IDENTIFICATION, CHARACTERIZATION AND ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA SPECIES USING CONVENTIONAL METHODS FROM ORAL THRUSH IN HIV SEROPOSITIVE PATIENTS.

By

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In
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Under the guidance of

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LIST OF ABBREVIATIONS

ABC- Adenosine Triphosphate Binding Cassette

AG-Acid / gas production

AIDS - Acquired Immunodeficiency Syndrome

ATCC- American Type Culture Collection

C. albicans- Candida albicans

C. dubliniensis- Candida dubliniensis

C. fomata- Candida fomata

C. glabrata- Candida glabrata

C. guilliermondii- Candida guilliermondii

C. kefyr- Candida kefyr

C. krusei- Candida krusei

C. parapsilosis- Candida parapsilosis

C .pelliculosa- Candida pelliculosa

C. stellatoidea- Candida stellatoidea

C. tropicalis- Candida tropicalis

C. vishwanathii - Candida vishwanathii

CA- Capsid

CARE- Candida DNA Repetitive Elements

CD- Cluster differentiation

CDC- Center for Disease Control and Prevention

Ce- Cellobiose

CFU- Colony forming units

CIE- Counterimmunoelectophoresis

CMA- Corn meal agar

CMI- Cell mediated immunity

Cms- Centimeters

DNA- Deoxyribonucleic acid

g-Grams

gp- Glycoprotein

GSOM- Glucose Serine Ornithine Methionine

GTT- Germ Tube Test

FDA- Food and Drug Administration

HAART - Highly Active Antiretroviral Therapy

HIV - Human Immunodeficiecy Virus

hr- hour

HWP- Hyphal wall protein

IFN – Interferon

IL- Interleukins

ID- Immunodiffusion

PMBC- Peripheral Blood Mononuclear Cells

KOH - Potassium hydroxide

Ma – Maltose

Mg- Milligrams

MF- Mc Farland's

ml- Milliliters

MHA - Mueller Hinton Agar

MIC - Minimum Inhibitory Concentration

NAC- Non albicans Candida

NACO – National AIDS Control Organization

NaCl- Sodium Chloride

NCCLS - National Committee for Clinical Laboratory standards

OC- Oral candidiasis

OPC - Oropharyngeal Candidiasis

P – Probability

Rf – Raffinose

RNA - Ribonucleic Acid

RPMI - Rosewell Park Memorial Institute

SAP- Specific secretary aspartyl proteunusual

SDA - Sabourauds Dextrose Agar

Spp- Species

Su – Sucrose

TB- Tuberculosis

Te- Trehalose

T- lymphocyte- Thymus dependent lymphocyte

TNF- Tumor necrosis factor

WHO- World Health Organization

Xy- Xylose

^{0}C- Degree Centigrade

χ^2 - Chi – square test

µ- Microgram

ABSTRACT

BACKGROUND - Oropharyngeal candidiasis continues to be a common opportunistic infection in patients infected with Human Immunodeficiency Virus (HIV). Though Candida albicans is the predominant isolate, rise in frequency of isolation of non albicans Candida (NAC) species is observed. Long term use of azoles for treatment as well as prophylaxis for Oropharyngeal candidiasis has lead to emergence of Azole resistant strains.

OBJECTIVE –The present study aimed at identification and characterization of Candida species and to derive their antifungal susceptibility pattern to Azole group of drugs from Oral candidiasis in HIV seropositive individuals.

MATERIALS AND METHODS - Oral swabs collected from 118 HIV seropositive patients was subjected for Gram stain and culture. Swabs yielding growth of Candida species are subjected to speciation by standard mycological methods like GTT, Dalmau-plate culture and sugar assimilation tests. Antifungal susceptibility testing done against Fluconazole, Voriconazole, Ketoconazole and Itraconazole by Disk Diffusion method according to CLSI guidelines (M44 A2).

RESULTS – Out of 118 samples 121 species were isolated. NAC(87.6%) were the predominant isolates, C. tropicalis(23.14%) is the most common species isolated among NAC followed by C. guilliermondii (19%), C. parapsillosis(13.22%), C. kefyr (12.4%), C. krusei(9.09%), C. glabrata(5.79%), C. fomata(2.48%), C. lusitaniae (1.65%) and C. pelliculosa (0.83%) whereas C. albicans were 12.39%. C. albicans were 53.33%, 66.66% susceptible to Fluconazole and Voriconazole respectively whereas NAC showed 46.22%, 58.49% susceptibility to the same drugs.

CONCLUSION –The present study underscores that, there is increased frequency of NAC in causing oral candidiasis in HIV seropositive individuals and decreased susceptibility to Fluconazole and Voriconazole among NAC than C. albicans.

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INTRODUCTION

Over 33 million people are diseased with Human Immunodeficiency Virus (HIV) globally and in India 2.31 million live with AIDS.[1]

Opportunistic infection continue to cause morbidity and mortality in patients with Human Immunodeficiency Virus infection throughout the world.[2]

Oropharyngeal Candida infections are the most common opportunistic diseases in Human Immunodeficiency Virus (HIV) infected individuals, occurring in up to 90% of patients during the course of their disease. Asymptomatic oral *C. albicans* carriage has been demonstrated in HIV-positive patients and an increased incidence of asymptomatic oral *C. albicans* carriage in HIV-positive patients compared to that in other at-risk groups has also been noted. Thus, a higher prevalence of oral *C. albicans* colonization may be a predisposing factor for the subsequent development of clinical thrush.[2]

The advent of Highly Active Antiretroviral Therapy (HAART) has permitted suppression of viral replication and a partial recovery of CD4 T-lymphocyte count in HIV infected patients. Although the incidence and prevalence of opportunistic infections have been reduced worldwide due to use of HAART, OPC remains the most frequent HIV-associated oral lesion in most developing countries including India.[3]

HIV infected patients will experience at least one episode of oropharyngeal candidiasis during the course of their illness. 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, general health and quality of life of these patients.[4, 5]

Earlier, it was considered that *C. albicans* was the most common species causing oral candidiasis, but the emergence of Human Immunodeficiency Virus (HIV) and AIDS, and the wide-spread use of azoles as prophylaxis and for treatment, however, lead to the increasing recovery of many other non Candida albicans species causing mucosal infections.[6]

Even though most respond well to a short course of azole therapy, up to 50% will experience a relapse within 1 month after the completion of therapy. Currently, the number of patients who experience multiple recurrences of mucosal Candidal infections and eventually fail to respond to azole therapy, is rising.[7]

Antifungal drug resistance and the emergence of novel species and species previously not associated with human disease as potential pathogens have also greatly contributed to the drastic increase in oral candidiasis.

Hence the study was done to isolate Candida from oral candidiasis in HIV seropositive cases, speciate them by various tests and derive their anti-fungal susceptibility pattern.

AIMS AND OBJECTIVES

Identification, characterization and antifungal susceptibility testing of Candida species isolate from oral thrush in HIV seropositive patients.

REVIEW OF LITERATURE

HUMAN IMMUNODEFICIENCY VIRUS

History and epidemiology

AIDS was first recognized in 1981, when the US centers 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's sarcoma. Within months, the disease became recognized 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.[8]

In 1982, the disease was named as AIDS.[8]

In 1983, Luc Montagnier isolated the virus from a patient with lymphadenopathy.[9]

In 1984 it was demonstrated clearly to be the causative agent of AIDS.[8]

In 1985, a sensitive Enzyme Linked Immunosorbent Assay was approved by FDA for use of blood donor screening in US. [10]

In 1986 the International committee for virus nomenclature decided on the generic name HIV for the virus causing AIDS. [8]

In 1986 HIV was detected for the first time in India among female sex workers in Chennai, Tamilnadu. [8]

In 1986 HIV-2 was detected in West African patients. [8]

In 1990, William G Merg published based on typing systems, phenotypic characteristics and genotypic methods. [11]

In 1990-91, CDC Atlanta, Georgia reported that AIDS patients suffer from opportunistic Candida infections with high prevalence and Candida species has been recognized has important cause for morbidity and mortality in AIDS patients.[12]

In 1992, National AIDS Control Organization (NACO) was established by Government of India.[11]

In 1993, Sentinel surveillance system was introduced in India.[11]

In 1996, antiretroviral drugs were available in India.[11]

In 2001, National AIDS Prevention and Control policy was started.[11]

In 2003, HAART was started in India.[11]

In 2004, ART was started in India.[10]

In 2005, first AIDS vaccine trial was initiated.[10]

Epidemiology of HIV-UNAIDS- Report on global AIDS epidemic 2008[1]

Total AIDS cases- 30-36 million globally.[1]

Total AIDS related deaths – 2 million globally.[1]

India- Report of HIV surveillance and HIV estimation in India 2008.[13]

In India, total cases 2.31 million.[1, 13] – Children - 3.5%

ANC cases – 0.48%

Female – 39%

Male – 57.02%

Origin and classification

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.[10]

Current knowledge places retroviral infection of humans as zoonoses that originated in primate-to-human species called jumping events. For HIV-1 and HIV-2, these events occurred in Central Africa and West Africa, most likely at multiple times, with more recent attaining major epidemic significance.[10]

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.[10, 11]

Structure and life cycle [12, 14]

The HIVs are spherical viruses of appropriately 100nm in diameter. The core or nucleocapsid is condensed into cylindrical, trapezoidal or triangular shape. HIV 1 and HIV 2 may be distinguished from each other by the degree of condensation of the nucleocapsid particularly as it separates from the envelope. Gag proteins compile the conical core of the mature HIV virion. MA protein lies underneath the lipid membrane and makes contact with it via amino-terminal myristylated positively charged segment. CA protein forms the capsid, the outer layer of the capsid. The complex of NC protein and viral RNA is in the center of the core, RT and IN molecules are associated with this complex by binding to the nucleic acid.

The HIV has a sophisticated replication cycle, involving at least nine functional genes. HIV envelope glycoprotein, Env binds to CD4 and a co-receptor, which is a member of chemokine receptor family. Fusion of the viral and cell membrane results in the entry of viral core carrying the HIV genome into the cytoplasm. Reverse transcription follows the entry and resulting proviral DNA is transported to the nucleus, where it integrates into the host genome. Transcription and slicing of the various messages occur in the nucleus using the integrated genome as the template. Genomic and non-genomic RNA are transported to the cytoplasm, where translation occurs. Viral genome and newly produced structural proteins are transported to assembly sites at the cytoplasmic membrane, where morphogenesis and budding of progeny virus occurs.

HIV 1/ HIV 2 Genes and their products [12, 14]

GENE		PROTEIN	FUNCTION
Structural	Gag	Gag MA, p17	Membrane anchoring, env protection, nuclear transport of viral core.
		Gag CA, p24	Core capsid.
		Gag NC, p7	Nucleocapsid binds RNA
		Gag p6 domain	Binds Vpr
	Pol	Protease, p15	Cleavage and maturation.
		RT	Reverse transcription
	env	gp120	External glycoproteins
		gp41	
Regulatory	Tat	Tat, p16/p14	Transcriptional transactivator
	Rev	Rev, p19	RNA transport, stability and utilization
Accessory	Nef	Nef p27-p25	CD4 & MHC-1 down regulation
	Vif	Vif p23	Promotes viral maturation and infectivity
	Vpr	Vpr p10-p15	Nuclear localization, arrests infected cells at G2/M phase.
	Vpu	Vpu , p16	Production of extracellular viral particles degrades CD4 in ER.
	Vpx	Vpx , p12-16	Analog Vpu in HIV 2.

Immune responses [14, 15]

Both humoral and cell mediated immune responses occur in the course of natural HIV infection. Although the immune system plays a key role in determining the viral set point and delaying disease progression, the overall HIV specific immune responses fail to eradicate integrated virus and without efficient therapy it is difficult to prevent the progression of HIV infection to development of AIDS.

CD8+ T-cells are important component of an HIV-1 specific immune response and play an essential role in the immune mediated containment of HIV-1 infection. The HIV-1 specific cytotoxic T-lymphocyte responses are involved in the control of viral infection including acute and chronic infection as described for rapid progressors, long term non-progressors and perhaps exposed uninfected individuals. The CD8+ Cytotoxic T-Lymphocytes are detectable by 2-3 weeks after the initial infection, reach a peak by 9-12 weeks and helps in declining the viral load in plasma and thus for partial control of the virus. The killing function of the CD8+ T-cells is very much efficient in killing, before infected cells produce new virions.

Differentiation of CD8+ T-cells requires cytokines produced by CD4+ T- cells and/or co-stimulators expressed on the infected cells.

Tropic and Biologic properties of HIV-1 isolates [15]

Chemokine Coreceptor	PMBC	Macrophage replication	T-cell line replication	Replicative phenotype	Synctium inducing phenotype
X4	+	-	+	Rapid / slow	++
R5	+	+	-	Slow / low	-
R5/X4	+	+	+	Rapid / high	+

Antibody response to HIV develop within 6-9 weeks after infection and peak at 12 weeks following the identification of CD8+ cytotoxic T-lymphocytes, although the increased titers of antibodies found later on in the course of HIV infection cannot efficiently control the virus. Neutralizing antibodies can neutralize free virus and target envelope glycoproteins or epitopes within gp120 and gp41 like V3 loop and the CD4 binding domain.

