ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Species Distribution and In vitro Antifungal Susceptibility of Oropharyngeal Yeast Isolates from HIV Patients in Zewditu Memorial Hospital, Addis Ababa, Ethiopia

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Abbreviations and Acronyms

AIDS	Acquired Immunodeficiency syndrome
ATCC	American type Culture Collection
API	Analytical profile Index
BMD	Broth Micro dilution
CLSI	Clinical Laboratory Standards Institute
CD4	Cluster of Differentiation 4
CI	Confidence Interval
DMLT	Department of Medical Laboratory Technology
DRERC	Departmental Research and Ethical Review Committee
FMHACA	Food Medicine and Healthcare Administration and Control Authority
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
MIC	Minimum Inhibitory Concentration
OPC	Oropharyngeal Candidiasis
SDA	Sabouraud Dextrose Agar
SDD	Susceptible Dose Dependent
SOP	Standard Operating Procedure
WHO	World Health Organization
YNB	Yeast Nitrogen Base
ZMH	Zewditu Memorial Hospital

Abstract

Background: Oropharyngeal Candidiasis (OPC) is the most frequent opportunistic fungal infection of the oral cavity in HIV infected patients. OPC is associated with increased morbidity and mortality in immunosuppressed patients. However, data on Species distribution and antifungal susceptibility profiles of yeast isolated from HIV-infected patients in Ethiopia is limited.

Objective: The aim of the study was to determine the spectrum of Oropharyngeal yeast isolates from HIV patients and to evaluate antifungal drug susceptibility profile of yeast isolates to commonly used antifungal drugs.

Method: A cross sectional study was conducted in Zewditu Memorial Hospital, Addis Ababa. Oral swabs were collected from 224 HIV patients and cultured on Sabouraud Dextrose Agar with chloramphenicol. All the isolates were characterized to a species level following standard microbiological techniques. The antifungal susceptibility profiles of all yeast were determined using disk diffusion method.

Result: Of 224 HIV patients screened, 139 (62.1%) were colonized by yeasts. The rate of colonization is higher in patients that non HAART than HAART initiated (72.3% versus 51.8%). A total of 155 yeasts were isolated of which 153 (98.7%) were accounted by *Candida* consisting of five species and 2(1.3%) *non-candida* yeasts. Out of all isolates C. *albicans* was the most frequently isolated species accounting 68.4% followed by C. *glabrata* (15.5%) and *C. tropicalis* (11%). The Antifungal susceptibility pattern of the yeast isolates for Fluconazole, ketoconazole, Amphotericin B, Clotrimazole, Nystatin and Miconazole showed a resistance of 12.3%, 8.4%, 5.8%, 2.6%, 1.3%, and 0.6% respectively. C. *albicans* were 5.7% resistant to ketoconazole and 6.6% resistant to Fluconazole. C. *glabrata* and *C. tropicalis* were 12.5% and 17.6% resistant to ketoconazole and 16.6% and 35.3% resistant to fluconazole respectively.

Conclusion: The spectrum of yeast isolated in the present study was relatively high even if *C. albicans* was the predominantly isolated yeast**.** The results of this study also revealed that development of drug resistance by yeast isolates were considerable. Isolation, characterization and evaluating drug susceptibility pattern of the isolates in this hospital in particular and in health institutions through out of the country in general is recommended.

1. Introduction

1.1. Background

Yeasts are recognized as major pathogens in human immunodeficiency virus (HIV) infected patients. The most predominant yeasts isolated from clinical samples include the *Candida* and *Cryptococcus* species [1-4]. *Candida* species are yeasts that cause a wide range of disease manifestations ranging from mild oral diseases to disseminated candidiasis [2, 3].

Oropharyngeal candidiasis (OPC) is the most frequent opportunistic infection encountered in HIV-infected individuals. The most frequently observed forms of oral candidiasis are erythematous candidiasis, a red, flat, subtle lesion either on the dorsal surface of the tongue and the hard or soft palates; pseudomembranous candidiasis that appears as creamy white curd-like plaques on the buccal mucosa, tongue and other oral mucosal surfaces that will wipe away, leaving a red or bleeding underlying surface; and angular cheilitis which is erythema or fissuring of the corners of the mouth [4-7]. It occurs in up to 90% at some point during the course of HIV disease [7-8]. Although the introduction of highly active antiretroviral therapy (HAART) has reduced the prevalence of most opportunistic infections [9, 10], OPC is still the most common HIV related oral lesion. About 90% of patients were found to suffer from Oropharyngeal or esophageal candidiasis in various stages of AIDS and clinical relapse is a common feature of OPC depending on duration of antifungal therapy and extent of immunosuppression [11, 12]. *Candida* species colonize the mucosal surfaces of all humans soon after birth and the risk of endogenous infection is ever-present. The presence of *Candida* in the oral cavities of HIV/AIDS patients predicts the subsequent development of oral candidiasis [13, 14].

The advent of antibacterial drugs with broad spectrum activity imbalances the host microbial flora and predispose for yeast infections. In addition the increased in the prevalence of

immunosuppressed patients as a result of disease progression, low CD4 T- lymphocyte count and increased viral load predispose HIV patients to Oropharyngeal candidiasis [14].

Although *Candida albicans (C. albicans)* is the most prevalent isolate accounting 70-95% of yeasts isolated from HIV infected individuals the rate of isolation of non *albicans Candida* species have been increasing significantly in the era of HIV/AIDS [8, 15]. OPC remains the most frequent HIV-associated oral lesion particularly in most developing countries where the prevalence of HIV is the high. The results obtained in studies conducted in African countries like Nigeria and Tanzania are good indicators of the magnitude of the problem in Africa [15].

Information concerning the antifungal susceptibility of *Candida* is important in the prediction of the likely efficacy of subsequent treatment. *C. albicans* is generally assumed to be susceptible to most antifungal agents. The recent increased use of antifungal therapy, however, has raised concerns over the potential for the emergence of resistance of *Candida* to antifungals. Indeed, the continued exposure of *Candida* to antifungal drugs in certain patient groups has already been shown to alter the susceptibility of strains [16, 17].

However, to the best of our knowledge, studies that have been published describing the distribution of yeast carriage and subsequent causing OPC in Ethiopian HIV patients and their antifungal susceptibility patterns are limited and inadequate. Fungal isolation using culture methods and in vitro antifungal susceptibility testing are not routinely performed in clinical laboratories in Ethiopia. This prospective cross-sectional study was, therefore, conducted with the aim of investigating the spectrum of yeasts colonizing HIV patients and causing OPC and their antifungal susceptibility profile in Zewditu Memorial Hospital in Addis Ababa, Ethiopia.

1.2. Statement of the Problem

Even though; *C. albicans* is one of the most frequently isolated yeasts in clinical laboratories from HIV/AIDS patients accounting up to 75% of the yeasts recovered from sites of infection [18, 19]; the incidence of opportunistic infections due to *C. albicans* and other *Candida* species has been increasing.

C. *albicans* was isolated from 52% samples and NAC species were isolated from 48% clinical specimens in Egypt [20] and resistance to fluconazole was found in 8 isolates (22.2%) of NAC species and 2 isolates (5.1%) of C. *albicans* isolates.

A study on the Common opportunistic infections among HIV-infected patients attending at ART clinic of Gondar University Hospital, Northwest Ethiopia [21] reported oral candidiasis was the second common opportunistic infection. Phenotypic and genotypic identification was performed from oral rinse samples from 13 HIV patients and isolated C. *albicans,* C. *tropicalis,* C. *krusei,* C. parapsilosis, C. *guilliermondi*, C. *glabrata*, and C. *dubliniensis* and the study indicated that HIV patients have high risk Candida infection in Ethiopia [22].

A variety of antifungal agents are now available for the treatment of *Candida* infections. However, worldwide reports indicated that pathogenic isolates of *C. albicans* have relatively high potentials for developing resistance [23]. The in vitro susceptibility profile of vaginal yeasts recorded a resistance of the isolates for Ketoconazole (32.9%), itraconazole (17%), Miconazole (8%), and Amphotericin B and Nystatin (1.2% each) [24]. A study by Mulu et al. [25], found that the *candida* species were resistance up to 12.2% to different azoles. The study by Wabe et al. [17] in Ethiopia shows resistance rates of C. *albicans* to Fluconazole shows 11.9%. To this effect, studying the spectrum of yeasts and their drug susceptibility patters in Ethiopia where the prevalence of HIV is high is timely and the highest priority.

1.3. Significance of the study

- The results of this study may be used as an input for the epidemiological studies of the distribution of Oropharyngeal yeast isolates in HIV patients.
- Determining species distribution of yeasts and the antifungal susceptibility provides information on the most prevalent etiologic agent of OPC.
- This study may indicate a simple method to perform drug susceptibility tests for fungi isolated from Ethiopian patients that is not currently limited in availability in the country.
- The study may also play significant role in the selection of appropriate antimicrobials for empiric treatment.
- Clinical samples were documented for further study.

2. Literature Review

Oropharyngeal candidiasis is a major opportunistic fungal infection in HIV patients. Therefore, determining the spectrum of yeasts implicated causing OPC and their drug susceptibility pattern is an active field of study.

Fleck et al. [26] in Mannheim University Hospital, Germany, isolated 512 *Candida* species from 427 patients; from which 174 were from primarily sterile sites including blood, intravasal catheter tips, abdominal cavity, CNS and pleural cavities. The frequency of the isolates was 43.0% C. *albicans,* 31.1% *C. glabrata,* 11.7% *C. tropicalis,* 5.7% *C. parapsilosis* and 3.7% *C. krusei* and 4.6% other rare species. The Antifungal susceptibility testing of this study demonstrated that 96.3% were susceptible to amphotericin B, 90.4% were susceptible to Fluconazole, and none of the isolates were resistant to voriconazole.

