3rd edition, 2008)

2.4.1.2 Immunological staging of HIV infection

Aside clinical staging, immunological staging can be done based on CD4 count measurement. The CD4 cell count and viral load are used for monitoring the progression of HIV infection. This supports and reinforces treatment decision-making. Low CD4 lymphocyte counts (<200 cells) are associated with severe immune depression. Patients are considered to have active viremia when their viral loads exceed 10,000 copies per milliliter (Saini, 2011). Tables 2.3 and 2.4 classifies the immunological staging for adults and children respectively.

Table 2.3: CD4 level in relation to the severity of immunosuppression in adult

Not significant immunosuppression	>500/mm³
Mild immunosuppression	350 – 499mm³
Advanced immunosuppression	250 – 349/mm³
Advanced immunosuppression	250 – 349/mm³
Severe immunosuppression.	250 – 349/mm³

Sourced from the guidelines for antiretroviral therapy in Ghana (3rd edition, 2008)

Table 2.4: CD4 level in relation to the severity of immunosuppression in children

HIV associated immune deficiency	Age-related CD4 values			
	<11 months (%)	12-35 months	36-59 months (%)	>5 yrs. (cells/mm ³)
Not significant	>35	>30	>25	>500
Mild	30-35	25-30	22-25	350-499
Advanced	25-30	20-25	15-20	200-349
Severe	<25	<20	<15	<200 or <15%

Sourced from the guidelines for antiretroviral therapy in Ghana (3rd edition, 2008)

2.4.2 Laboratory diagnosis of OPC

Proper identification of *candida* species have become necessary due to reports of reduced susceptibility to antifungals by *Candida* species. Various techniques for isolation and identification of yeast have been described and available for laboratories to adopt depending on resources available (Yang *et al.*, 2009). Conventional methods such as the assimilation and fermentation reactions have been described as slow, labour intensive and produces results that are sometimes not clear and conclusive (Williams *et al.* 1995).

Molecular identification has been described as more sensitive compared to the conventional methods (Williams *et al.* 1995). Other reliable methods of identification include commercial automated systems such as the VITEK 2.

Basic culture media used for isolating *Candida* species from clinical samples include blood agar, Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA) or broth (PDB) and Sabouraud brain heart infusion agar, or broth (SDB) (Yang *et al.*, 2009). Other media such as CHROMagar *Candida*, CandiSelect4, and Pharmamedia have been used as differential or selective media for isolation and identification of *Candida* species (Madhavan *et al.*, 2009). To differentiate closely related *C. albicans* from *C. dubliniensis*, Bird-seed agar have been used and has been found to be fast, sensitive and reliable (Pasligh *et al.*, 2010).

Microscopy can also be employed in identifying distinct features of yeast cells in clinical samples. Specimens obtained from exudates, sputum, urine and cerebrospinal fluid can be observed under reduced-light brightfield microscope or phase-contrast microscope (Aslanzadeh and Roberts, 1991). Stains such as potassium hydroxide solution (10-20%), lactophenol cotton blue, Gram and Giemsa can be used to reveal yeast cells in clinical samples under the microscope. Other histological stains such as Periodic acid Schiff, methenamine silver, hematoxylin-eosin and papanicolaou stains can also be used to detect fungi in tissues.

2.4.2.1 Automated and biochemical systems

Automated culturing systems are used to detect microbial growth. The principle behind these systems involve monitoring of CO₂ production released as a result of metabolic activities of the microbial cells. Duration of culture varies among *Candida* species. Reported mean time detection of *Candida albicans* varies from 35.3 to 85.8 hours. Yeasts cultures are usually held for 21 days (Fernandez *et al.*, 2009). Vitek-2 YST system are fully automated systems for yeast identification. It operates on the basis of enzymes detection in the yeast species. They could correctly identify *Candida* species in 18 hours (Loiez *et al.*, 2006). Although somewhat effective

in identifying most yeast species, limitations such as difficulty in identifying *Candida dubliniensis*, *C. kefyr* and differentiation of *C. haemulonii* from its sibling species *C. pseudohaemulonii* have been reported. (Cardenes-Perera *et al.*, 2004, Gomez-Lopez *et al.*, 2010). Automated systems are more sensitive compared to manual procedures and does not require manual inspection or examination of the culture (Han, 2006).

Biochemical tests are usually employed after initial identification of cultures on agar media and microscopy. These biochemical methods are based on carbohydrate utilization, analysis of protein profile and comparison of distinct isoenzymes. Changes among *Candida* species are detected through changes in colour indicator when the yeast cultures make use of 1% carbohydrates such as glucose, maltose, sucrose, trehalose and raffinose. Example of such system are API 20C, API 32C or RapID Yeast Plus. Unlike the API 20C and API 32C that utilizes carbohydrates, RapID Yeast Plus system hydrolysis 1% fatty acid ester, 0.05% aryl-substituted glycosides, 0.3% urea and 0.01% arylamide substrates. Colours produced at the end of incubation are coded and compared with differential Chart for species identification. These tests have been found to be economical, rapid and simple to perform and is also able to differentiate phenotypically alike *C. albicans* from *C. dubliniensis* (Aslanzadeh, 2006).

2.4.2.2 Molecular identification

Molecular identification of fungi targets internally transcribed spacer (ITS) regions nested in the nuclear rDNA. These ITS regions differ among distinct fungal species and even within the species. Ribosomal RNA (rRNA) is used for probe-based detection of species as its nucleotide sequence is well conserved within a species and varies between species (Pryce *et al.*, 2006). PCR techniques that are species-specific have been used for *Candida* species identification targeting several genes for species discrimination although commonly amplified are the sequences of the

ribosomal RNA operon. Following gel electrophoresis resolution, identification can be done based on obtained PCR product sizes or variation in PCR product sequence obtained either through direct sequencing or the use of restriction fragment analysis (Li *et al.*, 2003). Fluorescence in situ hybridization with peptide nucleic acid method (PNA Fish) is a novel technique that target highly conserved species-specific sequences in the rRNA of *C. albicans*. This system allows for the detection of *Candida* Asla. In differentiating phenotypically related *C. albicans* from *C. dubliniensis* a sensitivity of 98.7–100% and a specificity of 100% have been reported (Rigby *et al.*, 2002). Depending on the aim of the identification, Other techniques such as Ca3 genetic fingerprinting for *Candida albicans*, Multilocus Sequence Typing (MLST), Multilocus Enzyme Electrophoresis (MLEE), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) analysis, microsatellite-PCR and Amplified Fragment Length Polymorphism can be used for strain identification (Boldo *et al.*, 2003, Lopes *et al.*, 2007). Application of molecular technique as a means of routine identification has been challenging since it is expensive, require trained personnel and lack of sufficient species-specific primers (Lopes *et al.*, 2007).

2.5 MANAGEMENT OF OPC

Effective management of OPC is dependent on proper diagnosis based on detailed clinical history and presentation of lesion, laboratory confirmation of non-responding cases, keeping proper oral health and treatment using appropriate antifungal (Fathilah *et al.*, 2012).

Polyenes- nystatin and amphotericin B; the azoles -miconazole, clotrimazole, ketoconazole, itraconazole, and fluconazole, 5-fluorocytosine, and Echinocandins are antifungal agents used in

treating OPC (Pappas *et al.*, 2009). Antifungal agents are selected based on the severity of infection and immune status of patients.

Fluconazole, nystatin, clotrimazole and miconazole are the first line antifungal drugs used for treating OPC in HIV-patients (Thompson *et al.*, 2010; Lortholary *et al.*, 20112). Fluconazole usually comes in a form tablets and solution. Normal dosage is 50 mg daily for 7-14 days. Fluconazole has be reported to be effective and complies well during ART treatment (Lortholary *et al.*, 20112). Nystatin is usually dispensed in 100,000 U/mL suspension form. 4-6 ml is taken four times daily for 7-14 days.

Among the second line drugs are itraconazole, posaconazole and voriconazole. Posaconazole are used for treating fluconazole resistant *Candida* species (Vazquez, 2010). Ketoconazole is not widely used due its side effects and interaction with HIV protease inhibitors (Vazquez, 2010). Micafungin, caspofungin anidulafungin, amphotericin B and deoxycholate are used when OPC becomes refractory. With exception of Amphotericin B which could be administered orally or intravenously, micafungin caspofungin, anidulafungin, and deoxycholate are all dispensed as intravenous drugs. Normal dosage for caspofungin is 70mg as loading dose followed by 50mg daily. Micafungin is given 10-15mg daily. Amphotericin B is given 500mg every six hours (Thompson *et al.*, 2010; Lortholary *et al.*, 20112).

