A STUDY ON SPECIATION AND ANTIFUNGAL SUSCEPTIBILITY PATTERN OF *CANDIDA* ISOLATES FROM HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS AND CORRELATION WITH CD4 COUNT

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DECLARATION

I, **Dr. V.KALPANA DEVI**, solemnly declare that the dissertation titled "**A STUDY ON SPECIATION AND ANTIFUNGAL SUSCEPTIBILITY PATTERN OF *CANDIDA* ISOLATES FROM HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS AND CORRELATION WITH CD4 COUNT"** is a bonafide record of work done by me in the Institute of Microbiology, Madras Medical College and ART Center, Government General Hospital, Chennai under the guidance and supervision of **Prof. Dr. S. GEETHALAKSHMI, M.D, Ph.D.,** Institute of Microbiology, Madras Medical College, Chennai.

This dissertation is submitted to the TN Dr.MGR Medical University towards partial fulfillment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in March 2010.

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CONTENTS

INTRODUCTION

Infection with Human immunodeficiency virus (HIV) and its end stage Acquired immunodeficiency syndrome (AIDS) is the major public challenge of modern times, with over 25 million persons already dead and over 30 million living with HIV / AIDS, the majority of whom are without access to therapy[58].

Opportunistic infection continue to cause morbidity and mortality in patients with human immunodeficiency virus infection throughout the world[66].

Although the incidences of opportunistic infections have been reduced around the globe by Highly Active Anti Retroviral Therapy(HAART), the situation remains the same in most developing countries including India[52]. This is due to the fact that some patients do not have access to the treatment and others do not have sustained response to antiretroviral drugs for multiple reasons including poor adherence, drug toxicities, drug interactions or initial acquisition of a drug resistant strains[66].

Oropharyngeal candidiasis is the most common opportunistic infection observed in AIDS patients, occurring in an estimated 80 to 95% of these patients when the CD4 T lymphocyte counts are below 200 cells /mm^3 .Increased retroviral replication and an associated decline in immune defenses render these patients more susceptible to oropharyngeal candidiasis[23,26,13]. Presenting months or years before more severe opportunistic infection, oropharyngeal candidiasis may be a sentinel event indicating the presence or progression of HIV disease. Although usually not associated with severe morbidity, oropharyngeal candidiasis can be clinically significant. Severe

oropharyngeal candidiasis can interfere with the administration of medication and adequate nutritional intake[33].

The prolonged nature of AIDS predisposes these patients to recurrent episodes of oropharyngeal candidiasis that can increase in frequency and severity with progressive HIV disease.Therfore the prolonged management of Oropharyngeal Candidiasis in these patient population causes the development of drug resistant candidiasis[3,79].

Antifungal drug resistance is fast becoming a major problem[12,41]. Fluconazole has been successful in treating Oropharyngeal Candidiasis in HIV infected patients but its long term use and increased immunosuppression in HIV patients as assessed by the CD4 count raises the possibility of the acquisition of resistance by the yeast *Candida albicans*[45,68]. Many studies have estimated the incidence of clinical fluconazole resistance to be around 5-10%[63]. The increased reports of antifungal resistance and expanding drug therapy options prompted the need for clinically relevant antifungal susceptibility testing. Further the emergence of other *Candida species* such as *Candida krusei* and *Candida glabrata* with innately reduced susceptibilities to fluconazole[22] also results in treatment failure, emphasizing the need for speciation of the oral yeast isolates.

Considering the above the facts, the present study was conducted to determine the species distribution and antifungal susceptibility profiles of the *Candida* isolates from HIV patients with Oropharyngeal Candidiasis.

REVIEW OF LITERATURE

HUMAN IMMUNODEFICIENCY VIRUS

AIDS was first recognised in the summer of 1981, when the US centres of disease control and prevention reported the unexplained occurrence of *Pneumocystis jirovecii* among homosexual men in Los Angeles and an outbreak of a rare form of cancer among gay men in Newyork and California , medically known as Kaposi sarcoma. Within months , the disease became recognised in male and female injection drug users and soon thereafter in recipients of blood transfusions and in hemophiliacs. As the epidemiologic pattern of the disease unfolded, it became clear that a microbe transmissible by sexual contact and blood or blood products or from mother to infants was the most likely etiologic agent.

In 1983, Luc Montagnier isolated the virus from a patient with lymphadenopathy and by 1984 it was demonstrated clearly to be the causative agent of AIDS. In 1985, a sensitive enzyme linked immunosorbent assay was developed[37].

The etiologic agent of AIDS is HIV, which belongs to the family of human retroviruses and the subfamily of lentiviruses. There are two distinct types of human AIDS viruses namely HIV-1 and HIV-2.The two types are distinguished on the basis of genome organization and phylogenetic relationship with other primate lentiviruses[43].

The most common cause of HIV disease throughout the world is HIV-1. HIV-2 was first identified in 1986 in West African patients and was originally confined to West Africa. However a number of cases have been identified throughout the world[37].

HIV-1 is subdivided into three genetic groups designated M (Major), O (Outlier) and N (Non- M, Non – O) based on sequence diversity within the HIV –I gag and env genes.HIV-1 group is divided into 9 subtypes(A-D,F-H, J&K)[10].Subtypes differ in mode of transmission, geographical distribution and biologic characteristics. Subtype C viruses are the most common worldwide. The predominant subtype in Europe and the America is Subtype B. Subtype C is prevalent in India[70].

HIV-1 and HIV-2 have the same mode of transmission. The most common mode of HIV infection is sexual transmission – both homosexual and heterosexual contact. Infection may also occur through inoculation of infected blood products, via transfusion of infected blood products, transplantation of infected tissues or through contaminated needle[10].Large multi institutional studies have indicated the risk of transmission following skin puncture with needle contaminated with blood from a HIV infected person as 0.3% and after mucous exposure it is 0.09%.The other mode of transmission is from the infected mother to the child either intrapartum, perinatally or via breast milk[37].

The hallmark of HIV disease is a profound immunodeficiency resulting primarily from a progressive quantitative and qualitative deficiency of the

subset of T lymphocytes referred to as helper T cells or inducer T cells. This subset of T cells is defined phenotypically by the presence on its surface of the CD4 molecule which serve as the primary receptor for HIV.HIV uses two major coreceptors namely CCR5 and CXCR4 for fusion and entry[37].

After attachment, HIV RNA and enzymes are released into the host cell. Viral replication requires that reverse transcriptase (an RNA-dependent DNA polymerase) copy HIV RNA, producing proviral DNA; this copying mechanism is prone to errors, resulting in frequent mutations. These mutations facilitate the generation of HIV that can resist control by the host's immune system and by antiretroviral drugs. Proviral DNA enters the host cell's nucleus and is integrated into the host DNA in a process that involves HIV integrase. With each cell division, the integrated proviral DNA is duplicated along with the host DNA. Proviral HIV DNA is transcribed to viral RNA and translated to HIV proteins, including the envelope glycoproteins 40 and 120. The HIV proteins are assembled into HIV virions at the inner cell membrane and budded from the cell surface; each host cell may produce thousands of virions. After budding, protease, another HIV enzyme, cleaves viral proteins, converting the immature virion into a mature, infectious form .

The main consequence of HIV infection is damage to the immune system, specifically loss of CD4+ lymphocytes, which are involved in cell-mediated and, to a lesser extent, humoral immunity. CD4+ lymphocyte depletion may result from the following:

- Direct cytotoxic effects of HIV replication
- Cell-mediated immune cytotoxicity
- Thymic damage that impairs lymphocyte production

Infected CD4+ lymphocytes have a half-life of about 2 days, which is much shorter than that of uninfected CD4+ cells. Rates of CD4+ lymphocyte destruction correlate with plasma HIV level. Typically, during the initial or primary infection, HIV levels are highest (>10^6 copies/mL), and the CD4 count drops rapidly. The normal CD4 count is about 750/μL, and immunity is minimally affected if the count is > 350/μL. If the count drops below about 200/μL, a variety of opportunistic pathogens may produce clinical disease. The humoral immune system is also affected [2].

LABORATORY INVESTIGATIONS FOR HIV INFECTION

(i) Laboratory methods for the diagnosis of HIV infection

(ii) Laboratory methods for monitoring Stage and Progression of Infection[70] .

(i)**Laboratory methods for the diagnosis of HIV infection**

(I) Indirect Methods (Antibody detection methods)

(ii)Direct Methods

DETECTION OF SPECIFIC ANTIBODIES

Detection of anti –HIV antibodies is the main stay of diagnosing HIV.

Tests to detect specific antibodies can be classified into

- Screening tests
    - Conventional ELISA
    - Rapid tests

Rapid tests are in vitro qualitative tests for the detection of antibodies to HIV-1 and HIV-2 in serum, plasma, whole blood , saliva and urine.

-Immunoconcentration (Dot blot assays)

-Particle agglutination

-Immunochromatography(lateral flow)

-Immunocomb(Dipstick/comb tests)

- Supplemental tests(Confirmatory tests)

-Western Blot

-Immunoblot

-Lineimmuno assay

-Indirect fluorescent antibody test

-Radioimmunoprecipitation tests[69]

DIRECT DETECTION OF HIV INFECTION

HIV infection is diagnosed mainly by detecting the HIV antibodies. But there are situations where the serology is negative although there is definite evidence of exposure to HIV infection. Direct Detection methods are needed in the following settings

(i) To determine HIV status during the window period

(ii) In health care workers following accidental exposure to contaminated blood.

(iii) Also children born to HIV infected mother present dilemma as antibody positivity seen upto 18 months may be due to maternal antibodies.

The diagnosis in such situation can be made by direct detection method like

- Detection of p24 antigen
- Detection of HIV specific DNA
- Isolation of Virus by culture

(ii)Laboratory tests for monitoring stage and progession of HIV Infection

They are classified into

Viral markers	**Immunologic markers**
Plasma HIV RNA load	CD4 count

P24 antigenemia

Viral specific markers like antibodies to HIV antigens(p24, p17,nef) and soluble markers of immune activation like neopterin , β2 microglobulin previously recommended for monitoring the course of HIV infection are no longer recommended.

The close relationship between clinical manifestations of HIV infection and CD4+ T cell count has made measurements of the CD4 count as a routine part of the evaluation of HIV infected individuals[70].

CD4 T cell counts

The progressive depletion of CD4+ T lymphocytes is the cardinal event in the pathogenesis of infection by the human immunodeficiency virus. The number of these cells in the peripheral blood is the single most important parameter for monitoring the disease associated with HIV infection.

Determinants of CD4+ T cell counts and measurements of the HIV-RNA levels in serum or plasma provides a powerful tools for determining prognosis and monitoring response to therapy[37].

Throughout the course of the disease, the total T cell levels remain fairly constant despite a fall in CD4 T lymphocyte count, due to a concomitant rise in CD8 T lymphocytes .Therefore, the ratio of CD4 T lymphocytes to CD8 T lymphocytes is an additional important measure of disease progression. Measurement of lymphocyte subsets is done by flow cytometry which is the gold standard for enumeration of CD4 T lymphocytes.

CD4 T lymphocytes enumeration is also utilized as a surrogate marker for HIV-induced damage .The declining slope of CD4 T lymphocytes counts, indicates the speed of progression towards AIDS. While on therapy, improvement in CD4 T lymphocytes counts is indicative of the success of therapy. In resource-poor countries, with the arrival of generic drugs for anti-retroviral therapy, the need for CD4 T lymphocytes counts has dramatically increased. CD4 T lymphocytes counts are the criteria for initiating ART as well as monitoring the therapeutic response in a patient[16].

Flow Cytometry:

Flow cytometry uses the principles of light scattering , light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles .The cellular suspension is incubated previously with monoclonal antibodies conjugated with different fluorochromes and it is introduced in the hydraulic system so that the cells pass individually through the flow chamber, where the laser beam intercepts the cells. These contact produces two different dispersion patterns : the frontal dispersion (Forward Scatter, A) gives us information about the cellular size , while lateral dispersion (Side Scatter, B) informs us about cellular complexity. The monoclonal antibodies linked to cellular surface is at the same time excited by the laser and generate fluorescences that are collected by photomultipliers (up to 4 at the moment) that transform the optic to electric signal, then cytometer send all information to the computer system for later analysis. (FACS facility)

Fluorescence-activated cell sorting is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It provides quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of interest[40].

CD4 cell count is extremely important in the staging of HIV infection and revised classification of the Center of Disease Control divides HIV positive patients into three categories.

CDC CLASSIFICATION SYTEM OF HIV INFECTION

CD4+ T-cell count (cells/µl.) (CD4%)	Asymptomatic, acute (primary) HIV or PGL	Symptomatic, not A or C conditions	AIDS-indicator conditions
> 500 (28%)	A1	B1	C1
200–499 (15–28%)	A2	B2	C2
< 200 (14%)	A3	B3	C3

Category A: asymptomatic HIV infection, persistent generalized lymphadenopathy (PGL).

Category B: oropharyngeal and vulvovaginal candidiasis, constitutional symptoms such as fever (38·5°C) or diarrhea lasting >1 month, herpes zoster (shingles).

Category C: Mycobacterium tuberculosis (pulmonary and disseminated), Pneumocystis carinii pneumonia, candidiasis of bronchi; trachea or lungs, extrapulmonary cryptococcosis, CMV, HIV-related encephalopathy, Kaposi's sarcoma, wasting syndrome due to HIV.

When the number of CD4+ T cells declines below a certain level , the patient is at high risk of developing a variety of opportunistic diseases, particularly the infections and neoplasms that are AIDS defining illness[37].