The spectrum and drug susceptibility profile of *Candida* species with invasive candidiasis in Kuala Lumpur Hospital, Malaysia were studied by Amran et al. [27]. In this study a total of 159 clinical isolates of *Candida* species were isolated of which *C. albicans* were the most prevalent (71 isolates) followed by, *C. parapsilosis* (42 isolates), *C. tropicalis* (27 isolates) and *C. glabrata* (12 isolates). The *in vitro* antifungal susceptibility of the isolates against amphotericin B, fluconazole, voriconazole, itraconazole and caspofungin was conducted by using E-test. Over 90 % *C. albicans, C. parapsilosis* and *C. tropicalis* were susceptible to fluconazole while the susceptibility of *C. glabrata* to same drugs was 83.4%. Amphotericin B was found to be the most effective.

A cross sectional study to assess the prevalence of oral candidiasis in AIDS patients in Brazil, South America was conducted by Back-Brito et al. [28]. About 45 HIV positive individuals and 45 control healthy individuals were involved in this study. Oral rinse samples were collected and phenotypic identification was performed. The result of this study showed that the most

frequently isolated species in AIDS patients were *C. albicans* followed by *C. glabrata, C. lipolytica, C. krusei, C. guilliermondii*, and *C. parapsilosis*, while *C. tropicalis* and *C. dubliniensis* were the most frequent isolates in the control group.

A study assessing the distribution and drug susceptibility pattern of *Candida* species in Mexican HIV/ AIDS patient and health individuals was carried out by Sánchez-Vargas et al. [23] for over 3 years. Oral swabs were collected from 111 HIV infected and 201 non HIV infected study subjects. The results of this study depicted that, of the total 85 yeast isolates, *C. albicans* was the most frequent isolate followed by *C. glabrata, C. tropicalis* and *C. parapsilosis*. The antifungal susceptibility testing of the study implied from all the isolates 10.8% of the yeasts were resistant to one or more azole antifungal agents and 29% were intermediate susceptible.

Sánchez-Vargas et al. [29] also studied the oral carriage rate of yeasts among 312 Mexican AIDS patients by collecting oral swabs from oral lesions. The result of this study showed that *C. albicans* was the most frequently isolated species while the isolation rate of other *Candida* specie ranges from 16.5 to 38.5% of the isolates. The antifungal susceptibility test indicated that most isolates were susceptible to fluconazole, but 10.8% were resistant to one or more azoles.

A study conducted in India by Kantheti et al. [6] involving a total of 150 HIV positive and HIV negative study subjects by taking oral rinse samples. The overall culture positivity was found to be 35.33% of which *C. albicans* was the most prevalent species (16.67%) followed by *C. tropicalis* (8.00%), *C. glabrata* (4.67%), *C. parapsilosis* (1.33%), *C. kefyr* (0.67%), *C. guilliermondii* (0.67%), *C. stellatoidea* (0.67%). Similar study conducted by Furtado [19] in Bangalore, India reported that among yeast isolates the prevalence of *C. tropicalis and C. albicans* was 44% and 34% respectively. The study further indicated an increase in non albicans species and antifungal resistance was high in non albicans species ranging from 1% for both voriconazole and Amphotericin B to 12.5% for fluconazole.

Another study in Southern India; conducted by Umadevi et al. [30] evaluated the effect of HAART on the prevalence of oral candidiasis. The study included 100 HIV-seropositive subjects, 50 in the HAART group and 50 in the non HAART group. In this study, it has been indicated that the prevalence of oral candidiasis decreases in patients who had access to HAART than those who did not have access to HAART.

A five year study in Iran was conducted by Badiee and Alborzi [31] to identify *Candida* species and determine antifungal susceptibility patterns of the isolates. In this study a total of 595 *Candida* species were isolated. Of the isolates the most prevalent species was *C. albicans* (48%), followed by *C. krusei* (16.1%), *C. glabrata* (13.5%), *C. kefyr* (7.4%), *C. parapsilosis* (4.8%), *C. tropicalis* (1.7%) and other species (8.5%). The drug susceptibility pattern of the yeast isolates to fluconazole, amphotericin B, ketoconazole, itraconazole, voriconazole was determined, and showed that resistance varies depending on the species and the respective antifungal agents.

Similar studies were also conducted in the African continent. Hodgson et al. [15] reviewed oral fungal infections in HIV infected individuals in Africa. The reviewers indicated that oral candidiasis has been used as a marker of HIV progression in African HIV-infected individuals. OPC has been the significant cause of morbidity in HIV diseases. The susceptibility of the isolates to different antifungal agents ranges from 80-100% in some of the studies. Further; more many researches on OPC have been conducted in various countries in Africa.

A study conducted by Kwamin et al. [32] in Accra, Ghana isolated 201 *Candida* species from oral swabs of 267 HIV patients of which *C. albicans* is the predominant isolate (68.5%), followed by *C. tropicalis* (7.4%), *C. krusei* (6.4%), *C. parapsilosis* (3.0%), *C. sake* (2.5%) and other species (0.5- 1.5%). The study further depicted that prevalence of Candida species isolates was independent of HAART status.

The prevalence of C. *albicans* in the oral cavity in HIV seropositive patients in Abakaliki, Nigeria was studied by Okonkwo et al. [33]. In this study a total of 30 *Candida* species were isolated and of these 24 were *C. albicans* and the remaining were non albicans species including C. *tropicalis(1), C. pseudotropicalis (3), C. parapsilosis (1), and Candida guilliermondii* (1). Similar study in Nigeria was also conducted by Nweze et al. [34]. The study involved 200 Oropharyngeal swabs from HIV patients with 100 age matched healthy controls. The study depicted that 120 out of 200 were colonized by yeasts, *C. albicans* accounting the highest frequency (45%) followed by non albicans species such as *C. tropicalis* (18.3%), *C. parapsilosis* (15%), *C guilliermondii* (9.2%),*C dubliniensis* (7.5%),*C lusitaniae* (1.7%),*C krusei* (1.7%), and *C. kefyr* (1.7%).The study further showed that 11.7% of the isolates were resistant to fluconazole, 7.5% to itraconazole, 8.3% to flucytosine, and 1.7% to voriconazole. *C. albicans* was the most resistant species (17.5%) followed by *C. parapsilosis* (7.5%) and *C. tropicalis* (4.2%). This study showed no statistical significance association between HAART and species of *Candida* isolated in the study.

A study conducted by Hamza et al. [35] in Tanzania isolated 296 yeasts from 292 HIV patients with primary and recurrent OPC. The isolation rate of yeast species in descending order was *C. albicans* (84.5%), *C. glabrata* (6.8%), and *C. krusei* (3.4%). Of all the isolates 5% were resistant to antifungal drugs.

Another study carried out by Hamza et al. [36] involving 532 HIV infected patients of which 23.5% of the patients showed oral candidiasis. According to the study, adult patients receiving HAART had significantly lower prevalence of oral lesions.

There is limited number of studies conducted in relation to the distribution of yeasts and drug susceptibility pattern of fungal isolates from oral cavities of HIV patients in Ethiopia. Wabe et al. [17] conducted In vitro antifungal susceptibility of *Candida albicans* isolates from oral cavities

of patients infected with human immunodeficiency virus in Jimma University Specialized Hospital and isolation and experimentation were conducted in the Laboratory of Medical Microbiology of Jimma University. The overall objective of the study was to isolate *C. albicans* from HIV patients with OPC and to study its drug susceptibility profiles. The isolates were tested against Fluconazole, Amphotericin B, Nystatin, Miconazole and ketoconazole employing broth dilution method. Among the isolates of *C. albicans* 97.7% were found out to be susceptible to amphotericin B, 95.3% to Nystatin, 92.9% to ketoconazole and Miconazole and 88.1% to Fluconazole. The study shows a relative highest rate of resistance to fluconazole (11.9%) and a significant cross resistance among azoles.

Another study undertaken by Mulu et al, [25] to determine the species diversity and in vitro susceptibility of Candida isolates from late presenting AIDS patients in northwest Ethiopia in University of Gondar teaching Hospital; collected oral rinse samples from 215 HIV patients and cultured on CHROMagar plates at 37°C for 48 hours. *Candida* species identification was made following standard microbiological techniques and In vitro drug susceptibility tests were made using broth micro dilution method. The colonization rate of *Candida* species was found to be 82.3%. C. *albicans* was the predominant species isolated from 81% of patients but there was a diversity of other species. The most frequent non-albicans species was C. *glabrata* isolated in 22.5% of the patients followed by C. *tropicalis* in 14.1%, C. *krusei* in 5.6% and other unidentifiable *Candida* species in 4% of HIV patients. The *Candida* species identified were 12.2%, 7.7%, and 4.7% resistant to fluconazole, ketoconazole and itraconazole, respectively.

3. Objective

3.1. General Objective

- To determine the spectrum of Oropharyngeal yeast isolates from Zewditu Memorial Hospital HIV patients and to evaluate antifungal drug susceptibility profile of yeast isolates to commonly used antifungal drugs.

3.2. Specific Objectives

- To determine the species distribution of yeasts from HIV patients.
- To determine the antifungal susceptibility profile of the isolates to commonly used antifungal agents.