Oral candidiasis can be prevented through good oral hygiene practices. Regular cleaning of the teeth and rinsing the mouth with antiseptics such as Chlorhexidine digluconate and cetylpyridinium chloride helps to prevent oral mucositis (Fathilah *et al.*, 2012).

2.6 ANTIFUNGAL AGENTS: MODE OF ACTION AND MECHANISM OF RESISTANCE

The azoles are broad-spectrum fungistatic antifungals. It comprises of the imidazoles (Ketoconazole, miconazole and clotrimazole) and the triazoles (Fluconazole, itraconazole, voriconazole, and posaconazole). The triazoles are less toxic, and are commonly used to treat invasive fungal infections. The imidazoles normally come in a form of creams, ointments, and suppositories to treat vaginal yeast infections and dermatophytes infections. The imidazoles have been associated with more side effects compared to the triazoles (Bodey, 1993).

Azoles exert antifungal activity by inhibiting the C14 α demethylation of lanosterol in fungi resulting in the interference of synthesis of ergosterol in the fungal cell membrane (Xiao *et al.*, 2004). Four major mechanisms of resistance to azoles have been described in *Candida* species. These include decreased drug concentration, target site alteration, Up-regulation of target enzyme and development of bypass pathways (Loffler *et al.*, 1997).

Interaction of Polyene with the membrane sterol of fungal cells causes development of porin channels which leads to loss of transmembrane potential and impaired cellular function of the fungal cell. The mechanism of resistance involve the linkage of molecules hydrophobically to the membrane sterol of the fungal cell forming a configuration which produces pores in the fungal cell leading to altered permeability, leakage of important cytoplasmic contents and eventual death of the fungal cell (Kerridge 1995). In *Candida* and *Cryptococcus* species resistance to Polyene has been attributed to defects in the *ERG3* gene involved in ergosterol biosynthesis (Dick *et al.*, 1980). Resistance to amphotericin B have also been linked to high levels of catalase activity, with low susceptibility to oxidative damage (Sokol-Anderson *et al.*, 1986).

The echinocandins are semi-synthetic lipopeptides that competitively inhibit β -glucan synthetase. Echinocandins prevent the formation of β -1, 3-d glucan, an important structural and functional component of the fungal cell wall. This causes altered structure of the cell wall leading to cell rupture in yeast and aberrant hyphal growth in molds. Although highly effective against *Candida* and *Aspergillus* species, no antifungal activity has been found against Zygomycetes, *Cryptococcus*, *Trichosporon*, *Scedosporium*, and *Fusarium* species (Espinel-Ingroff, 2003). Even among the *Candida* species, *C. parapsilosis* and *Candida guilliermondii* isolates have been reported to be associated with higher MIC values compared to *C. albicans* (Mora-Duarte *et al.*, 2002). Caspofungin is approved for the treatment of invasive *Aspergillosis* in patients not responding to amphotericin B and itraconazole (Mora-Duarte *et al.*, 2002). Although the mechanisms of echinocandins resistance is still under investigation, in *Candida* species, secondary resistance has been attributed to a point mutations commonly found at the Ser645 position (a highly conserved region) in the *Fks1* gene of the β -1,3-d-glucan synthase complex (Balashov *et al.*, 2006).

5-Fluorocytosine (5FC) is a fluorinated pyrimidine which inhibits the activities of most yeast cells. The mechanism of action of 5FC was described by Polak and Scholer, (1975) and Diasio *et al.*, (1978). This involve the conversion of 5FC into 5-fluorouracil through the catalytic action of cytosine deaminase. The 5-fluorouracil is further converted to 5-fluorouridylic acid by UMP pyrophorylase enters the RNA of the fungal cells and inhibits protein synthesis. The 5-fluorouracil also inhibits DNA synthesis by interfering with the activity of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division in fungi cells. Intrinsically, some yeast strains are resistant to flucytosine due to impaired cellular uptake secondary to a mutation in cytosine permease. Resistance to flucytosine could also be acquired through mutations in

cytosine deaminase or uracil phosphoribosyl transferase. Prevalence of primary resistance to flucytosine by *candida* and *C. neoformans* species has generally been low (Price *et al.*, 1994, Brandt *et al.*, 2001). This notwithstanding, resistance by some *Candida* species has been documented occasioning the use of flucytosine only in combination with other antifungal agents, mostly amphotericin B (Hospenthal *et al.*, 1998).

2.7 REVIEW OF ANTIFUNGAL SUSCEPTIBILITY TESTING METHODS

Antifungal susceptibility testing (AFST) constitute an important role in the field of medical mycology. It is used to monitor the susceptibility patterns and resistance rates of pathogenic strains against mostly used antifungal agents. It is also used to determine antifungal activity of novel compounds and predict clinical result. Establishment of Standard methods by institutions such as the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) have made it possible to determine the susceptibilities of *Candida* species to some antifungal drugs. So far interpretive breakpoints have been established for fluconazole, itraconazole, voriconazole, and 5-fluorocytosine. A new designation, susceptible dose dependent (SDD) have also been introduced for species with intermediate MICs that could be successfully treated with higher doses of antifungal drugs in vivo (Rex *et al.*, 1997). Just as in bacterial susceptibility testing, factors that interfere with the ability to obtain optimum concentration of the antifungal drug at the site of target is important when interpreting in vitro susceptibility results (Sanders and Sanders 1982).

Examples of standard susceptibility testing methods include the Clinical and Laboratory Standards Institute (CLSI) reference susceptibility testing assays, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) standard susceptibility testing assays and other

methods such as the E test, colorimetric microdilution and agar dilution. The availability of these standardized methods have facilitated the performance of large-scale surveillance studies that have documented the potency and spectrum of antifungal drugs against clinical isolates of *Candida* spp. (Espinel-Ingroff, 2003; Otrrosky-Zeichner *et al.*, 2003; Pfaller *et al.*, 2005; Pfaller *et al.*, 2006).

2.7.1 The CLSI reference susceptibility testing method

Standardization of antifungal susceptibility testing was initiated by the Clinical and Laboratory Standards Institute (CLSI) formerly National Committee for Clinical Laboratory Standards (NCCLS) in 1983 through a multicenter studies. Following these studies was the description and documentation of reference microdilution methodologies for yeasts including *Candida* species and *Cryptococcus neoformans* in NCCLS document M27-A2 (2002). NCCLS document M38-A describes reference methods for moulds including *Aspergillus* species and *Fusarium* species.

The CLSI guideline recommends the broth microdilution method (BMD) as the best method to perform antifungal susceptibility testing (NCCLS, 1997). This method provides reproducible MIC results and employs RPMI 1640 broth medium. Incubation is at 35°C for 24 hours, and an MIC endpoint criterion of prominent reduction in growth ($\geq 50\%$ inhibition relative to control growth) (Odds *et al.*, 2004; Pfaller *et al.*, 2004). Although described as the best method for performing antifungal susceptibility testing, limitations such as delays in getting results for management of patient because of 24-72 hours required for reporting, failures to discriminate amphotericin B-resistant isolates from the susceptible ones, lack of interpretative breakpoints for many fungus-antifungal drug combinations and difficulty in reading and interpreting minimum

inhibitory concentrations for azole and flucytosine especially for heavy trailing isolates have been reported in routine laboratory practice (NCCLS, 2002, Rex *et al.*, 1995, Pfaller *et al.*, 2004).

Other methods that correlate with the CLSI microdilution methods have since been described for yeasts (Pfaller *et al.*, 2006, Pfaller *et al.*, 2005). Examples of such methods include the standard disk diffusion susceptibility testing method for *Candida* described in CLSI document M44-A (NCCLS, 2004). This method employs Mueller-Hinton agar, supplemented with 2% glucose and methylene blue as the test medium.

2.7.2 The EUCAST standard susceptibility testing assays

The EUCAST reference method was developed by the Subcommittee on Antifungal Susceptibility Testing (AFST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and described in the EUCAST Discussion Document E.Dis 7.1. The method was developed primarily for the validation of yeast species excluding *Cryptococcus neoformans* and other non-fermentative yeasts. The major difference between the EUCAST and CLSI dilution methods is based on modifications of some test parameters such as the medium used, density of inoculum used, microdilution plates, MIC reading time point and MIC reading method. Findings from various validation studies have found the EUCAST microdilution assay as reproducible and correlate well with the CLSI method (Cuenca-Estrella *et al.*, 2003).