Opportunistic infections in HIV patients

Bacterial Infections

- Bacterial Diarrhea
- Bacterial Pneumonia
- Mycobacterium Avium Complex (MAC)
- Mycobacterium Kansasii
- Syphilis & Neurosyphilis
- Tuberculosis

Malignancies (Cancers)

- Anal Dysplasia/Cancer
- Cervical Dysplasia/Cancer
- Kaposi's Sarcoma, Lymphomas

Viral Infections

- Cytomegalovirus (CMV)
- Hepatitis C
- Herpes Simplex Virus (oral & genital herpes)
- Herpes Zoster Virus (shingles)
- Human Papilloma Virus (HPV, genital warts, anal/cervical dysplasia/cancer)
- Molluscum Contagiosum
- Oral Hairy Leukoplakia (OHL)
- Progressive Multifocal Leukoencephalopathy[72]

Fungal Infections

- Candidiasis
- Aspergillosis
- Coccidioidomycosis
- Cryptococcal Meningitis
- Histoplasmosis

Protozoal Infections

- Cryptosporidiosis
- Isosporiasis
- Microsporidiosis
- Toxoplasmosis

Neurological Conditions

- AIDS Dementia Complex[79]
- Peripheral Neuropathy

Others

- Aphthous Ulcers
- Thrombocytopenia
- Wasting Syndrome

The first overt indication of AIDS may be opportunistic infection with the fungus *Candida albicans*, which causes appearances of sores in the mouth (Thrush)[49].

CANDIDA

History

Oral candidiasis has been recognised as a clinical entity since the time of Hippocrates in his “Epidemics” described aphthae or thrush in debilitated patients[84]. The first binomial to gain wide acceptance was *Monilia albicans*

which was suggested by Zopf in 1890. Berkhout in 1923, after recognising the difference between *Monilia sp* isolated from rotting plants and those isolated from medical cases established the genus *Candida* to accommodate the later[77]. This was accepted as the official name of the genus by the Eight botanical congress in Paris in 1954.

Two major medical events have revived the interest in fungal diseases in general and *Candida* infections in particular .

The first was the introduction of antibacterial drugs in the second half of the twentieth century. These drugs may act as a predisposing factor for mycotic infections causing an imbalance of host's natural microbial flora in favour of fungi, upon which they have inhibitory activity .The second event was the increase in the prevalence of immunosuppressed patients during the last few decades, as a result of chemotherapy or disease [AIDS], which led to a parallel increase in the incidence of *Candida* infections in general and the less pathogenic non- *albicans Candida* species in particular[94].

Taxonomy

The Genus *Candida* belongs to the phylum Ascomycota, in class Ascomycetes , in order Saccharomycetales and family Saccharomycetaceae[94,9].

Genus *Candida* includes more than 150 species. Frequent human pathogens are[83]

Candida albicans (Robin) Berkhout 1923

Synonymy : *Oidium albicans / Monilia albicans*

Candida guilliermondii (castellani)1938 (Teleomorph : *Pichia guilliermondii*)[50]

Synonymy : *Endomyces guilliermondii/ Monilia guilliermondii*

Candida glabrata (Anderson) Meyer& Yarrow 1978

Synonymy: *Torulopsis glabrata*

Candida Krusei(castellani) Berkhout 1923

Synonymy : *Saccharomyces Krusei / Endomyces Krusei*

Teleomorph: *Issatchenkia orientalis*

Candida parapsilosis(Ashford) 1959

Synonym: *Monilia parapsilosis*

Candida tropicalis(Castellani)Berkhout 1923

Synonymy : *Monilia tropicalis / Oidium tropicalis*

Candida Kefyr (castellani) Basgal 1931

Synonymy : *Candida pseudotropicalis / Monilia pseudotropicalis*

Teleomorph: *Kluvyveromyces marxianus*

Candida lusitaniae Teleomorph: *Clavispora lusitaniae*[62]

Candida dubliniensis [Sullivan et at 1993,1995]

Candida viswanathii Sandhu et randhawa 1959[87]

Epidemiology

Although most *Candida spp.* have been isolated from lower animals and environmental sources, human infections are usually endogenous .They reside primarily in the gastrointestinal tract but can also be found as commensals in the vagina and on the skin. *Candida albicans* is the most commonly isolated species from all these sites[51].

As summarized by Odds, the range of *C.albicans* isolation from the oral cavity is 1.9-41.4%, the gastrointestinal tract is 0-55% and the vagina 2.2-68%.The same study indicates that the mean values of data from a number of epidemiological surveys reveal that *C.tropicalis* and *C.glabrata* are the second most frequent species isolated ; the former from the oropharynx and the latter from the vagina and the GI tract[72,94].

Oral carriage rates may be higher in certain settings such as HIV infected patients with CD4 count less than 500/µl, denture users with denture stomatitis, persons with diabetes, patients receiving chemotherapy for malignant conditions and children. Among persons with diabetes , local factors such as smoking and the presence of dentures additionally promotes *Candida* colonization of the mouth[77].

Most epidemiological studies indicates that infections caused by non-*C.albicans* yeast species tend to become more prevalent relative to those caused by *C.albicans*[38]. Nevertheless, *Candida albicans* is still the most frequent etiological agent of candidiasis[91]. Oropharyngeal Candidiasis is the most frequent form , occurring in upto 90% of persons with advanced untreated HIV infection[33].Oesophageal Candidiasis occurs less frequently (10-20%) but

is the leading cause of oesophageal disease[67]. Vaginal Candidiasis has been noted in 27-60% of women. However the incidence of vaginal Candidiasis appears to be similar in HIV and HIV uninfected women[96].

Pathogenicity and pathogenesis

Virulence factors

Candida sp that are normal flora can invade tissues and produce life threatening infections in patients whose immune defenses have been altered by diseases and iatrogenic intervention[72,57].*Candida albicans* the most common species isolated from all forms of Candidiasis appears to possess a number of virulence determinants. These includes

(i) The ability of the fungus to adhere to host tissues

(ii) Production of proteolytic enzyme like secretory aspartyl proteases and phospholipase enzymes

(iii) Yeast hyphal morphogenetic transformation

(iv) Phenotypic switching

(v) Various immunomodulatory effects of fungal determinants[94].

Pathogenesis of Candidiasis

Candidiasis is mostly an endogenous infection arising from overgrowth of the fungus inhabitating the normal flora[6]. The Gastrointestinal tract is considered a major reservoir for candidiasis from which the fungus can invade the blood stream following damage to the GI mucosa causing deep seated / disseminated infection[93].It is believed that Candida can cross the intact GI

mucosa by a process called persorption following fungal overgrowth due to excessive antibiotic treatment[48].

However it may be occasionally acquired from exogenous sources (such as catheters or prosthetic devices)[6].This is of particular importance in the development of deep seated and systemic candidiasis as most of these therapeutic modalities are used in compromised hosts whose defense system are unable to combat the introduced pathogen.

Once *Candida* entered blood stream whether from exogenous or endogenous source, the microorganisms adheres to the endothelial surface before dissemination into tissues[93,74]. Person to person transmission may be occasionally seen in cases such as oral candidiasis in neonates of mothers with vaginal candidiasis or endophthalmitis following corneal transplantation from an infected donor[9].

Pathology

The macroscopic and microscopic appearance of lesions caused by *Candida spp.* is primarily influenced by the interaction of three factors:

- The site of infection
- The pathogenicity of the infecting microorganism and to a lesser extent its species
- The competence of the host's immune system

Consequently the pathological and histopathological features of *Candida* infections are highly variable ; the same microorganism can cause

dissimilar pathological lesions in patients whose immune system is intact and those who are in a state of immunosuppression.

Superficial Infections

Superficial infections result from invasion of the superficial layers of the skin or mucosa by the microorganism. Macroscopically , characterized by formation of a grayish plaque, surrounded by edema[94] .In oral thrush, the pseudomembanous lesion is composed of necrotic material, white blood cells, commensal bacteria, yeasts, pseudohyphae and loose epithelial cells.The fungus is restricted to stratum corneum of the affected area[84].

Deep infections

Several parenchymatous organs may be involved .These infections are characterized by microabscesses. Granulomata with giant cells and lymphocytes may be formed in chronic infections[94].

Clinical Manifestations

As the frequency of diseases caused by *Candida* has increased, a relatively large number of manifestations have become well documented. Clinical entities may be divided into two large groups[57,94]

1. Mucocutaneous Candidiasis

(i)Mucosal

- Oral Candidiasis(Thrush)
- Vaginal Candidiasis
- Candida oesophagitis

(ii)Cutaneous

- Generalised Cutaneous Candidiasis
- Erosio interdigitalis ,Candida folliculitis , balanitis
- Candida balanitis- Intertrigo ,Diaper rash
- Chronic mucocutaneous Candidiasis

(iii) Nail - Paranychia , Onychomycosis

2.Deep Seated Candidiasis

- Central Nervous System Candidiasis
- Respiratory tract Candidiasis
- Candida infection of Vasculature
- Ocular Candidiasis
- Candida arthritis, Osteomyelitis, Costochondritis, Myositis
- Urinary tract Candidiasis
- Cardiovascular Candidiasis
- Disseminated Candidiasis and Candidemia[51]
- Candidiasis of Peritonum , Liver, Spleen and Gall bladder

ORAL CANDIDIASIS

Oral candidiasis is the most prevalent opportunistic infections affecting the oral mucosa. *Candida spp* are part of the normal mouth flora in 25-50% of healthy individuals. *Candida albicans* is the most frequent colonizer (70-80%) but any of the *non- albicans spp* may be seen .Oropharyngeal Candidiasis on the other hand , goes beyond mere carriage to symptomatic infection [94]. A number of predisposing factors have the capacity to convert Candida from the common flora(saphrophytic stage) to a pathogenic organism[60]-

Predisposing factors for oral candidiasis

Local factors	General factors
Denture wearing	Immunosuppressive drugs/diseases
Smoking	Chemotherapy
Inhalation steroids	Endocrine disorder
Topical steroids	Hematinic malignancies
Hyperkeratosis	Malignancies[60]

CLASSIFICATION

Oral candidiasis can be classified as follows[1,25]

A- Acute candidiasis

1) Acute pseudomembranous candidiasis (Thrush)

2) Acute atrophic candidiasis (Erythematous)

B- Chronic candidiasis

1) Chronic hyperplastic candidiasis (candidial leukoplakia)

2) Denture induced candidiasis (chronic atrophic candidiasis)

3) Median rhomboid glossitis

C- Angular cheilitis

Oral Candidiasis and HIV

Oropharyngeal Candidiasis occurs in three quarters of all those who have HIV infection. In about one-third it tends to be recurrent and becomes

progressively more severe with increasing immunodeficiency[98]. Oral Colonisation with inherently drug resistant organisms is more common in advanced HIV infection (CD4 <50 cells/ µl)[32].

Candidal infection in AIDS is almost exclusively mucosal, systemic invasion is rare and late event .Oropharyngeal and Vulvovaginal disease are the most common forms of mucocutaneous candidiasis in HIV patients[33].

Candida albicans has been identified as the most common causative agent of oral Candidiasis which was recognized from the beginning of AIDS pandemic as an early expression of immunodeficiency that occurs in HIV infected patients. Less frequently , *C. glabrata, C.parapsilosis, C.tropicalis, C.krusei* and several other spp may cause the disease[33]. Recurrent disease is caused by the same strain of *Candida* in about 50% of case , remaining 50% are caused by the new strains of *Candida albicans* or new species[32].The majority of disease is caused by organisms that are part of normal flora, although rare cases of person to person transmission have been documented. Indeed , unexplained oral candidiasis in previously healthy adults has been considered an important clinical predictor of AIDS as well as highly predictive of worsening immunodeficiency[7].

Upto 90% of persons with advanced untreated HIV infection develop Oropharyngeal Candidiasis, with 60% having atleast one episode per year with frequent recurrences(50-60%)[32,11].Symptoms of Oropharyngeal Candidiasis include burning pain, altered taste sensation, difficulty in swallowing liquids / solids. Many patients are asymptomatic. Most commonly present with

pseudomembranous candidiasis and less commonly with other forms like erythematous, hyperplastic / angular cheilitis[33,86].

LABORATORY DIAGNOSIS OF ORAL CANDIDIASIS

SPECIMEN COLLECTION

Specimens from patients with oral candidiasis can be collected by swabs[63] and kept , preferably , in transport medium before being processed in the laboratory. Specimens from oral lesions can also be obtained by scraping[94].

DIRECT EXAMINATION

The preferred method for the direct microscopic examination of oral candidiasis incudes (i) Wet mount (ii) Fixed mount . Processing of the specimen does not generally require treatment with keratinolytic substance.

Wet mounts can be unstained , prepared in saline or stained with lactophenol cotton blue or calcofluor white.

Microscopic examination of specimens from oral candidiasis will demonstrate the presence of budding yeast cells, pseudohyphae and/ or hyphae. Demonstration of hyphal elements in direct microscopic examination is important as *Candida spp* normally colonises the oral mucosa and the presence of the hyphal elements in addition to the yeasts , is an indicator of infection. However *C.glabrata*, a significant non-albicans species does not produce hyphae or pseudo hyphae in clinical specimens[94].

CULTURAL CHARACTERISTICS:

Candida spp grow on almost all common laboratory media particularly blood agar and Sabouraud's dextrose agar with antibacterial antibiotics for primary isolation[27].