4. Materials and Methods

4.1. Study Area and Period

The study was carried out in the Zewditu Memorial Hospital, Addis Ababa, Ethiopia from February, 2014 to May, 2014. The hospital as a teaching referral hospital has a referral status and is located in the Addis Ababa, Ethiopia. The hospital provides HIV/AIDS interventions including free diagnosis, treatment and monitoring. The center diagnoses new cases and monitors those on therapy. The hospital possesses high patient flow and is a multiple service center. The hospital provides services including Condom/Female Condom/Dental Dam Distribution, family Planning, STD Prevention, TB Prevention, Care of Orphans, Health-facility based medical care, Prevention of mother-to-child transmission, Universal precautions, Psycho-Social counseling and support and HIV/AIDS Research.

The VCT center provides screening of volunteer individuals directly come to get the service or referred from other departments and establish link to the ART clinic to those who have positive results. Adherence team facilitates the link and strengthens the physician-client relation and follows up.

4.2. Study Design

A single institutional hospital based cross-sectional study was conducted to determine the species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolated from HIV patients in Zewditu Memorial Hospital.

4.3. Population

Source of population:

Source of population of the present study were HIV seropositive patient attending the ART clinic of the Hospital.

Study population: Patients from which clinical samples were taken within the study period

4.4. Sample Size and Sampling Technique

The worldwide prevalence of OPC among HIV patients ranges from 80-95%. About 90% of HIV patients were found to suffer from Oropharyngeal or esophageal candidiasis in various stages of AIDS [32, 37]. The colonization rate of Candida species among AIDS patients of 0.University of Gondar teaching Hospital was found to be 82.3% [25].

So by taking the prevalence of 82.3% (p=0.823), a confidence interval of 95% ($Z_{\alpha/2}$ =1.96, where z is a statistic for a level of confidence) and a sampling error of 5% (d=0.05), the total sample size (n) was calculated using the formula; $n = \frac{(Z\ \alpha/2)^2\ P(1-P)}{d^2}$. Therefore a total of 224 participants were enrolled using consecutive sampling technique [38] and HAART initiated and Non HAART initiated patients were balanced conveniently.

4.5. Measurement

4.5.1. Dependent variables and Independent variables

Dependent variables: Yeast species isolates, Susceptibility pattern.

Independent variables: Socio-demographic factors (age, sex), HAART status (HAART initiated and Non HAART).

4.5.2. Inclusion criteria and Exclusion criteria

Inclusion criteria: All HIV seropositive patients came to the hospital during the study period provide consent for participation.

Exclusion criteria: Patients with antifungal treatment history with in two weeks before sample collection and who didn't give consent to participate were excluded.

4.6. Data Collection and Processing

Specimen Collection

Oropharyngeal swabs were taken by the principal investigator after a written informed consent obtained from adult participants and for the participants less than the age of 18 years; consent from the guardian and assent the children obtained. The specimens were collected from oral lesions when present, the oral cavity mucosa, the dorsal surface of the tongue, and the floor of the mouth as this has been found to be the most colonized site in the oral cavity by firmly swabbing using sterile rayon tipped applicator stick swabs and placed to Amie's transport medium (Oxoid Transystem Amie's, Copan, Italy) as performed elsewhere [39-41] and transported at a temperature of 2-8^{o}c with a minimum of delay. Microbiological investigations were performed in the Microbiology Laboratory of the Food, Medicine and Health Care Administration and Control Authority of Ethiopia.

Identification of Isolates

Clinical specimens were inoculated on Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (Fluka Analytical, India) plates by using streaking method. SDA was prepared according to manufacturer's instructions and specification.

Inoculated plates were incubated at 35-37^{o}c for 24-48hrs. Plates with no growth after 48 hours were re incubated for a further one week. Color, shape, and texture of each colonies

were studied as preliminary identification. Each colony was then sub- cultured onto Potato Dextrose Agar (Lab M limited, Lancashire, UK) to obtain pure culture.

Each isolate was then identified to a species level by performing Germ tube test, carbohydrate assimilation test and temperature tolerance test described by Kangogo et al. [42], Marinho et al. [43], kim et al. [44], Kurtzman et al. [45] and other studies [46-47].

Antifungal Susceptibility Test

Antifungal susceptibility test were done by agar diffusion method following Clinical Laboratory Standards Institute (CLSI) guidelines, the Neo-sensitabs guidelines of the Rosco Diagnostica and other previously studied methods [48-51] against 6 drugs (Clotrimazole, Fluconazole, Ketoconazole, Miconazole, Nystatin and Amphotericin B).

Impregnation of Antifungal Disks

Six mm blank antibiotic assay discs (Sigma Aldrich, Germany) were impregnated with each drug (reference standards of the United States Pharmacopeia (USP), and supplied by EFMHACA) following the procedures implemented elsewhere [49-50]. Briefly, 20 mg of each antifungal drug was dissolved in 20ml of DMSO and a known concentration of each drug (Clotrimazole (10µg/disk), Fluconazole (25µg/disk), Ketoconazole (10µg/disk), Miconazole (10µg/disk), Nystatin (50µg/disk), and Amphotericin B (10µg/disk)) was adsorbed on to blank antibiotic discs and air dried.

Susceptibility Testing

A suspension of the yeasts was prepared by emulsifying 3-5 colonies in a 5mL NaCl (0.85% saline) and the turbidity of the suspension was adjusted to a 0.5 McFarland standard by adding sufficient sterile saline or more colonies. The suspensions were evenly streaked over the entire agar surface of SDA (Lab M limited, Lancashire, UK) as performed elsewhere [4, 16].

The antifungal disks prepared as described above were placed on SDA plates seeded with yeast isolates and incubated at 35 °C (±2 °C) for 24-48hrs. The zone diameter was measured and categorized to susceptible (S), susceptible dose dependent(SDD) and resistant (R) according to the M44 guidelines of the Clinical Laboratory Standards Institute and Rosco Diagnostica Company (Neosensitabs, Denmark) instructions.

4.7. Operational Definitions of Terms

Oropharyngeal candidiasis: an opportunistic fungal infection in immune compromised individuals caused by yeast like fungi *Candida* species [7].

Resistance: Resistant strains are those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules or when zone diameters have been in a range where clinical efficacy has not been reliable in treatment studies [48].

Susceptible-Dose Dependent (SDD): isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates [48]**.**

Susceptible: The susceptible category implies that an infection due to the strain may be appropriately treated with the dose of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated [48].

Opportunistic infections (OIS):- Opportunistic infections are late complications of HIV infection for the most part in patients with reduced CD4+ T-cells in the blood of the patients.

Sugar assimilation test: The assessment of the ability of yeast to utilize carbohydrates is based on the use of carbohydrate- free yeast nitrogen base agar and observing for the presence of growth around carbohydrate impregnated filter paper disks after an appropriate period of incubation [52].

4.8. Data Quality Control

To enhance the quality of the data, site assessment and pretest were done. Follow up of performance of autoclaves, incubator, and refrigerator were done regularly. ATCC species of C. *albicans* ATCC 90028 was used and run as a control at each level of processing of test. Inoculum size was monitored by using 0.5 McFarland standards visually.

4.9. Statistical Analysis

Data were collected, summarized, tabulated and analyzed using SPSS software 20. Categorical variables were summarized by proportions and percentages. Patients were categorized into age groups and into those on HAART and those not receiving HAART. Comparisons of proportions between groups were done by chi-square test, and the significant level set at $p < 0.05$.

4.10. Ethical Considerations

Research and Ethical Review Committee of Department of MLS, College of Health Sciences of AAU reviewed and approved prior to patient participation. Ethical clearance was obtained from Addis Ababa Health Bureau. Then a letter informing the hospital was written and permission was obtained from ZMH. Informed written consents were obtained from participants before data collection. The respondents were given the right to refuse to take part in the study as well as to withdraw at any time during the study period. All the information obtained from the study subjects was coded to maintain confidentially.

4.11. Dissemination of Results

After conducting the research, the results of the study was submitted to Department of Medical Laboratory Sciences, Addis Ababa University. Oral presentation of the thesis was made. Reports were also be submitted to ZMH, annual conferences of professional societies and other concerned bodies. The manuscript will be submitted to peer reviewed

5. Result

Demographic Characteristics

Study subjects enrolled in the present study were 224 HIV-seropositive individuals of which 155 (69.2%) were females and 69 (30.8%) were males. The median age of the study participants was 34.5 years with age ranged from 3 to 78 years (Table 1). As one can depict from the table, majority of the participants were in the age group of 30-39 years (37.5%) followed by 40-49 (21%) and 0-19 years (19.2%).

Table1: Age and Sex distribution of HIV positive patients Participated in the study at Zewditu Memorial Hospital, Addis Ababa, Ethiopia, February - May 2014 (n=224)

Age group (in years)	Number of Male screened	Number of female screened	Total
0-19	14 (32.6%)	29 (67.4)	43 (19.2%)
20-29	4 (12.1%)	29 (87.9%)	33 (14.3%)
30-39	26 (31.0%)	58 (69.0%)	84 (37.5%)
40-49	21 (44.7%)	26 (55.3%)	47 (21.0%)
>50	4 (23.5%)	13 (76.5%)	17 (7.6%)
Total	69 (30.8%)	155 (69.2)	224 (100%)

* Age group adopted from (Mulu et al., 2013)

Among the study participants 112 (50%) were under Highly Active Antiretroviral Therapy (HAART) while the remaining 112 (50%) were not under Highly Active Antiretroviral Therapy. Out of 155 female participants 76 (49%) were under antiretroviral therapy. Similarly among 69 male study participants 36 (52.2%) under antiretroviral therapy.