Depending on the species, drug and incubation period, MIC correlation of 85- 95% has been reported between the EUCAST and CLSI methods (Espinel-Ingroff *et al.*, 2005, Cuenca-Estrella *et al.*, 2002). This notwithstanding, CLSI MIC breakpoints are not used to interpret EUCAST MIC data due to findings of poor agreement between the two methods when used for the susceptibility for fluconazole, itraconazole, and voriconazole against some *Candida* species (Espinel-Ingroff *et al.*, 2005).

2.7.3 E-TEST and other colorimetric microdilution assays

E-TEST is an agar-based diffusion method used for the determination of MIC values. They are commercially available for amphotericin B, fluconazole, itraconazole, flucytosine, voriconazole, Posaconazole and caspofungin. Correlation studies between E-TEST and CLSI methods have produced variable results. However, in most cases percentage of agreement were above acceptable limits.

Percentage agreements of 96% for fluconazole (Barry *et al.*, 2002), 95% for voriconazole (Maxwell *et al.*, 2003) and 83% for posaconazole (Sims *et al.*, 2006) against *Candida* have been reported. Between the EUCAST microdilution method and E-TEST, 90.4% agreement for fluconazole and *Candida* species has been reported (Cuenca-Estrella *et al.*, 2005). Break point for CLSI MIC is used for interpretation of the E-TEST results. The E-TEST is easier to use and have been reported to be more efficacious compared to the reference microdilution method in detection of amphotericin B resistance in *Candida* (Peyron *et al.*, 2001).

Colorimetric microdilution assays such as the Sensititre Yeast One (TREK Diagnostic Systems, Cleveland, Ohio) and ASTY colorimetric microdilution panel (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo) contain colorimetric dyes and are read visually. Compared to the reference microdilution, percentage agreement of 92.3 to 98 has been reported for amphotericin B, fluconazole, itraconazole, ketoconazole and flucytosine against filamentous fungi (Carrillo-Munoz *et al.*, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY SITES

Samples were collected from seventeen (17) health facilities in the central region of Ghana between May, 2017 and June, 2018 using non-probability sampling methods. Among these facilities, Cape Coast Teaching hospital (CCTH) was the largest health facility which serves as a referral hospital for people in Cape Coast metropolis and other surrounding districts. It is also a teaching facility for the school of medical sciences, University of Cape coast. All the other health facilities provide primary health care services. Provision of ART services in the region started around 2006. Details of health facilities where participants were recruited and number of participants drawn from each facility is shown in Table 3.1.

Table 3.1: Sites of recruitment and number of participants

HEALTH FACILITY	#Samples collected	# Positives
Cape Coast Teaching Hospital	14	5
Ewim Polyclinic	11	8
Elmina Health Centre	10	9
Cape Coast Municipal Hospital	9	7
University of Cape Coast Hospital	23	13
Ankaful Prisons	12	11
Centre of Awareness (Cape Coast)	15	11
Frame Health Centre	5	4
Dunkwa Municipal Hospital	39	19
Diaso Health Centre	18	15
Abura Dunkwa Hospital	8	5
Mfantsiman Hospital Saltpond	18	8
Mercy Women Catholic Hospital	5	5
Our Lady of Grace Hospital	24	18
Apam Catholic Hospital	33	21
Winneba Trauma Specialist Hospital	11	11
Kasoa Polyclinic	31	24

3. 2 STUDY DESIGN AND PARTICIPANTS

The study was an analytical and cross-sectional. The study participants were recruited from HIV-infected patients showing symptoms of OPC across all gender and age. Patients suspicious of having OPC based on established criteria such as observation of typical whitish-yellow, curdlike, easily removable plaques or lesions on the oropharyngeal mucosa or tongue, red lesions on the tongue or dorsal palate, burning pain in the mouth, altered taste sensation, and difficulty swallowing liquids and solids (Greenspan *et al*; 1994) were recruited after they consented to be part of the study. Demographic data including year of HIV diagnosis, history of previous fungal infections, history of use of antifungal therapy, history of ART treatment and duration on ART prior to sampling were obtained from folders of participants.

3.2.1 Inclusion and exclusion criteria

HIV-patients with presumptive diagnosis of OPC across all ages and gender irrespective of their ART status were included in the study.

Patients who were too ill and unconscious to participate or undergoing emergency procedures were excluded from the study.

3.2.2 Sample size

Sample size was determined using the formula. $[Z^2 \cdot P \cdot (1-P)]/C^2$

Where

Z = Standard score corresponding to 95% Confidence Level = 1.96.

P = Prevalence = 75.3% (0.753) obtained from a study by Kwamin *et al.*, (2013)

C = Precision limit or proportion of sampling error of 5% = 0.05

Sample size= $(1.96^2 \times 0.753 \times 0.247) / 0.05^2 = 286$ samples

3.3 SAMPLE COLLECTION

Oral swabs were aseptically taken from patients by gently rotating the sterile swab over the suspected area of infection. The swabs were placed into a transport medium immediately after swabbing and labelled. Specimen were transported on ice pack to the Microbiology Department at CCTH as soon as possible. Specimens that could not be transported or processed immediately were refrigerated at 2-8°C. Blood samples were also collected in EDTA tubes for CD4+ T-lymphocyte estimation using the BD FACS count machine.

3.4 LABORATORY METHODS AND PROCEDURES

The study was divided into three main phases:

- Quantitative analyses- Examination of specimen, culture and isolation
- Presumptive and definitive identification
- Antifungal susceptibility testing

3.4.1 Quantitative analysis

This part of the work was done at the Microbiology department of the Cape Coast teaching hospital laboratory and consisted of culture, isolation of *candida* species and maintenance of the isolates.

3.4.1.1 Culture and isolation

Oral swabs obtained from participants were aseptically cut into 10 ml Sabouraud brain heart infusion (SBHI) and incubated at $35\pm 2^{\circ}\text{C}$ for 18 to 24 hours. The broth was sub-cultured onto Sabouraud dextrose Agar (SDA) plates supplemented with broad spectrum antibiotics and incubated for 24-48 hours to obtain pure yeast isolates. *Candida* species were isolated based on their colonial morphology on the SDA together with distinguishing characteristics microscopic features and Gram stain.

3.4.1.2 Maintenance of isolates

Confirmed isolates were stored in sterile SDB and refrigerated at $2-6^{\circ}\text{C}$ until they were further used in the study. Working stocks were sub-cultured on SDA every 4-12 weeks to obtain pure cultures and then restocked. Slant stock on SDA was also maintained at a temperature of $2-8^{\circ}\text{C}$.

3.4.2 Presumptive and definitive identification

This phase of the work was carried out at the Department of Medical Microbiology Laboratory, University of Ghana. Presumptive identification was done using germ tube test and *Candida* differential agar (HICROME) from HIMEDIA laboratories PVT. India. Definitive identification was done using HiCandida identification kit also from HIMEDIA laboratories PVT. India.

3.4.2.1 Germ tube test

Germ tube test was performed to differentiate germ tube positive (*Candida albicans*, *Candida stellatoidea* and *Candida dubliniensis*) from other *Candida* species. In performing the germ tube test, a small portion of the *Candida* isolates were inoculated into 0.5 ml serum and incubated for 2-3 hours at 35°C and observed under the microscope for the presence or absence of germ tube formation.

3.4.2.2 Culture on *Candida* differential agar

Working stock was sub-cultured onto SDA to obtain pure cultures. The fresh and pure cultures were sub cultured onto the *Candida* differential agar plate and incubated aerobically at 35 °C for 48 hours. Species were identified based on the colour and morphology of the colonies on the differential agar plate.

3.4.3 Definitive identification

Definitive identification was done using HiCandida identification kit following manufacturer's instructions on test procedure (HIMEDIA laboratories PVT. India).

Test procedure

- Homogenous suspension was prepared by picking 2-4 identical colonies into 2-3ml sterile saline
- The density of the suspension was adjusted to 0.50 D at 620nm.
- Test kit was open aseptically and inoculated with 50µl of the suspension and incubated at 22.5 °C +/- 2.5 °C for 24-48 hours.
- Results were interpreted as per the standards given in the identification index.

3.4.4 Antifungal susceptibility testing

This aspect of the work was carried out using the Disk Diffusion method described in CLSI document M44-A. This method uses Mueller-Hinton agar supplemented with 2% Glucose and 0.5µg/mL Methylene Blue Dye (GMB) and is considered to be a good choice for routine susceptibility testing of yeasts. MIC determination strips (fluconazole 0.016-256 µg/ml, Amphotericin B 0.002-32 µg /ml, and Flucytosine 0002-32 µg /ml) obtained commercially from

HiMedia Laboratories Pvt. (India) was used for the antifungal susceptibility testing. These strips give reproducible MIC values that are equivalent to the standard reference MIC obtained by broth dilution method.