Antibodies in the sera of HIV infected patients effectively neutralize the autologous viral variant but not the heterologous viruses.

However, there are number of factors limiting the efficient immune control of HIV infection that include immune exhaustion, lack of adequate T- helper function, escape from immune recognition, host genetics, protected viral reservoirs, potential infection of CD8+ cells and defects in antigen presenting cells.

Pathogenesis [16, 17, 18]

HIV is transmitted by exposure of the oral, rectal or vaginal mucosa during sex, by transfusion of contaminated blood products, use of contaminated syringe or materno-fetal circulation or by breast feeding. Sexual transmission accounts for >90% of HIV infections.

In majority of individuals, infection with HIV-1 results in gradual sustained decrease in CD4 T-cells and the consequent development of immune dysfunction and opportunistic infections, hallmark of AIDS. In most case, progression to AIDS occurs after a prolonged period of clinical stability that lasts on average from 8-10 years. However, in few people disease progression occurs rapidly within 1-3 years.

Variation in the natural history of disease in humans reflects the complex role of viral and host cell factors in HIV infection. These differences may emerge as a result of variation in the host immune response and difference in the cytopathicity or trophism of the virus.

Although infection with HIV -2 may also cause AIDS, the period of clinical progression is considerably longer, suggests that HIV- 2 is less virulent.

Despite variation in the rate of clinical progression, infection with HIV commonly proceeds in three states: 1. Primary infection, 2.Clinically asymptomatic infection and 3.Symptomatic disease progression. [17]

LABORATORY INVESTIGATIONS FOR HIV INFECTION [19]

I. Laboratory methods for the diagnosis of HIV infection

II. Laboratory methods for monitoring Stage and Progression of Infection.

I. Laboratory methods for the diagnosis of HIV infection

a. Indirect Methods - Antibody detection methods.

b. Direct Methods - Antigen detection 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

1. Conventional ELISA

2. 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)

Confirmatory tests

-Western Blot

-Immunoblot

-Lineimmuno assay

-Indirect fluorescent antibody test

-Radioimmunoprecipitation tests.[20]

DIRECT METHODS FOR DETECTION OF HIV ANTIGEN[19]

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 up to 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

B. LABORATORY TESTS FOR MONITORING STAGE AND PROGESSION OF HIV INFECTION[19]

a. Viral markers - Plasma HIV RNA load

p24 antigenemia

b. Immunologic markers- CD4 count

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.[19]

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. [9]

CD4 T cell counts

The progressive depletion of CD4+ T lymphocytes is the hallmark 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 provide powerful tools for determining prognosis and monitoring response to therapy. The ratio of CD4 T-lymphocytes to CD8 T- lymphocytes is an additional important measure of disease progression. [9]

Measurement of lymphocyte subsets is done by flow cytometry which is the gold standard for enumeration of CD4 T-lymphocytes. The rapid decrement of CD4 T-lymphocyte counts, indicates the speed of progression towards AIDS. While on therapy, improvement in CD4 T lymphocytes counts is indicative of the success of therapy. CD4 T-lymphocytes counts are the criteria for initiating ART as well as monitoring the therapeutic response in a patient.[11, 17]

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 opportunistic infections and neoplasm's that are AIDS defining illness.[21]

The first overt indication of AIDS may be opportunistic infection with the fungus Candida albicans, which causes oral candidiasis.[22]

CORRELATION BETWEEN CD4 COUNT AND HIV ASSOCIATED DISEASES [22]

1. >500 cells/ mm^3 • Acute primary infection • Persistent generalized lymphadenopathy • Recurrent vaginal candidiasis
2. <500 cells/mm^3 • Pulmonary TB • Herpes zoster • Extra-intestinal salmonellosis • HIV associated ITP • Lymphoid interstitial pneumonitis • Pneumococcal pneumonia • Oropharyngeal candidiasis • Kaposi's sarcoma • Cervical intraepithelial lesions • Interstitial pneumonitis
3. <200 cells/mm^3 • Pneumocystis carinii pneumonia • Cryptosporidiosis • Oesophageal candidiasis • HIV associated wasting • Mucocutaneous Herpes simplex • Microsporidiasis • Extra pulmonary TB • Peripheral neuropathy
4. <100 cells/mm^3 • Cerebral Toxoplasmosis • Progressive Multifocal leukoencephalopathy • Cryptococcal meningitis • HIV associated dementia
5. <50 cells/mm^3 • CMV retinitis • Disseminated mycobacterium avium intracellulare

WHO Clinical Staging[9, 23]

Clinical stage 1	Clinical stage 2	Clinical stage 3	Clinical stage 4
▪ Asymptomatic Persistent generalized lymphadenopathy	▪ Unexplained moderate weight loss (<10% body weight) ▪ Recurrent URTI ▪ Herpes zoster ▪ Angular cheilitis ▪ Recurrent oral ulceration ▪ Popular pruritic eruptions ▪ Seborrheic dermatitis ▪ Fungal nail infection	▪ Unexplained severe weight loss(>10% body weight) ▪ Unexplained chronic diarrhea >1 month ▪ Unexplained persistent fever ▪ Persistent oral candidiasis ▪ Oral hairy leukoplakia ▪ Pulmonary TB ▪ Severe bacterial infection ▪ Acute necrotizing ulcerative stomatitis, gingivitis or peridontitis ▪ Unexplained anemia (<8g/dl), Neutropenia &/or thrombocytopenia (<50000 cells/µl)	▪ HIV wasting syndrome ▪ Pneumocystis pneumonia ▪ Recurrent bacterial pneumonia ▪ Chronic herpes infection ▪ Oesophageal candidiasis ▪ Extra pulmonary TB ▪ Kaposi's sarcoma ▪ CMV infection ▪ CNS toxoplasmosis ▪ HIV encephalopathy ▪ Extrapulmonary Cryptococcosis ▪ Disseminated non-tuberculous Mycobacterial infection ▪ Progressive multifocal leukoencephalopathy ▪ Chronic Cryptosporidiosis ▪ Chronic Isospordiasis ▪ Disseminated mycosis ▪ Recurrent septicemia ▪ Lymphoma ▪ Invasive cervical carcinoma ▪ Atypical disseminated Leishmaniasis ▪ Symptomatic HIV- associated neuropathy or HIV associated cardiomyopathy.

Yeast like Fungi-CANDIDA

History

History of Oral candidiasis has been recognized since 4th century, the time of Hippocrates in his book "Epidemics" who described oral thrush in debilitated patients.[24, 25]

The word "thrush" is derived from ancient Scandinavian or Anglo-Saxon words for the disease.[25, 26] The French word for the condition is 'le Muguet', meaning 'lily of the valley'.[24] "torsk" is the Swedish synonym for oral thrush.[25]

In 1890, Zopf suggested the name of the fungi as *Monilia albicans* which derived Moniliasis (clinical entity), the early name of Candidiasis.[27]

Berkhout in 1923, after recognizing the difference between *Monilia species* isolated from rotting plants and those isolated from medical cases established the genus *Candida* to accommodate the later.[24]

This was accepted as the official name of the genus by the Eighth botanical congresses in Paris in 1954.[28]

In 1911, Castellani suggested yeast species other than C. albicans and described their characters. His report of "tea-tasters cough" has been considered to be an early account of bronchopulmonary candidiasis.[27]

According to modern taxonomy, Moniliasis is now reserved for fungal infections in plants.[29] "Candidiasis" came into common use in United States but in Canada, England, France and Italy the term "Candidiosis" has been accepted as the more descriptive term. Since the other mycotic diseases end with –osis, namely Histoplasmosis, Cryptococcosis and so forth, the term "Candidiosis" is preferable for consistency, but candidiasis is used presently in most of the literature on the disease.[26]

In 1940, Joachim and Polayes described Candidal endocarditis as an opportunistic infection in heroin abused patients. At the same time, the association of candidiasis and steroid therapy, immunosuppressive drugs, cytotoxic agents and immune defects became apparent.[24, 30]

In 1945, Conant et al described identification of Candida species based on fermentation of Glucose, Maltose, Lactose and Sucrose.[27]

In 1948, Wickerham et al described the assimilation method.[27]

In 1956, Reynolds and Braude described the germ tube test for identification of C.albicans.[27]

In 1959, Vishwanathan and Randhwana isolated C. vishwanthii from India.[24]

In 1960, Taschdjian et al, described chalmydospore formation by C. albicans in Cornmeal agar.[24]

In 1968 Brown Thompson in Denmark observed that different strains of C. albicans produced varying morphologies when streaking on Malt agar.[31]

In 1971 Dolan C T gave the identification scheme for yeasts.[31]

In 1975 Holt R J gave details of methods for evaluation of sensitivity of the pathogenic fungi to therapeutic agents, media, incubation temperature, drug solution and time.[31]

Warnock (1979), Odds and Abbot (1980) worked on resistogram patterns and applied them for epidemiological studies.[31]

In 2002, CLSI document M27- A2 for antifungal susceptibility testing of yeast by broth dilution method, in 2003 M44- A for antifungal susceptibility of yeast by disk diffusion was released.[16]

Taxonomy

The Genus *Candida* belongs to the phylum Deuteromycota, in class Blastomycetes, in order Moniliasis and family Cryptococcaceae.[32]

Genus *Candida* includes more than 163 anamorphic species.[24]

Frequent human pathogens are[28] -

1. Candida albicans (Robin, Berkhout) 1923
Synonym : *Oidium albicans / Monilia albicans/ C. intestinalis*

2. Candida guilliermondii (Castellani)1938
Synonym : *Endomyces guilliermondii/ Monilia guilliermondii*

3. Candida glabrata (Anderson ,Meyer& Yarrow)1978
Synonym: *Torulopsis glabrata*

4. Candida krusei (Castellani, Berkhout) 1923
Synonym : *Saccharomyces krusei / Endomyces krusei/ Monilia parakrusei, Candida lobata*

5. Candida parapsilosis (Ashford) 1959
Synonym: *Monilia parapsilosis*

6. Candida tropicalis (Castellani, Berkhout) 1923
Synonym : *Monilia tropicalis / Oidium tropicalis/Candida vulgaris*

7. Candida kefyr (Castellani, Basgal)1931
Synonym : *Candida pseudotropicalis / Monilia pseudotropicalis/* Castellani pseudotropicalis

8. Candida lusitaniae

9. Candida dubliniensis (Sullivan et al) 1993,1995

10. Candida viswanathii (Sandhu et al)1959-87

11 .Candida stellatoidea (Jones et Martin) 1939
Synonym : Monilia stellatoidea, Paraconidia stellatoidea

12. Candida fomata

Less common species[28]

C. pelliculosa	C. inconspicua
C. pintolopesii	C. lambia
C. pulcherrima	C. lipolytica
C. rugosa	C. catenulata
C. utilis	C. chiropterorum
C. fermentati	C. ciferri
C. zeylanoides	C. haemulonii
C. hunsicola	C. norvegensis

Ecology

Candida species are recognized to be commensal or normal flora of alimentary tract, upper respiratory tract, female genital tract especially vagina and on the skin. It is known that these species serve to cause endogenous infection due to its commensal nature.[17]

According to many literatures the source of candidiasis in humans is mostly endogenous; studies have largely focused on the distribution of yeast flora in patients and in healthy persons.[33] The prevalence of Candida species reported from different anatomic sites varies greatly depending on the subjects sampled and the isolation method used, although C. albicans is most common.[25] In healthy individuals the commensal strain and the infecting strain are same, usually single species. Even recurrent candidiasis is caused by single persistent strain unique to particular patient.[2,34]

Odds isolated C. albicans from oral cavity (1.9-41.4%), alimentary tract (0-55%) and from vagina (2.2-6.8%). His study also showed C. tropicalis and C. glabrata are second most common species isolated from oropharynx and vagina respectively.[16,26]

Cohen et al reported the prevalence of yeast as 35% in oropharynx, 50% in jejunum, 60% in ileum and 70% in colon. Among them again, C. albicans is the most prevalent species in alimentary tract (79%), followed by C. glabrata (9.1%), C. parapsilosis (5.4%), C. krusei (2.9%) and C. tropicalis (2.3%).[25]

Patel M, Shacklenton J and Coogan M M studied on 100 normal individuals and showed total carriage of 63%, out of which 70.3% is C. albicans followed by 4.7% C. dubliniensis, 3.2% each of C. parapsilosis, C. cerevisiae and 3.1% C. tropicalis.[35]

Oral carriage of C. albicans in healthy non hospitalized individuals has been reported to vary between 2-37%. But the incidence of oral candidiasis increases as a consequence of hospitalization. In HIV infection, oral carriage rate increases to 81.3%. High prevalence of oral Candida colonization is the predisposing factor for the subsequent development of clinical oral candidiasis.[16, 36]

Oral candidiasis in HIV seropositive patients

Candidiasis is undoubtedly the most common fungal infection in HIV seropositive patients, manifests most commonly as oral thrush, oropharyngeal candidiasis and even skin rash.