Out of 224 cases (one specimen per patient) yeast growth was observed in 139 (62.1%) cases or clinical samples. No fungal growth was found in 85 (37.9%) of the samples. Among 139 positive culture patients 93 (66.9%) were females and 46 (33.1%) were males indicating females had a higher frequency of colonization than males. However, the frequency of yeast colonization was not significantly associated with sex [x^2=0.640, df =1, P = 0.342]. The colonization rate of yeast were higher in study subjects that were not under antiretroviral therapy 81 (72.3%) than patients that were HAART initiated 58 (51.8%) indicating that patients that were not under drug treatment were more colonized, and affected. Isolation rate of yeasts from patients under drug treatment and those that were not under treatment was statistically significant [x^2=9.176, df= 1 P = 0.002]. The higher the frequency of colonization of yeasts in immunocompromised patients implied that the occurrence of subsequent infection unless interventions have been made.

Distribution of Yeasts

One hundred and fifty five (155) yeast isolates belonging to three genera (*Candida, Rhodotorula* and *Cryptococcus*) were isolated from 139 culture positive patients. Out of a total 155 yeast isolates 91(58.7%) were recovered from patients who were not under HAART while 64 (41.3%) isolates were obtained from patients who were under HAART. As shown in table 2, species of yeast recovered in their descending order were *C. albicans (*106), *C. glabrata* (24), *C. tropicalis* (17), *C. krusei (*4), *C. kefyr* (2), *Rhodotorula* species (1) and Cry*ptococcus laurentii*(1).

Candida albicans was isolated from 106 (47.3%) of the 224 patients examined followed by *C. glabrata* 24 (10.7%), C. *tropicalis* 17 (7.6%) and C. *krusei 4(1.8)* (Table 2).

Table 2: Species of yeasts isolated from Oropharyngeal swab samples of 139 HIV positive patients at Zewditu Memorial Hospital, Addis Ababa, Ethiopia, February - May 2014 (n=224).

Species of yeast	isolates (n)	Patients' positive (%)
C. albicans	**106**	**47.3**
C. glabrata	24	**10.7**
C. tropicalis	17	**7.6**
C. krusei	4	**1.8**
C. kefyr	2	**0.9**
Rhodotorula species	1	**0.45**
Cryptococcus laurentii	1	**0.45**
Total	**155**	**Patients, n=224**

Furthermore; out of 139 culture positive samples, mixed cultures were recovered from 16 samples. C. *albicans* was isolated in all mixed cultures in combination of C. *glabrata*, appeared 7 times, C. *tropicalis* appeared 5 time, and C. *krusei*, C. *kefyr*, *Cryptococcus laurentii* and *Rhodotorula* species appeared once with C. *albicans*. The mixed cultures were harbored from 6 samples collected from HAART initiated and 10 pre HAART patients. Similarly, 9 of the mixed cultures were recovered from samples collected from Females and 7 were from samples collected from male participants.

Among the total of 155 isolates the most common yeast species was C. albicans, comprising 68.4% followed by C. glabrata (15.5%), C. tropicalis (11%), C. krusei (2.6%) and C. kefyr (1.3%) (Figure1).

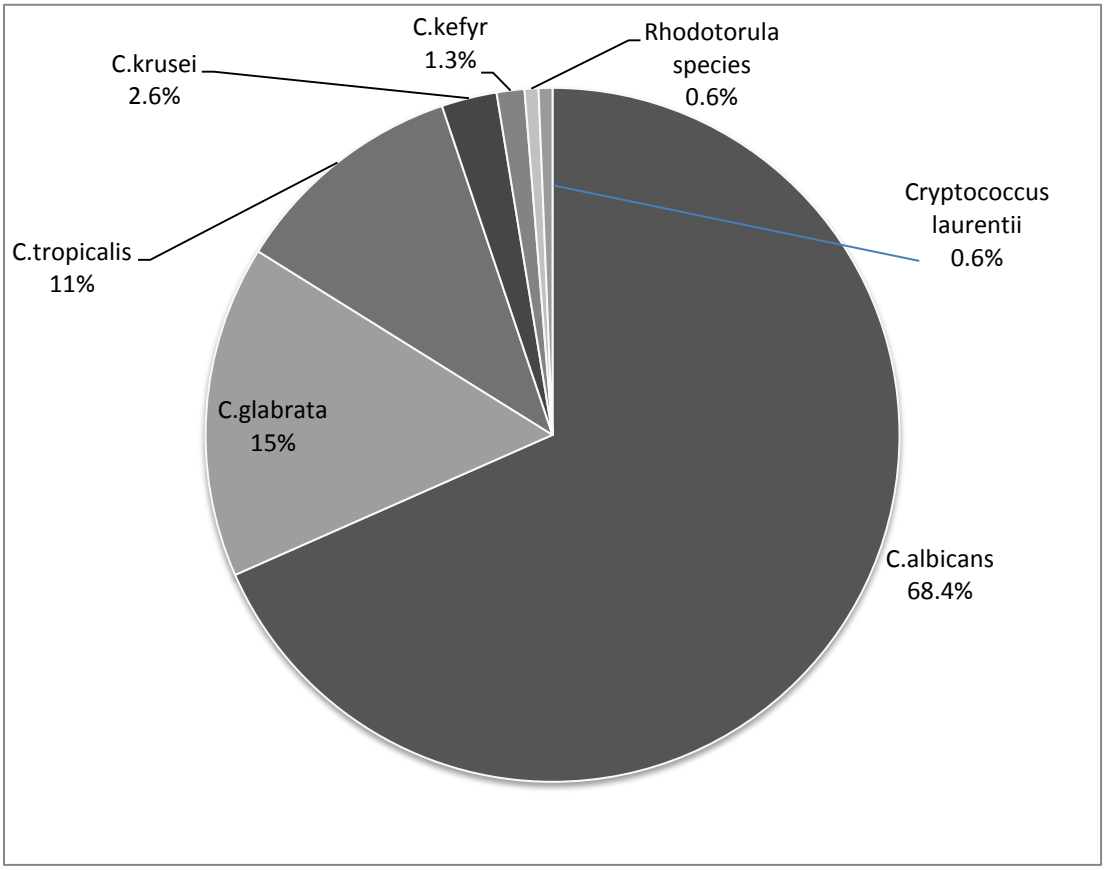

Figure 1: Distribution of yeast species among 155 isolates recovered from HIV patients.

The distribution of the yeast isolates in relation to sex is showed in table 3. From the three predominant Candida isolates (C. albicans, C. glabrata and C. tropicalis) the two common yeasts from the 105 yeasts isolated from female patients were C. albicans and C. glabrata accounting 69.5 % and 17.1% respectively. Similarly the two common yeasts out of 50 yeasts isolated from male patients were C. albicans and C. tropicalis amounting 66.0% and 16.0 % respectively. No significant association observed between sex and the type of yeast isolated (P=0.655).

Table 3: Distribution of yeast species in relation to sex among patients at Zewditu Memorial Hospital, Addis Ababa, Ethiopia; February - May 2014 (n=155).

Isolates	Female N (%)	Male N (%)	Total
C. albicans	73 (68.9)	33 (31.1)	106
C. glabrata	18 (75.0)	6 (25.0)	24
C. tropicalis	9 (52.9)	8 (47.1)	17
C. krusei	2 (50)	2 (50)	4
C. kefyr	1 (50)	1 (50)	2
Rhodotorula species	1 (100)	0	1
Cryptococcus laurentii	1 (100)	0	1
Total	105 (67.7)	50 (32.3)	155

The distribution of yeast isolates in patients under antiretroviral drug treatment and those that were not under HAART is shown in table 4. As can be seen in the table C. *albicans* was isolated in 63 (59.4%) patients that were not under antiretroviral drug treatment while in patients under antiretroviral drug treatment (HAART) was 43 (40.6%). Similarly, C. *glabrata* was isolated in 13 (54.2%) patients that were not under HAART and 11(45.8%) patients were under antiretroviral drug treatment. Nine (52.9%) and 8(47.1%) C. *tropicalis* were isolated from patients not under HAART and under HAART respectively. Both the non-Candida yeasts isolates *Rhodotorula* species and *Cryptococcus laurentii* were isolated from patients not under antiretroviral drug treatment (non HAART initiated patients (Table 4). But the species of yeasts isolated were no significantly associated to the HAART status (P=0.883).

Table 4: Distribution of oral yeast isolates among HIV-infected patients based on HAART status at Zewditu Memorial Hospital, Addis Ababa, Ethiopia; February - May 2014 (n=155).

Isolates	Not on HAART N (%)	HAART Initiated N (%)	Total
C. albicans	63 (59.4)	43 (40.6)	106
C. glabrata	13 (54.2)	11 (45.8)	24
C. tropicalis	9 (52.9)	8 (47.1)	17
C. krusei	3 (75.0)	1 (25.0)	4
C. kefyr	1 (50)	1 (50)	2
Rhodotorula species	1 (100)	0	1
Cryptococcus laurentii	1 (100)	0	1
Total	91 (58.7)	64 (41.3)	155

In Vitro Antifungal Susceptibility

The results of antifungal susceptibility, as summarized in table 5 showed that out of the total 155 yeast isolates the highest resistance rates observed were against the action of fluconazole with 12.3% resistant and to the activities of Ketoconazole with a resistant of 8.4% from the total isolates. Similarly, 9 (5.8%) of isolates were resistant to Amphotericin B, and 2.6%) of isolates were resistant to Clotrimazole. The least rate of resistance from all isolates was against Miconazole (1 (0.6%)) and, 2 (1.3%) were resistant to Nystatin. According to the data in table 5, it was revealed that Nystatin and Miconazole were the most effective antifungal drugs and fluconazole had the poorest activity against all species of yeast to inhibit the in vitro growth of yeasts isolated in this study.

Table 5: Antifungal Susceptibility of yeast isolates as determined by CLSI M44 and Neo-sensitabs guidelines breakpoints, ZMH, Addis Ababa, 2014 (n=155).