Prior to AFST, all isolates in suspension were sub-cultured unto SDA plates again to ensure their purity, viability and to obtain fresh isolates. About five distinct colonies obtained from 24hrs incubation at 35°C was suspended in physiological saline (0.85% NaCl) vortex for 15 seconds and adjusted by adding sufficient sterile saline or more colonies to attain 0.5 McFarland standard (corresponds to 1×10^6 to 5×10^6 CFU/ml).

A sterile cotton swab was dipped into the suspension within 15 minutes after adjusting the turbidity of the inoculum suspension. The swab was rotated many times and pressed against the inside wall of the tube to remove excess fluid from the swab. The dried surface of a sterile Mueller-Hinton + GMB agar plate was inoculated by evenly streaking the swab over the entire agar surface. The inoculum was allowed to dry for 5-15 minutes with the lid in place.

The MIC strip was lifted with the help of an applicator and placed at a desired position on the agar plate swabbed with test culture and allowed for 60 seconds to be absorbed and firmly adhere to the agar surface. The plates were incubated at 35°C for 24 hours.

Each plate was examined after 20-24 hours of incubation and read only when sufficient growth was observed. MIC was read as where the ellipse intersects the MIC scale on the strip. Where the ellipse intersects the strip in between two dilutions, the MIC was read as the value which is nearest to the intersection. Isolated colonies, pinpoint colonies and hazes appearing within the zone of inhibition were ignored as recommended by the manufacturer. MIC reading in this case was determined at a point on the scale at which prominent reduction of growth was seen. Where

growth was seen along the entire strip, the MIC was reported as greater than the highest values on the strip and where the inhibition ellipse is below the strip MIC was reported as less than the lowest value on the strip. Results were interpreted based on CLSI interpretive criteria (M 60, 2017).

Antifungal drug	Sensitive ($\mu\text{g /ml}$)	SDD ($\mu\text{g /ml}$)	Resistance ($\mu\text{g /ml}$)
Amphotericin B	< 1	-	> 1
Fluconazole	≤ 2	4 ≤ 32 (<i>C. glabrata</i>)	≥ 8 ≥ 64 (<i>C. glabrata</i>)
Flucytosine	≤ 4		> 16

Quality control strains were set in similar way for every batch of isolate that was tested. As recommended by NCCLS, quality control strains *C. albicans* ATCC 90028 *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were used.

3.5 DATA HANDLING AND ANALYSIS

All subjects who took part in the study were given coded numbers for identification instead of being identified by name or personal data for security and confidentiality. Data obtained from the study were entered into Microsoft Excel 2016 and imported into IBM SPSS Statistics Version 25 for statistical analysis. Categorical variables were summarized as frequencies and percentages, and continuous variables as means and standard deviations. Pearson Chi Square test was used to determine associations between *C. albicans*-associated OPC and categorical variables, and point

biserial correlation was used to determine association between the former and continuous variables at an alpha level of 0.05. Binary logistic regression was used to determine the predictive value of ART for *C. albicans*-associated OPC, when the variable showed significant associations with the latter in the Chi Square test.

3.6 ETHICAL CONSIDERATION

Ethical approval was sought from the Ethics and Protocol Review Committee (EPRC) of the School of Biomedical and Allied Health Sciences (SBAHS), College of Health Sciences of the University of Ghana (**CHS –Et/M.4-P2.9/2017-2018**). Written informed consent was also obtained from all participants.

CHAPTER FOUR

RESULTS

4.1 DEMOGRAPHIC CHARACTERISTICS OF STUDY SUBJECTS

Table 4.1 summarizes the general characteristics of the study participants, and comprised 33.6% males ($n=71$) and 63.4% females ($n=123$). Their mean age was 40.711 ± 15.157 years, with 68.6% ($n=133$) of them being on antiretroviral therapy (ART) and 10.3% ($n=20$) having previous exposure to fluconazole. None of the participants was on prophylactic antifungal agents at the time of recruitment. The mean CD4 counts of the study participants was low, being 211.062 ± 235.584 cells per microliter.

Table 4.1 General characteristics of HIV/AIDS participants with OPC

Parameter	Frequency (N)	Percentage (%)
Gender		
Males	71	36.6
Females	123	63.4
ART		
Yes	133	68.6
No	61	31.4
Antifungal therapy		
Yes	20	10.3
No	174	89.7
CD4 Counts		
≤ 200	112	63.3
201 - 499	48	27.1
≥ 500	17	9.6

Mean age = 40.711 ± 15.157 years; Mean CD4 count = 211.062 ± 235.584 cells/μl ART = Active antiretroviral therapy

4.2 CANDIDA SPECIES AND DISTRIBUTION

The *Candida* isolates were identified to the species level. In all, 67.8% (194/286) samples were culture positive, with seven different *Candida* species. Figure 4.1 shows the distribution of *Candida* species isolated with *C. albicans* 134 (69.1%) topping the list.

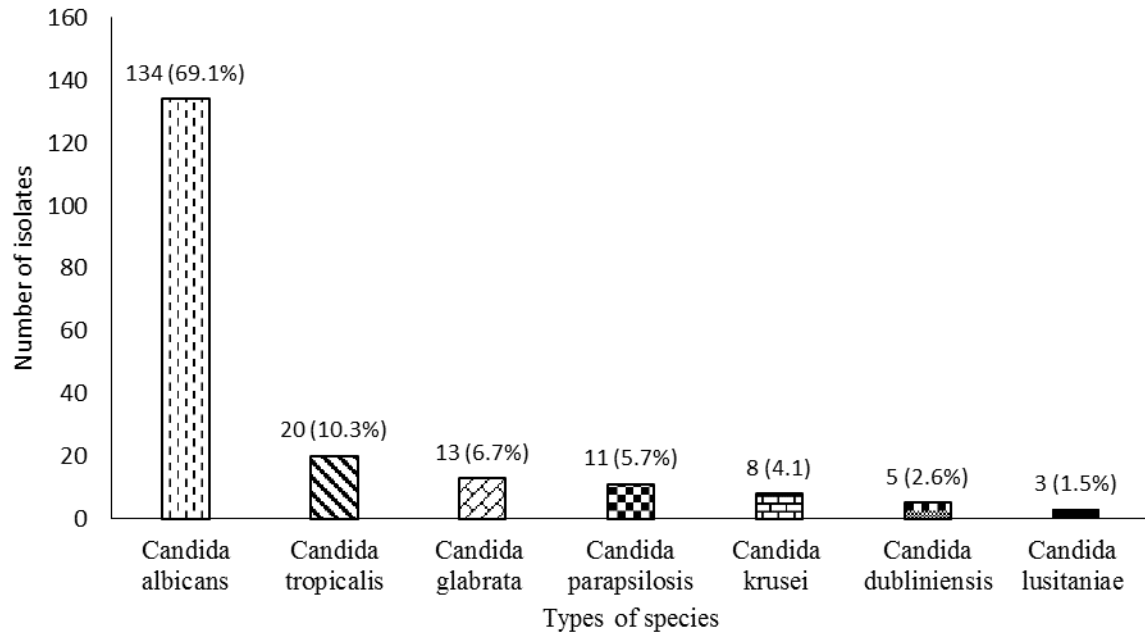


Figure 4.1: Distribution of *Candida* species isolated from culture

4.3 ASSOCIATION BETWEEN CHARACTERISTICS OF PARTICIPANTS AND CANDIDA INFECTION.

The demographic characteristics of the study participants were statistically not different in gender [(OR=1.01, 95% CI, 0.535-1.6) ($p = 0.981$)], previous exposure to antifungal drugs [(OR=1.05, 95% CI, 0.353-2.880) ($p = 0.924$)], duration of ART [R=-1.03; 95% CI, 0.402-2.66) ($p=0.993$)] and CD4 count despite being selected from different sites.

However, *C. albicans*-associated OPC was significantly higher among HIV-infected patients on ART compared to ART-naïve patients [(73.9% vs. 26.1%; OR=2.16, 95% CI, 1.141-4.101) ($p=0.018$)]. The odds for an HIV-infected patient to develop *C. albicans*-associated OPC was found to be about 2.2 times higher for a patient on ART than ART-naïve patient. Details of the associations are presented in Table 4.2.