ISOLATION OF CANDIDA

Sabourauds Dextrose Agar with antibiotics

The routine medium used for isolation of fungi in culture from mucocutaneous infections is Sabouraud's dextrose agar supplemented with antibiotics like gentamycin , chloramphenicol or tetracycline to prevent bacterial overgrowth[38]. The addition of cycloheximide to inhibit fungal contaminants permits the growth of *Candida albicans* but inhibits most strains of *C.tropicalis, C.krusei* and *C.parapsilosis*.Use of two SDA containing chloramphenicol supplemented with or without cycloheximide is recommended[94].

Cultures can be incubated at 28^{O}C or / and at 37^{O}C.*Candida* colonies appears in two to three days and more than three days for some *Candida spp* like *C. guillermondii* and *C.glabrata*.

Colony Characteristics and Microscopic Morphology of Candida on SDA

- *Candida albicans*

Macroscopically – Colonies are smooth , creamy , pasty , glistening Microscopically – globose or short ovoid cells(5 - 7 µm),

- *Candida tropicalis*

Macroscopically – white to cream coloured colonies with peripheral fringe
Microscopically – globose – ovoid or short ovoid cells (4 -8 µm X 5-11 µm)

- *Candida krusei*

Macroscopically - Colonies are flat ,dry becoming dull, smooth or wrinkled with the dense of mycelium extending as lateral fringe around the colony

Microscopically - Cylindrical , few ovoid cells / elongated cells (3-5 µm x 6-20 µm)

- *Candida parapsilosis*

Macroscopically – Soft, smooth, white sometimes lacy

Microscopically – Short ovoid to long ovoid cells (2.5 - 4µm x 2.5 -9 µm)

- *Candida guilliermondii*

Macroscopically – Thin , flat, glossy , cream to pinkish colonies

Microscopically - short ovoid / ovoid cells(2-5 µm x 3-7 µm)

- *Candida glabrata*

Macroscopically-Cream coloured, soft, glossy ,smooth colony

Microscopically-small, round yeasts (2.5 – 4.5 µm x 4-6 µm)

- *Candida kefyr*

Macroscopically- Smooth , creamy appearance

Microscopically- short, ovoid with a few elongate cells(2.5 -5 µm x 5-10 µm)[34,84]

CHROM agar

A new differential culture that allows selective isolation of yeasts and presumptive identification of most commonly isolated *Candida spp* especially *Candida albicans, Candida tropicalis* and *Candida krusei*[65].Identification of yeast pathogen by traditional methods requires several days and specific mycological media. Chromogenic media contain chromogenic substrates which react with enzymes secreted by the target microorganisms to yield colonies of varying colours after incubation at 37^{0} C for 48 to 72 hours[39].

Cornmeal Agar

The commonly used differential medium both for genus identification as well as speciation is the Corn meal Agar plate(Dalmau plate) supplemented with Tween 80 (Polysorbate 80) or Rice starch agar[24].

Corn meal agar is a general purpose medium used for the isolation of fungi . In 1960, Walker and Huppert modified the basic formulation by adding polysorbate 80 which stimulated rapid and abundant chlamydospore formation. Dextrose is added to Corn meal agar to enhance fungal growth and pigment production.

Subcultures made by furrowing the Corn meal agar plates with coverslips applied on the streak line and incubated at 28 ^{0}C for 2-5 days.Cover slips applied provides an anaerobic environment which enhances the formation of hyphae and blastoconidia and the polysorbate 80 reduces the surface tension.

After 2-5 days of incubation , plates are examined directly under the microscope for the presence of pseudo hyphae/ true hyphae, chlamydospores , arthroconidia, blastoconidia.

Chlamydospore formation are seen in the isolates of *C.albicans* and *C.dubliniensis. C.dubliniensis* often produces chlamydospores abundantly in clusters.

Microscopic Morphology of different species on Corn Meal Agar

C.albicans –Hyphae and pseudohyphae formed with clusters of blastospores at internodes and large thick walled terminal chlamydospores

C.tropicalis- Abundant long , branching pseudohyphae .Ovoid / elongate pseudohyphae found anywhere along the pseudohyphae .True hyphae may be present.

C.krusei – Pseudohyphae with elongated blastoconidia in tree like arrangements / Crossed match stick appearance

C.parapsilosis- Fine pseudomycelium with single or small clusters of blastoconidia.Thick pseudo mycelium and giant cells also found.

C.glabrata –No pseudohyphae.Small, spherical and tightly compacted blastoconidia

C.guilliermondi –Pseudohyphae with blastospores in small chains or in clusters

C.kefyr –Abundant pseudomycelium of elongate cells that lie parallel giving log in stream appearance. Blastoconidia are infrequent[84,34,4].

Growth in Sabourauds Dextrose broth

Serves as an important differentiating method for various *Candida species*. Ring around the surface of the tube at the broth interface indicates *Candida tropicalis* , a thick pellicle creeping along the sides indicates *C.krusei* while growth occurring at the bottom indicates other *Candida species*[84].

Germ Tube Test (Reynolds Braude Phenomenon)

A germ tube is defined as a filamentous extension from a yeast cell that is about half the width and three to four times the length of the cell[28]. The principle of the test is the ability of *Candida albicans or Candida dubliniensis* to produce blastospores when incubated in serum at 37^{O}C for two hours[94]. It helps in the presumptive identification of *Candida albicans*[43] or *Candida dubliniensis*[94,44]. A true germ tube has no constriction at the point of origin. Early pseudohyphae of *Candida tropicalis* may be confused but characteristically show a point of constriction adjacent to the mother cell[28].

Taschdjian et al in 1960 reported that *C.albicans* and its variants are able to produce germ tube when incubated with various substances like human or sheep serum, rabbit plasma , egg albumin, thioglycollate broth and various peptone medium at 37^{O}C for 2 hours[61].

A new Monoclonal Ab specific for *C.albicans* germ tube has been described. Hydrophobic components of the germ tube of *C.albicans* were used as antigens to prepare monoclonal antibodies. MAb-16B1F10 was shown by indirect IF to be specific to the surface of the mycelium phase of the C.albicans and C.stellatoidea but not with the species *C.dubliniensis*. The identification of

this antigen on the surface of the mycelial phase of *C.albicans* cells could be powerful tool for diagnostics and especially for differentiation of *C.albicans* and C.dubliniensis[53] .

Biochemical Characterisation

Biochemical speciation of candida is based on

1) Assimilation 2) Fermentation tests.

Candida species can utilize carbohydrates both oxidatively (assimilation) & anaerobically (fermentation) .Yeasts possessing the ability to ferment a given carbohydrate do also assimilate that substance but not necessarily vice versa[94].

1. **Sugar assimilation tests:**

Sugar assimilation determines the ability of a particular yeast to utilize a particular carbohydrate as the sole source of carbon in the presence of oxygen[35]. Carbohydrate utilization patterns are the most commonly used conventional methods for the definitive identification of yeast recovered in a clinical laboratory[4].

Various techniques:

- Classic Wickerham method (By Wickerham & Burton 1948) , assesses assimilation by determining the ability of a given Yeast isolate to grow in a broth supplemented with carbohydrates[97].
- Auxanographic methods:-

This technique employs minimal media agar plates on which paper disc impregnated with different carbohydrates are placed and the growth ability of

the yeast around a specific disc is an indication of the yeast ability to assimilate the carbohydrate[94,44].

- Commercial Kits

Modification of the auxanographic assimilation technique and have replaced the conventional methods. Commonly used are API 20c, API 32c, micro-drop assimilation test system and uni-yeast-tek system ,Vitek, Minitek etc[46,94].

2. **Sugar fermentation test:**

The classic tests involved liquid media supplemented with different carbohydrates, a colour indicator to assess pH changes to measure acid formation and the Durham's tube to assess gas production .There are several modifications for assessment of gas production such as use of semisolid media or a wax layer on top of liquid medium. Production of gas and not the change of colour in the fermentation fluid is considered as the indicator of positive fermentation[94,56].

OTHER METHODS

SEROLOGY

Candida spp. can also be identified serologically, using specific antisera. A commercial available set of sera (iatron system), prepared against cell wall antigens enables quick species identification of the commonly encountered pathogenic *Candida spp.* by simple slide agglutination tests.

MOLECULAR METHODS

i) Use of specific DNA probes like those encoding for actin gene, 14-lanosterol demethylase, part of the 18s RNA gene complex, chitin synthetase gene, mitochondrial DNA or Candida DNA Repititive Elements (CARE)

ii) Electrophoretic patterns of DNA

iii) RNA profiling

iv Restriction enzyme analysis

v Amplification techniques like Polymerase chain reaction[94].

ANTIFUNGAL SUSCEPTIBILITY TESTING

The rising prevalence of serious fungal infections and antifungal drug resistance has created an increased demand for reliable methods of in vitro testing of antifungal agents that can assist in their clinical use[46].

Invitro susceptibility tests

1. Provides a reliable measure of the relative activities of two or more antifungal agents
2. Correlate with invivo activity and predict the likely outcome of therapy
3. Provides a means with which to monitor the development of resistance among a normally susceptible population of organisms.
4. Predict the therapeutic potential of newly discovered investigational agents[67].

Methods used for in vitro antifungal susceptibility testing of Yeasts are

1) Agar based method
   - Disk Diffusion
   - Agar macrodilution
   - E-test
2) Broth based methods
   - Macrodilution
   - Microdilution
   - Colorimetric microdilution
3) Others

   Neosensitabs tablets

   EUCAST method, Spectrophotometric

   Flowcytometer, Fungitest[67]

The CLSI (formerly NCCLS) subcommittee on Antifungal susceptibility testing has developed reference methods for broth macro and microdilution susceptibility testing of Yeast (CLSI M27-A3 document) and more recently a disk diffusion method for Yeasts (CLSI M44 A document).

Broth microdilution:

Although the antifungal broth macrodilution test was the first method proposed by the CLSI, this test is cumbersome for use in clinical laboratory. The broth microdilution has become the most widely used technique.

CLSI recommends RPMI 1640 medium with L-glutamine, without sodium bicarbonate and buffered with morpholine propane sulfonic acid at 0.165M as the test medium .Yeast inocula standardized spectrophotometrically and diluted in RPMI medium to obtain a final concentration of 0.5 to 2.5 * 10^3 CFU/ml .

In microbroth dilution, 100µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of flat bottom 96 well micro titre plates. A constant volume (100 µl) of inoculum was added to each micro dilution well containing 100 µl of serial dilution of the antifungal agents to reach final concentration and incubated at 35ºC for 48 hrs for *Candida*[36,21]. In macrobroth, 0.1 ml of antifungal drug, containing 10 times the final targeted concentration was dispensed in test tubes and 0.9 ml of the inoculums added to the serial dilution of the antifungal agents[12].

Drug dilution range

1) Fluconazole 64 to 0.12 µg/ml
2) Amphotericin, itraconazole 16 to 0.03 µg /ml

Clotrimazole, Nystatin[68]

MIC of polyene fungals (Amphotericin, nystatin) defined as the lowest concentration that inhibit growth by 100%.

MIC of azoles were defined as the lowest concentration that inhibit growth by 50% in microbroth & 80% in macrobroth dilution[21,46].

Interpretation[21] :

	Susceptible	Susceptible Dose Dependent	Resistant
Fluconazole	≤ 8µg/ml	16-32 µg/ml	≥ 64 µg/ml
Itraconazole	≤ 0.125 µg/ml	0.25-0.5 µg/ml	≥ 1 µg/ml

Broth based alternative approaches for yeasts

Colorimetric methods

A novel alternative to the standard method of visual grading of turbidity is the use of colorimetric methods or fluorescent dyes for the determination of MIC endpoints. Commercial (Sensititre Yeast one and fungitest) and noncommercial (tetrazolium salt methods and substrate uptake indicator has been adapted for susceptibility testing)[30,46].

Spectrophotometric methods

The spectrophotometric approach to MIC end point determination provides an objective and rapid MIC reading and eliminates the subjective judgements concerning minimal turbidity .Spectrophotometric MICs reflecting either a ≥ 50% inhibition or a ≥ 70% inhibition of growth relative to control growth provides a good agreement with the NCCLS reference methods for fluconazole and other azoles[76].

Flow cytometry

Flow cytometric methods have been adapted for Antifungal susceptibility testing by introducing DNA- binding vital dyes into the culture to detect fungal cell damage after exposure to an antifungal agent. Inspite of faster results,the need for flow cytometer preclude their use in small laboratories[68].

Agar based methods

Disk Diffusion method

Agar disk diffusion is a simple, flexible and cost effective alternative to broth dilution testing [67]. CLSI subcommittee has proposed a standard disk diffusion method for susceptibility testing of *Candida spp* to the Fluconazole, Posaconazole and Voriconazole. The subcommittee has established zone interpretative criteria for Fluconazole and Voriconazole . Recommends MHA medium supplemented with 2% glucose and 0.5µg/ml of methylene blue and the inoculums size of 0.5 Mcfarland standard and incubation temperature of 35^{o} C for 20 to 24 hr and some strains may require 48 hr incubation[68,20].Addition of a low concentration of methylene blue (0.5µg/ml) make the zones of inhibition clearer and easier to measure precisely[8,60].

Agar based alternative approaches

1) Neosensitabs tablets

Tablets of established and some of the new antifungal agents are available. Preliminary comparisons with both M27-A2 and M44A methods have provided promising results[29].

2) Epsilometer test (E- test)

E-test uses a non-porous plastic strip immobilised with a predefined gradient of a given antimicrobial agent on one side and printed with an MIC on the other side. The medium that provides the best performance for E-test MICs is solidified RPMI supplemented with 2% dextrose. When the strip is placed on an inoculated agar plate, a continuous , stable and exponential antimicrobial

gradient is established along the side of the strip. After incubation, the MIC value can be read directly from the MIC scale printed on the strip[68].