Antifungal	Disk Potency	S		SDD		R		Zone diameter in mm		
		N	%	N	%	N	%	R	SDD	S
Amphotericin B	10µg/disk	130	(83.9)	16	(10.3)	9	(5.8)	≤10	10-14	≥15
Clotrimazole	10µg/disk	148	(95.5)	3	(1.9)	4	(2.6)	≤11	12-19	≥20
Fluconazole	25µg/disk	123	(79.4)	13	(8.4)	19	(12.3)	≤14	15-18	≥19
Ketoconazole	15µg/disk	134	(86.5)	8	(5.2)	13	(8.4)	≤20	21-27	≥28
Miconazole	10µg/disk	133	(85.8)	21	(13.5)	1	(0.6)	≤11	12-19	≥20
Nystatin	50µg/disk	150	(96.8)	3	(1.9)	2	(1.3)	No	10-14	≥15

[S, susceptible; SDD, susceptible dose dependent; R, resistant]

Results of antifungal susceptibility profile of each of the 153 *Candida* isolates based on their in vitro susceptibility to the studied 6 antifungal drugs are shown in Table 6. Two of the non-*candida* isolates were found to be susceptible to the six antifungal drugs assessed and not shown in table 6.

The highest rates of resistance to Amphotericin B were seen in *C. tropicalis* (17.6%) *and C. glabrata* (8.3%). The two Nystatin resistant species were *C. glabrata* and *C. kefyr* while a single species of C. *albicans*, C. *glabrata* and C. *tropicalis* were SDD against the anti-fungi. Only one isolate of C. *albicans* was observed to be resistant to Miconazole. No susceptible isolates of *C. krusei a*nd *C. kefyr* to Amphotericin B were found.

Clotrimazole showed greater activity against C. albicans; 105 (99.1%) were susceptible. Only 4 *candida* species consisting one isolate from each of C. *albicans*, C. *glabrata*, C. *tropicalis* and *C. krusei* were resistant to Clotrimazole. All the evaluated C. *krusei* (4) were susceptible to Nystatin and both isolates of C. *kefyr* were susceptible to Clotrimazole.

Table 6: In vitro antifungal susceptibility of Candida species to Antifungal Agents as determined by CLSI Disk diffusion Method

Species	Antifungal agent	Susceptible	SDD	Resistant
		Number (%)	Number (%)	Number (%)
C. albicans (106)	Amphotericin B	98 (92.4)	6 (5.7)	2 (1.9)
	Clotrimazole	105 (99.1)	0 (0)	1 (0.9)
	Fluconazole	92 (86.8)	7 (6.6)	7 (6.6)
	Ketoconazole	95 (89.6)	5 (4.7)	6 (5.7)
	Miconazole	101 (95.3)	4 (3.8)	1 (0.9)
	Nystatin	105 (99.1)	1 (0.9)	0 (0)
C. glabrata (24)	Amphotericin B	21 (87.5)	1 (4.2)	2 (8.3)
	Clotrimazole	21 (87.5)	2 (8.3)	1 (4.2)
	Fluconazole	19 (79.2)	1 (4.2)	4 (16.6)
	Ketoconazole	20 (83.3)	1 (4.2)	3 (12.5)
	Miconazole	19 (79.2)	5 (20.8)	0 (0)
	Nystatin	22 (91.6)	1 (4.2)	1 (4.2)
C. tropicalis (17)	Amphotericin B	9 (53.0)	5 (29.4)	3 (17.6)
	Clotrimazole	15 (88.2)	1 (5.9)	1 (5.9)
	Fluconazole	10 (58.8)	1 (5.9)	6 (35.3)
	Ketoconazole	13 (76.5)	1 (5.9)	3 (17.6)
	Miconazole	11 (64.7)	6 (35.3)	0 (0)
	Nystatin	16 (94.1)	1 (5.9)	0 (0)
C. krusei (4)	Amphotericin B	0 (0)	3 (75.0)	1 (25.0)
	Clotrimazole	3 (75.0)	0 (0)	1 (25.0)
	Fluconazole	0 (0)	2 (50.0)	2 (50.0)
	Ketoconazole	3 (75.0)	1 (25.0)	0 (0)
	Miconazole	0 (0)	4 (100)	0 (0)
	Nystatin	4 (100)	0 (0)	0 (0)
C. kefyr (2)	Amphotericin B	0 (0)	1 (50)	1 (50)
	Clotrimazole	2 (100)	0 (0)	0 (0)
	Fluconazole	0 (0)	2 (100)	0 (0)
	Ketoconazole	1 (50)	0 (0)	1 (50)
	Miconazole	0 (0)	2 (100)	0 (0)
	Nystatin	1 (50.0)	0 (0)	1 (50.0)

Fluconazole showed the poorest activity by which 7 (6.6%) C. *albicans* 4 (16%) C. *glabrata,* 6 (35.3%) C. *tropicalis,* and 2 (50%) C. *krusei* were resistant. Similarly, 6 (5.7%) C. *albicans*, 3 (12.5%) *C. glabrata*, 3 (17.6%) *C. tropicalis* a*nd one C. kefyr* were resistant to ketoconazole. In contrast to fluconazole and ketoconazole 4 (3.8%) C. *albicans*, 5 (20.8%) *C. glabrata*, 6 (35.3%) *C. tropicalis*, 4 (100%) *C. krusei* and both of *C. kefyr* were found to be SDD to Miconazole.

Nystatin exhibited greatest activity against C. *albicans*, C. *tropicalis, and* C. *krusei* with no resistant strain found; only one species of each of C. albicans (0.9%), C. *glabrata* (4.2%), C. *tropicalis* (5.9%) and C. *kefyr* (50%) were found to be SDD.

In the present study, from 19 fluconazole resistant isolates 6 (31.6%) were resistant to Amphotericin B, 3 (15.8%) were resistant to Clotrimazole, 9 (47.4%) were resistant and 4 (21.1%) were SDD to ketoconazole and one resistant to Nystatin. Among the 123 Fluconazole susceptible isolates, no (0%) resistant isolate to amphotericin B as well as ketoconazole and only 1 (0.8%) resistant to Clotrimazole were isolated. There was a significant difference in frequency of resistance to ketoconazole, Clotrimazole and Amphotericin B between fluconazole resistant and susceptible Candida isolates ($p < 0.05$). All 9 Amphotericin B resistant isolates were susceptible to Clotrimazole and 4 susceptible and 5 resistant to ketoconazole.

6. Discussion

The present study was undertaken to determine the spectrum of yeasts isolated from oral swabs collected from HIV patients and find out the drug susceptibility profile of yeast isolates. A total of 224, study participants were enrolled in the present study of which 155 (69.2%) were females and 69 (30.8%) were males. The highest number of women screened is probably because most men rarely go for routine checkup, before the disease has reached symptomatic stage and the highest health seeking by females. The ages of study subjects ranged from 3 to 78 years. Out of the 224 study subjects 139 (62.1%) were culture positive indicating Oropharyngeal carriage of yeasts is a common finding in HIV positive patients.

The findings of this study was similar to that reported by Junqueira et al. [53], Costa et al. [54], Nweze et al.[34] and Gugnani et al. [55] where a prevalence rate of positive culture (oral carriage) documented were 62%, 62.6 %, 60% and 65.3% respectively. However, the result obtained in this study (62.1 %) was higher when compared with those of Kantheti et al. [6], Okonkwo et al. [33], Manikandan et al. [56] and Hamza et al. [36] and these authors documented 35.3%, 12.5%, 43% and 23.5% respectively. On the other hand a positive culture of yeasts obtained in this study was much less than those reported by Patel et al. [3], Mulu et al [25] and Erkose et al. [57] which were 82%, 82.3% and 82.8% respectively. The difference in frequencies of isolations may be from differences in the characteristics of sample population and sampling methods used.

One hundred fifty five yeast isolates were recovered from this study. They were C. *albicans*, C. *glabrata, C. tropicalis,* C. *krusei*, C. *kefyr,* C*ryptococcus laurentii* and *Rhodotorula* specie. The pattern of isolation of *Candida* species from Oropharyngeal swabs of HIV patients was similar with earlier study conducted in Ethiopia [25]. However, in the present study C*ryptococcus laurentii* and *Rhodotorula* species were isolated.

These species were isolated by Gugnani et al [55], Kwamin et al [33], Badiee et al. [31] and Junqueira et al. [53]. Of these, C. *albicans was* the most common yeast isolate accounting 106 (68.4%) of the total isolates followed by C. *glabrata* 24 (15.5%), *C. tropicalis* 17 (11%), C. *krusei* 4 (2.6%), C. *kefyr* 2 (1.3%), C*ryptococcus laurentii* and *Rhodotorula* species 1 (0.6%) each.

Of the total yeast isolates the three dominant yeasts were *C. albicans*, *C. glabrata* and C. *tropicalis*, accounting 147 (94.8%) out of the total isolates. Our finding in this regard was consistent with many earlier studies [29, 35]. Among yeast isolates, other *Candida* species accounted 23 (14.8%) of the total isolates indicating other *candida* species are becoming increased. Out of the 153 Candida isolates C. albicans accounted 69.3% and the non *albicans Candida* species covered 30.7% potion of the Candida species isolated.

In the present study the distribution of yeast isolates in patients under antiretroviral therapy (HAART) and those were not under HAART was studied. The results of this study revealed that of the total yeast isolates 91 (58.7%) isolates were recovered from samples collected from patients that were not under HAART while 64 (41.2 %) were from study subjects receiving HAART. A reduction in yeast isolates in patients under HAART was observed in many earlier studies [21, 30, 36]. C. *albicans* was isolated in 63 (59.45%) cases in patients that were not under antiretroviral therapy while in patients under antiretroviral therapy was 43 (40.6%). Similarly, C. *glabrata* was isolated in 13 (54.2%) cases of patient that were not under drug treatment and 11(45.8%) in patient were receiving HAART. *Rhodotorula* and *Cryptococcus laurentii* were the isolated from patients not receiving HAART.