Table 4.2: Logistic regression model with respect to Candida infection in HIV patients

Parameter	<i>C. albicans</i> (N= 134)	Non- <i>C. albicans</i> (N= 60)	P-value
	OR (95% CI)	OR (95% CI)	
Gender			
Males	1.01(0.535-1.896)	0.99(0.527-1.868)	0.981
Females*	Reference	Reference	-
Antifungal Hist.			
Yes	1.05(0.383-2.880)	0.95(0.347-2.612)	0.924
No*	Reference	Reference	-
ART			
Yes	2.16(1.141-4.101)	0.46(0.244-0.877)	0.018
No *	Reference	Reference	-
Duration on HAART			
Below 3 months	1.03(0.402-2.660)	0.967(0.376-2.486)	0.994
3-6 months	0.58(0.186-1.816)	1.719(0.551-5.364)	0.351
Above 6 months*	Reference	Reference	-
CD4 Count			
≤ 200	0.845(0.277-2.576)	1.184(0.388-3.611)	0.767
201 – 499	0.917(0.274-3.070)	1.09(0.326-3.654)	0.888
≥ 500*	Reference	Reference	-

4.4 ANTIFUNGAL SUSCEPTIBILITY PATTERN

Tables 4.3 and 4.4 show the antifungal profile of *Candida* species and their MICs tested against fluconazole, amphotericin B and flucytosine.

Susceptibility of *Candida* isolates to fluconazole

Overall resistance shown by *C. albicans* isolates tested against fluconazole was 29.1% (39/134). Comparatively, the non-*Candida albicans* isolates were significantly more resistance to fluconazole than the *C. albicans* isolates [Non- *albicans* = 45 % (27/60) vs. *C. albicans* = 29.1 % (39/134); p= 0.033] Figure 4.2.

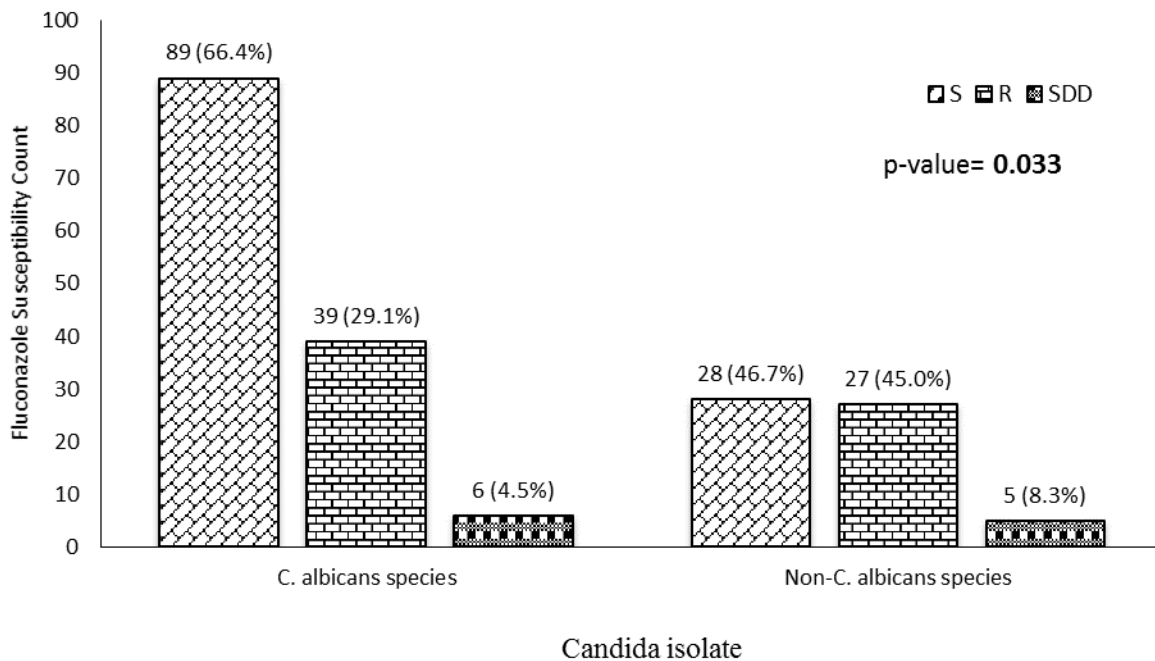


Fig 4.2: Graph comparing susceptibilities of *C. albicans* and non-*albicans* to fluconazole

Susceptibility of *Candida* isolates to Amphotericin B

1.5% (2/134) of *Candida albicans* isolates tested against amphotericin B were resistant. Non- *C. albicans* showed a more reduced susceptibility to amphotericin B, but this difference was not statistically significant [1.5% vs. 3.3%, $p= 0.266$].

Susceptibility of *Candida* isolates to flucytosine

Overall resistance among isolates of *C. albicans* tested against flucytosine was 2.7% (3/134). The highest resistance was shown by isolates of *C. krusei* 3(37.5%). None of the *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis* isolates tested against flucytosine was resistant.

Table 4.3: Antifungal susceptibility profile of HIV/AIDS patients with OPC

Parameter	Fungi Species	S. N (%)	S-DD. N (%)	I. N (%)	R. N (%)	P-value
Fluconazole						0.000
	<i>C. albicans</i>	89(66.4)	6(4.5)	-	39(29.1)	
	<i>C. krusei</i>	0(0.0)	0(0.0)	-	8(100.0)	
	<i>C. parapsilosis</i>	8(72.7)	0(0.0)	-	3(27.3)	
	<i>C. glabrata</i>	0(0.0)	4(30.8)	-	9(69.2)	
	<i>C. lusitaniae</i>	3(100.0)	0(0.0)	-	0(0.0)	
	<i>C. dubliniensis</i>	5(100.0)	0(0.0)	-	0(0.0)	
	<i>C. tropicalis</i>	12(60.0)	1(5.0)	-	7(35.0)	
Amphotericin B						0.266
	<i>C. albicans</i>	132(98.5)	-	-	2(1.5)	
	<i>C. krusei</i>	7(87.5)	-	-	1(12.5)	
	<i>C. parapsilosis</i>	11(100.0)	-	-	0(0.0)	
	<i>C. glabrata</i>	12(92.3)	-	-	1(7.7)	
	<i>C. lusitaniae</i>	3(100.0)	-	-	0(0.0)	
	<i>C. dubliniensis</i>	5(100.0)	-	-	0(0.0)	
	<i>C. tropicalis</i>	20(100.0)	-	-	0(0.0)	
Flucytosine						0.007
	<i>C. albicans</i>	130(97.0)	-	1(0.7)	3(2.3)	
	<i>C. krusei</i>	5(62.5)	-	0(0.0)	3(37.5)	
	<i>C. parapsilosis</i>	11(100.0)	-	0(0.0)	0(0.0)	
	<i>C. glabrata</i>	13(100.0)	-	0(0.0)	0(0.0)	
	<i>C. lusitaniae</i>	3(100.0)	-	0(0.0)	0(0.0)	
	<i>C. dubliniensis</i>	5(100.0)	-	0(0.0)	0(0.0)	
	<i>C. tropicalis</i>	18(90.0)	-	0(0.0)	2(10.0)	

S. = Sensitive, R. = Resistance, S-DD = Susceptible Dependent Dose, I. = Intermediate, P-value<0.05 implies statistically significant

Table 4.4 MICs of susceptibility test

Parameter	Fungi	MIC Range (Sensitive)	Mean (Sd) Sensitive MIC	Mic Range (Resistance)	Mean (Sd) Resistance MIC	p-value
Fluconazole						0.041
	<i>C. albicans</i>	0.016-2.00	0.58(0.62)	16.00-256.00	173.54(75.63)	
	<i>C. krusei</i>	-	-	64.00-256.00	88.00(67.88)	
	<i>C. parapsilosis</i>	0.016-2.00	0.61(0.87)	16.00-64.00	37.33(24.44)	
	<i>C. glabrata</i>	-	-	96.00-256.00	149.33(67.88)	
	<i>C. lusitaniae</i>	0.023-1.50	0.84(0.75)	-	-	
	<i>C. dubliniensis</i>	0.016-2.00	1.04(0.94)	-	-	
	<i>C. tropicalis</i>	0.016-1.00	0.40(0.37)	24.00-256.00	131.43(117.20)	
Amphotericin B						0.018
	<i>C. albicans</i>	0.002-1.00	0.11(0.19)	1.50-4.00	2.75(1.77)	
	<i>C. krusei</i>	0.004-1.00	0.25(0.36)	≥6.00	16.00(0.00)	
	<i>C. parapsilosis</i>	0.004-0.75	0.11(0.22)	-	-	
	<i>C. glabrata</i>	0.002-1.00	0.26(0.40)	≥32.00	32.00(0.00)	
	<i>C. lusitaniae</i>	0.004-0.05	0.02(0.02)	-	-	
	<i>C. dubliniensis</i>	0.004-0.064	0.02(0.02)	-	-	
	<i>C. tropicalis</i>	0.002-0.75	0.12(0.23)	-	-	
Flucytosine						0.000
	<i>C. albicans</i>	0.002-3.00	0.41(0.65)	≥32.00	32.00(0.00)	
	<i>C. krusei</i>	0.016-0.75	0.26(0.30)	≥32.00	32.00(0.00)	
	<i>C. parapsilosis</i>	0.004-0.75	0.14(0.23)	-	-	
	<i>C. glabrata</i>	0.002-2.00	0.26(0.54)	-	-	
	<i>C. lusitaniae</i>	0.094-0.50	0.26(0.21)	-	-	
	<i>C. dubliniensis</i>	0.008-1.00	0.42(0.53)	-	-	
	<i>C. tropicalis</i>	0.006-2.00	0.51(0.67)	≥32.00	32.00(0.00)	