Multiple strains are present in HIV individuals; these patients developing oral candidiasis have shown four different scenarios from carriage stage to disease.[2]

I. Single strain maintenance
II. Multiple strain maintenance
III. Strain replacement
IV. Species replacement

Carolina Rodrigues, Costa et al studied 99 oral swabs from HIV seropositive patients and could isolate Candida albicans (50%) as the most common species. Candida tropicalis (29.9%) is the second most common species isolated. Candida parapsilosis (19.3%), Candida guilliermondii (4.8%), Candida krusei (1.61%), Candida lusitaniae (1.61%) and Candida kefyr (1.61%) were also reported in their study.[3]

Shobha D Nadagir et al could isolate Candida albicans (66.6%) and non albicans Candida (33.3%), Candida dubliniensis (48.9%), Candida krusei (20%), Candida parapsilosis (11%), Candida stellatoidea (6.7%), Candida tropicalis (8.9%) and Candida guilliermondii (4.9%).[37]

Vaishali Wabale et al concluded Oropharyngeal Candidiasis is the most common (90%) opportunistic infection in HIV seropositive patients. Candida albicans (74.3%), Candida dubliniensis (17.8%), Candida parapsilosis (6.7%), Candida tropicalis (2.2%) were isolated in their study.[38]

Arati Mane et al studied 210 HIV seropositive individuals & 125 HIV seronegative individuals. Out of which 60 had lesions of Oral Candidiasis and 28 HIV seronegative individuals have Candidal colonization. Candida albicans was the predominant isolate & higher frequencies of non albicans Candida were observed in HIV infected individuals.[39]

Vargas KG and Joly S showed an association between yeast carriage/CD4 cell count and development of oral candidiasis in follow up of 2 years.[2]

His co-relation of yeast carriage with CD4 count as follows-

CD4 count range	**Median intensity of yeast carriage/ ml of saliva**
>400 cells/ µl	309 colonies (no oral thrush within 2 years)
200- 400 cells/ µl	1090 colonies
<200 cells/ µl	4351 colonies
With treatment	415 colonies

His observation was that, 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. 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. Up to 90% of persons with advanced untreated HIV infection develop Oropharyngeal Candidiasis, with 60% having at least one episode per year with frequent recurrences (50-60%).[40]

Virulence factors

The state of the host is of primary importance in determining Candida pathogenicity. There must be a breakdown of mucosal surfaces or in the host defense for diseases to occur. However, there are factors associated with the organism rather than the host that contribute to its ability to cause disease and explained the differences among species in their pathogenicity.

I. Adherence of Candida species to host cell

Adherence of Candida species to a wide range of tissue types and inanimate surfaces is essential and important in the early stages of colonization and tissue

invasion. Germinated C. albicans cells adhere to host tissue more readily than do yeast phase cells.[9]

Hyphal dimorphism status of Candida species is still inconclusive, **dimorphism** may have role as a virulence factor. Hyphal wall protein coded by HWP1 gene and other hyphal growth factors interacts with host receptors (Flucosyl glucosamine, Fibronectin, Arginine-glycine-aspargine) with specific ligand receptor interaction and non specific electrostatic forces, Vanderwaal's forces. Dimorphism is important but not essential in causing disease. In HIV HWP1 is increased and more adhesive strains are selected under antifungal stress.[41, 42]

Hyphae of C. albicans have a sense of touch so that they grow along grooves and through pores **(thigmotropism)**. This may aid infiltration of epithelial surfaces during tissue invasion. This is controlled by Efg1 – transcriptional regulator for adhesion.[41]

II. Enzymes

Production of hydrolytic enzymes is important determinant for tissue invasion and Candida species are able to produce 14 different hydrolytic enzymes.[25]

a. Specific secreted Aspartyl Proteinase (SAP)

   SAP is an extracellular enzyme. This enzyme coded by SAP gene is important component of pathogenecity and also correlates with the active disease process. It helps in tissue invasion by degradation of keratin, collagen and mucin.[43]

   SAP2, it degrades immunoglobulins, complements and cytokines. Produced by C. albicans, C.dubliniensis, C. tropicalis and C. parapsilosis.[44]

   In HIV- virulent strains are formed with higher SAP production.[45]

b. Phospholipase production

Coded by PLB1 gene expression. This enzyme concentrated at hyphal tips have greater potential for invasion by hydrolyzing phospholipids in host cell membrane. In HIV there will be increased production of phospholipases by Candida species and strains.[45]

c. Acid phosphatases
d. Alkaline phosphatases
e. Peptidases
f. β-glucosidase
g. Plasma coagulases
h. Leucine amino peptidases
i. Metallopeptidases
j. Haemolytic factors
k. Siderophores

III. Yeast hyphae-Dimorphism

Hyphae transformation occurs in active disease and facilitate penetration. These are regulated by regulators *Cph1, Efg, CaTec* which regulate the expression of **Hyphal dependent gene** - SAP4 and SAP6 and **Hyphal independent gene**- SAP1 and SAP3 respectively.[46, 47]

IV. Immunomodulation

An immunosuppression of host defenses with C. albicans is observed in experimental animals particularly attributed to mannan.[17]

V. Phenotypic switching

Soll et al described this phenomenon, which is the reversible morphological variation among the strains of organisms due to various contributing factors like synthetic media, repeated cultivation and prolonged incubation. Also there is

change in epitopes expressed on their surfaces, regulated by switch regulatory gene. This contributes to virulence of C. albicans by facilitating its ability to survive, invade tissues and escape from host defenses.[48]

VI. Others[48]

Mannan

Thrombin induced platelet induced microbicidal protein

Temperature

Azole resistance

Biofilms

Pathogenesis and Pathology of Oral Candidiasis

Opportunistic infections occur due to breach in immunity of host leading to shift of the commensal organism to cause parasitic invasion. Candidiasis is mostly an **endogenous infection** arising from overgrowth of the fungus inhabitating the normal flora.[27]

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.[49] It is believed that Candida can cross the intact GI mucosa by a process called **persorption** following fungal overgrowth due to excessive antibiotic treatment.[50] However it may be occasionally acquired from **exogenous** sources (such as catheters or prosthetic devices).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.[51]

Pathogenesis of candidiasis is mainly due to adhesion and tissue invasion.

Candida favoring colonization in HIV patients [34, 52, 53]

- The changing oral environment and host immune factors cause inefficiency of existing bacterial population to compete effectively with the increased yeast cells on epithelial cells. Yeast cell count >1000CFU/ ml of saliva.
- Strains produce increased transcriptional factors.
- Azole resistance
- Altered composition of saliva, decreased mucin.
- Secretary IgA level decreases
- Lowered CD4 count
- Perturbation in the T-helper cell function and lack of T cell recognition of immunodominant Candida antigen.

In HIV seropositive patients the yeast cell number increases and its adhesion also increases. Adhesion of Candida occurs by specific and non-specific interaction between Candidal cell surface adhesions and various binding sites on the host cells.

On adhesion infected tissue increases cytokines TNF-α, IL-10, IL-12 and chemokine MIP-2 production. There is prominent chronic granulocyte dominated inflammatory response. Adherence avoids flushing action of saliva. Enzyme production is attenuated, enzyme produced causes lysis of keratinocytes and collagen helps in tissue invasion. Hyphal morphogenesis, phenotypic switching and antigenic modulation occurs. Pseudohyphae pertaining to its larger size resist phagocytosis. Phospholipases produced at hyphal tips helps in tissue penetration.

Lowered CD4 count and decreased function of neutrophils and macrophages helps in dissemination.[52, 53]

Pathology

The macroscopic and microscopic appearance of lesions 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.[17]

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 .The fungus is restricted to stratum corneum of the affected area.[17, 26]

Deep infections

Several parenchymatous organs may be involved .These infections are characterized by micro abscesses. Granuloma with giant cells and lymphocytes may be formed in chronic infections.[17]

Spectrum of diseases by Candida[8, 17]

Clinical entities may be divided into large groups

1. Mucocutaneous Candidiasis

(i)Mucosal

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

(ii)Cutaneous

- Generalised Cutaneous Candidiasis
- Erosio interdigitalis

-Candida folliculitis

- Candida balanitis

- Intertrigo

-Diaper rash

- Chronic mucocutaneous Candidiasis

(iii) Nail - Paronychia , Onychomycosis

2. Deep Seated Candidiasis

- Central Nervous System Candidiasis

- Urinary tract Candidiasis

- Respiratory tract Candidiasis

- Cardiovascular Candidiasis

- Candida infection of Vasculature

- Disseminated Candidiasis and Candidemia

- Ocular Candidiasis

- Candida arthritis, Osteomyelitis, Costochondritis, Myositis

- Candidiasis of Peritoneum, Liver, Spleen and Gall bladder

ORAL CANDIDIASIS

Oral candidiasis is the most prevalent opportunistic infections affecting the oral mucosa. 57% of the HIV infected patients and 90% of the AIDS patients develop oral candidiasis.[54]

Predisposing factors for oral candidiasis[9, 55]

Local factors	**General factors**
Denture wearing	Chemotherapy
Smoking	Hematologic malignancies
Inhalation steroids	Immunosuppressive drugs/diseases
Hematologic malignancies	Endocrine disorder

Oral candidiasis types[9, 17, 55]

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

Oral Colonization with inherently drug resistant organisms is more common in advanced HIV infection (CD4 <50 cells/µl).[56] Candidal infection in AIDS is almost exclusively mucosal, systemic invasion is rare and late event.[57] Unexplained oral candidiasis in previously healthy adults has been considered an important clinical predictor of AIDS as well as highly predictive of worsening immunodeficiency.[57]

Up to 90% of persons with advanced untreated HIV infection develop Oropharyngeal Candidiasis with 60% having at least one episode per year with frequent recurrences (50-60%). 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.[9, 58]

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 species may cause the disease. Recurrent disease is caused by the same strain of Candida in about 50% of cases; remaining 50% are caused by the new strains of Candida albicans or new species.[53] 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.[9]

LABORATORY DIAGNOSIS OF ORAL CANDIDIASIS

SPECIMEN COLLECTION

Specimens from patients with oral candidiasis can be collected by swabbing or scraping the surface of the lesions under aseptic precautions.[17]

DIRECT EXAMINATION[17]

The preferred method for the direct microscopic examination of oral candidiasis includes (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 species normally colonizes 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 pseudohyphae in clinical specimens.

CULTURAL CHARACTERISTICS:

Candida species grow on almost all common laboratory media particularly blood agar and Sabourauds dextrose agar with antibacterial antibiotics for primary isolation.[17]

ISOLATION OF CANDIDA

Sabourauds Dextrose Agar with antibiotics

The routine medium used for isolation of fungi in culture from mucocutaneous infections is Sabouraud dextrose agar supplemented with antibiotics like Gentamycin, Chloramphenicol or Tetracycline to prevent bacterial overgrowth. 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.[17]

Cultures can be incubated at 37^0 C. Candida colonies appears in two to three days and more than three days for some Candida species like C. guilliermondii and C. glabrata.[17]

Growth in Sabouraud 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.[17]

SPECIATION OF CANDIDA

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. The principle of the test is the ability of Candida albicans or Candida dubliniensis to produce blastospores when incubated in serum at 37^0C for two hours. It helps in the presumptive

identification of Candida albicans or C. dubliniensis. A true germ tube has no constriction at the point of origin. These are also called hand mirror forms.[17, 54]

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, [24] egg albumin, Thioglycollate broth and various peptone medium like Trypticase soya broth, GSOM (Glucose, Serine, Ornithine, Methionine) medium, Lee's medium, egg white, CSF and plasma at 37^0C for 2 hours.[47, 59]

Factors affecting germ tube formation-growth medium, inoculum size, incubation temperature, concentration of simple carbohydrates, microaerophilic conditions presence of albumin and presence of peptide fraction.[60]

Germ tube test is positive if 30% of yeast cells produce germ tubes.

False positive results occur on prolonged incubation by elongated blastoconidia. Pseudogerm tube has constriction at the origin of the filamentous structure seen in C. tropicalis, C. parapsilosis.[61]

>5% of C. albicans are germ tube test negative.[61]

A new Monoclonal Antibody 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 Immuno-Fluroscence 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.[62]

Cornmeal Agar

The commonly used differential medium for speciation is the Corn meal Agar plate supplemented with Tween 80 (Polysorbate 80).[24]

In 1960, Walker and Huppert modified the basic formulation by adding polysorbate 80 which stimulated rapid and abundant chlamydospores formation and could appreciate better morphology of yeasts.[63]

Kelly and Funigello showed addition of 1% Tween 80 enhances chlamydospore formation.[64]

Subcultures made by Dalmau plate technique on the Corn meal agar plates and incubated at 28^0C for 2-5 days. After 2-5 days of incubation, plates are examined directly under the microscope for the presence of pseudo hyphae/ true hyphae, chlamydospores, arthroconidia and blastoconidia. Chlamydospore formations are seen in the isolates of C. albicans and C.dubliniensis. C.dubliniensis often produces chlamydospores abundantly in clusters.