TREATMENT OF ORAL CANDIDIASIS

(i)Mild:

Clotrimazole troches at a dosage of 10mg 5 times daily,

Nystatin at a concentration of 10000 U/ml, dosage of 4-6ml 4 times daily

(ii)Moderate to severe:

Oral Fluconazole:100-200mg daily for 7-14 days

(iii)Fluconazole refractory Candidiasis

Itraconazole: 200mg daily for 14 days

Intravenous echinocandins or Amphotericin –B at a dosage of 0.3mg/kg daily

(iv) Chronic suppressive therapy is usually not necessary for HIV infection.If needed, fluconazole at a dosage of 100mg 3 times daily is recommended.

In HIV patients, the use of HAART has been associated with decreasing rates of oral carriage of *Candida albicans* and reduced frequency of symptomatic oropharyngeal candidiasis.Thus, HAART should be used as adjunctive therapy whenever possible for all HIV infected patients with oropharyngeal or oesophageal Candidiasis[75].

ANTI FUNGAL DRUG RESISTANCE

The antifungal drug resistance can be divided into two types:

(a) Clinical resistance

(b) InVitro resistance: Intrinsic /primary resistance

Acquired / Secondary Resistance[42]

Clinical Resistance

Clinical failure to respond to an antifungal agent occurs due to many causes. A major problem for patients with HIV infection is the improper use of prescribed medications . Clinical failures may also result from inadequate absorption of an antifungal medication. Drug interaction can also result in decreased level of antifungal metabolites[31]

Intrinsic Resistance

Intrinsic resistance is an inherited characteristic of a species or a strain.This innate level of susceptibility is thought to be a drug organism characteristic and independent of drug exposure.

Acquired /Secondary Resistance:

Acquired resistance occurs when a previously susceptible isolate develops a resistant phenotype, usually as a result of prolonged treatment with antifungals[93].

Azole Resistant Candidiasis

The development of resistance to azole antifungals in yeasts is a recent phenomenon.It is usually associated with *Candida albicans*.

- **Acquired azole resistance**

One of the consequences of continuous suppressive antifungal therapy in HIV patients has been the emergence of resistant disease .Resistance has been described both to fluconazole and itraconazole and tends to occur in persons with advanced HIV disease with CD4+ count < 50/mm , who have

been exposed to antifungal therapy on a chronic basis[58]. Resistant strain of *Candida albicans* were found in 20 to 33% of symptomatic oral candidiasis patients with HIV and upto 14% of asymptomatic patients with HIV[93].

- **Intrinsic azole resistance**

Patients on fluconazole suppressive therapy are more likely to have infection caused by *non –albicans species* such as *Candida krusei* and *Candida glabrata* which are intrinsically resistant to fluconazole[58].

AIMS AND OBJECTIVES

1. To isolate *Candida species* from HIV patients with oropharyngeal Candidiasis.
2. To speciate the *Candida* isolates.
3. To determine the antifungal susceptibility pattern of the isolates by disk diffusion and microbroth dilution method.
4. To find out the correlation of various *Candida* isolates and their antifungal susceptibility pattern to the CD4 count of the patients.

MATERIALS AND METHODS

STUDY PERIOD

This is a cross sectional study undertaken over period of June 2008 – May 2009

STUDY PLACE

This study was carried out at the Institute of Microbiology , Madras Medical College , Chennai and Antiretroviral therapy (ART) centre , Madras Medical College and Government General Hospital, Chennai.

ETHICAL CONSIDERATIONS

The study was reviewed and approved by the Institutional Ethical Committee, Madras Medical College and General Hospital, Chennai-3.

STATISTICAL ANALYSIS

Statistical analysis were carried out using statistical package for Social sciences and Epi- software by statistician .The proportional data of this cross sectional study were tested using Pearson's Chi Square Analysis test and Binomial Proportion test.

STUDY GROUP

Study group includes 150 HIV positive patients presenting with clinical picture of oral candidiasis

Inclusion Criteria

1. HIV positive patients: Seropositivity status of the patients were determined as per NACO guidelines.

2. Patients with clinical picture of candidiasis on the oral / pharyngeal mucosa.
3. Patients above 18 years of age group.
4. Both Males and Female were included in the study.
5. Not on any antifungals or corticosteroids 30 days prior to the study.

Patients were included in the study only after getting informed written consent.

All patients satisfying the inclusion criteria were only once documented and were assigned serial numbers.

Patients were interviewed by structured questionnaire and their hospital records were used to know about the previous episodes of Oropharyngeal Candidiasis, use of any antifungal agents and past medical conditions.

SPECIMEN COLLECTION

Oropharyngeal specimens were collected by firmly swabbing the lesion site with two steile cotton swabs under strict aseptic precautions.

Samples were transported immediately to the laboratory and subjected to various mycological tests.

Blood was collected in EDTA vacutainer tubes from each patients for enumeration of their CD4 count using BD FACS Counter.

SPECIMEN PROCESSING

Specimens collected were subjected to standard mycological procedures.

Direct Microscopic examination

One swab was used for the direct microscopic examination by Gram stain. For each specimen , smears were made on a dry, clean glass slide, air dried and heat fixed . The fixed smears were stained by Gram staining method and observed under the oil immersion for the presence of gram positive budding yeast cells with or without pseudohyphae[94].

Culture on Sabourauds Dextrose Agar

The culture medium used was Sabourauds Dextrose Agar with pH 5.6 supplemented with antibiotics like gentamycin and chloramphenicol to prevent bacterial overgrowth .Second swab was inoculated immediately into the plates and incubated at 28^{O}C and 37^{O}C for 24-72 hours[94].

Isolates were identified by colony morphology on SDA plates. Growth appears in 2 to 3 days as creamy, white pasty colonies .The microscopic morphology of the colony on Gram stain was noted.

Species identification

Isolates were speciated based on the following tests:

Germ Tube Test

- 0.5ml of human serum was taken in a sterile test tube.
- A small portion of an isolated colony of the yeast to be tested was inoculated into the human serum.
- The test tube was incubated at 37^{O}C for two hours.

- After two hours of incubation , a drop of the yeast serum suspension was placed on a microscopic slide , overlaid with a cover slip and examined microscopically for the presence of germ tube under low power microscope.
- If the test was positive within two hours, the isolate was considered presumptively as *Candida albicans / Candida dubliniensis*[28,94].

Growth at 42^{o}C

All germ tube positive isolates were subcultured on SDA and incubated aerobically at 42^{o}C, to distinguish between *Candida albicans* and *Candida dubliniensis*[91,99].

	Candida albicans	***Candida dubliniensis***
Growth at 42^{o}C	Present	Scanty / Absent

Corn Meal Tween 80 Agar (Dalmau Plate Culture Technique)

- An isolated colony from the primary culture media was taken using a straight wire and inoculated into cornmeal agar plate containing 1% Tween 80 by making three parallel lines about half an inch apart at an 45^{o} angle to the culture media.
- A sterile cover slip was placed over the surface of the agar, covering a portion of the inoculated streaks.
- Plate was incubated at 28^{o}C for 48 hours.

- After 48 hours , the areas where the cuts into the agar made, were examined first under the low power objective and then under the high power objective for the presence of

  * Hyphae - true or pseudohyphae * Blastoconidia
  * Arthroconidia * Chlamyoconidia[4]

Candida CHROM agar

- Isolates were subcultured twice on Sabourauds Dextrose agar prior to inoculation on chromogenic media.
- A single yeast colony was streaked onto the plates.
- Plates were incubated at 37^{o} C in the dark.
- The results were read after 48 hours and the colour of the colony was noted.
- This helps in the presumptive identification of most commonly isolated *Candida species* especially *Candida albicans, Candida tropicalis, Candida krusei.*

Candida albicans - Light green
Candida tropicalis - Steel blue with pink hallow
Candida krusei - Pink
Candida parapsilosis - Cream coloured
Candida glabrata - Purple
Candida dubliniensis - Dark green
Candida guilliermondii - Pale pink to purple[65].

Sugar fermentation test (APPENDIX XII)

- A loopful of 24-48 hours culture from a sugar free media was suspended in sterile distilled water.
- 0.2 ml of this suspension was added to 2% sugar fermentation media with bromothymol blue indicator.
- Dextrose, Lactose, Sucrose and Maltose sugars were tested.
- Inoculated sugar tubes were incubated at 30^{o} c for 48 to 72 hrs.
- The ability to ferment a sugar was noted by the presence of acid and gas trapped in Durham's tube[56].

Sugar Assimilation test (APPENDIX XI)

- A yeast suspension was made from a 24-48 hrs culture grown in a sugar free media, in to 2 ml of Yeast Nitrogen base by adding heavy inoculums.
- The suspension was added to 18 ml of molten agar cooled to 45^{o} c and mixed well and the entire volume was poured in to a 90 mm sterile petri plate.
- The plate was allowed to set at room temperature until the agar surface hardens.
- With the help of sterile forceps ,carbohydrate discs [Hi-media] like dextrose, sucrose, lactose, raffinose, trehalose were placed on the surface of the inoculated agar.

- The plates were incubated at 30^{o} c for 24-48 hr and were observed for the growth of yeast around the sugar discs, indicating assimilation of that particular carbohydrate.

- Each *Candida species* utilizes specific carbohydrate substrate and the characteristic carbohydrate profiles were used to identify the species[27,4].

Antifungal Susceptibility testing

Antifungal susceptibility test for the *Candida* isolates was done by

(i) Disk Diffusion method

(ii) Microbroth dilution method, as per the CLSI guidelines on antifungal Susceptibility testing in M44-A document and M27-A2 document repectively.

Inoculum Preparation (Stock Suspension Preparation)

- *Candida* isolates to be tested were grown on SDA at 35^{o} C and subcultured atleast twice to ensure purity and viability.

- The inoculum suspension was prepared by picking five colonies of 1mm diameter from a 24 hour old culture and suspended in 5ml sterile 0.85% NaCl.

- The turbidity of the cell suspension was adjusted to 85% transmittance at 530nm using a spectrophotometer by adding sterile 0.85% NaCl as necessary.

- The resulting stock suspension contains 1×10^{6} to 5×10^{6} yeast cells/ml.

Disk Diffusion Method

Medium :

Mueller Hinton Agar supplemented with 2% dextrose and 0.5µg/ml methylene blue was used

Procedure:

- Optimally within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton was dipped into the undiluted inoculum suspension.
- The excess fluid from the swab was removed by firmly pressing against the inner wall of the tube above the fluid level .
- The dried surface of the plate was inoculated by evenly streaking the surface in three different directions , rotating the plate approximately 60^{o} each time to ensure an even distribution of inoculums .As the final step , the rim of the agar was swabbed.
- The plate was allowed to dry for 3 to 5 minutes and not more than 15 minutes.Using a pair of flame sterilised forceps, the antifungal discs were applied on the surface of the inoculated plates.
- The following antifungal discs were used

Fluconazole 25µg	Nystatin10 µg
Amphotericin 100U	Ketaconazole10 µg
Itraconazole 10 µg	Clotrimazole10 µg

- The plates were inverted and incubated at 35^{o} C within 15 minutes after the disks were applied.

- Plates were examined after 20 to 24 hours of incubation. In case insufficient growth, plates were read at 48 hours .
- The resulting zones of inhibition was measured to the nearest whole millimeter at the point where there was prominent reduction in growth[20].
- Interpretation[71]

Drug	Susceptible	SDD	Resistant
Fluconazole (25 µg)	≥ 19 mm	15-18 mm	≤ 14mm
Itraconazole (10 µg)	≥ 20mm	12- 19mm	≤ 11 mm
Amphotericin B(100 U)	>10mm	Not applicable	≤ 10mm

Susceptibility pattern of the *Candida* isolates for ketaconazole , clotrimazole and nystatin were determined by comparison with the standard control strains[42].

Susceptible : Zone diameter of test strain is more than 80% of control strain

Intermediate : Zone diameter of test strain is less than 80% of control strain

Resistant : No zone of inhibition[42.]

* Following standard strains were tested each time to ensure quality control

Candida albicans ATCC 90028 *Candida parapsilosis* ATCC 22019

Candida tropicalis ATCC 750 *Candida krusei* ATCC 6258 [20]

Microbroth Dilution Method

Medium

RPMI 1640 medium with glutamine , without bicarbonate in MOPS (3N-Morpholino propane sulphonic acid), buffer sterilized by membrane filtration.

Inoculum preparation

- The stock suspension (1×10^6 to 5×10^6 yeast cells/ml) prepared was further diluted to 1:1000 in RPMI medium to obtain a inoculum which contains 1×10^3 to 5×10^3/ml.This inoculum was further diluted 1:1 when the wells are inoculated to achieve the final desired inoculums size containing 0.5×10^3 to 2.5×10^3/ml

Antifungal Stock Solution

The MIC range of antifungal agents used were

(i) Fluconazole : 64 -0.125µg/ml

(ii) Itraconazole , Amphotericin -B: 16-0.031 µg/ml

Fluconazole was dissolved in sterile distilled water whereas amphotericin B and itraconazole were dissolved in DMSO. From the working dilution, the desired concentrations were prepared

Procedure

- The test was perfomed in a sterile , disposable ,96 well microdilution plate using standard RPMI 1640 medium.

- The 2 x drug concentration in 100µl volume was dispensed into the wells of row 1 to of the microdilution plate using a multichannel pipette.
- Row 1 contains the highest drug concentration and row 10 contains the lowest drug concentration.
- Each well was inoculated with the 100 µl of 2 x inoculums suspension which brings the inoculums densities and drug dilutions to the final concentration.
- The growth control well contains 100 µl of sterile drug free medium and was inoculated with corresponding diluted 2 x inoculums suspensions.
- Row 11 was used to perform the sterility control containing only the drug free medium only
- The Quality control strains was tested in the same manner.
- The plates were incubated at 35^{o} C for 48 hours[21].