4.5 ASSOCIATION BETWEEN ANTIFUNGAL EXPOSURE AND RESISTANCE

As observed in Table 4.5, fluconazole resistance was significantly higher in participants previously exposed to fluconazole compared to fluconazole-naïve patients [Fluconazole exposed patients = 70% (14/20) vs. Fluconazole naïve patients = 29.9 % (52/174); $p= 0.001$]. No significant association was however found between antifungal use and resistance to Amphotericin B ($p=1.000$) or flucytosine ($p=0.925$).

Table 4.5: Association between exposure to antifungal drug and resistance

Parameter	Antifungal History N (%)		P-value
	Yes (N= 20)	No (N= 174)	
Fluconazole			0.001
Sensitive	6(30.0)	111(60.3)	
Resistance	14(70.0)	52(29.9)	
S-DD	0(0.0)	11(6.3)	
Amphotericin			1.000
Sensitive	20(100.0)	170(97.7)	
Resistance	0(0.00)	4(2.3)	
Flucytosine			0.925
Sensitive	19(95.0)	166(95.4)	
Resistance	1(5.0)	7(4.0)	
Intermediate	0(0.0)	1(0.6)	

P-value < 0.05 implies statistically significant

4.6 RELATIONSHIP BETWEEN ART AND RESISTANCE

Fluconazole resistance was significantly higher among ART-naïve patients compared to patients on ART [ART = 25.6% (34/133) vs. ART naïve = 52.5% (32/61); $p=0.001$ Table 4.6].

Table 4.6: Association between ART intake and antifungal resistance

Parameter	ART intake		<i>p</i> -value
	Yes (N= 133)	No (N= 61)	
Fluconazole			0.001
Sensitive	89(66.9)	28(45.6)	
Resistance	34(25.6)	32(52.5)	
S-DD	10(7.5)	1(1.6)	
Amphotericin B			1.000
Sensitive	130(97.7)	60(98.4)	
Resistance	3(2.3)	1(1.6)	
Flucytosine			0.311
Sensitive	127(95.5)	58(95.1)	
Resistance	6(4.5)	2(3.3)	
Intermediate	0(0.0)	1(1.6)	

P-value<0.05 implies statistically significant

4.7 RELATIONSHIP BETWEEN CD4 COUNTS AND RESISTANCE

Significantly high fluconazole resistance was observed among patients with lower CD4 count (\leq 200 cells/ μ l) compared to patients with higher CD4 counts. [CD4 (\leq 200 cells/ μ l) = 44.6% (50/112) vs. CD4 (201-409 cells/ μ l) = 20.8% (10/48); vs. CD4 ($>$ 500 cells/ μ l) = 23.5% (4/17) $p=0.004$]. Table 4.7

Table 4.7: Association between CD4 counts level and antifungal resistance

Parameter	CD4 count N (%)			<i>p</i> -value
	\leq 200 (N= 112)	201 – 499 (N= 48)	\geq 500 (N= 17)	
Fluconazole				0.004
Sensitive	59(52.7)	32(66.7)	13(76.5)	
Resistance	50(44.6)	10(20.8)	4(23.5)	
S-DD	3(2.7)	6(12.5)	0(0.0)	
Amphotericin				0.783
Sensitive	109(97.3)	47(97.9)	17(0.0)	
Resistance	3(2.7)	1(2.1)	0(0.0)	
Flucytosine				0.808
Sensitive	105(93.8)	46(95.8)	17(100.0)	
Resistance	6(5.4)	2(4.2)	0(0.0)	
Intermediate	1(0.9)	0(0.0)	0(0.0)	

P-value $<$ 0.05 implies statistically significant

CHAPTER FIVE

DISCUSSION

5.1 SPECIES IDENTIFICATION AND DISTRIBUTION

C. albicans accounted for majority of OPC in the study participants. This is consistent with results obtained from similar studies in Ghana and elsewhere (Kwamin *et al.*, 2013; Feglo *et al.*, 2012; Sánchez-Vargas *et al.*, 2005; Hamza *et al.*, 2006; Mokaddas *et al.*, 2007; Amran *et al.*, 2011; Nweze *et al.*, 2011).

The spectrum of non-*Candida albicans* species and rate of isolation in the current study however differed from those reported in previous studies. The present study identified seven *Candida* species, compared to twenty and eight reported in Accra and Kumasi respectively (Kwamin *et al.*; 2013; Feglo *et al.*; 2012). Rate of isolation was also lower compared to 75.3%, and 82.3% respectively obtain by Kwamin *et al.*; and Mulu *et al.* Geographical differences may account for the variations between the present and previous findings (Budhavari, 2009). Patient characteristics, source of sample, and variations in sampling methods could also account for the differences in the rate of isolation and spectrum of non-*Candida albicans* species.

Non-*albicans* constituted about 31% of isolated cases in the present study. This was consistent with 30.5% reported in Accra by Kwamin *et al.*, (2013) but higher than the 15%, 22% and 16.5% reported in Tanzania, USA and Mexico respectively (Hamza *et al.* 2006, Redding *et al.* 1999; Sánchez-Vargas; 2005). This rate appears to follow the rising trend of 55% and 50% reported in Nigeria and Brazil (Nweze *et al.*; 2011, Costa *et al.*; 2006). The increased isolation of non-*albicans* species reported in the current study has serious clinical implications and underscores the need for routine identification before treatment, especially, since majority of them have been demonstrated to be less susceptible to commonly administered antifungals (Arendrup *et al.*,

2013). The present study did not establish any correlation between *Candida* infections and exposure to antifungal drugs. This is consistent with earlier findings, such as that of Patel *et al.* (2006).

C. albicans-associated OPC was found to be higher among HIV-infected patients on ART compared to ART-naïve patients. Key limitations of this study such as the absence of information on ART regime and level of immune suppression of the patients makes it difficult to explain the high trend of OPC among HIV-patients on ART. However, in Southern Brazil OPC development was found to be associated with severe immunodeficiency and high viral loads irrespective of ART use. Alcohol consumption and smoking were also found as high risk factors (Petruzzi, *et al.*; 2013).

5.2 ANTIFUNGAL SUSCEPTIBILITY PROFILE OF CANDIDA ISOALTES

In the present study, the prevalence of fluconazole resistance among the *C. albicans* isolates was about 30%. This prevalence falls within the 5-50% reported by related studies carried out in some Africa countries (Hamza *et al.*, 2008; Abrantes *et al.*, 2014; Mulu *et al.*, 2013; Wabe *et al.*, 2011; Nweze *et al.*, 2011; Enwuru *et al.*, 2008). However in Texas, Taiwan and Turkey, rates less than 4% were reported (Satana *et al.* 2010; Wu *et al.* 2012; Patel *et al.* 2012). The disparities in fluconazole resistance between the current and previous studies could be attributed to geographical variations, as suggested by Arendrup *et al.* (2013).

The present study also observed significantly higher fluconazole resistance among patients whose CD4 counts were low (≤ 200 cells/ μ l) compared to patients with higher CD4 counts. This was consistent with findings of related study where significant correlation was established

between CD4+ T lymphocytes and fluconazole resistance. In that study, *Candida* isolates from HIV-patients with reduced CD4+ cells (< 200 cells/ μ l) were significantly resistant to fluconazole (Lortholary *et al.*, 2012).

Non-*albicans* species were significantly more resistant to fluconazole than *C. albicans* in the present study. This trend could be attributed to inherent resistance exhibited by isolates of *C. krusei* and *C. glabrata* to fluconazole (Terças *et al.*, 2017). Fluconazole resistance was significantly higher in participants previously exposed to fluconazole compared to fluconazole naïve patients. Selective pressure due to short- or long-term exposure to azole could explain the high resistance among participants previously exposed to fluconazole (Pelletier *et al.*, 2000).