Plates are never incubated at 37^0 C, may result in failure of chlamydospore formation.[24]

Positive control- C. albicans ATCC 10231[63]

Negative control-Saccharomyces cerevisiae ATCC 9763[63]

Other media used for chlamydospore production are Rice starch agar with Tween 80, Casein milk agar, Staib agar, Caffeic acid, Ferric citrate agar.[17]

Biochemical Characterization

Biochemical speciation of Candida is Fermentation tests and Assimilation tests. Candida species can utilize carbohydrates both anaerobically (fermentation)

and oxidatively (assimilation) .Yeasts possessing the ability to ferment a given carbohydrate does also assimilate that substance but not necessarily vice versa.[17]

1. **Sugar fermentation test:**

Yeast contains specific enzyme system for anaerobic degradation of specific carbohydrates with the production of acid and gas as end products. Yeasts metabolize carbohydrates via Embden Meyerhoff pathway in anaerobic conditions. The classic tests involved liquid media supplemented with different carbohydrates, a color 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 color in the fermentation fluid are considered as the indicator of positive fermentation. [17]

CANDIDA SPECIES	***GLUCOSE***	**MALTOSE**	**SUCROSE**	***LACTOSE***
C. albicans	AG	AG	-	-
C. tropicalis	AG	AG	AG	-
C. guilliermondii	AG	-	AG	-
C. parapsilosis	AG	-	-	-
C.krusei	AG	-	-	-
C. glabrata	AG	-	-	-
C. kefyr	AG	AG	AG	-

2. **Sugar assimilation tests:**

Sugar assimilation determines the ability of particular yeast to utilize a particular carbohydrate as the sole source of carbon in the presence of oxygen.[63] Carbohydrate utilization patterns are the most commonly used conventional methods for the definitive identification of yeast recovered in a clinical laboratory.[65]

Various techniques:

1. 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.[64]

2. 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.[47]

3. Commercial systems and kits

Modification of the auxanographic assimilation technique and have replaced the conventional methods. [24, 64, 65]

Commonly used are[24] - API 20c

- API 32c
- Micro-drop assimilation test system
- Uuni-yeast-tek system
- Vitek
- Minitek
- Candifast
- Candicheck kit
- Mycotube
- Auxodisk
- Fungichrome
- Microscan yeast identification panel

CHROM AGAR MEDIUM

CHROMagar is a selective and differential medium for identification of yeast directly from clinical samples. This medium allows selective isolation of yeasts and simultaneously identifies species of Candida.[66] This medium contains chromogenic substrate which reacts with specific enzymes secreted by yeast and produces colonies of various pigmentation. It also facilitates the detection and identification of yeasts from mixed cultures and provides results within 24-48 hr sooner than standard isolation methods.[67, 68]

In 1996, M A Pfaller, A Houston and S Coffmann were able to identify 95% of C. albicans, 100% of C. tropicalis and C. krusei, 94% of C. glabrata accurately on CHROMagar. 92% of yeast was able to grown on CHROM agar and indeed 94% of yeast was grown on SDA. They found that 18% of positive cultures contained mixtures of yeasts and 47% of these mixed culture were not able to detect with SDA alone.[66]

In 2006, Duane R Hospenthal showed colours of the colonies appeared intense at 37^0 C and intensified daily peaking at 72hrs. Most of the non-Candida isolates produced variable shades of ivory, pink and lavender.[69]

Ribot W R, Kirkpatrick et al suggests a higher likelihood of developing OPC in patients receiving concomitant chemoradiotherapy. Chromogenic media is helpful to screen for relatively, commonly occurring non- albicans yeasts causing OPC in head & neck cancer patients.[70]

Hiroshi Isogai et al reported that there was no significant difference of CFU among symptomatic and asymptomatic patients. Most frequently isolated species

were C. albicans followed by C. tropicalis (80%), C. krusei (70%), C. parapsilosis (30%), C. guilliermondii, C. glabrata and C. dubliniensis (15%).[71]

CANDIDA SPECIES	COLOR OF COLONIES
C. albicans	Light green
C. tropicalis	Blue with pigment diffusion
C. guilliermondii	Small pink- purple
C. parapsilosis	Pale pink
C.krusei	Pink
C. glabrata	Dark pink to purple
C.dubliniensis	Dark green

Tetrazolium reduction medium

This medium is used to differentiate various Candida species. Tetrazolium is reduced to different gradients by the species of Candida.[24]

Species	Growth on Tetrazolium reduction medium
C. albicans	Pale pink
C. tropicalis	Maroon
C. guilliermondii	Pink and pasty
C. parapsilosis	Rose pink
C.krusei	Pink and Dry
C. glabrata	Pale pink
C. krusei	Salmon pink

Immunodiagnosis[17, 26]

Antibody detection

Tests used to detect antibodies against Candida are Gel Immunodiffusion (ID), Counter-ImmunoElectrophoresis (CIE), Enzyme Linked Immunosorbent Assay (ELISA), Latex agglutination test.

These tests are oriented to detect anti-mannan antibodies and antibodies against internal antigens (cytoplasmic antigens, enolase, Hsp 90).

False negative results are the major limitations of these tests due to less sensitivity and poor immune status of the patients.

Antigen detection

Detection of mannan using commercial ELISA, Pastorex Latex agglutination systems incorporating monoclonal antibodies (Sansosi diagnostics). Detection of glycoprotein antigen using Cand-Tec Latex agglutination test (Ramco laboratories).

MOLECULAR METHODS[17]

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.

vi) Pyrolysis mass spectrometry

Treatment for Oropharyngeal candidiasis in HIV patients[72]

a) Early uncomplicated infection

- Topical Clotrimazole 10mg troches, 4-5 times/day for 14 days
- Nystatin suspension 4-6 lakh units/ml, 4-6 times/ day for 7-14 days
- Amphotericin B suspension

b) Systemic therapy

- Tab Fluconazole 200-400mg
- Tab Ketoconazole 200-400mg
- Tab Itraconazole 200-400mg

} for 10-14 days

c) Fluconazole refractory OPC

- Tab Itraconazole
- Amphotericin B oral suspension

d) Severe refractory OPC

- IV Amphotericin B

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.[9, 73]

ANTIFUNGAL SUSCEPTIBILITY TESTING[74]

Methods used for in vitro antifungal susceptibility testing of Yeasts are

1) Agar based method

1. Disk Diffusion
2. Agar macrodilution
3. E-test

2) Broth based methods

1. Macrodilution
2. Microdilution

3) Others[24]

EUCAST method

Spectrophotometric

Flowcytometer

Fungi test

Sensititre Yeastone Test Panel (TREK)

E-yeast broth microdilution method

The CLSI, Pennsylvania, USA, 2002 subcommittee on Antifungal susceptibility testing has developed reference methods for broth macro and microdilution susceptibility testing of Yeast (CLSI M27-A2 document) and more recently a disk diffusion method for Yeasts (CLSI M44-A document) 2003.

Broth microdilution[74]:

The broth microdilution has become the most widely used technique, it is cumbersome to adapt. CLSI recommends RPMI 1640 medium with L-glutamine, without sodium bicarbonate and buffered with Morpholine Propane Sulfonic acid at 0.165mol/L as the test medium .Yeast inocula standardized spectrophotometrically and diluted in RPMI medium to obtain a final concentration of 0.5 to 2.5 X 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 titer 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 35^0C for 48 hrs for Candida.

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.

Drug dilution range

1) Fluconazole - 64 to 0.12 µg/ml

2) Amphotericin, Itraconazole - 16 to 0.03 µg /ml

3) Ketoconazole - 0.0313 to 16 µg /ml

4) Voriconazole - 0.008 to 16 µg /ml

Interpretation[74]:

Broth dilution well read with reading mirror and growth in each well compared with that of the growth control.

0- Optically clear

1- Slightly hazy

2- Prominent reduction in turbidity compared with drug free growth control

3- Slightly reduction in turbidity compared with drug free growth control

4- No reduction in turbidity compared to control

MIC for Amphotericin- score 0

MIC for Azoles- score 2

Agar based methods[74]

Disk Diffusion method

Agar disk diffusion is a simple, flexible and cost effective alternative to broth dilution testing. CLSI subcommittee has proposed a standard disk diffusion method for susceptibility testing of Candida species to the Fluconazole 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 inoculum size of 0.5 Mcfarland standards and incubation temperature of 35^0 C for 20 to 24 hr and some strains may require 48 hours incubation. Addition of a low concentration of methylene blue (0.5µg/ml) makes the zones of inhibition clearer and easier to measure precisely.

Agar based alternative approaches[74]

1) Neosensitabs tablets (Dutec diagnostics) – agar diffusion method

Tablets of established and some of the new antifungal agents are available.

Preliminary comparisons with both M27-A2 and M44-A methods have provided promising results.

2) Epsilometer test (E-test) [75]

E-test uses a non-porous plastic strip immobilized 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.

ANTI FUNGAL DRUG RESISTANCE

Oral azoles are the drugs more extensively used in the therapy of oral candidiasis in HIV infected patients. Despite their pharmacological and clinical effectiveness, persistent and/ or recurrent episodes of oral candidiasis are observed in HIV infected patients. Even though most respond to short course of Azole therapy, up to 50% of patients will experience a relapse within 1 month after the completion of therapy. Currently, the number of patients who experience multiple recurrences of mucosal Candidal infections and eventually fail to respond to azole therapy is rising.[74]

Oral candidiasis not responsive to Fluconazole has been reported both in HIV infected and in other immunocompromised patients. Consequence of this is the emergence of Fluconazole resistant strains of Candida especially C. glabrata and C.krusei.[76]

Risk factors suggested to be of importance in the development of Fluconazole-resistant mucosal Candidal infection include duration of exposure to Fluconazole and degree of immunosuppression.[75, 76]

Other possible reasons for the failure of Azole therapy includes inadequate patient compliance, decreased drug absorption or increased drug metabolism due to the use of concomitant medications, infection with an azole resistant organism, selection or induction of resistance in the infecting organism during therapy.[75] Furthermore , several studies indicates that failure of therapy due to selection or induction of resistance is becoming more common and demonstrated a correlation between invitro Fluconazole resistance of Candida species and clinical failure.[76,77,78,79]

The antifungal drug resistance can be divided into two types[17]:

(a) Clinical resistance: In-vivo resistance

(b) InVitro resistance: a) Intrinsic / Primary resistance

b) Acquired / Secondary Resistance

Clinical Resistance[9, 17]

Clinical resistance is defined as persistence or progression of an infection despite appropriate antimicrobial therapy. 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.

Intrinsic Resistance

Resistance is considered primary when an organism is resistant to the drug before exposure. 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. Patients on Fluconazole suppressive therapy are more likely to have infection caused by non –albicans species such as C. krusei, C. glabrata,

C. inconspicua and C. norvegenesis which are intrinsically resistant to Fluconazole.[80,81]

Acquired /Secondary Resistance

Secondary resistance is that which develops in response to exposure to the drug, i.e., resistance occurs when a previously susceptible isolate develops a resistant phenotype, usually as a result of prolonged treatment with antifungals. This mechanism of resistance accounts for the emergence of resistance to azoles , increased use of the Azoles, coupled with the fact that they are fungistatic drugs has likely resulted in the emergence of resistance to Azoles. [9, 17]

Mechanism of drug resistance

There are several possible mechanisms of resistance to Azole antifungal agents.

1. Failure to accumulate drug intracellularly may result either from lack of drug penetration due to change in membrane lipids or sterols or perhaps more commonly active efflux of drug. The upregulation of these multidrug efflux pumps results in multidrug- resistant phenotype strains. Two different types of efflux pumps are seen 1. Adenosine triphosphate-binding cassette (ABC) transporters encoded by the CDR genes (CDR1 and CDR2) and major facilitators encoded by the MDR genes. Overexpression of MDR1 genes results in exclusive Fluconazole resistance.[7, 75]

2. Increased production of the target enzyme 14α-demethylase leads to increased metabolism of drugs.[24]

3. Point mutation of the 14α-demethylase gene, potentially leading to diminished affinity of Azoles for the enzyme.[7]

4. Alteration in membrane sterol and/or lipid content also confers resistance. Several resistant isolates have been found to accumulate nontoxic 14α-methyl fecosterol instead of the toxic compound 14α-methyl-3, 5-diol suggesting that these organisms have a mutation in the sterol -5-6-denaturase.[82]

5. Production of Candida Biofilms also poses antifungal drug resistance by restricting penetration of antifungals due to exopolymeric material. Growth rate has been considered as an important modulator of drug activity in bacterial biofilms. Biofilms are thought to grow slowly because nutrients are limited, resulting in decreased metabolism of the microorganisms. A slow growth rate is frequently associated with the adoption of a different phenotype by organisms such as change in cell envelope, which in turn affect the susceptibility of the Candida to antifungals. In addition, synthesis of new proteins occurs after C. albicans attaches to surfaces, which suggests that drug resistance might also arise as a consequence of specific surface-induced gene expression.[83]

MATERIALS AND METHODS

STUDY PERIOD

This is a cross sectional study undertaken over period of December 2010-December 2011

STUDY PLACE

This study was carried out at the Department of Microbiology, BLDEU'S Sri B M Patil Medical college and research centre, Bijapur, Karnataka.