Variable antifungal susceptibility profile of yeasts tested against the six drugs was observed in the present study. In this study we found that irrespective of the species of yeasts assessed 12.3% were resistant to fluconazole and 8.3% were resistant to ketoconazole which is similar

to a recent study in Ethiopia [25] that reported 12.2% and 7.7% of the isolates were resistant to fluconazole and ketoconazole respectively. The increase in the resistance of the isolates against the action of fluconazole may be associated with the accessibility of the drug with free of charge and the frequent usage of the drugs as drug of choice.

The resistance of the isolates to the effect of fluconazole obtained in this study was similar with previous studies conducted in Taiwan [58], in Nigeria [34], in Bangalore, India [19] which reported a frequency of resistance 11.7%, 11.7% and12.5% respectively.

In contrast to the highest frequency of resistance to fluconazole and amphotericin B (12.3% and 5.8% respectively) in this study, previous investigations conducted by Gugnani et al. [55] and Zomorodian et al. [59] documented that no resistant yeast was reported in their studies. But a recent worrisome result of resistance to fluconazole, ketoconazole and amphotericin B reported by Aher [60]. The author reported that from all 148 *candida* species isolates 14.9% were resistant to amphotericin B, 39.1% were resistant to fluconazole and 37.2% resistant to ketoconazole.

The susceptibility of the isolates to the action of Miconazole and Nystatin were 85.8% and 96.8%, demonstrating activity against all isolates respectively. However, one C. *kefyr* and one C. *glabrata* were resistant to Nystatin and only one isolate of C. albicans was resistant to Miconazole. The remaining isolates were intermediately susceptible to the two drugs. The frequency of non-*albicans candida* species isolated in HIV patients is increasing [6, 30]. The highest resistant to fluconazole of non *albicans Candida* isolates (26.5%) than C. *albicans* (6.6%) in this study and many others [25, 61] is suggestive of species identification and antifungal susceptibility testing for successful treatment.

7. Conclusion and Recommendations

This study showed C. albicans was the most frequently isolated species (68.4%) followed by (30.3%) non *albicans* Candida isolates and (1.3%) non-*Candida* yeasts. The antifungal susceptibility profiles of all isolates were from 79.4-96.8% susceptible, 1.9-13.5% intermediate, and 0.6-12.3% resistant depending of variation in yeast and the six antifungal drugs assessed.

The distribution of oral yeasts including *Candida* species is changing and the resistance to antifungal drugs is increasing. A national surveillance to study the epidemiology and the susceptibility pattern of Oropharyngeal yeast isolates to antifungal drugs is recommended. Simple and accessible technologies to identify and examine the susceptibility pattern of yeasts are recommended to accessible in Ethiopia, so that early isolation, speciation and antifungal susceptibility will aid the clinicians to institute proper antifungal therapy thus decreasing morbidity and mortality.

A combination of phenotypic methods can be useful for the presumptive identification of yeasts. It is recommended to investigate different samples from HIV patients and control health individuals for the spectrum of yeasts and the susceptibility pattern of candida species to different antifungal agents employing simple and rapid emerging identification and susceptibility testing methods. As shown in this study and many others, the use of HAART decreases the colonization by yeasts and incidence of OPC. As a result, early initiation of HAART is encouraged.

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Annexes

Annex I. English Versions of Participant Information Sheet

My name is BIRHAN MOGES. I am a laboratory technologist postgraduate student at Addis Ababa University. Now I am conducting a study entitled Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in **Zewditu Memorial Hospital, Addis Ababa, Ethiopia**.

You are invited to participate in this study. Please read the following statements and ask any unclear points before you agree to participate. If you agree to be included in this study, I would like to ask you to sign on a document to show your agreement; participate accordingly, and give clinical specimen.

Introduction

The topic of this study is Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in Zewditu Memorial Hospital, Ethiopia. Since Oropharyngeal yeasts are the major opportunistic health problems in the world, the result of the study can be helpful in planning and intervention to solve the problem in our country. Participation in this study is exclusively voluntarily. If you are not interested to participate or if you once decide to participate and withdraw yourself at any time, there will be no consequences and you will get all the services provided in the hospital with no problem. If you decide to participate, you have to sign on the assent/ permission template form and you may obtain a copy of this information sheet.

Expected from participants

As a participant of this study, you are expected to give Oropharyngeal swab or oral rinse. Being asked to give sample does not neccessarly mean that you have the disease.When you are found to be positive for the micro-organisms, you will be informed by the health worker and receive proper treatment. You need to know that your results might be discussed with other appropriate individual out of this hospital. But your name, address will not be disclosed rather an identification code will be used in such conditions.

Time required for participation

You will spend 2-5 minutes until the specimen is collected and permission form is signed.

Risks of participant

Specimen collection will be done using sterile swabs that will have no effect and you will not get any risk as the sample will be collected by well trained professionals.

Confidentiality

The information in your records is strictly confidential. All information that you give and the results from your specimen will be used for this study only. Only limited numbers of professional will have access the information. The information will be encoded in a computer and saved with password protection.

Benefits of participation

By participating, you will get no financial benefits. Even though there is no direct benefit due to participation in this study, the findings of the study is useful for better understanding of the problems Oropharyngeal yeast infections. You will also obtain all the results of the analysis for free and communicated to your physician for the appropriate management.

Rights of participants

Your participation is completely voluntary, and you can refuse to participate or withdraw from the study at any time. Refusal to participate will not result in loss of medical care provided or any other benefits. You can get your results of the analysis.

Communication

In case if you have any questions, unclear ideas and doubt about the project, contact addresses are: **Investigator:** Birhan Moges (BSc), DMLS; AAU, +251913695895, Email-ebmoges@gmail.com

Advisor: Adane Bitew (PhD), DMLT, AAU, +251911039162

For additional information, please contact Addis Ababa University, College of Health Sciences; Department of Medical Laboratory Sciences at: Telephone +251112755170.

Your signature below indicates that you have read /or listened, and understand the information provided for you about the study. Before you sign,please understand purpose of the study, procedure,risks and benefits of participation, right to refuse or withdraw, confidentiality and privacy, and who to contact if you have question. I have read /or listened to the description of the study and I understand what procedures are and what will happen to me in the study.

Agree to participate? Yes---------- No----------

Annex II: Amharic Versions of Participant Information Sheet

እኔ ብርሃን ሞገስ እባላለሁ፡፡ በአዱስ አበባ ዩኒቨርሲቲ፣ ጤና ሳይንስ ኮሌጅ፣ የህክምና ላቦራቶሪ ሳይንስ ትምህርት ክፍል የሁለተኛ ዲግሪ ተማሪ ስሆን የምርምር ስራየን በመስራት ላይ እገኛለሁ፡፡ እርስዎም በዚህ ጥናት ላይ እንዲሳተፉ ተጋብዘዋል፡፡ በጥናት ለመሳተፍ ፈቃደኛ ሆነዉ ከተስማሙ መስማማትዎን የሚያሳይ ዶክመንት ላይ እንዲፈርሙ እጠይቃለሁ፡፡

መግቢያ: የጥናቱ ርዕስ "በአፍ ውስጥ የሚገኙ ፈንገሶች የዝርያ ስርጭትና ከጸረ-ፈንገስ መድሃኒቶች ጋር ያላቸዉን መስተጋብር በዘዉዲቱ መታሰቢያ ሆስፒታል ከሚታከሙ የኤች አይ ቪ ታካሚዎች፣ ኢትዮጵያ" በሚሌ ርዕስ እያጠናሁ እገኛለሁ፡፡ ይህ ጥናትም በተሳታፊ ሙሉ ፈቃደኝነት ላይ ተመስርቶ በአፍ ውስጥ የሚገኙ ፈንገሶች የዝርያ ስርጭትና ከጸረ-ፈንገስ መድሃኒቶች ጋር ያላቸዉን ተላምዶ ለማወቅ እና አማራጭ መንገዶችን ለመጠቆም ያስችላል፡፡

ከጥናቱ ተሳታፊ የሚጠበቁ: በዚህ ጥናት ለመሳተፍ የሚስማሙ ከሆነ ከአፍ ውስጥ ናሙና ተጠርጎ እንዲወሰድ እና ለጥናቱ እንዲዉል መስማማት ይጠበቅቦታል፡፡ የጤና ባለሙያ ከእርሰዎ ናሙናዉን ይሰበስባል፡፡ ከተወሰደዉም ናሙና ላይ የሚገኙ መረጃዎች የእርሶን ማንነት የማይገልጹ ማስረጃዎን ማለትም ስም፣ አድራሻና የመሳሰለት መረጃዎች ሳይጨምርና ለዚህ ጥናት አገልግሎት ብቻ የሚዉል መለያ ቁጥር በመጠቀም ከዚህ ሆስፒታል ዉጭ ለሚገኙ ለሥራዉ አግባብነት ላላቸዉ ሰዎች ቢነገር የማይቃወሙ መሆኑን መስማማት ይጠበቅቦታል፡፡ ናሙና ሰጡ ማለት በሽታው ይገኝብዎታል ማለት አይደለም፡፡ በእርሰዎ ናሙና ውስጥ የበሽታ አምጭ ተህዋስ ቢገኝ ከጤና ባለሙያዉ አስፈሊጊውን ህክምና ያገኛሉ፡፡