The resistance displayed by both *C. albicans* and non-*albicans* species against amphotericin B was low, and the difference in resistance between the two was not statistically significant. The relatively low resistance prevalence of *Candida* to amphotericin B reported in the current study falls within 0% and 3% reported by Anderson *et al.*, (2005) and Barchiesi *et al.* (2003) respectively. The results also indicate that amphotericin B is still an effective drug for treating *Candida* infections. The reason for the low amphotericin B resistance could be because usage of the drug is restricted in the country, and so is not easily obtained over the counter.

Prevalence of resistance among *C. albicans* and non-*albicans* tested against flucytosine was less than 9%. With the exception of *C. krusei* which showed a high resistance (37.5%), all the other non-*albicans* species (*C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis*) were susceptible to flucytosine. Flucytosine drug is rarely administered in isolation; it is combined with other antifungal agents. Moreover, resistance to the drug among *Candida* species have been generally low, and differ geographically (Gualco *et al.*, 2007). Resistance of 2.3% among *C. albicans* observed in this study is higher than the 0% and 0.6% reported by Barchiesi *et al.*

(2000) and Cuenca-Estrella et al. (2001) respectively and could be attributed to geographical variations or co-resistance with other antifungal drugs.

CHAPTER SIX

CONCLUSION AND RECOMMEDATION

6.1 CONCLUSION

C. albicans was the most isolated *Candida* species in OPC among the HIV-infected participants, with non- *Candida albicans* species also showing a rising trend. Non- *Candida albicans* species were more resistance to fluconazole, and prior fluconazole therapy and ART were associated with reduced susceptibilities to fluconazole. Without any contraindication, flucytosine and Amphotericin B may be considered for OPC not responding to fluconazole therapy.

6.2 RECOMMENDATIONS

Findings of the study revealed that fluconazole resistance was prevalent among non-*albicans* species isolated from the HIV-infected patients we surveyed. This result further emphasizes the need for speciation of causative organisms and antifungal susceptibility testing to be done for patients who do not respond to routine treatment. Furthermore, a nationwide surveillance of antifungal resistance should be carried out to provide information on the efficacy of these drugs and emergence of resistance.

6.3 LIMITATIONS OF THE STUDY

A key limitation of the study is the absence of control group to help ascertain if the susceptibility pattern and distribution of *Candida* species observed in this study is peculiar to HIV patients. Another limitation is the absence of key information such as the ART regime and suppression level of patients that could have help explain some findings observed in this study.

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APPENDICES

APPENDIX 1: Informed consent of participants

University of Ghana
School of Biomedical and Allied Health Sciences
Department of Microbiology
P.O.BOX 4236
Korle-Bu, Accra.

Participant ID Number:

Name of Participant;

Study Title: Distribution and susceptibility profile of candid isolates from HIV patients with oropharyngeal candidiasis

Dear Participant,

INFORMED CONSENT FORM

I seek your permission to be part of a study described below. You are encouraged to seek further understanding of the study before you decide to be part of this study. Participants of this study will be voluntarily enrolled and all obtained information before or after the analysis of the study result will be treated as confidential. You can choose to redraw your participation during the course of the study without any penalties.

The study in few words

The aim of the study is to determine the distribution and prevalence of antifungal resistance among *Candida* isolates from HIV/AIDS patients with Oropharyngeal candidiasis (OPC). OPC is a common opportunistic fungal infection that normally affect people who have impaired immune system such as HIV-positive patients. It is caused mostly by *Candida albicans* and some non-albicans species. Symptoms of OPC include white, curd-like, discrete plaques seen on the buccal mucosa, throat, tongue, or gingivae. OPC can gradually develop into complications like local discomfort, changes in taste, poor food intake leading to malnutrition and wasting and early death when not treated early. OPC is treated with a host of antifungal drugs such as fluconazole, Amphotericin B, and flucytosine. However, there have been reports of some OPC cases not responding to these drugs when applied. Hence it is important to determine periodically the efficacy of these antifungal drugs.

Procedure

A sterile cotton wool swab will be used to collect samples from your mouth for culture, identification and antifungal susceptibility testing. About 3-4 ml of blood sample will also be collected from you for CD4 estimation.

Risks

You may feel a slight pain at the site where blood sample will be drawn. This pain is mild and does not last for long. Apart this pain, the procedure will not pose any health risk to you.

Benefit

No immediate or direct benefit will be derived from this study except to communicate any clinical findings to your health worker. However, findings of this study will help clinicians make better therapeutic decisions during treatment.

Confidentiality

All information derived from you will be treated confidentially. This will include coding all information you give to us and avoiding using your personal name

Contact

Address all questions with regards to this study to Henry Asare Quansah (or Dr Japheth Opintan (0244 789209) of the Department of Medical Microbiology, University of Ghana, School of Biomedical and Allied health Sciences, Korle-Bu.

Participant: I understand all the above and hereby agree to participate or allow my ward to participate in this study.

_____	_____	_____
Name of participant	Signature/Thumbprint	Date
_____	_____	_____
Name of witness	Signature/Thumbprint	Date
_____	_____	_____
Name of investigator	Signature/Thumbprint	Date

STRUCTURED QUESTIONNAIRE

(FORM A)

TITLE

DISTRIBUTION AND SUSCEPTIBILITY PROFILE OF CANDID ISOLATES FROM HIV
PATIENTS WITH OROPHARYNGEAL CANDIDIASIS

SECTION A;

SOCIO-DEMOGRAPHICS

1. Name of Health Facility ()
2. Name/Id of participant.....
3. Age of participant () Yrs.
4. Sex of participant: MALE/FEMALE
5. Place of Residence ()
6. Marital Status;
 - a. Single
 - b. Married
 - c. Divorced/ Separated
7. Educational Status
 - a. No education
 - b. Primary
 - c. J.H.S
 - d. S.H.S
 - e. Tertiary and Above
8. Occupation of Participant.....

B. CLINICAL AND FOLDER REVIEW

1. Retroviral Status & Year of diagnosis	Positive	Negative		
Does the patient present clinical manifestation of OPC?	Yes	No		
Form of OPC	Pseudomembranous Erythematous Angular cheilitis			
History of recurrent OPC?	Yes	No		
History of Antifungal Treatment and name	Yes	No		
Date/ Year ART started?				
History of Antiretroviral treatment (ART)?	Yes	No		
Date/ Year ART started?				
Current CD4+ count (cells/mm ³)	≥500 (type 1) >201 to<499 (type2) ≤200(type3)			

C. LABORATORY RESULT

a. Culture Result.....

b. Identification result

c. MIC Result	Susceptibility/ Resistant
Fluconazole.....
Flucytosine.....
Amphotericin B.....

Appendix 2: Request to ART centres

Cape Coast Teaching Hospital,
P.O. BOX 1363,
Cape coast

Dear Sir/Madam

REQUEST TO CARRY OUT A CLINICAL SURVEY ON CLIENTS OF ART CENTRE

I write seeking your approval to carry out a clinical survey on client attending ART clinic at your facility. The study seeks to isolate *Candida* species from HIV/AIDS patients presenting with OPC and evaluate them against Fluconazole, Amphotericin B and Flucytosine. Attached is an official ethical clearance obtained from the University of Ghana, College of Health sciences sanctioning the study and participant consent form.

Thanks very Much.

Yours faithfully,

.....

Henry Asare Quansah
(Principal Investigator)

Cape Coast teaching Hospital
Mobile: 024614826

The In-Charge,

ART centre,

.....