Interpretation

The plates were observed for the presence or absence of visible growth. Agitations of the microdilution plates prior to MIC determination is recommended . The growth in each well was compared with the growth control drug free well.

A numerical score which ranges from 0 to 4 was given to each well

1 - Optically clear

2 - slightly hazy or approximately 25% of growth control

3 - prominent decrease in turbidity or approximately 50% of growth control

4 - slight reduction in turbidity or approximately 80% of growth control

5 – no reduction in turbidity

End point of MIC

Fluconazole and Itraconazole - Score 2 or less

Amphotericin - Score 0

Quality control strains used were

Candida parapsilosis ATCC 22019

Candida krusei ATCC 6258[21].

Enumeration of CD4 + T cells

- Estimation of CD4+ T lymphocyte of the HIV infected patients was done by Fluorescent Activated Cell Sorter (FACS) count system.
- Blood sample was collected aseptically in a K3 EDTA Vacutainer tube.
- 50µl of whole blood was added to the reagent tube containing fluorescent labelled antibodies which results in the binding of labelled monoclonal antibodies to the lymphocyte surface antigen.
- After adding fixative solution to the reagent tube, the sample was run in the instrument.

- The cell comes in contact with the laser beam which causes the fluorochrome labelled antibodies to fluoresce.
- The fluorescent light provides the information necessary for the instrument to count the cells.
- The software identifies the T- lymphocyte subpopulation and correlates with the absolute count
- Results provides the absolute counts of CD4+ lymphocyte[23].

RESULTS

This cross sectional study was carried out during the period June 2008 to May 2009 in the Institute of Microbiology, Madras Medical College, Chennai .Speciation of the Candida isolates from 150 HIV patients with Oral Candidiasis was done and antifungal susceptibility pattern of the isolates were determined by disk diffusion and micro broth dilution method.

TABLE - 1

AGE DISTRIBUTION OF HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS (n=150)

Age(Years)	Total No. of cases	Percentage
18-28	26	17.33
29-39	64	42.66
40-50	48	32
51-61	09	6
>62	03	2
Total	150	100

Majority of the study population belonged to the age group of 29-39 years(42.66%)

TABLE-2
GENDER DISTRIBUTION OF HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS (n=150)

GENDER	NUMBER	PERCENT
MALE	103	69
FEMALE	47	31

TOTAL	150	100

Table I shows that male patients outnumbered female patients.

TABLE - 3
TYPE OF LESIONS IN HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS (n=150)

Type of Lesion	No of Patients	Percentage
Pseudomembranous lesions	93	62
Pseudomembranous & Erythematous	42	28
Pseudomembranous with Angular cheilitis	7	4.6
Hyperplastic	4	2.7
Erythematous	4	2.7
Total	150	100

Majority of the patients had pseudomembranous type of lesion (62%), followed by pseudomembranous and erythematous type of lesion(28%).

TABLE - 4
DISTRIBUTION OF HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS WITH/WITHOUT HAART

TREATMENT	No.of Patients	Percentage
On HAART	43	28.7
Not on HAART	107	71.3
Total	150	100

28.7% of the patients were under HAART and 71.3% of the patients were not on HAART. Majority of the patients were on HAART for duration below 3 months.

TABLE-5
CULTURE POSITIVITY IN HIV PATIENTS WITH OROPHARYNGEAL CANDIDIASIS (n=150)

Total no. of samples	No. of culture positive samples	Percentage of culture positivity	Total no of isolates
150	150	100	152

Out of 150 samples, 152 isolates were isolated with a culture positivity of 100%

TABLE-6
DISTRIBUTION OF THE *CANDIDA* ISOLATES (n=152)

	Number of isolates	Percent
Single	148	97.4
Mixture	4	02.6
Total	152	100

Of the 150 samples, two revealed a mixture of two species accounting for the 152 isolates.

TABLE - 7
DISTRIBUTION OF THE C*ANDIDA ALBICANS* AND NON –*ALBICANS* ISOLATES (n=152)

	No.of isolates	Percentage
Candida albicans	118	78
Non *albicans Candida(NAC)*	34	22
Total	152	100

Candida albicans was the most frequently isolated species accounting for 78% and the remaining were non- *albicans* accounting for the 22%.

TABLE - 8
SPECIES DISTRIBUTION OF THE *CANDIDA* ISOLATES (n=152)

Species	Total no	Percentage
Candida albicans	118	78
Candida tropicalis	17	11
Candida krusei	8	5
Candida parapsilosis	6	4
Candida glabrata	2	1
Candida guilliermondii	1	1
Total	152	100

Among the non-*albicans Candida sp, Candida tropicalis* was the most common species isolated.

TABLE-9
DISTRIBUTION OF PATIENTS WITH PRIMARY AND RECURRENT OROPHARYNGEAL CANDIDIASIS (n=150)

	No.of patients	Percentage
Primary OPC	102	68
Recurrent OPC	48	32

68% of the patients presented with primary oropharyngeal candidiasis and remaining 32% presented with recurrent oropharyngeal candidiasis.

TABLE-10
DISTRIBUTION OF ORAL *CANDIDA* ISOLATES AMONG PATIENTS WITH PRIMARY AND RECURRENT OROPHARYNGEAL CANDIDIAISIS (n=152)

SPECIES	Patients with Primary OPC		Patients with Recurrent OPC	
	N	%	N	%
Candida albicans	84	55.2	34	22.3
Candida tropicalis	11	7.2	6	3.9
Candida krusei	4	2.6	4	2.6
Candida parapsilosis	2	1.3	4	2.6
Candida glabrata	2	1.3	-	-
Candida guilliermondi	1	1	-	-
Total	104	68.6	48	31.4

There was no statistically significant difference in species distribution between patients with primary and recurrent OPC.(P value = 0.281)

TABLE -11

ANTIFUNGAL SUSCEPTIBILITY BY DISK DIFFUSION METHOD (n=152)

	Susceptible		SDD/ I		Resistant	
	n	%	n	%	n	%
Fluconazole	121	79.6	10	6.6	21	13.8
Itraconazole	115	75.7	13	8.6	24	15.8
Amphotericin B	150	100	NA	NA	-	-
Ketaconazole	125	82.2	21	13.8	6	4
Clotrimazole	123	81	15	10	14	9
Nystatin	123	81	22	14	7	5

NA –Not applicable

By disk diffusion method, 21 (13.8%) isolates were resistant to fluconazole, 24(15.8%) were resistant to itraconazole. All the isolates were susceptible to amphotericin.6(4%) were resistant to ketaconazole, 14(9%) were resistant to clotrimazole and 7(5%) were resistant to nystatin.

TABLE - 12
DISTRIBUTION OF THE SUSCEPTIBITY PATTERN AMONG THE *CANDIDA* ISOLATES BY DISK DIFFUSION METHOD (n=152)

Species	Total n	Fluconazole			Itraconazole			Ampho-B		Ketaconazole			Clotrimazole			Nystatin		
		S	SDD	R	S	SDD	R	S	R	S	I	R	S	I	R	S	I	R
C.albicans	118	103	7	8	99	7	12	118	-	98	17	3	102	9	7	103	11	4
C.tropicalis	17	11	2	4	7	3	7	17	-	12	3	2	11	2	4	7	7	3
C.parapsilo	6	6	-	-	5	-	1	6	-	6	-	-	1	3	2	5	1	-
C.Krusei	8	-	-	8	3	2	3	8	-	7	-	1	7	1	-	5	3	-
C.glabrata	2	-	1	1	-	1	1	2	-	1	1	-	2	-	-	2	-	-
C.guilliermo	1	1	-	-	1	-	-	1	-	1	-	-	-	-	1	1	-	-
Total	152	121	10	21	115	13	24	152	-	125	21	6	123	15	14	123	22	7
Percentage	100	79.6	6.61	13.8	75.7	8.5	16	100	-	82.2	14	4	81	10	9	81	14	5

TABLE-13
ANTIFUNGAL SUSCEPTIBILITY BY MICROBROTH DILUTION METHOD (n=152)

	Susceptible		SDD		Resistant	
	N	%	N	%	N	%
Fluconazole	121	79.6	13	8.6	18	11.8
Itraconazole	116	76.3	13	8.6	23	15.1
Amphotericin B	152	100	-	-	-	-

11.8% of the *Candida* isolates were resistant to fluconazole and 15.1% were resistant to itraconazole. All the isolates were susceptible to amphotericin B.

TABLE -14
MIC OF FLUCONAZOLE BY MICRO BROTH DILUTION METHOD (n=152)

Species	No of isolates	Fluconazole by broth dilution					
		Susceptible <8µg/ml		SDD 16-32µg/ml		Resistant >64µg/ml	
		n	%	n	%	n	%
C.albicans	118	103	87.3	9	7.6	6	5.1
C.tropicalis	17	11	64.7	2	11.8	4	23.5
C.parapsilosis	6	6	100	-	-	-	-
C.Krusei	8	-	-	1	12.5	7	87.5
C.glabrata	2	-	-	1	50	1	50
C.guilliermondi	1	1	100	-	-	-	-
Total	152	121	79.6%	13	8.6	18	11.8

5.1% of the *Candida albicans,* 23.5% of the *C.tropicalis,* 87.5% of the *C.krusei* and 50% of the *C.glabrata* isolates showed MIC in the resistant range of >64µg/ml for fluconazole.

TABLE - 15
MIC OF ITRACONAZOLE BY MICROBROTH DILUTION METHOD (n=152)

Species	No. of isolates	Susceptible ≤0.125µg/ml		SDD 0.25-5µg/ml		Resistant ≥ 1µg/ml	
		n	%	n	%	n	%
C.albicans	118	99	83.9	7	5.9	12	10.2
C.tropicalis	17	08	47.1	3	17.6	6	35.3
C.parapsilosis	6	5	83.3	-	-	1	16.7
C.krusei	8	3	37.5	2	25	3	37.5
C.glabrata	2	-	-	1	50	1	50
C.guilliermondii	1	1	100	-	-	-	-
Total	152	116	76.3	13	8.6	23	15.1

10.2% of the *Candida albicans*, 35.3% of the *C.tropicalis* ,16.7% of *C.parapsilosis*, 37.% of the *C.krusei* and 50% of the *Candida glabrata* showed MIC in the resistant range of ≥ 1µg/ml for itraconazole.

TABLE – 16
ANTIFUNGAL SUSCEPTIBILITY OF AMPHOTERICIN –B BY MICROBROTH DILUTION (n=152)

Species	No of isolates	Susceptible n (%)	Resistant >1µg/ml
C.albicans	118	118(100%)	-
C.tropicalis	17	17(100%)	-
C.parapsilosis	6	6 (100%)	-
C.Krusei	8	8 (100%)	-
C.glabrata	2	2 (100%)	-
C.guilliermondi	1	1 (100%)	-
Total	152	152(100%)	

All the 150 isolates showed MIC in the susceptible range to amphotericin.

TABLE – 17
COMPARISON OF RESISTANCE OF CANDIDA ISOLATES BY DISK DIFFUSION AND MICROBROTH DILUTION

Species	Fluconazole		Itraconazole	
	Disk diffusion	Microbroth dilution	Disk Diffusion	Microbroth Dilution
C.albicans	8	6	12	12
C.tropicalis	4	4	7	6
C.parapsilosis	-	-	1	1
C.Krusei	8	7	3	3
C.glabrata	1	1	1	1
C.guilliermondi	-	-	-	-
Total	21	18	24	23
Percentage	13.8	11.8	15.8	15.1

By disk diffusion 21(13.8%) isolates were found to be fluconazole resistant and 24 (15.8%)isolates were found to be itraconazole resistant whereas by microbroth dilution 18 (11.8%) isolates were found to be fluconazole resistant and 23 (15.1%) isolates were itraconazole resistant.

TABLE - 18
DISTRIBUTION OF FLUCONAZOLE AND ITRACONAZOLE RESISTANCE *CANDIDA* ISOLATES AMONG PRIMARY AND RECURRENT CASES

Drug	Primary		Recurrent	
Fluconazole (n = 18)	7	39 %	11	61 %
Itraconazole (n = 23)	7	30.5%	16	69.5 %

61% of the fluconazole resistant isolates were isolated from patients with recurrent candidiasis and 39% from primary cases.

About 69.5% of the itraconazole resistant isolates were isolated from recurrent cases and 30.5% from the primary case. There is a significant difference in itraconazole resistance between primary and recurrent cases(Chi square test :p value<0.001).

TABLE -19
CORRELATION OF HIV PATIENTS WITH ORAL CANDIDIASIS WITH THE CD4+ T CELL COUNT (n=150)

CD4 Cells/ µl	No of Patients	Percent
<50	23	15.3
51-200	83	55.4
201-350	30	20
351-500	12	8
>500	2	1.3
Total	150	100

Among the 150 HIV patients with Candidiasis , 106 (70.7%) patients had CD4 count < 200 Cells/µl. This was found to be statistically significant by proportional test(p<0.001)

TABLE - 20
CD4 DISTRIBUTION AMONG THE *CANDIDA* ISOLATES (n=152)

CD4 cells /µl	*Candida albicans*		*Non albicans*	
	n=118	%	n=34	%
<50	18	15.2	5	14.7
51-200	64	54.2	21	61.7
201-350	26	22	04	11.8
351-500	9	7.6	03	8.8
>500	1	01	01	2.9
Total	118	100	34	100

70.1% of the *Candida albicans* and 76.4% of the *non albicans Candida sp*. were isolated from the patients CD4 count <200 cells / µl.