ተሳታፊዉ የሚያጠፋዉ ጊዜ: የተዘጋጀዉን የስምምነት ቅጽ ለመፈረምና ናሙና ለመስጠት 2-5 ደቂቃ ያስፈልጋል፡፡

በጥናቱ በመሳተፍ የሚያስከትላቸዉ ችግሮች: ናሙና የሚወሰደው ፍጹም ንጹህ በሆነ ዕቃና በሰለጠነ ባለሙያ ስለሆነ በሚሰበሰብበት ወቅት ምነም አይነት ችግር አያስከትልብዎትም፡፡

የመረጃዉ ሚስጥራዊነት: ማንኛዉም የሰጡት መረጃ እና ከተወሰደዉ ናሙና ላይ የተገኘዉ የላቦረቶሪ ዉጤት የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ፡፡ ይህን ማህደር ሊያገኙ የሚችሉ የተወሰኑ የጥናቱ ተባባሪ ሠራተኞች ብቻ ናቸዉ፡፡ ከዚህም በላይ ስለ እርስዎ ያለዉን ማንኛዉንም መረጃ የይለፍ-ቃለ ባለዉ የኮምፒዉተር የመረጃ ማህደር ዉስጥ እንዲቀመጥ ይደረጋል፡፡

በጥናቱ በመሳተፍ የሚያስከትላቸዉ ጥቅሞች: በዚህ ጥናት በመሳተፍዎ የሚያገኙት የገንዘብ ጥቅማጥቅም የለም፡፡ ለወደፊት በተመሳሳይ ሁኔታ ዉስጥ ላሉ በሽተኞች በመረጃ ላይ የተመረተ ህክምና ለመስጠት ያግዛል፡፡ የላቦራቶሪ ዉጤቶችን በነፃ ያገኛሉ፤ እንዲሁም ስለ አስፈላጊዉ ህክምና ከሀኪምዎ ጋር ይነጋገራሉ፡፡

የጥናቱ ተሳታፊዎች መብት: ትብብርዎ ሙሉ በሙሉ በፍቃደኝነት ላይ የተመሠረተና ተሳትፎዎን መተውና በማንኛውም ሰዓት ጥናቱን ማቆም ይችላሉ፡፡ በጥናቱ ዉስጥ ያሎትን ተሳትፎ በማንኛዉም ጊዜ የማቐረጥ ሙሉ መብትዎ የተጠበቀ ከመሆኑም በላይ ራስዎን ከጥናቱ በማግለልዎ ምክንያት የሚቀርብዎት ምንም ዓይነት የሆስፒታል አገልግሎት አይኖርም፡፡ ከዚህም በተጨማሪ ጥናቱን በተመለከተ ማንኛዉንም ዓይነት ጥያቄ የመጠየቅና ገለፃ የማግኘት መብት አለዎት፡፡ የላቦራቶሪ ምርመራ ዉጤቱንም በነፃ ማግኘት ይቻላል፡፡

ግንኙነትና ጥያቄ

ይህን ጥናት በተመለከተ ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥመዎ ድንገተኛ ችግር ወይም ጥያቄ ካሎት በሚከተለዉ አድራሻ ይጠቀሙ፡፡

ተመራማሪ፤ ብርሃን ሞገስ (ቢ.ኤስ.ሲ)

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የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል፤ የጤና ሳይንስ ኮሌጅ፣ አዲስ አበባ ዩኒቨርሲቲ

ለተጨማሪ መረጃ

አዲስ አበባ ዩኒቨርሲቲ፤

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል

ስልክ: +251112755170 ይጠይቁ::

ከዚህ በታች የሚገኘው ፊርማዎ ለእርስዎ የተሰጠውን መረጃ ማንበብዎን፣ መስማትዎን እና መገንዘብዎን የሚያሳይ ነው፡፡ ከመፈረምዎ በፊት እባክዎትን የጥናቱን ዓላማ፣ የተሳትፎ ጉዳትና ጥቅሙ፣ የመተው፣ የማቋረጥ፣ መብትና ነፃነት እንዳለዎት ይረዱ፡፡ ተስማምተዋል? የጥናቱን መግለጫ አንብቤያለሁ/ ሰምቻለሁ እናም ተረድቻለሁ፡፡ መመሪያው ምን እንደሆነና በእኔ ምን ሊከሰት እንደሚችል ተረድቻለሁ፡፡

በጥናቱ ላይ ለመሳተፍ፤

እስማማለሁ ---------- አልስማማም ----------

Annex III: English Versions of Consent form

This page contains an agreement signature to participate in the study entitled with "**Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in Zewditu Memorial Hospital, Ethiopia"**. So please read the following points and sign your signature at the end in the space provided.

1. I understand the objective of the study "Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in **Zewditu Memorial Hospital**, Ethiopia" and I can communicate with the investigators when I want them.
2. I know that the information/ specimen that I will give used for this study only.
3. I understand that, all the information given for the study and the results are confidential.
4. I understand that I will not get any money for my participation.
5. I understand that I have a right to stop from participation any time in the study.
6. I understand all the information which is explained by specimen collector.

Signature of the participant: _____

Address of the participant: _____

Date: _____

Evidences of the agreement

1. _____
2. _____

Please direct any questions or problems you may encounter during this study to:

Birhan Moges

Department of Medical Laboratory Sciences, College of Health Sciences

Addis Ababa University

Mobile: +251913695895

Email:ebmoges@gmail.com

For additional information, please contact Addis Ababa University, College of Health Sciences,

Department of Medical Laboratory Sciences at: Telephone +251112755170

Annex IV: Amharic Versions of Consent form

የተሳታፊ ስምምነት ቅጽ

ይህ ገጽ "Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in Zewditu Memorial Hospital, Ethiopia" ማለትም" በአፍ ውስጥ የሚገኙ ፈንገሶች የዝርያ ስርጭት ናከጸረ-ፈንገስ መድሃኒቶች ጋር ያላቸዉን መስተጋብር በዘዉዲቱ መታሰቢያ ሆስፒታል ከሚታከሙ የኤችአይቪ ታካሚዎች፣ ኢትዮጵያ" በሚል ርዕስ የተሳታፊ ስምምነት ቅጽ ነዉ፡፡ በመሆኑም እባክዎን በዚህ በታች የተዘረዘሩትን ነጥቦች ይረዱና፤ ለመሳተፍ ፈቃደኛ ሆነዉ ከተስማሙ መስማማትዎን የሚያሳይ ፊርማዎን ከታች በተሰጠው ቦታ ላይ እንዲፈርሙ እጠይቃለሁ፡፡

1. እኔ "በአፍ ውስጥ የሚገኙ ፈንገሶች የዝርያ ስርጭትና ከጸረ-ፈንገስ መድሃኒቶች ጋር ያላቸዉን መስተጋብር በዘዉዲቱ መታሰቢያ ሆስፒታል ከሚታከሙ የኤችአይቪ ታካሚዎች፣ ኢትዮጵያ" የሚለዉ ጥናት አላማ በደንብ ተገንዝቤአለሁ፡፡
2. ከእኔ የሚወሰደዉ ናሙና ለጥናቱ አላማ ብቻ እንደሚዉል ተረድቻለሁ፡፡
3. ሁሉም መረጃዎች እና የናሙና ዉጤቱ ምስጢራዊ መሆኑን ተገንዝቤአለሁ፡፡
4. በጥናቱ ላይ በመሳተፌ ምንም የገንዘብ ክፍያ እንደማላገኝ ተረድቻለሁ፡፡
5. በጥናቱ ያለመሳተፍ እንዲሁም በማንኛዉም ጊዜ የማቋረጥ መብት እንዳለኝ አዉቄአለሁ፡፡
6. ሁሉም መረጃዎች በአስተባባሪዉ/ዎች ተገልጾልኝ በደንብ ተረድቻለሁ፡፡

የተሳታፊ ፊርማ፡ ----------

የተሳታፊ አድራሻ፡----------

ቀን፡----------

<u>በስምምነቱ ወቅት የነበሩ ምስክሮች</u>

1. _____
2. _____

ይህን ጥናት በተመለከተ ጥያቄ ቢኖርዎት ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥመዎት ድንገተኛ ችግር በሚከተለዉ አድራሻ ይጠቀሙ፡፡

ብርሃን ሞገስ

ሞባይል፡ +251913695895

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል

የጤና ሳይንስ ኮሌጅ፤

አዲስ አበባ ዩኒቨርሲቲ

ኢ-ሜይል፤ebmoges@gmail.com

ለተጨማሪ መረጃ፡ አዲስ አበባ ዩኒቨርሲቲ፤ የሕክምና ላብራቶሪ ሳይንስ ት/ክፍል ይጠይቁ፡፡

ስልክ፡ +251112755170

Annex V: English Version of Assent form for Under 18 Years Participants

This page contains an agreement signature for family members or other adult guard for participants under the age of 18 years to participate in the study entitled with "Species distribution and in vitro antifungal susceptibility of Oropharyngeal yeast isolates from HIV patients in Zewditu Memorial Hospital, Addis Ababa, Ethiopia".

For this study oral swab samples will be collected using sterile swab. The sample collection procedure does not induce pain or does not have any danger.

The sample collected will be used only for the study purpose and the result is strictly kept confidential and if the disease causing agent is found in your sample the results will be communicated to your physician for the appropriate management.

You will get no financial benefits. Participation in the study is completely voluntarily. You can refuse to participate or withdraw from the study at any time. Refusal to participate will not result in loss of medical care provided or any other benefits.

Do you allow the (name) to participate in the study?