Appendix 3: Distribution of Candida species

	Number	Percentage
	(%)	
C.albicans	134	69.1
C. tropicalis	20	10.3
C. glabrata	13	6.7
C. parapsilosis	11	5.7
C. krusei	8	4.1
C. dubliniensis	5	2.6
C. lusitaniae	3	1.5
TOTAL	194	100

Appendix 4; MICs of susceptibility test

Parameter	Fungi	Mic Range (Sensitive)	Mean (Sd) Sensitive Mic	Mic Range (Resistance)	Mean (Sd) Resistance Mic	p-value
Fluconazole						0.041
	<i>C. albicans</i>	0.016-2.00	0.58(0.62)	16.00-256.00	173.54(75.63)	
	<i>C. krusei</i>	-	-	64.00-256.00	88.00(67.88)	
	<i>C. parapsilosis</i>	0.016-2.00	0.61(0.87)	16.00-64.00	37.33(24.44)	
	<i>C. galbrata</i>	-	-	96.00-256.00	149.33(67.88)	
	<i>C. lusitaniae</i>	0.023-1.50	0.84(0.75)	-	-	
	<i>C. dubliniensis</i>	0.016-2.00	1.04(0.94)	-	-	
	<i>C. tropicalis</i>	0.016-1.00	0.40(0.37)	24.00-256.00	131.43(117.20)	
Amphotericin B						0.018
	<i>C. albicans</i>	0.002-1.00	0.11(0.19)	1.50-4.00	2.75(1.77)	
	<i>C. krusei</i>	0.004-1.00	0.25(0.36)	≥6.00	16.00(0.00)	
	<i>C. parapsilosis</i>	0.004-0.75	0.11(0.22)	-	-	
	<i>C. galbrata</i>	0.002-1.00	0.26(0.40)	≥32.00	32.00(0.00)	
	<i>C. lusitaniae</i>	0.004-0.05	0.02(0.02)	-	-	
	<i>C. dubliniensis</i>	0.004-0.064	0.02(0.02)	-	-	
	<i>C. tropicalis</i>	0.002-0.75	0.12(0.23)	-	-	

Flucytosine

0.000

<i>C. albicans</i>	0.002-3.00	0.41(0.65)	≥32.00	32.00(0.00)
<i>C. krusei</i>	0.016-0.75	0.26(0.30)	≥32.00	32.00(0.00)
<i>C. parapsilosis</i>	0.004-0.75	0.14(0.23)	-	-
<i>C. galbrata</i>	0.002-2.00	0.26(0.54)	-	-
<i>C. lusitaniae</i>	0.094-0.50	0.26(0.21)	-	-
<i>C. dubliniensis</i>	0.008-1.00	0.42(0.53)	-	-
<i>C. tropicalis</i>	0.006-2.00	0.51(0.67)	≥32.00	32.00(0.00)

Appendix 5: Atlas of Laboratory Result

A. Appearance of Candida species on Candida differential Agar.

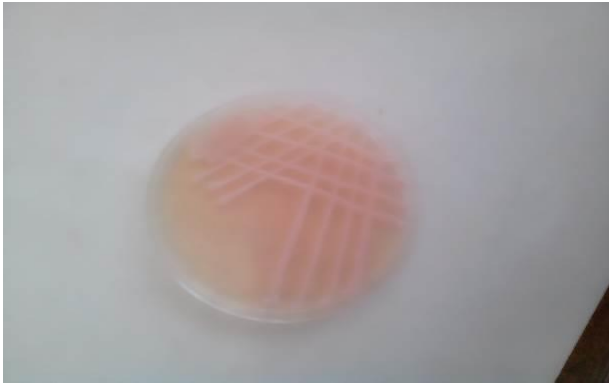


Plate 1: *C. glabrata*

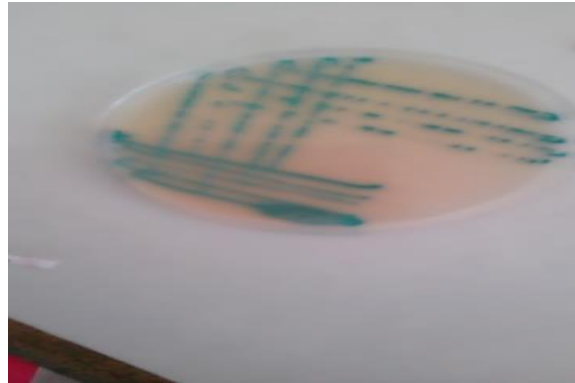


Plate 2: *C. albicans*

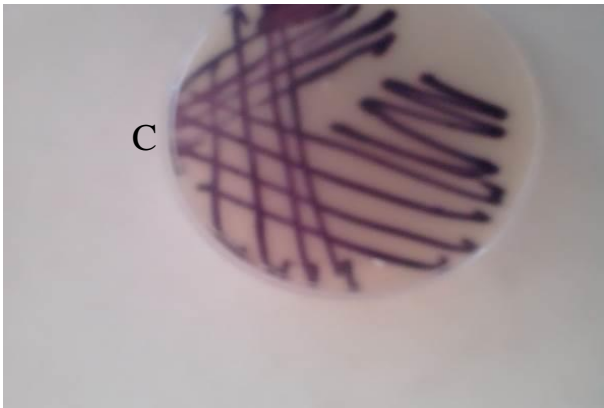


Plate 3: *C. lusitanae*

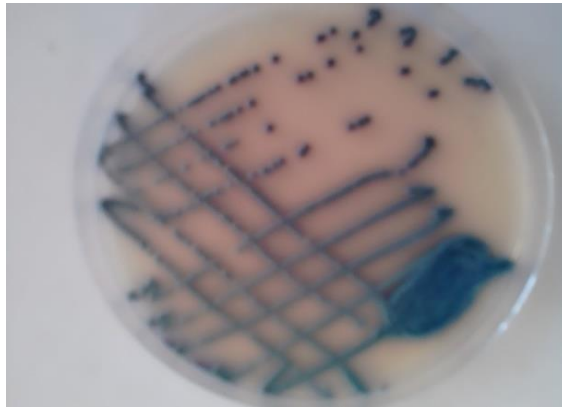


Plate 4: *C. tropicalis*

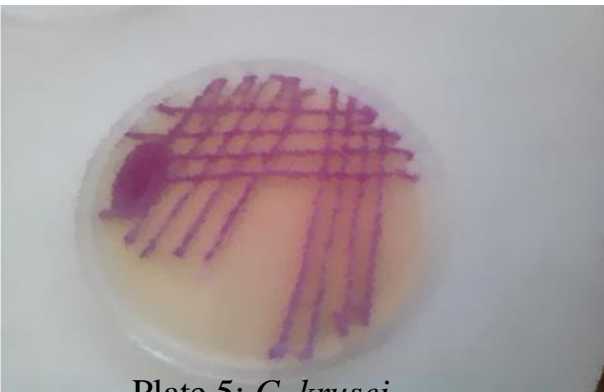


Plate 5: *C. krusei*

B. Antifungal sensitivity plates



Plate 6: FLUCY-sensitive *C. albicans*

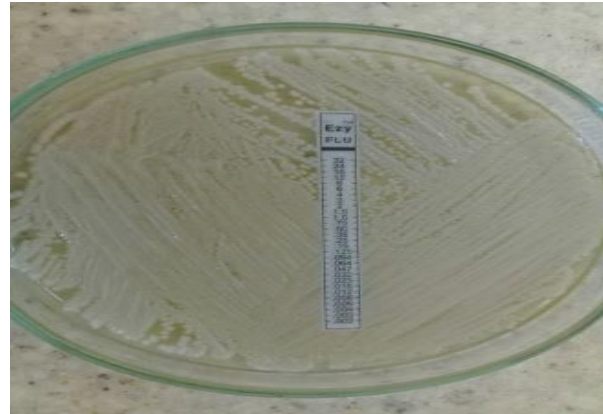


Plate 7: FLUC-resistant *C. krusei*

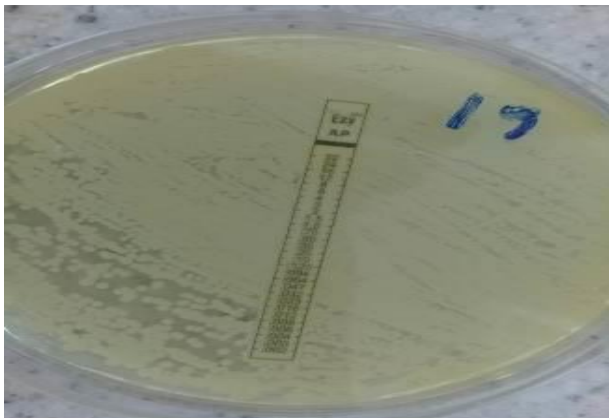


Plate 8: AMB-resistant *C. krusei*

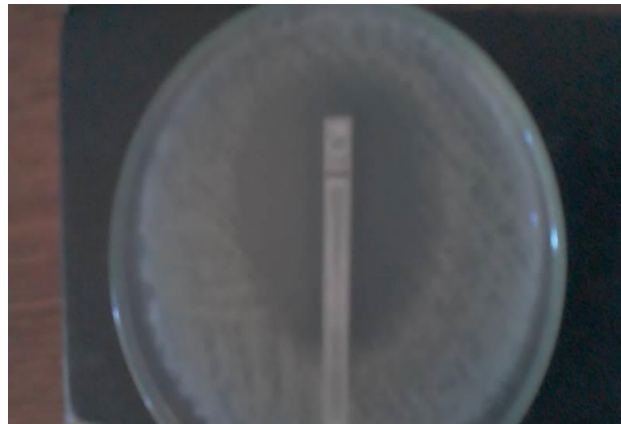


Plate 9: AMB-sensitive *C. albicans*