ETHICAL CONSIDERATIONS

The study was reviewed and approved by the Institutional Ethical Committee, BLDEU'S Sri B M Patil Medical College and Research Centre, Bijapur, Karnataka.

STUDY GROUP

Study group includes 118 HIV positive patients presenting with oral candidiasis from all departments of our hospital.

Inclusion Criteria

1. HIV seropositive patients including both males and females of all age group: Seropositivity status of the patients was determined as per NACO guidelines.

2. Patients with clinical picture of candidiasis on the oral/pharyngeal mucosa.

3. Not on any antifungals within one month of the study.

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

SPECIMEN COLLECTION[16, 84]

Oropharyngeal specimens were collected by firmly swabbing the lesion site with two sterile cotton swabs under strict aseptic precautions. Samples were transported immediately to the laboratory and subjected to various mycological tests.

SPECIMEN PROCESSING[17, 24]

Specimens collected were subjected to standard mycological procedures.

Direct Microscopic examination[17, 24]

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 or 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.

Culture on Sabourauds Dextrose Agar[17]

The culture medium used was Emmon's modified Sabourauds Dextrose Agar (supplemented with antibiotics like Gentamycin 5µg and Chloramphenicol 50µg) with pH 6.6 to prevent bacterial overgrowth. Second swab was inoculated immediately into the plates and incubated at 37^0C for 24-72 hours.

Isolates were identified by colony morphology on SDA plates. Growth appears in 2 to 3 days as creamy, white pasty colonies. From the culture Gram's stain done to note the microscopic morphology.

Species identification

Isolates were speciated based on the following tests:

Germ Tube Test (Reynolds-Braude phenomenon) [24]

A small portion of an isolated colony of the yeast to be tested was inoculated into the 0.5ml human serum and incubated at 37^0C for two hours.

After two hours of incubation, a drop of the yeast serum suspension was placed on a glass slide, overlaid with a cover slip and examined microscopically for the presence of germ tube under low power microscope.

Test said to be positive , if tube like extension from the parent cell half the width and three to four times the length and no constriction at the point of attachment to yeast cell is seen in >30% of total yeast cells within 2hrs of inoculation, the isolate was considered presumptively as Candida albicans / Candida dubliniensis.[24]

Growth at 42^0C[73, 84]

All germ tube positive isolates were sub cultured on SDA and incubated aerobically at 42^0C, to distinguish between Candida albicans and Candida dubliniensis.

	Candida albicans	***Candida dubliniensis***
Growth at 42^0C	Present	Scanty / Absent

Corn Meal Tween 80 Agar (Dalmau Plate Culture Technique)[24]

An isolated colony from the primary culture media was picked using a straight wire and inoculated into cornmeal agar plate containing 1% Tween 80 at 45^0 angles to the culture media.

A sterile cover slip was placed over the surface of the agar, covering a portion of the inoculated streaks. The streak project beyond cover slip provides partial anaerobic environment. Plate was incubated at 28^0C for 48 hours.

The areas where the agar was streaked were examined first under the low power objective and then under the high power objective.

Observation – Chlamydospore: Large, highly refractile thick walled cell, single or multiple, terminal or intercalary seen in C. albicans and C.dubliniensis.

Based on typical morphological arrangement of pseudohyphae and blastoconidia as explained in table below, species are identified.

Morphology and microscopy on SDA and CMA[24, 32, 54, 82, 85]

Species	Morphology and microscopy on SDA	Morphology and microscopy on CMA
C.albicans	White, cream, soft smooth to wrinkled pasty colonies. Grow at 42^0 C. Microscopy- oval, globose budding yeast cells.	Abundant branched pseudohyphae with 3.5-6 x 4-8 µ blastoconidia in clusters along pseudohyphae and majority at septa. Chlamydospore- spherical thick walled refractile structures arranged singly of size 8-12µm.
C.dubliniensis	White cream soft smooth to wrinkled colonies. No growth or pools growth at 42^0 C. Microscopy – globose, oval yeast cells.	Abundant branched pseudohyphae with 3.5-6 x 4-8 µ blastoconidia. Produce abundant Chlamydospores arranged in singles,chains or clusters terminal or intercalary.
C. tropicalis	Cream White glistening to dull soft smooth wrinkled with mycelial border.	Long pseudohyphae branch abundantly. Blastospores oval single or in small groups (sparse) at point of constriction.
C. guillermondii	Flat moist smooth cream thin dull colony.	Fine pseudohyphae, ovoid blastoconidia arranged in clusters along the pseudohyphae.
C. kefyr	Cream to brown glossy smooth dull colonies.	Elongated few blastoconidia with abundant pseudohyphae lying in parallel (log in stream appearance).
C. krusei	Off- white dull smooth flat dry wrinkled colonies with lateral fringe. Grow at 42^0 C.	Elongated few blastoconidia with abundant pseudohyphae with cross match stick appearance.
C. glabrata	White smooth Grow at 42^0 C.	Only blastoconidia of 2.5-4 x 3-6µ, no pseudohyphae.
C. parapsilosis	White cream soft smooth to wrinkled colonies. Develop lacy pattern satelliting 'spider colonies' along streak line. Microscopy – oval elliptical elongated.	Blastoconidia single / small clusters along pseudohyphae which are curved with large regularly branched hyphal elements.gaint cells seen. Pine forest or sage brush appearance.
C. fomata	White- cream soft smooth colonies.	Small blastospores, no pseudohyphae.
C. vishwanathii	No typical morphology.	No typical features.
C. rugosa	White cream smooth.	Branching pseudohyphae.
C. pelliculosa	Cream soft smooth glistening butyrous.	Pseudohyphae seen.
C. lusitaniae	White- cream smooth.	Abundant pseudohyphae wavy, blastoconidia at septa or between septa.

Sugar Assimilation test[24]

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 of 4MF.The suspension was added to 18 ml of molten agar cooled to 45^0 c and mixed well and the entire volume was poured in to a 90 mm sterile petriplates. The plate was allowed to set at room temperature until the agar surface sets. With the help of sterile forceps, carbohydrate discs [Hi-media] Sucrose, Maltose, Trehalose, Xylose, Lactose, Cellibose, Dulcitol were placed on the surface of the inoculated agar. The plates were incubated at 35^0 c for 7 days.

Observation - Growth of yeast colonies around the sugar discs, indicating assimilation of that particular carbohydrate. The species identified as table[24] below**.**

Species	**Glu**	**Mal**	**Suc**	**Lac**	**Celli**	**Gal**	**Tre**	**Raffi**	**Melli**	**Xyl**	**Ino**	**Dul**
C. albicans	+	+	+	+	+	+	+	-	-	+	-	-
C. tropicalis	+	+	+	-	+	+	+	-	-	+	-	-
C. guilliermondii	+	+	+	-	+	+	+	+	+	+	-	+
C. kefyr	+	+	+	+	+	+	-	+	-	+	-	-
C. krusei	+	-	-	-	-	-	-	-	-	+	-	-
C. glabrata	+	-	-	-	-	-	+	-	-	+	-	+
C. parapsilosis	+	+	+	-	-	+	+	-	-	+	-	-

Antifungal Susceptibility testing[74]

Antifungal susceptibility test for the *Candida* isolates was done by Disk Diffusion method according to CLSI M44-A2 document.

The inoculum suspension was prepared by picking four- five colonies from a 24 hour old culture and suspended in 5ml sterile 0.85% NaCl. Turbidity matched to 0.5MF.

Medium:

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

Procedure:

Within 15 minutes after adjusting the turbidity of the inoculum suspension, sterile cotton swab was dipped into the 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 60^0 each time to ensure an even distribution of inoculum. 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 sterilized forceps; the antifungal discs were applied on the surface of the inoculated plates.

The following antifungal discs were used

Fluconazole 25µg

Ketaconazole10µg

Itraconazole 10µg

Voriconazole 1µg

The plates were inverted and incubated at 35^0 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.

Interpretation

Drug	Susceptible	SDD	Resistant
Fluconazole (25µg)	≥19mm	15-18mm	≤ 14mm
Voriconazole (1µg)	≥42mm	31-42mm	≤30mm
Ketoconazole (10µg)	≥20-32	-	≤19mm
Itraconazole (10µg)	≥ 20mm	12- 19mm	≤ 11 mm

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

Resistant: No zone of inhibition.

Following standard strains were tested each time to ensure quality control

Candida albicans ATCC 90028

Candida tropicalis ATCC 750

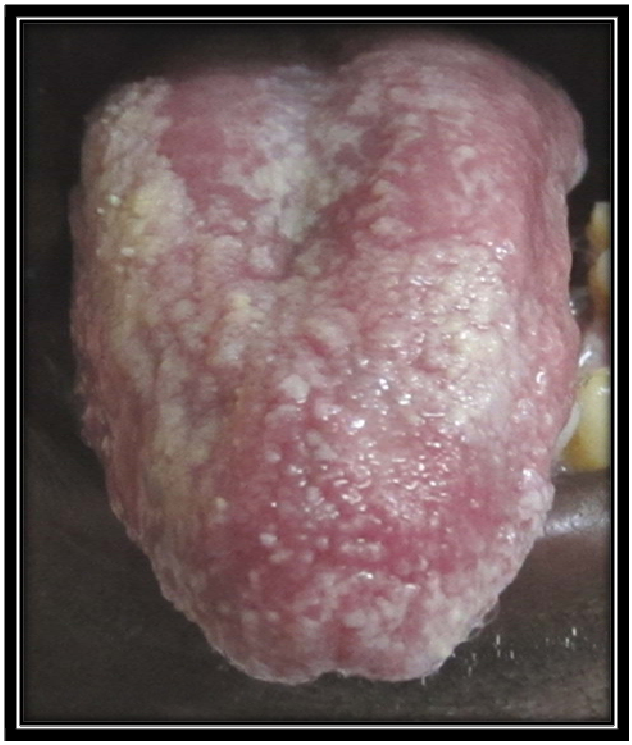


Figure 1: Oral Candidiasis Pseudomembranous type

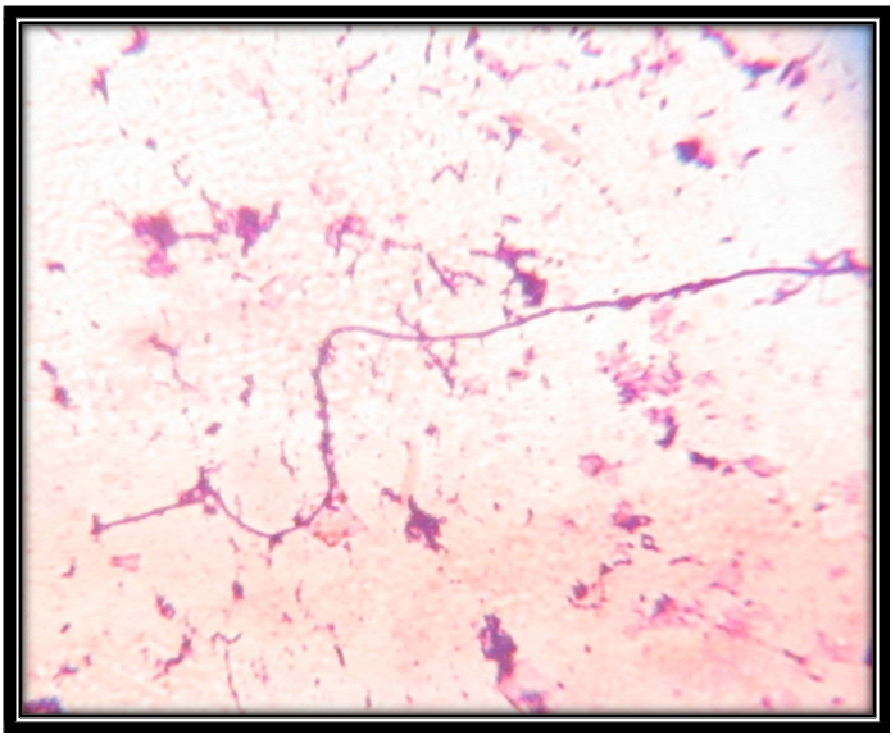


Figure 2: Gram's staining showing budding yeast cells with pseudohyphae.