Allow Participation

Signature of the participant: _____

Signature of the Guardian:_____

Address of the participant: _____

Date: _____

Evidences of the agreement

1._____

2._____

Please direct any questions or problems you may encounter during this study to:

Birhan Moges

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Mobile: +251913695895

Email:ebmoges@gmail.com

For additional information, please contact Addis Ababa University, College of Health Sciences,

Department of Medical Laboratory Sciences: Telephone +251112755170

Annex VI: Amharic Version Assent form for Under 18 Years Participants

ይህ ገጽ “Species Distribution and In vitro Antifungal Susceptibility of Oropharyngeal Yeast Isolates from HIV patients in Zewditu Memorial Hospital, Addis Ababa, Ethiopia” ማለትም “በአፍ ውስጥ የሚገኙ የፈንገስ ዝርያዎች ስርጭትና ከጸረ-ፈንገስ መድሃኒቶች ጋር ያላቸዉን መስተጋብር በዘዉዲቱ መታሰቢያ ሆስፒታል ከሚታከሙ የኤች አይ ቪ ታካሚዎች” በሚል ርዕስ ለሚካሄደው ጥናት ናሙና ለመዉሰድ ከቤተሰብ ወይም ሌላ አዋቂ ሰው እድሜያቸው ከ18 አመት በታች ለሆናቸው ተሳታፊዎች ስምምነት የመጠየቂያ ገጽ ነው፡፡

ለዚህ ጥናት ከአፍ ውስጥ በመጥረግ ናሙና እንወስዳለን፡፡ ናሙና የምንወስድበት መሳሪያ ንጽህናው ሙሉ በሙሉ አስተማማኝና ከዚህ በፊት ጥቅም ላይ ያልዋለ ነው፡፡ ናሙና በምንወስድበት ጊዜ የሚደርስ ህመም የሚፈጥር ስሜትም ሆነ አደጋ የሚያስከትል ሂደት የለውም፡፡

ለጥናት የሚወሰደው ናሙና ለጥናት አላማ ብቻ ይውላል፡፡ የናሙናው ውጤት ምስጥራዊነት የተጠበቀ ሲሆን በናሙናው ውስጥ የበሽታ አምጭ ተህዋስ ቢገኝከጤና ባለሙያዉ አስፈሊጊውን ህክምና ያገኛሉ፡፡በጥናቱ ላይ በመሳተፍዎ ምንም የገንዘብ ክፍያ አያገኙም፡፡ በጥናቱ ለመሳተፍ የመፍቀድም ሆነ ያለመፍቀድ እንዲሁም በማንኛዉም ጊዜ የማቋረጥ መብት አለዎት፡፡ (ስም) በጥናቱ እንዲሳተፍ/ እንድትሳተፍ ይፈቅዳሉ?

ፈቃደኛ ከሆኑ፤

የተሳታፊ ፊርማ፣---------- የፈቀደው ግለሰብ ፊርማ፣----------

አድራሻ፣---------- ቀን፣----------

በስምምነቱ ወቅት የነበሩ ምስክሮች

1._____2._____

ይህን ጥናት በተመለከተ ጥያቄ ቢኖርዎት ወይም ከዚህ ጋራ በተዛመደ መልኩ ስለሚያጋጥመዎት ድንገተኛ ችግር በሚከተለዉአድራሻ ይጠቀሙ፡፡

ብርሃን ሞገስ ሞባይል፡ +251913695895፣ ኢ-ሜይል፤ ebmoges@gmail.com

የሕክምና ላብራቶሪ ሳይንስ ትምህርት ክፍል፣ የጤና ሳይንስ ኮሌጅ፤ አዲስ አበባ ዩኒቨርሲቲ

ለተጨማሪ መረጃ፡ አዱስ አበባ ዩኒቨርሲቲ ፤ የሕክምና ሊብራቶሪ ሳይንስ ት/ክፍል ይጠይቁ፡፡

ስልክ፡ +251112755170

Annex VII: Laboratory test Procedure for Specimen Collection and Processing

Sample collection, processing, inoculation, biochemical and drug susceptibility testing will carried out using different microbiological methods described by different researchers [62].

A. Collection and processing of Oropharyngeal Swabs

1. Firmly swab the oral lesions when present, the oral cavity mucosa using sterile cotton swab, the dorsal surface of the tongue, and the floor of the mouth [14, 18].
2. The collected swab samples were placed in Amie's transport medium aseptically immediately after collection.
3. Transport the samples with appropriate temperature and minimum of delay to the Microbiology laboratory.
4. Inoculate the swabs on to Sabouraud Dextrose Agar media supplemented with chloramphenicol.
5. Incubate at 35^0c for 24-48hrs [32]. Plates with no growth after 48 hours will be re-incubated for a further one week [29].
6. Use a pure colony for the identification of the species using standard conventional mycological methods. Morphological and biochemical properties.

B. Identification Tests for Yeasts

1. Germ Tube Test:

The germ tube test provides a simple, reliable and economical procedure for the presumptive identification of *Candida albicans*. About 95% of the clinical isolates produce germ tubes when incubated in serum at 35^0C for 2.5-3 hours.

Procedure:

1.1. Two to three fresh colonies of yeast were inoculated into 0.5 ml of human serum.

1.2. Incubate at 37º C for 2-3hrs.

1.3. After desired period of incubation, place a loop-full of culture on a glass slide and overlaid with a cover-slip and observe under microscope for germ tube formation [43].

1.4. The appearance of small filaments projecting from the cell surface confirmed formation of germ tubes [4].

2. Growth at 45^{o}C /Temperature tolerance/:

i. Identify isolates with positive Germ tube test.

ii. Inoculate into SDA plates.

iii. Incubate at 45°C in aerobically.

iv. Observe for any growth daily for 10 days.

Unlike *C. albicans, C. dubliniensis* lacks the ability to grow at 45^{o}C [43].

3. Carbohydrate Assimilation Test:

The ability of yeasts to use a particular carbohydrate as a sole source of carbon in a medium can be used to isolate the species. Yeast Nitrogen Base contains all essential nutrients and vitamins necessary for the cultivation of yeasts except a source of carbohydrate.

i. Prepare Yeast Nitrogen Base (YNB) (Difco Laboratories, USA) as manufacturer's specification.

ii. Using a sterile cotton swab, remove the growth from the subculture and suspend in 9 mL sterile normal saline.

iii. Using a new sterile swab, uniformly inoculate the medium with the yeast suspension.

iv. Following inoculation, paper disks soaked in 1% w/v solution of different carbohydrates including Glucose, Maltose, Sucrose, Lactose, Raffinose, Mellibiose, Xylose, Galactose, Inositol, Dulicitol, Cellobiose and Trehalose were placed onto the surface of the medium.

v. Press each disc with sterile forceps to make good contact with the agar surface.

vi. Incubate the plates in an inverted position (agar side up) at 25°C for 48–72 hours.

vii. The carbohydrate assimilation was observed with a presence of a halo of growth around each carbohydrate.

C. Antifungal Disk preparation

The powder of the disks were dissolved using dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany). Manufacturer's recommended concentrations, other previous researches and the CLSI guidelines were used for the preparation process, storage and use of antifungal susceptibility disks.

Procedure:

i. Dissolve the antifungal powders in their specific solvents (Fluconazole, Clotrimazole, Nystatin, Miconazole, ketoconazole and Amphotericin B with DMSO).
ii. Twenty mg of the antifungal drug powder was soaked in 20ml of DMSO in six different test tubes (1mg/ml antifungal agent solution).
iii. Load 10µl Clotrimazole (10µg/disk), 25 µl Fluconazole (25µg/disk), 10 µl Ketoconazole (10µg/disk), 10 µl Miconazole (10µg/disk), 50 µl Nystatin (50µg/disk), and 10 µl Amphotericin B (10µg/disk) according to the Rosco Diagnostica Company (Neosensitabs, Denmark), (Hi-media, India), and CLSI.
iv. Dry the disks and store at 4°C until use within 1 to 2 weeks [4, 16, 50, 59].

D. Antifungal susceptibility Testing

Procedure

i. Prepare a suspension of the yeast by emulsifying 3-5 colonies in a 5mL normal saline.
ii. Adjust the turbidity of the suspension to a 0.5 McFarland standard by adding sufficient sterile saline or more colonies [12, 31, 41].

iii. Dip a sterile cotton swab in to the suspension and rotate several times and press firmly against the inside wall of the tube above the fluid level to remove excess fluid from the swab.

iv. Evenly streak the swab over the entire agar surface SDA (Lab M limited, Lancashire, UK) and exposed to air dry [4].

v. Dispense the antifungal disks onto the surface of the inoculated agar plate and press down to ensure its complete contact with the agar surface.

vi. Place the plates in an incubator set to 35 °C (±2 °C) within 15 minutes after the disks are applied.

vii. After 24-48hrs measure zone diameter.

viii. Inhibitory zone diameters were measured at the transitional point where growth abruptly decreased, as determined by a marked reduction in colony sizes.

ix. Interpret the reaction of the test to each antifungal agent used as susceptible, susceptible dose dependent, or resistance as per the CLSI standard.

E. **Criteria of specimen rejection**

- ✓ Inappropriate specimen transport device,
- ✓ Mislabeled specimen and unlabeled specimen.

Annex VIII. Declaration

Title of project: Species Distribution and In vitro Antifungal Susceptibility of Oropharyngeal Yeast Isolates from HIV Patients in Zewditu Memorial Hospital, Addis Ababa, Ethiopia.

I the undersigned, declare that this is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been acknowledged.

Principal investigator:

Name: Birhan Moges (BSc).

Place: Department of Medical Laboratory Sciences, AAU

Signature _____

Date of submission _____

Advisor:

This thesis has been submitted with my approval as University advisor.

Name: **Adane Bitew (MSc, PhD)**

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