TABLE- 21
CD4 DISTRIBUTION AMONG THE FLUCONAZOLE RESISTANT *CANDIDA* ISOLATES

CD4 cells/µl	*Candida albicans*(n=118)		*Non albicans*(n=34)	
	No of Resistant isolates n=6	Percentage of isolates resistant to fluconazole	No of Resistant isolates n=12	Percentage of isolates resistant to fluconazole
<50	03	2.5 %	02	05.8 %
51-200	02	1.6 %	09	26.4 %
201-350	01	0.8 %	01	02.9 %
351-500	-	-	-	-
>500	-	-	-	-

Among the 34 *non albicans* isolates, 11 (32.2%) fluconazole resistant isolates were seen in patients with CD4 <200 cell/µl and 1 (2.9%) fluconazole resistant isolate was seen in patients with CD4>200 cells/ µl. Among the 118 *Candida albicans* isolates ,5 (4.1%) were seen in patients CD4 <200 cells/ µl and 1 (2.9%) fluconazole resistant isolate was seen in patients with CD4>200 cells/ µl.

DISCUSSION

Oral Candidiasis is the most common fungal infection in HIV infected patients and has been identified as a clinical predictor for progression to AIDS.

There have been several studies on Oral Candidiasis in HIV infected patients. In the recent past, these studies shows an increasing incidence of non-*albicans Candida spp.* in Oral Candidiasis and increasing rates of antifungal drug resistance particularly with the immunocompromised patients. Hence this study was done to speciate and to determine the antifungal susceptibility pattern of the *Candida* isolates from HIV patients with Oral Candidiasis and to correlate it with the CD4 count of the patients.

In the present study, majority of the patients were in the age group of 29-39 years (43%).(Table-1) This correlated with the study of CA Enwuru et al 2008, in which majority of the patients were within 29-39 years of age accounting for 42%.In contrast , in the study done by Bandar et al 2006, majority of the patients were in the age group of 20-30 years(85%)

There was a male preponderance accounting for 69% in this study (Table -2) VP Baradkar et 2009 and Prinscilla da et al 2002, also reported similar results in their studies.In contrast, Latiff et al 2004 showed female preponderance constituting 74.7% of the study group.

In the present study, Pseudomembranous candidiasis was the predominant type of Oral candidiasis seen in 93 patients(62%) followed by pseudomembranous and erythematous lesions in 42 patients(28%).(Table-3). This was similar to the study of Hamza et al, 2008, in which 66.4% patients had pseudomebranous type of lesion followed by 22.6% patients with

pseudomembranous and erythematous type of lesion whereas Ranganathan et al , 2008, reported pseudomembranous as the most common type followed by erythematous type.

In the current study, among the 150 HIV patients with Candidiasis, 43 (28.7%) were on HAART and majority of them were on HAART for less than 3 months duration.107(71.3%) were not on HAART at the time of enrollment of the study.In the study done by Hamza et al 2008, 36.6% were on HAART and the study by Princilla et al, 37% were under HAART at the time of enrollment of the study.

Out of 150 samples processed, *Candida* was isolated from SDA culture in all 150 (100%) samples. Of these , two revealed a mixture of two species constituting for the 152 isolates.(Table-5 & 6) This correlated well with the study of Hamza et al 2008 and Shoba D Nadagir 2008 et al where there was 100% Culture positivity on SDA agar. The study of Prinscilla et al 2002 showed 100% culture positivity with 11 patients harboring more than one *Candida spp*.

Out of the 152 *Candida spp.* isolates obtained in the present study, species identification revealed that 118(78%) were *Candida albicans* which was the most frequently isolated species and the remaining 34(22%) were *non-albicans Candida spp.*(Table-7) This was comparable to the studies done by Usha arora et al 2009 , Shoba D Nadagir et al 2008 , VP Baradkar et al 2009 where *Candida albicans* was the major isolate accounting for 75% , 66.6% and 70% respectively.In his study, Latiff et al 2004, reported 86% of isolates as *Candida albicans* which was found to be slightly higher than the present study .

In this study, among the 32 *non-albicans Candida spp., Candida tropicalis* was the most common isolate seen in 17(11%) isolates followed by *Candida krusei* in 8(5%), *Candida parapsilosis* in 6(4%), *Candida glabrata* in 2(1%) and *Candida guilliermondi* in 1 (1%) isolate. *Candida dubliniensis* was not isolated in this study.(Table-8) This correlated with the studies of Usha Arora et al 2009, and CA Enwuru et al 2008, where *Candida tropicalis* was the most common *non albicans species*. In contrast, Bharadkar et al 2008 and Latiff et al 2004 has showed *Candida parapsilosis*, as the most common non albicans isolate accounting for 15% and 8% respectively. Hamza et al 2008, and Prinscilla et al 2002, reported *Candida glabrata* as the commonest non albicans isolate in their studies.

In the present study, out of 150 patients with oral Candidiasis, 102 (68%) patients presented with primary oral candidiasis and about 48(32%) patients presented with recurrent candidiasis. (Table-9). Majority of the patients with recurrent candidiasis had a history of only one previous episode of oral candidiasis.In the study done by Hamza et al 2008, 56.2% of the patients presented with primary candidiasis and 43.8% with recurrent Candidiasis.

In this study, out of the 152 isolates, 104 (68.6%) were isolated from patients with primary candidiasis and 48(31.4%) from recurrent candidiasis. Species distribution of the Candida isolates among the patients with primary Candidiasis reveals that , *Candida albicans* was the most common isolate 84 (55%) followed by *Candida tropicalis* which accounts for 11 isolates(7%) .Similarly among the patients with recurrent candidiasis .*Candida albicans* was the frequent isolate 34 (22%) followed by *Candida tropicalis* 6 (3.9%).There was no statistically significant difference in species distribution between

patients with primary and recurrent Candidiasis (P = 0.281).(Table-10) This correlated very well with the study done by Hamza et al 2008 where 55.7% of the candida isolates were isolated from patients with primary candidiasis and 44.3% from recurrent Candidiasis. *Candida albicans* was the most frequent isolate followed by *Candida glabrata* in both primary and recurrent candidiasis patients with no statistically significant difference.

Fichtenbaum et al 2000 and Cartledge et al 1999 suggests that recurrent infections resulted in repeated exposure to antifungal agents and this might predispose to a shift to non albicans species.In the present study , however *Candida albicans* was the most common isolate in both primary and recurrent candidiasis .This could be explained by the fact that majority of the patients with recurrent candidiasis had only one previous episode of oropharyngeal candidiasis.

The antifungal susceptibility pattern of the Candida isolates determined by the disk diffusion method revealed that 21(13.8%) of the isolates were fluconazole resistant, 24(15.8%) isolates were itraconazole resistant,6(4%) isolates were ketaconazole resistant, 14 (9%) were clotrimazole resistant and 7(5%) were nystatin resistant.(Table 11)

The current study revealed that by disk diffusion, fluconazole resistance was observed in 7% of the *Candida albicans*, 23.5% of *C.tropicalis* , 100% of *C.krusei* and 50% of *C.glabrata*.The higher incidence of resistance in *C.krusei* and *C.glabrata* was due to the fact that they are intrinsically resistant to fluconazole.

In the present study, the antifungal susceptibility pattern determined by Microbroth dilution method shows that about 18 (11.8%) isolates were resistant to Fluconazole and 23 (15.1%) were resistant to itraconazole.All the isolates were susceptible to amphotericin(100%).(Table-13) This was similar to the study done by Usha et al 2009 where 11.5% of the isolates were fluconazole resistant. CA Enwuru et al 2008 showed that fluconazole resistance was observed in 9.5% of the *Candida* isolates where the resistant rates were found to be slightly lower than the current study .The study conducted by Hamza et al 2008, reveals that 5% and 8.4% of the *Candida* isolates were resistant to fluconazole and itraconazole respectively. Carolina et al 2006 reported 8.1% of the isolates were fluconazole resistant and about 8.1% were resistant to itraconazole and no resistance to amphotericin B. Many studies done by Bailey et al 1994, Chavanet et al 1994 have estimated the incidence of clinical fluconazole resistance to be from 6 to 36%.

In the current study, fluconazole resistance was observed in 5.1% of the *Candida albicans.Candida krusei* shows 87.5% resistance to the fluconazole .*Candida glabrata* shows 50% and *C.tropicalis* shows 23.5% resistance to fluconazole (Table -14). Resistance was mainly among *non albicans Candida spp.* that are primarily / intrinsically resistant to fluconazole whereas in the study conducted by CA enwuru et al, fluconazole resistance by microbroth dilution method revealed resistance in 10% of the *C.albicans* , 7.7% of the *C.tropicalis* and 40% of the *C.krusei*. Shoba Nadagir et al showed fluconazole resistance in 12.2% of *C.albicans* which was higher than the resistant rate reported in this study.

In this study, itraconazole resistance by broth dilution was 10.2% in *C.albicans*, 35.3% in *C.tropicalis*, 16.7 % in *C.parapsilosis*, 50% of *C.glabrata* and 37.5% of *C.krusei*. (Table-15) whereas Vargas et al 2005, showed itraconazole resistance in 11% of *C.albicans*, 7% of the *C.glabrata* , 18.2% of *C.tropicalis* and no resitance to *C.parapsilosis*.

In the present study , there has been increased resistance to azoles particularly among non-albicans isolates. This emphasizes the need for speciation of the candida isolates from HIV patients with oral Candidiasis.

Comparison of the susceptibility pattern of the isolates detected by disk diffusion and microbroth dilution showed minor discrepancies in the current study. (Table-17) Disk diffusion zone diameters were highly reproducible and correlated well with microdilution, making agar based methods as a viable alternate to broth dilution. Disk diffusion is an easy and practical technique to screen antifungal agents when compared to the broth dilution which is a costly and cumbersome procedure. However data on agar based tests for itraconazole and amphotericin B are scarce, emphasizing the further research to standardize other antifungal agents.

In this study, among the itraconazole resistant isolates, 69.5% were isolated from patients with recurrent candidiasis as compared to 30.5% from primary cases. 61% of the fluconazole resistant isolates were from patients with recurrent candidiasis and 39% from primary cases. (Table-18) The association between azole resistance and recurrent candidiasis was found to be statistically significant.(p value=0.001).This was similar to the study done by Hamza et al

where there was significant association between itraconazole resistance and recurrent candidiasis.

In the present study, among the 150 HIV patients with oropharyngeal candidiasis, 106 (70.7%) patients had CD4 count <200 cells /mm^3 which was statistically significant by proportional test (p= <0.001) (Table-19) This correlated well with the study conducted by Usha et al where 76.66% patients with orophayngeal candidiasis had CD4 count <200 cells/ µl. Singh et al 2007 also reported that there is increased risk of oropharyngeal candidiasis in HIV patients with progressive immunodeficiency . Therefore even when patient's CD4 count is not known, presence of oral lesions of candidiasis may be considered as a marker of immunosuppression.

In the current study there was significant association between non albicans and fluconazole resistance with CD4 count (p value-0.004). About 32.2% of the fluconazole resistant, non albicans isolates were seen in patients with CD4 less than 200 cells/µl. (Table- 20 & 21).This correlated well with the study done by Usha et al 2009 indicating that the level of immunosuppression is an important risk factor in the emergence of resistance in yeast isolates.

SUMMARY

- In the present study, majority of the study population belonged to the age group of 29 – 39 years (43%).
- There was a male preponderance accounting for 69%.

- Majority of the HIV patients with oropharyngeal Candidiasis presented with pseudomembranous type of lesions constituting for 62%.

- 150 samples from HIV patients with oral Candidiasis were studied in detail and *Candida species* were isolated from all the 150 samples yielding a culture positivity of 100%

- Out of the 150 patients , two patients showed more than two *Candida* isolates resulting in a total of 152 *Candida* isolates.

- Majority of the isolates were *Candida albicans* (78%) and remaining were non-*albicans Candida species* (22%)

- Among the non-*albicans*, *Candida tropicalis* (11%) was the most common isolate followed *Candida krusei* (5%), *Candida parapsilosis* (4%), *C.glabrata* (1%) and *Candida guilliermondii* (1%).

- Among the 150 HIV patients with oropharyngeal candidiasis , 32% presented with recurrent Oropharyngeal Candidiasis.

- By Disk Diffusion Method, 13.8% of the *Candida* isolates were resistant to fluconazole, 15.8% were resistant to itraconazole, 4% were resistant to ketaconazole, 9% to clotrimazole and 5% were resistant to nystatin.

- By microbroth dilution, 11.8% of the *Candida* isolates exihibited MIC in the resistant range (> 64µg/ml) for fluconazole and 14 % of

the isolates exihibited MIC in the resistant range(>1 µg/ml) for itraconazole.

- Itraconazole resistant isolates were more common in patients with recurrent candidiasis.
- All the isolates were susceptible to amphotericin B.
- Antifungal susceptibility results by disk diffusion method were comparable with that of microbroth dilution method.
- Out of the 150 HIV patients with oropharyngeal candidiasis, 70.6% had CD4 count less than 200 cells/ µl indicating the association of candidiasis with immunosuppression.
- Azole resistance and non *albicans Candida* isolates were more common in HIV patients with CD4 count <200 cells/ µl indicating the emergence of resistant *Candida* isolates with immunosuppression.