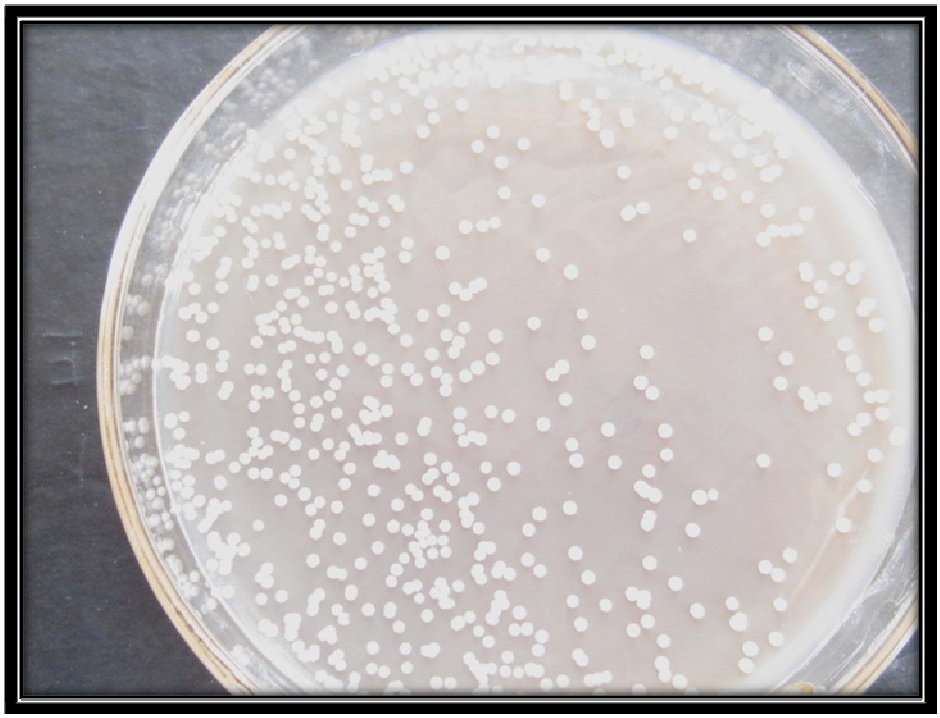


Figure 3: Growth on Sabouraud Dextrose Agar

Figure 4: Dalmau Plate Culture on Corn meal agar

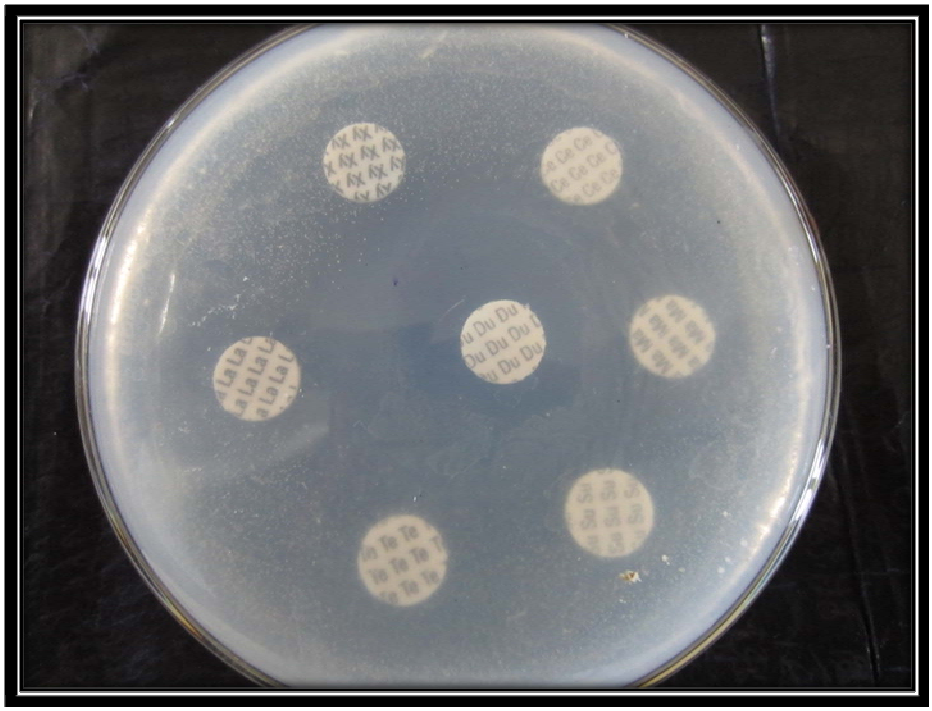


Figure 5: Auxonographic sugar assimilation test

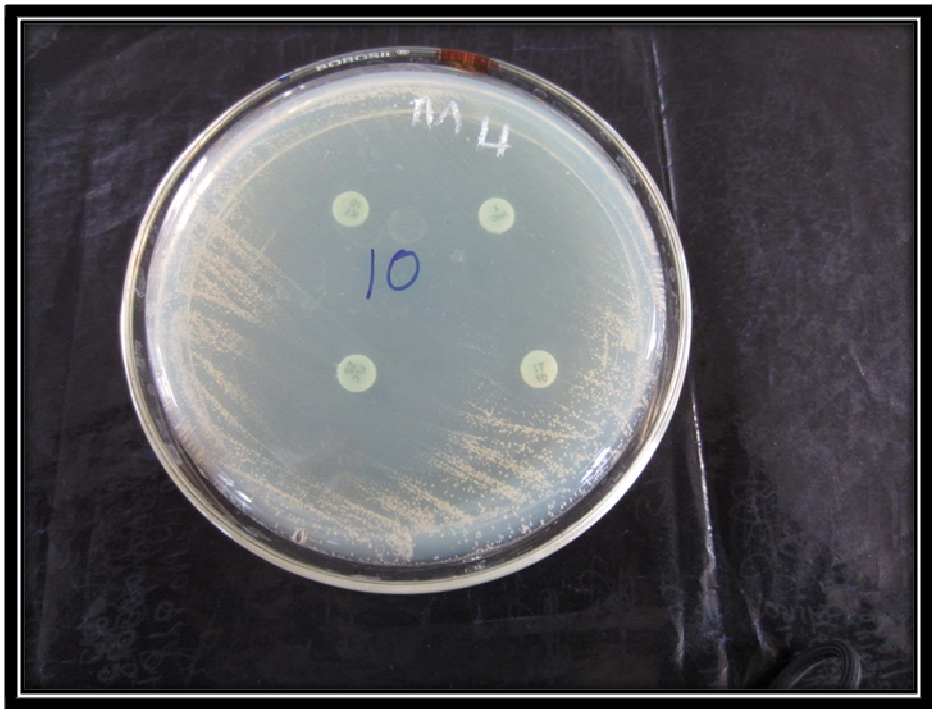


Figure 6: Anti-Fungal susceptibility testing by Disk diffusion method

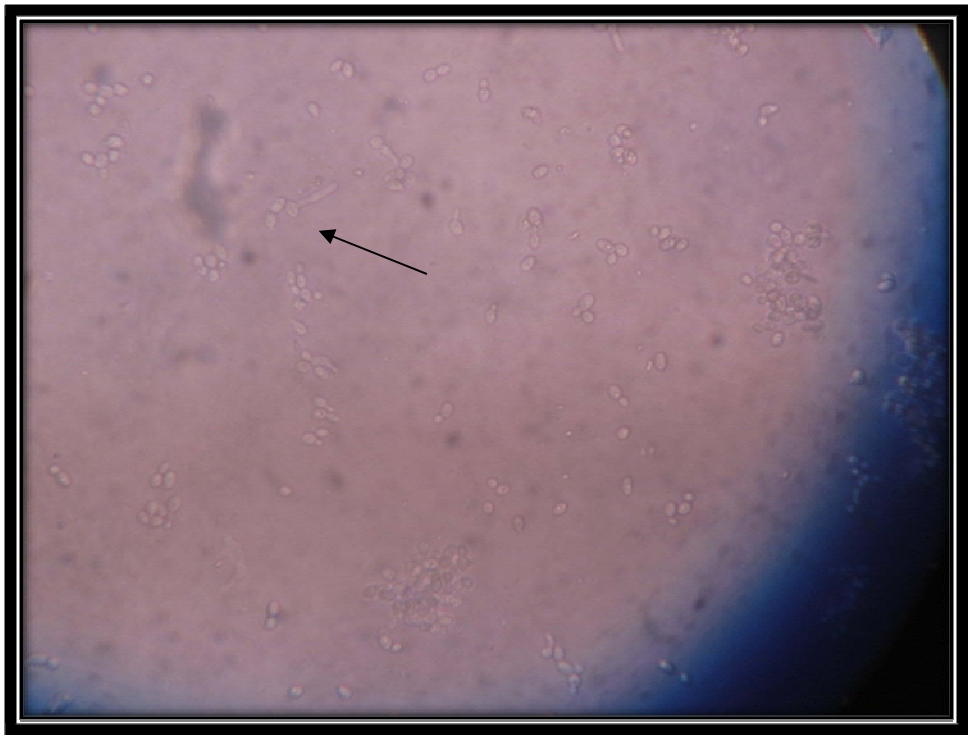


Figure 7: Germ Tube Formation

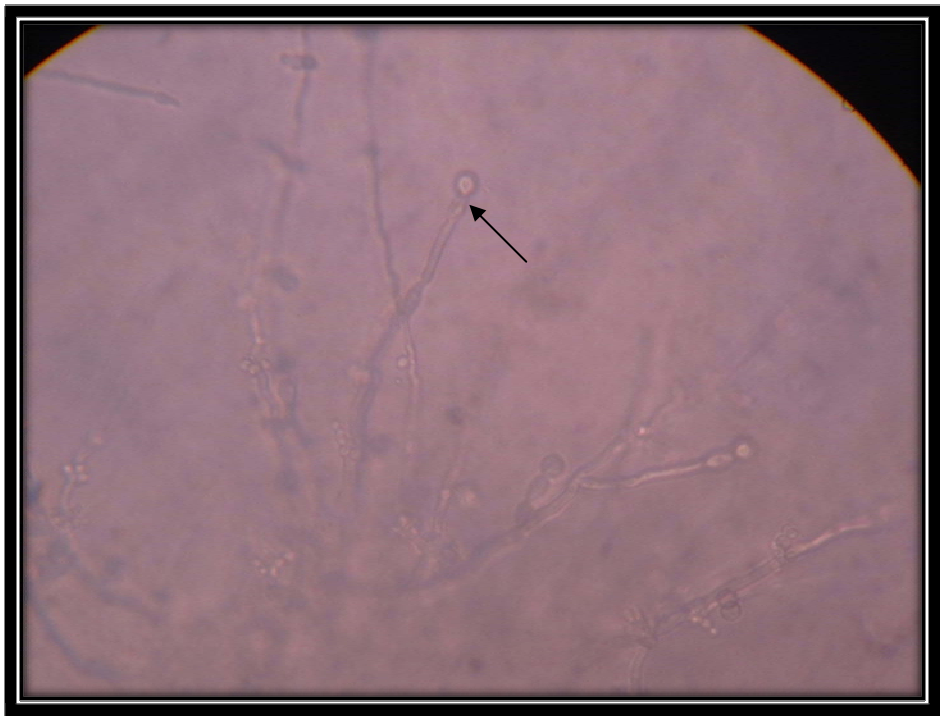


Figure 8: Terminal Chlamydospores formation of C. albicans

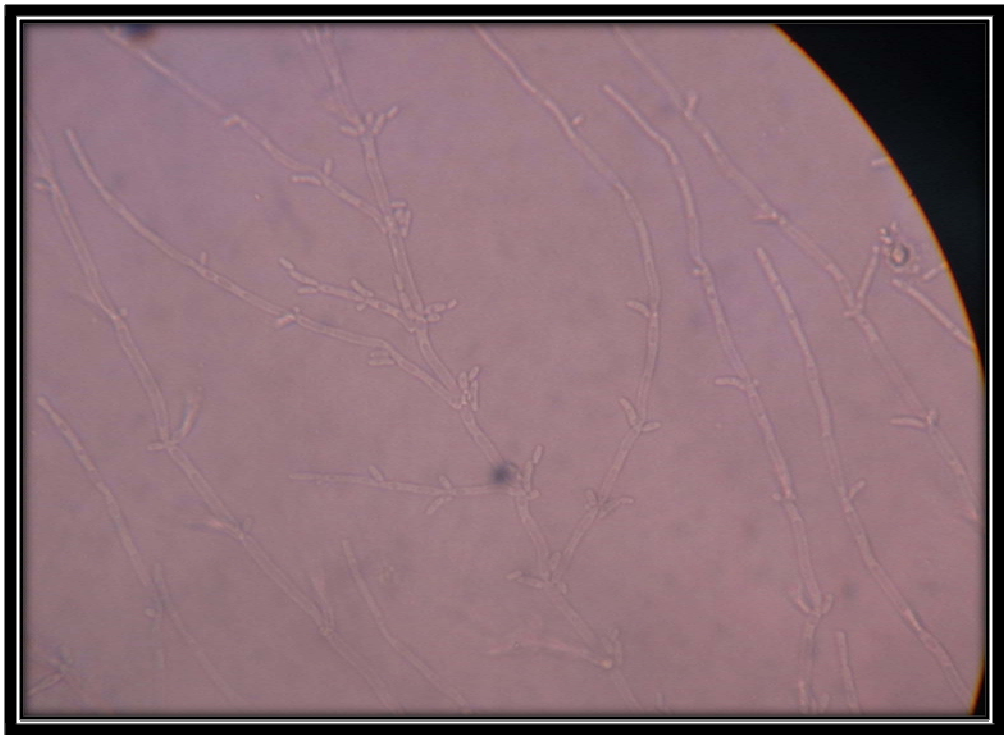


Figure 9: Microscopic appearance of C .tropicalis on CMA

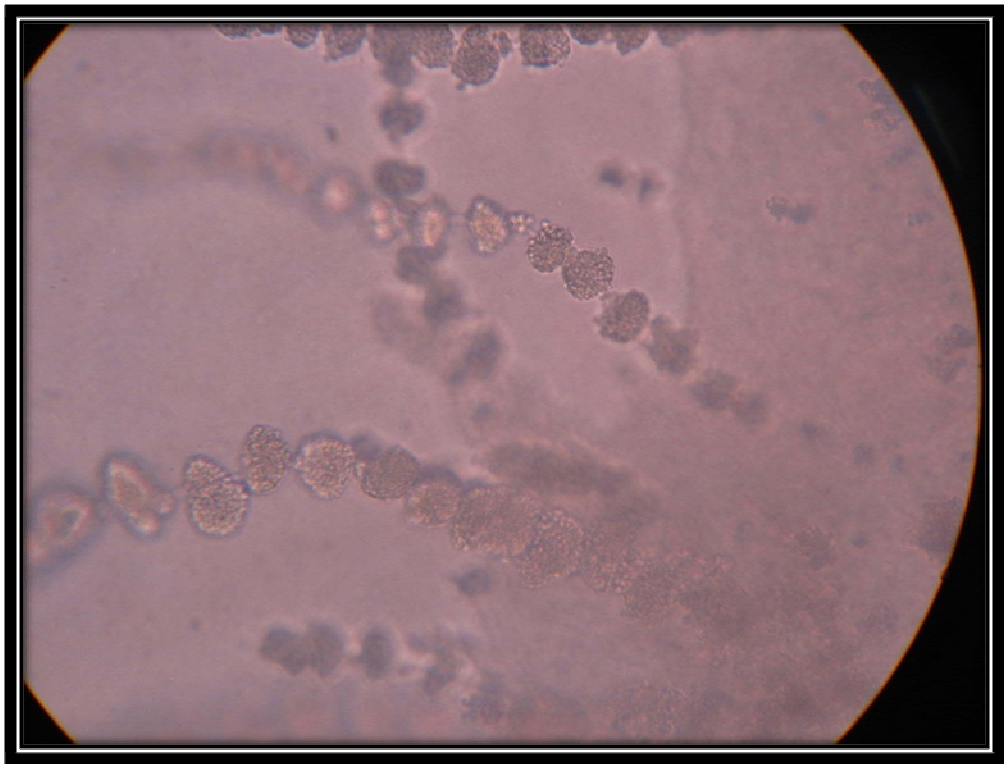


Figure 10: Microscopic appearance of C. guilliermondii on CMA

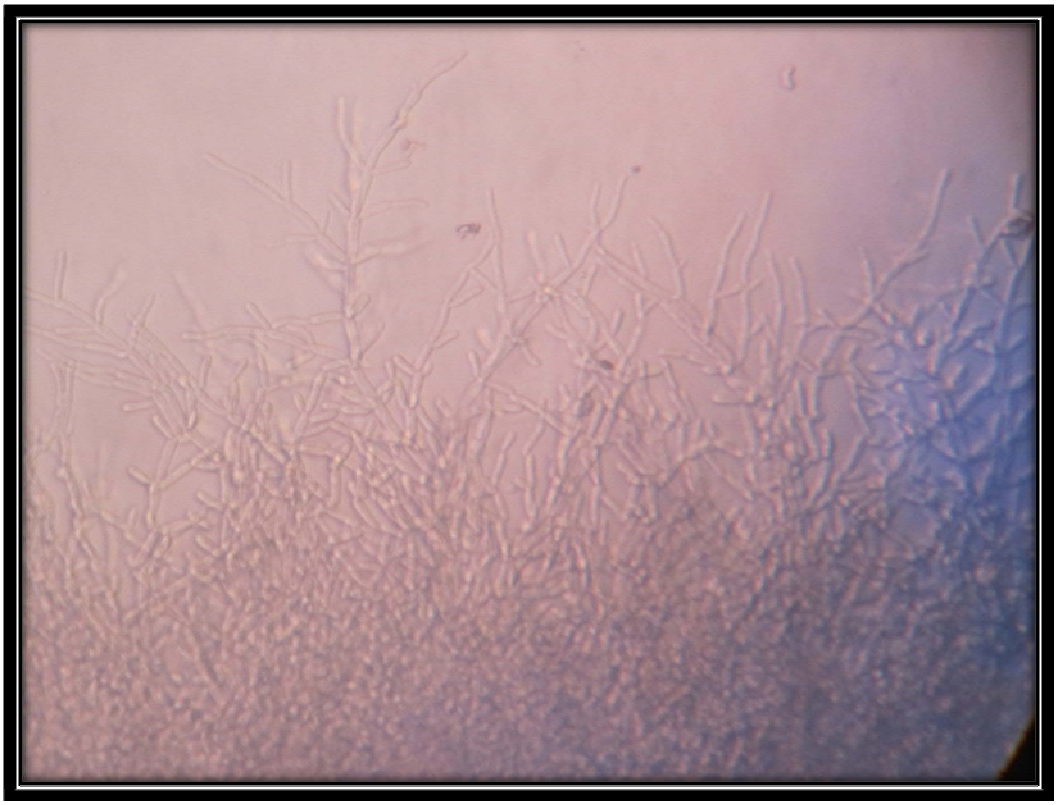


Figure 11: Microscopic appearance of C.krusei on CMA

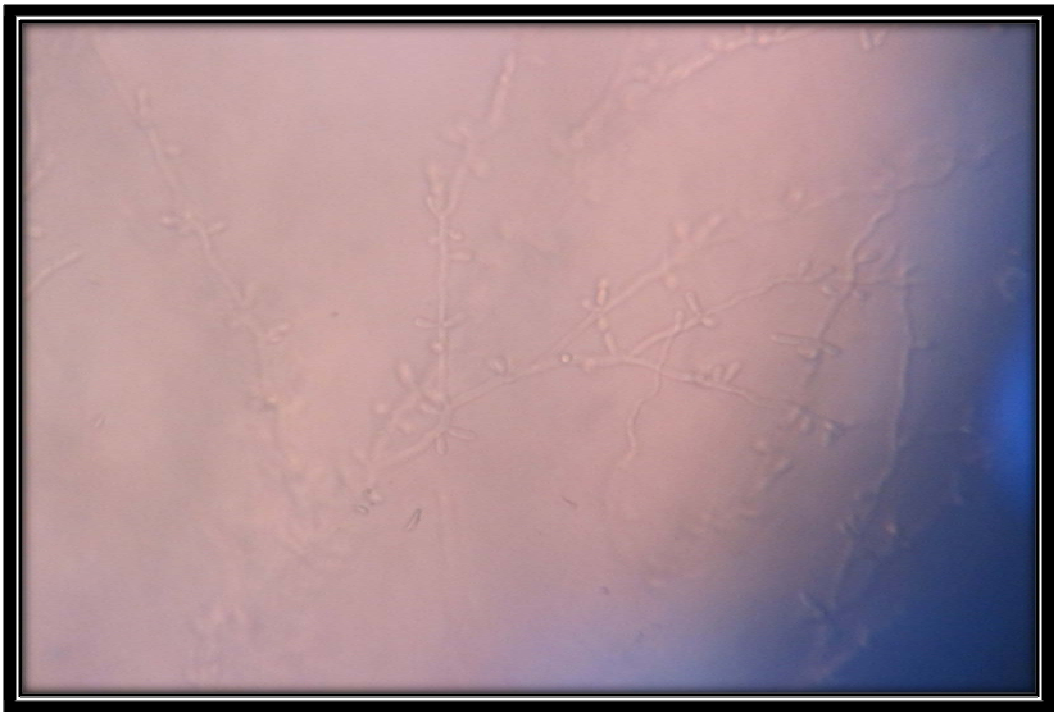


Figure 12: Microscopic appearance of C. kefyr on CMA

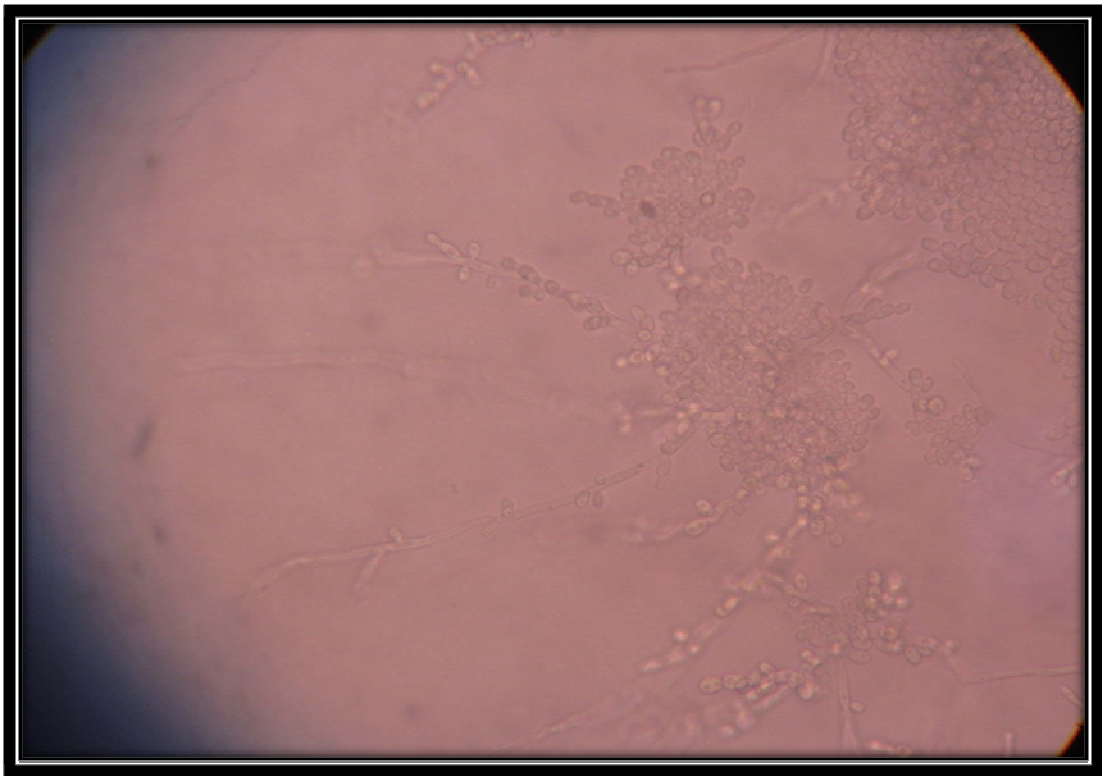


Figure 13: Microscopic appearance of C. parapsilosis on CMA

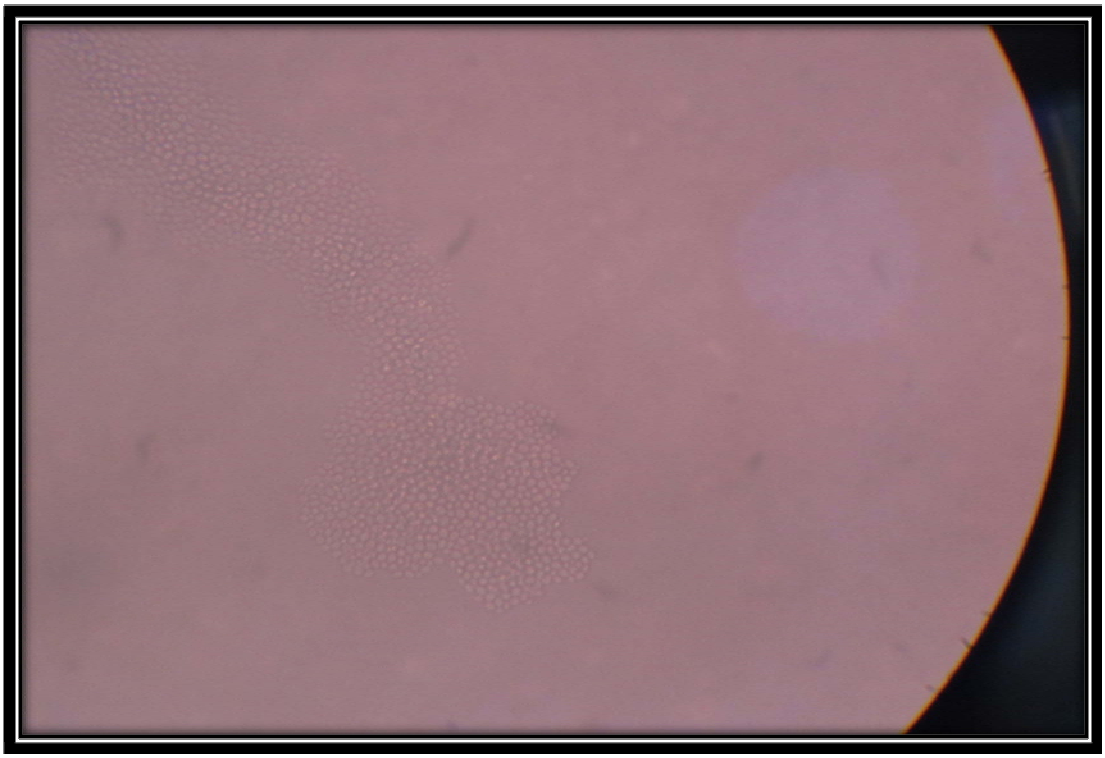


Figure 14: Microscopic appearance of C. glabrata on CMA

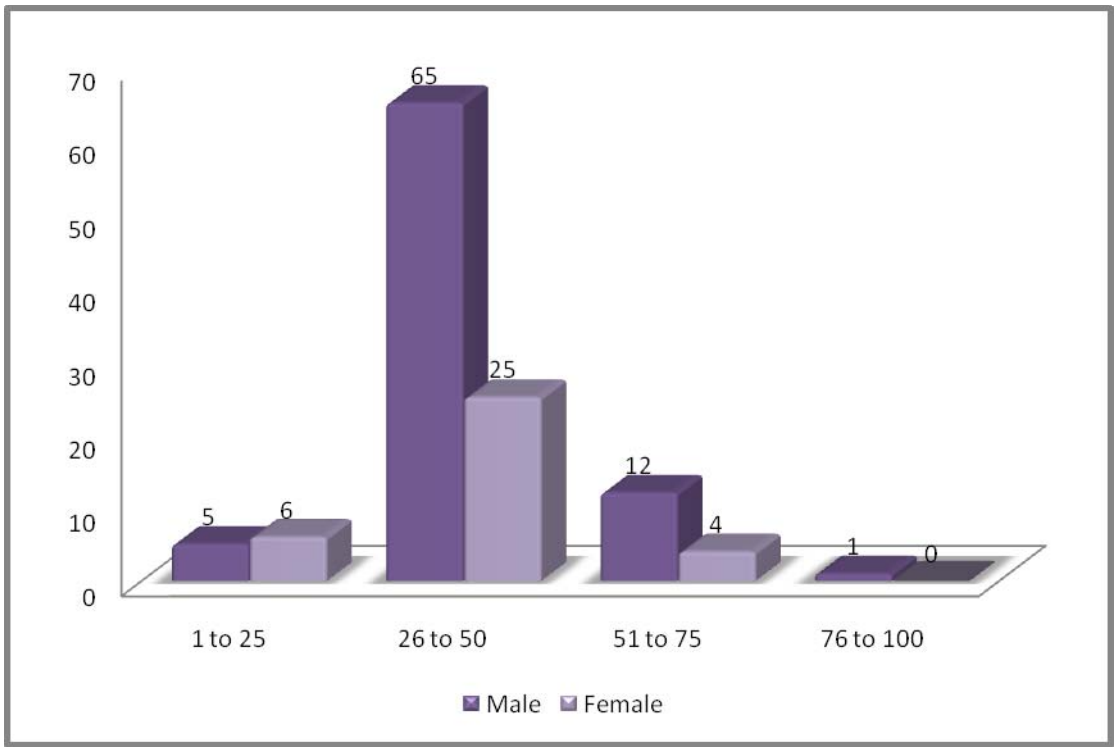
RESULTS

Table 13: Age wise distribution

Age	Male		Female		Total
	Number	Percentage	Number	Percentage	
1-25	5	6.02%	6	17.14%	11
26-50	65	78.31%	25	71.42%	90
51-75	12	14.45%	4	11.42%	16
76-100	1	1.20%	0	0.00	1
Total	83	100%	35	100%	118

Mean age of the study is 44.25 years.

Figure 1: Age wise distribution

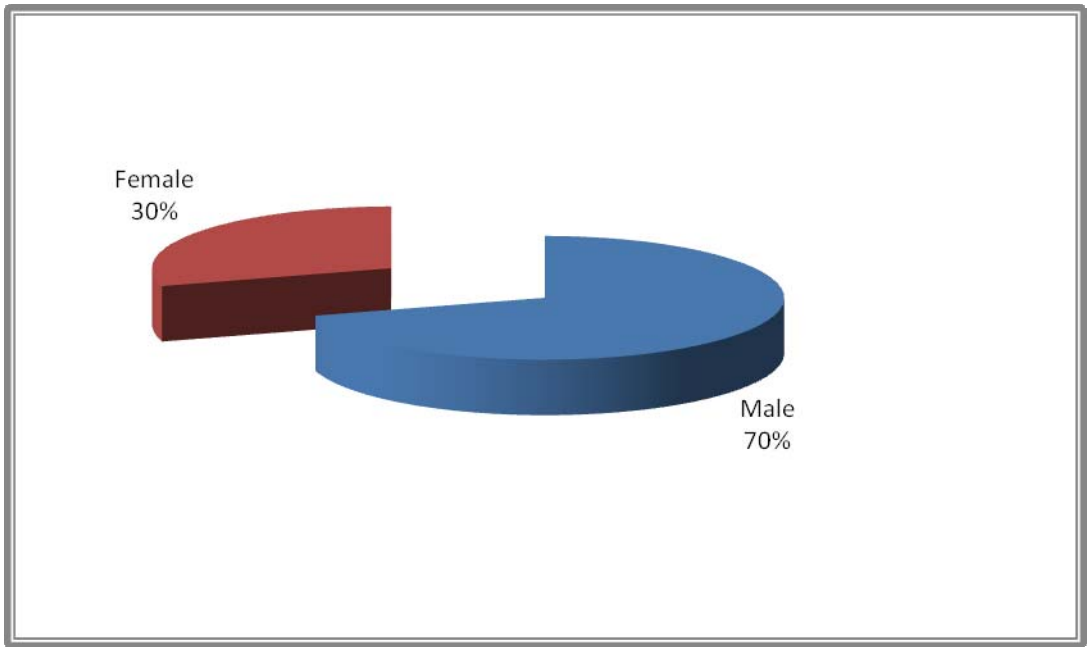


The above table shows study group consists of 118 HIV seropositive patients. 76.27% of patients are between 26-50 years. Mean age of study subjects is 44.25 years**.**