CONCLUSION

In conclusion, *Candida albicans* is the most frequently isolated species from HIV seropositive patients with primary as well as recurrent Candidiasis. Non- *albicans Candida species* are emerging as important pathogens with increasing rates of azole resistance and with increased immunosuppression. Since oral candidiasis may be considered as indirect marker of immunosuppression in HIV patients, regular oral check up of these patients could be an indicator of immunosuppression. Further the increasing rates of resistance particularly among the *non- albicans Candida spp.* emphasizes the need for speciation and determination of antifungal susceptibility pattern of the oral candida isolates from HIV patients with oropharyngeal candidiasis.

BIBLIOGRAPHY

1. Akpan A, R.Morgan; Oral Candidiasis, *Post graduate Medical journal* 2002 ;78 : 455 -459

2. Allen .J, McCutchen, Merck Manual for health care professionals, Merck Manual Online library, Infectious diseases, Human immunodeficiency virus, 2009.

3. Andrew.B; More common opportunistic infections, their symptoms, diagnosis , prophylaxis and treatment, Positive Magazine issue ; 2003; 42 : 1-10

4. Bailey and Scott's Diagnostic Microbiology, 12th edition, chapter 50, Part V, 2007: Pg 629-717

5. Baily GG, Perry FM , Denning DW , Mandal BK 1994 . Fluconazole resistant candidosis in an HIV cohort. AIDS 8 :787 -792

6. Band, J.D., and D.G.Maki.1979.Infections caused by arterial catheters used for hemodynamic monitoring. *American Journal of Medicine*, 67: 735-741

7. Barchiesi F et al ; Transmission of fluconazole resistant *Candida albicans* between patients with AIDS and oropharyngeal candidiasis documented by pulsed- field gel electrophoresis ,*Clini Infect Disease* 1995 ; 21:561-4

8. Barry, A.L., M.A.Pfaller, R.P.Rennie ,P.C.Fuchs and S.D.Brown.2002 .Precision and accuracy of fluconazole susceptibility testing by broth

microdilution, E test, and disk diffusion methods. *Antimicrob. Agents .Chemother*. 35:2302-2305.

9. Berkhout.C.M, 1923,De Schimmelgeschlachten Monilia, Oidium, Oospora, en torula.*Candida spp*. doctor fungus.org.

10. Brigitte P Griffith, Sheldon Campbell and Donald R Mayo, Murray's Manual of Clinical Microbiology, 9th edition, Vol II, Chapter 83, 2006 : Pg 1310.

11. Bruatto M et al. *Candida albicans* biotypes in human immunodeficiency virus type 1 infected patients with oral candidiasis before and after antifungal therapy, *J Clin Microbiology* 1991; 29; 726-30.

12. CA Enwuru et al, Fluconazole resistant oppurtunistic oesophageal *Candida* and non- *Candida* yeast like isolates from HIV infected patients attending ARV clinics in Lagos, Nigeria, African Health sciences, Sep:2008; 8(3);142-148

13. Calvet et al; Reversible fluconazole resistance in *Candida albicans* : a potential in vitro model. *Antimicrob Agents Chemother* 41: 535 -539.

14. Carolina et al , Species distribution and antifungal susceptibility profile of oral *Candida* isolates from HIV infected patients in the antiretroviral therapy era, *Mycopathologia* 2006 , 162 : 45 -50.

15. Cartledge JD, Middle J, Gazzard BG : Non *albicans* oral candidosis in HIV positive patients, *Journal of Antimicrobial Chemotherapy* 1999, 43 ; 419 -422.

16. CD4 lymphocyte enumeration techniques, Report of a regional workshop, Bangkok, Thailand; 24-27, Nov 2003

17. CDC staging based on absolute CD4+ count and CD4 percentage in an HIV -1 infected Indian population : treatment implications ,*J.Clinical Exp. Immunology*, 2005 Sep:141(3): 485-490

18. Ceballos Salobrena, A., L.Gaitan Cepeda, L.Cebellos-Garcia, and L.P.Samaranayake.2004.The effect of antiretroviral therapy on the prevalence of HIV associated oral candidiasis in a spanish cohort. *Oral Surg.Oral Med.Oral Patho.Oral Radiol.Endod*, 97:345-350.

19. Chavanet et al 1994. Cross sectional study of the susceptibility of Candida isolates to antifungal drugs and in vitro-in vivo correlation in HIV infected patients. AIDS 8 :945 -950.

20. Clinical and Laboratory Standards Institute(CLSI). 2004. Method for antifungal disk diffusion susceptibility testing of yeasts. Approved standard- M44A.

21. Clinical Laboratory Standards Institute(CLSI).2002.Reference method for Broth dilution antifungal susceptibility of yeasts .Approved standard-Second edition, M27-A2

22. Coppola, S et al1997; Efficacy of itraconazole in treating AIDS associated infections due to Candida krusei. *Europ. J. Epidemiolog*. 11 :243-244.

23. Crowe et al ; Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV infected persons. *J Acquire Immune defici Syndr* 4 : 770 -776.

24. De Hoog, G.S., Guarro , J et al. 2000.Ascomycetous yeasts genus: *Candida* .In Atlas of clinical fungi, 2nd edition, 180 -226.
25. Deporan Greenspen, Oral manifestations of HIV, HIV insite knowledge, Base Chapter, 1998.

26. Dupont B ,Denning DW, Marriot D , Sugar A ,Vivani MA, Srisanthana T 1994. Mycosis and AIDs patients . *J Med Mycol* 32 :19 -28

27. Edouard Drouhet ; Tropical Infectious Diseases, Principle, Pathogens and Practice, Chapter 64, Candida Infections, Pg 655

28. Elmer Koneman, Stephen Allen, William Janda, Colour Atlas and Textbook of diagnostic Microbiology, 6th edition 2006 ;

29. Espinel Ingroff, E.Canton, D.L . Gibbs, and A.Wang, 15th Europ. *Congress Clinic. Microbiol. Infect. Diseases*, Abstract, 2005, Pg : 1736 .

30. Espinel Ingroff, M.A .Pfaller, S.A.Messer, C.C.Knapp, N.Holliday and S.B.Killian ,2004.Multicentre comparison of the sensititre yeast one colorimetric antifungal panel with NCCLS M27A2 reference method for testing new antifungal agents against clinical isolates of *Candida spp*. *Journal of Clinic Microbiol* 40 : 2101 -2102.

31. Fichtenbaum CJ and William J Powderly, Refractory Mucosal Candidiasis in patients with HIV infection, Clinical Infectious Diseases , 1998, March,:Pg 556-565.

32. Fichtenbaum CJ et al ;Refractory Mucosal Candidiasis in advanced human immunodeficiency virus infection, Clinical Infectious Disease 2000; 30 : 749- 56.

33. Fichtenbaum CJ, Judith A berg, Candidiasis and HIV, HIV Insite Knowledge Base Chapter, Feb 2006

34. Fisher and Cook 's Fundamentals of Diagnostic Mycology, 1998, Chapter-7, Yeast and Yeast like organisms, Pg: 197 -222.

35. Gary S. Moore, Mycology for the Clinical Laboratory, Yeast Identification, Pg 169 -198

36. Hamza et al; Species distribution and in vitro antifungal susceptibility of oral yeast isolates from Tanzanian HIV infected patients with primary and recurrent oropharyngeal candidiasis;*BMC Microbiology* 2008;8:135

37. Harrison's Principles of Internal Medicine , 17th Edition , Vol I, Section 14, Chapter 182 , 2008 : Pg 1137 -03.

38. Hazen.K.C and Howell, S.A.2003. *Candida, Cryptococcus,* and other yeasts of medical importance.In Murray, P.R., Baron , E.J et al, Manual of Clinical Microbiology, 8th edition .Washington .D.C: ASM press, 1693-711.

39. Hospenthal et al, Presumptive identification of *Candida species* other than *Candida albicans, Candida krusei ,Candida tropicalis* with the chromogenic medium CHROM agar *Candida*, Annuals of Clinical Microbiology and Antimicrobials, 2006, 5.

40. Howard M Shapro, Flow cytometry principles, From practical flow cytometry, Third edition, P:245.

41. Jabra Rizk et al, Fungal biofilms and drug resistance .Emerging infectious diseases 2004; 10(1)

42. Jagdish Chander, Textbook of Mycology, 3rd edition 2009, Antifungal drugs : Pg 71-91.

43. Jawetz,Melnick, and Adelbergs, Medical Microbiology, 24th edition, Section IV , Chapter -44, AIDS and Lentiviruses, Pg :604-620.

44. Jha BK, Dey S, Tamang MD, Shivananda PG,Brahmadatan KN; Characterisation of *Candida species* isolated from cases of lower respiratory tract infection, *Kathmandu University Medical Journal*, 2006,Vol.4,Issue 15,290-294.

45. Johnson, E.M., D.W.Warnock, J.Luker, S.R.Porter, C.Sully.1995.Emergence of azole drug resistance in *Candida species* from HIV infected patients receiving prolonged fluconazole therapy for oral Candidosis. *J. Antimicrobial Chemother*. 35 :103-114.

46. Kate G Davey et al, Comparative evaluation of Fungitest and broth microdilution methods for antifungal drug susceptibility testing of *Candida species* and *Cryptococcus neoformans* , *Journal of Clini Microbiol*, 1998, 36(4): 926- 930.

47. Kevin C Hazen and Susan A Howell ; Murray's Manual of Clinical Microbiology, 9th edition, 2006, Volume II, chapter 119, *Candida, Cryptococcus,* and other yeasts of Medical importance, Pg 1777.

48. Krause, W., Matheis and Wulf,k. 1969.Fungemia and funguria after oral administration of *Candida albicans*. *Lancet*, 1, 598 -9.

49. Kuby ‘s Immunology; Kindt, Goldsby, Osborne; 6th edition, 2006, Part IV, AIDS and other immunodeficiencies, Pg: 513.

50. Kurtzman, C.P.1998. Pichia. E.C.Hansen emend. The yeasts , a taxonomical study,4th edition.Amsterdam:Elsevier, 273-352.

51. Kwong chung, K.J. and Bennett, J.E 1992.Medical Mycology Philadelphia: Lea and Feiberger,81-104,768-770.

52. Latiff et al, Susceptibility pattern and molecular type of species specific *Candida* in Oropharyngeal lesions in Indian HIV positive patients , *Journal of Clinical Microbiology* , March 2004, Vol 42, No 3, Pg 1260-1262

53. Leblond et al, New Monoclonal antibody specific for *Candida albicans* germ tube, *Jounal of Clini Microbiol*, Jan 2000,Vol:38,No-I,Pg 61-67

54. Luis Vargas et al, Oral *Candida* isolates colonizing or infecting HIV infected and healthy persons in Mexico; *Journal of Clinical Microbiology*, 2005, 43(8):4159-4162.

55. Mackenzie.D.W.R, Serum tube identification of *Candida albicans* , *Journal of Clinical Pathology*,1952,15,563-565

56. Mackie and McCartney Practical Medical Microbiology, 14th edition, Chapter 41,Section D, 2007:Pg 721-756

57. Mandell ,Douglas and Bennett’s Principles and Practice of infectious diseases,6th edition,2005, Vol-1

58. Mary M Horgan , William G Powderly, Pfaller's Clinical Mycology, Chapter 21, Oral fungal infections, Pg:446.

59. Masur H, Michelis MA , Green JB ,et al ;An outbreak of community acquired *Pneumocystis carinii* pneumonia, Initial manifestation of cellular immune dysfunction, *N Eng J Med* 1981,305; 1431

60. Mats Jonell, Burket's Oral Medicine , 2008, 11th edition

61. Meis,J.,M.Petrou, J.Bille,D.Ellis,D.Gibbis, and the Global antifungal surveillance Group 2000. A global evaluation of the susceptibility of *Candida species* to fluconazole by disk diffusion. Diag . Microbiol. Infect.Dis. 36:215-223.

62. Merz ,W.G.,Khazan,U., et al . 1992. Strain delineation and epidemiology of *Candida lusitaniae* . *Journal of Clin Microbiology*,30,449-54.

63. Merz.W.G and Roberts.G.G.2003.algorithms for detection and identification of fungi : In Murray , P.R. and Baron,E.J.et al, *Manual of clinical Microbiology*, 8th edition . Washington, DC:ASM press, 668-85.

64. Million L, fluconazole resistant recurrent oral Candidiasis in HIV positive patients ;persistence of *Candida albicans* with the same genotype .*J Clini Microbiol* 1994; 32(4); 1115-1118

65. Mine Yucesoy and Serhat Marol,Performance of CHROM agar *Candida* and BIGGY agar for identification of yeast species, *Annuals of Clinical Microbiology and Antimicrobials* ; 2;8

66. MMWR, Recommendations and Reports, Treating OI Among HIV infected Adults and Adolescents, Dec 17, 2004, 53,1-112

67. Moore RD,Chaisson RE.Natural history of oppurtunistic disease in an HIV infected urban clinical cohort, *ANN Intern Med* 1996; 124 : 633-42.

68. Murray, Manual of Clinical Microbiology, 9 th edition,2006, Vol-II,chapter 131, Antifungal agents and Susceptibility test methods ,Pg : 1980

69. Natalya U Makarova et al, Persistence of Oropharyngeal *Candida albicans* strains with reduced susceptibilities to fluconazole among HIV Seropositive children, adults in along term care facility, *Journal of Clin Microbiol* 2003, May, 41(5);1833-1837

70. National AIDS Control Organisation, Guidelines for HIV testing, March 2007, Laboratory tests for monitoring , staging and progression of HIV diseases, pg 91 -96

71. Negri et al ,Correlation between E test , Disk Diffusion , and Microdilution methods for antifungal susceptibility testing of *Candida species* from Infection and Colonisation , *Journal of clinical laboratory Analysis*,2009,23 :324 -330.

72. Odds FC ,1988;*Candida* and Candidosis, 2nd edition London Balliere Tindall

73. Oppurtunistic infections, AIDS med.com, April 1, 2009

74. Orozco,A.S., Zhou,X. and Filler ,2000.Mechanisms of the proinflammatory response of endothelial cells to Candida albicans infection. *Infect Immunol*, 68, 1134 – 41.

75. Peter G Pappas et al ; Clinical Practice guidelines for the management of Candidiasis,2009 , Update by the Infectious diseases society of America, CID 2009 : 48 , Pg 503 -535

76. Pfaller M. A ,Messer, Coffman, Comparison of Visual and Spectrophotometric methods of MIC endpoint determinations by Using Broth microdilution methods to test five antifungal agents,including the new triazole D0870, *Journal of Clin Microbiol*, May 1995,vol.33,No.5,Pg 1094-1097

77. Pfaller,Clinical Mycology ,Chapter 6 ,*Candida* ,Pg 201

78. Pfaller.M.A ,Houston.A,Coffman.S, Application of CHROM agar *Candida* for rapid screening of clinical specimens for *Candida albicans, Candida tropicalis, Candida krusei* and *Candida glabrata*, *Journal of Clinical Microbiology*,1996(Jan); 58-61

79. Powderly WG et al, 1999 ;Oropharyngeal Candidiasis in patients with HIV : suggested guidelines for therapy. AIDS Res Hum Retrovir 15 : 1619 – 1623.

80. Powderly WG: Mucosal Candidiasis caused by non *albicans species* of *Candida* in HIV positive patients.AIDS 6:604,1992.

81. Price,R.W.1996. Neurological complications of HIV infection. *Lancet* 348:445-452.

82. Princisilla et al, Multicenter Brazilian Study of Oral *Candida species* isolated from AIDS patients , March 2002, Vol.97(2);253-257

83. Ranganathan et al ; Oral *Candida species* in healthy and HIV infected subjects in Chennai, South India, *Tropical Medicine and Health*, 2008,Vol.36, No.2,pg 101 -106

84. Rippon JW , Medical Mycology , Second edition, Candidiasis and the Pathogenic yeasts, 1982, 484-531

85. Royce, R.A., A.Sena, W.Cates and M.S.Cohen,1997. Sexual transmission of HIV. *N. Engl. J. Med.* 336 :1072-1078

86. Samaranayake, Commensal oral Candida in Asian Cohorts, *International journal of Oral Sciences*, 2009, 1: 2-5

87. Sandhu,R.S.., and H.S.Rundhawa.1962.On the reisolation and taxonomic study of *Candida vishwanathii* , *Mycopathologia*, 18 : 179 -183.

88. Shoba D Nadagir et al ; Significance of isolation and drug susceptibility testing of non-*Candida albicans species* causing oropharyngeal candidiasis in HIV patients , *Southeast Asian J Trop Med Public Health*,2008, Vol 39 , No 3

89. Singh HR, singh NG, singh TB ; Estimation of CD4+ and CD8+ T-lymphocytes in HIV infection and AIDS patients in Manipur , *Indian Journal of Medical Microbiology* , 2007 25(2); 126-32

90. Sneha K Chuchanur et al , Detection and antifungal susceptibility of oral *Candida dubliniensis* from human immunodeficiency virus infected patients; *Indian Journal of Pathology and Microbiology* , 2009, Vol 52 , Pg : 501 – 504.

91. Sullivan, D.J. , Westerneng ,T.J., et al 1995 . *Candida dubliniensis spp.* nov., phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV infected individuals. *J Clin Microbiology*, 141, 1507-21.

92. Sullivan,D.,Benett , D et al.1993.Oligonucleotide fingerprinting of isolates of *Candida species* other than *Candida albicans* and of atypical *Candida species* from HIV positive and AIDS patients. *J Clin Microbiol* , 31, 2124-33.

93. Theodore C White, Murray's Manual of Clinical Microbiology,9th edition , 2006 ,Volume-II, Chapter 130, Mechanisms of Resistance to antifungal agents,Pg:1961-1971

94. Topley & Wilson's Microbiology and Microbial infections , Medical Mycology, 10th edition, 2005, Chapter-30, Candidiasis , Pg :579-620.

95. Vargas KG , Joly S. Carriage frequency , intensity of carriage , and strains of oral yeast species vary in the progression to oral candidiasis in Human immunodeficiency virus positive individuals. *J Clin Microbiol* 2002; 4 :341 -50.

96. White MH.Is vulvovaginal candidiasis an AIDS – related illness? *Clin Infect Dis* 1996: 22 Suppl 2: S124 -7.

97. Wickerham, L.J., and K.A.Burton . 1948.Carbohydrate assimilation for the classification of yeasts, *Journal of.Bacteriology*,56 :363 -371

98. William G Powderly, Jonathan Cohan ; Infectious Diseases, 2nd edition, Vol II , chapter 126 : Pg 1285.

99. William R Kirckpatrick et al, Detection of *Candida dubliniensis* in oropharyngeal samples from HIV infected patients in North America by primary CHROM agar *Candida* screening and susceptibility testing of isolates, *J Clini Microbiol*, 1998,Vol 36 , No 12: pg 3007-12.

ABBREVIATIONS

AIDS	-	Acquired Immunodeficiency Syndrome
CLSI	-	Clinical Laboratory Standard Institute
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic Acid
EDTA	-	Ethylene Diamine Tetraacetic acid
ELISA	-	Enzyme Linked Immuno Sorbent Assay
EUCAST	-	European Committee of Antimicrobial Susceptibility test
FACS	-	Fluorescent Activated Cell Sorter
HAART	-	Highly Active Antiretroviral Therapy
HIV	-	Human Immunodeficiecy Virus
KOH	-	Potassium hydroxide
MHA	-	Mueller Hinton Agar
MIC	-	Minimum Inhibitory Concentration
MOPS	-	3N-Morpholino propane sulphonic acid
NCCLS	-	National Committee for Clinical Laboratory standards
OPC	-	Oropharyngeal Candidiasis
RNA	-	Ribonucleic Acid
RPMI	-	Rosewell Park Memorial Institute
SDA	-	Sabourauds Dextrose Agar
SDD	-	Susceptible Dose Dependent

APPENDIX

I.POTASSIUM HYDROXIDE SOLUTION(10% KOH)

Potassium hydroxide - 10 gm

Glycerol - 10 ml

Distilled water - 80 ml

To solution of 10% KOH, 10% glycerol is added to prevent drying. The ingredients are mixed and stored at room temperature.

II GRAM STAINING

Methyl Violet(2%) - 10g Methyl Violet in 100ml absolute alcohol in 1 litre of distilled water(Primary stain)

Grams Iodine - 10g iodine in 20g KI (fixative)

Acetone - Decolourising agent

Dilute Carbol fuchsin (1%)- Secondary stain

III SABOURAUD DEXTROSE AGAR WITH ANTIBIOTICS

Composition:

Peptone - 10 gm

Dextrose - 40 gm

Agar - 20 gm

Distilled water - 1000ml

Chloramphenicol - 50 mg

Final pH was adjusted to 5.6

Media Preparation

The above ingredients were reconstituted in one litre of distilled water. Dissolve the powder in distilled water by boiling.Dissolve Chloramphenicol in 10ml of 95% alcohol and added to the boiling medium.The medium was then removed from heating , mixed well and then dispersed in tubes and autoclaved at 121^{o} C for 15 minutes and the final pH was adjusted to 5.6.The tubes were cooled in slanted position and later the slants were stored in refrigerator.

IV CHROM AGAR CANDIDA (Hi-CHROM Candida Differential Agar)

Ingredients	**Grams/ Litre**
HiVeg Special Peptone	15
Yeast Extract	4
Dipotassium Hydrogen phosphate	1
Chromogenic mixture	7.22
Chloramphenicol	0.50
Agar	15

Ph : 6.3±0.2

Suspend 42.72 grams in 1000ml of distilled water. Heat to boiling to dissolve the medium completely. Cool the medium to 50^{o}C and pour into sterile petridishes.

V.CORNMEAL-TWEEN 80 AGAR

Composition

Corn meal	50 gm
Agar	15 gm
Distilled Water	1000ml
Tween 80(1%)	3 ml

Suspend the ingredients in 1000ml distilled water. Boil to dissolve completely. Add Tween80 to the above medium.

Sterilise by autoclaving at 121^{o}C for 15 minutes.

VI.YEAST NITROGEN BASE AGAR MEDIUM (Dehydrated Media supplied by Hi-Media)

Ingredients	Grams/L	Ingredients	Grams/L
Ammonium sulphate	5.00	Thiamine hydochloride	0.004
L-Histidine	0.01	Boric acid	0.0005
hydrochloride	0.02	Copper sulphate	0.00004
DL-Methionine	0.02	Potassium iodide	0.0001
DL-Tryptophan	0.000002	Ferric chloride	0.0002
Biotin	0.00004	Manganese sulphate	0.0004
Calcium pantothenate	0.000002	Sodium molybdate	0.0002
Folic acid	0.02	Zinc Sulphate	0.0004
Inositol	0.0004	Monopotassium phosphate	1.00
Niacin	0.0002	Magnesium sulphate	0.50
Para amino benzoic acid	0.0004	Sodium chloride	0.10
Pyridoxine hydrochloride	0.0002	Calcium chloride	0.10
Riboflavin			

Dissolve 6.7 gms of the media in 100ml of distilled water. Sterilise by filtration and store at 4^{o}C.

VII CARBOHYDRATE FERMENTATION MEDIA

Peptone - 15 gms

Sugar to be tested - 20 gms

NaCl - 5 gms

Distilled water - 1000ml

Dissolve the peptone, Bromothymol blue indicator and sodium chloride in 1000ml of distilled water and 20 gms of the sugar to be tested. Distribute approx 5ml in sterile test tubes containing an inverted Durham's tube (for gas production).Sterilise by autoclaving at 10 pounds pressure.

VIII MUELLER HINTON AGAR

Beef infusion	-	300ml
Casein hydrolysate	-	17.5 gm
Starch	-	1.5 gm
Agar	-	10gm
Distilled Water	-	1000ml
pH	–	7.4

Sterilise by autoclaving at 121^{O}C for 20 minutes.

IX RPMI 1640 (ROSEWELL PARK MEMORIAL INSTITUTE) MEDIA

RPMI medium – 10.4 gm

MOPS buffer- 34.43gm

Dissolve powdered medium in 900ml distilled water .Add MOPS to a final concentration of 0.165mol/L and stir until dissolved. While stirring, adjust the pH to 7.0 at 25 ^{O}C . Add additional water to bring medium to a final volume of 1000ml .Filter sterilize and store at 4^{O}C

X Mc Farland 0.5 Turbidity standard

- Prepare this turbidity standard by adding 0.5ml of 1.175% BaCl2 to 99.5ml of 0.H2SO4 with constant stirring to maintain a suspension.
- Verify the correct density of the turbidity standard by using a spectrophotometer.The absorbance at 625nm should be 0.08 to 0.10 for the 0.5 McFarland standard
- Distribute 4 to 6ml into screw capped tubes and tightly seal thse tubes and store them in the dark at room temperature.
- Vigorously agitate this turbidity standard on a mechanical vortex just before use.

XI SUGAR ASSIMILATION PATTERN

Species	Glucose	Lactose	Sucrose	Raffinose	Trehalose
Candida albicans	+	-	+	-	+
Candida dubliniensis	+	-	+	-	+
Candida glabrata	+	-	-	-	+
Candida guilliermondii	+	-	+	+	+
Candida kefyr	+	+	+	+	-
Candida krusei	+	-	-	-	-
Candida parapsilosis	+	-	+	-	+
Candida tropicalis	+	-	+	-	+

(Mackie and McCartney Practical Medical Microbiology)

XII SUGAR FERMENTATION PATTERN

Species	Glucose	Lactose	Sucrose	Maltose
Candida albicans	+	-	-	+
Candida dubliniensis	+	-	-	-
Candida glabrata	+	-	-	-
Candida guillermondii	+	-	+	-
Candida kefyr	+	+	+	-
Candida krusei	+	-	-	-
Candida parapsilosis	+	-	-	-
Candida tropicalis	+	-	+	+

(Mackie and McCartney Practical Medical Microbiology)

PROFORMA

S.No: IP/OP No.

Name of the patient : Occupation :

Age : Marital status :

Sex : Address :

Chief Compliants: Duration

Presenting illness:

H/o Difficulty in swallowing

H/o pain and burning sensation

Past History :

H/o similar illness in the past : Yes/No

No. of episodes

Treatment details

H/o past medical illness

Personal History : Smoking : Dietary habits:

Treatment History :

HAART: Yes/ No

Duration of treatment

Type of regimen

General Examination :

Local Examination : Oral Ulcer /patch, Site, Distribution

Laboratory Investigations :

HIV Status CD4 count

Mycological Investigations

Specimen – Oral Swab

Microscopy

Gram's Stain :

Culture on SDA

Colony morphology

Gram stain

Culture on CHROM agar

Germ tube test

Growth on Cornmeal agar

Biochemical Reactions

Sugar Fermentation test

Glucose	Lactose
Sucrose	Maltose

Sugar assimilation test

Dextrose	Sucrose
Lactose	Trehalose
Raffinose	

Antifungal Susceptibility Test:

Disk Diffusion test :

Fluconazole	Amphotericin B
Itraconazole	Ketaconazole
Clotrimazole	Nystatin

Micro broth dilution test

Fluconazole	Itraconazole	Amphotericin B