Table 14: Sex wise distribution

Gender	Number	Percentage
Male	83	70 %
Female	35	30 %
Total	118	100 %

Figure 2: Sex wise distribution

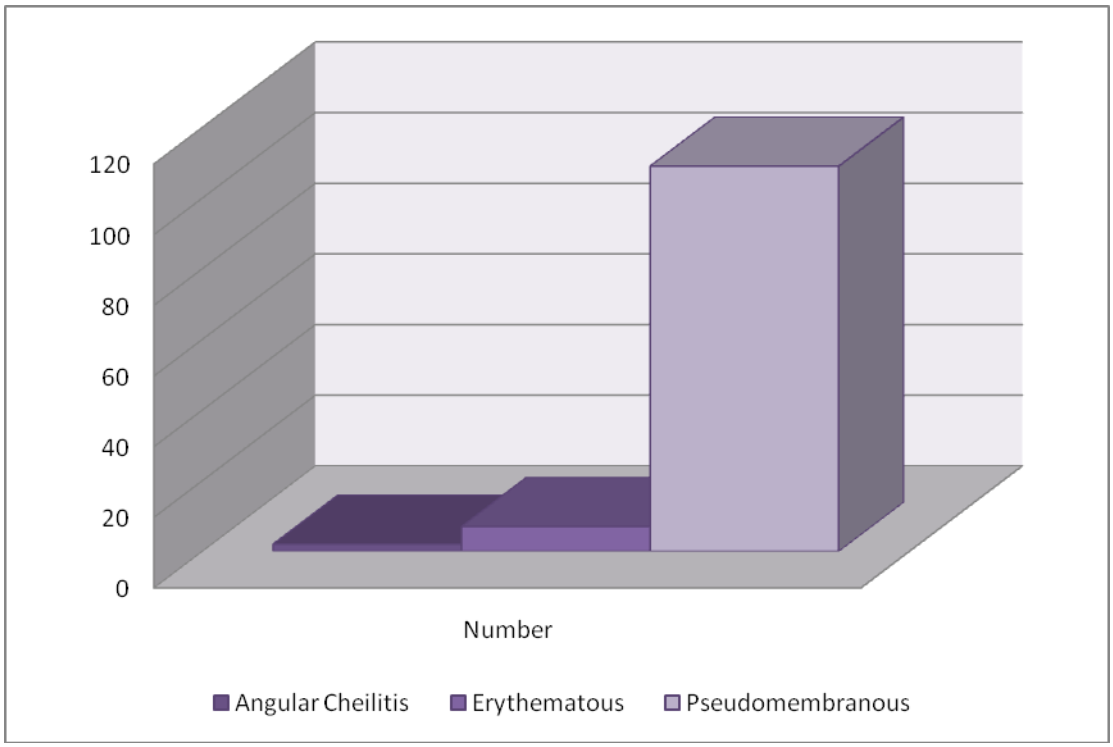


The above table shows study group predominantly constituted of males 70% and remaining 30% are constituted by females. Male to female ratio is 2.6: 1.

Table 15: Types of Lesions

Types of Lesions	Number	Percentage
Angular Cheilitis	2	1.69%
Erythematous	7	5.93%
Pseudomembranous	109	92.3%

Fig 3: Types of Lesions

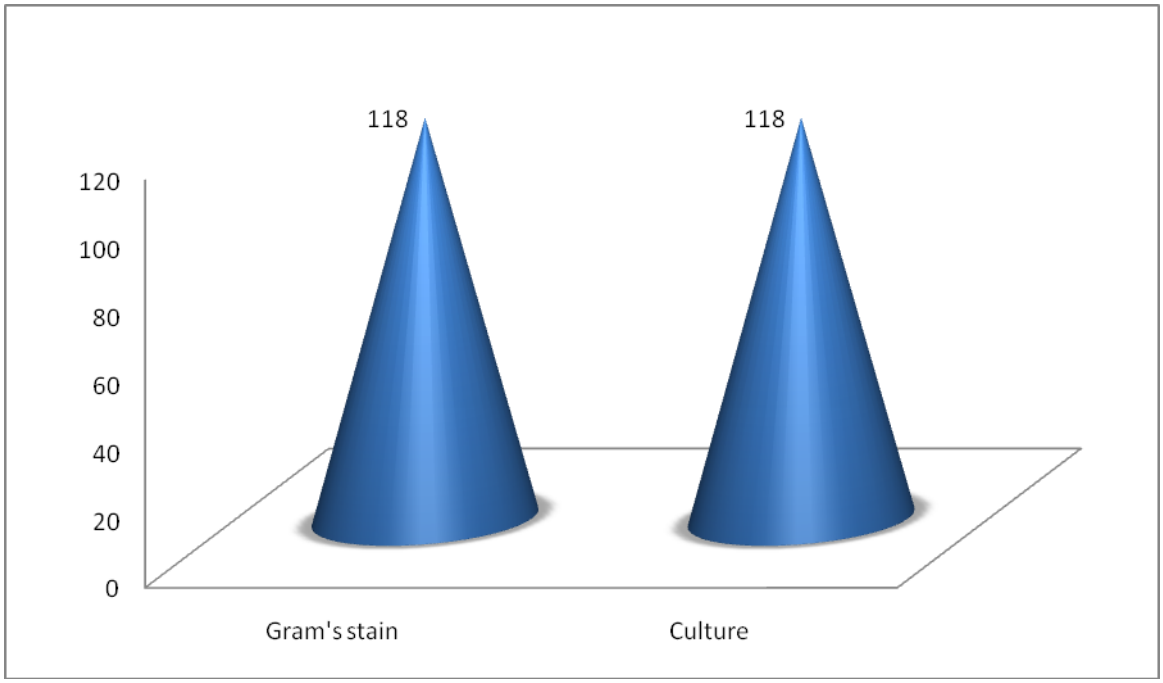


The above table shows 92.3% of patients presented with pseudomembranous type of lesion.

Table 16: Smear positive Candida growing on SDA

Method	Number	Percentage
Gram's stain positive	118	100%
Culture positive	118	100%

Figure 4: Smear positive Candida growing on SDA

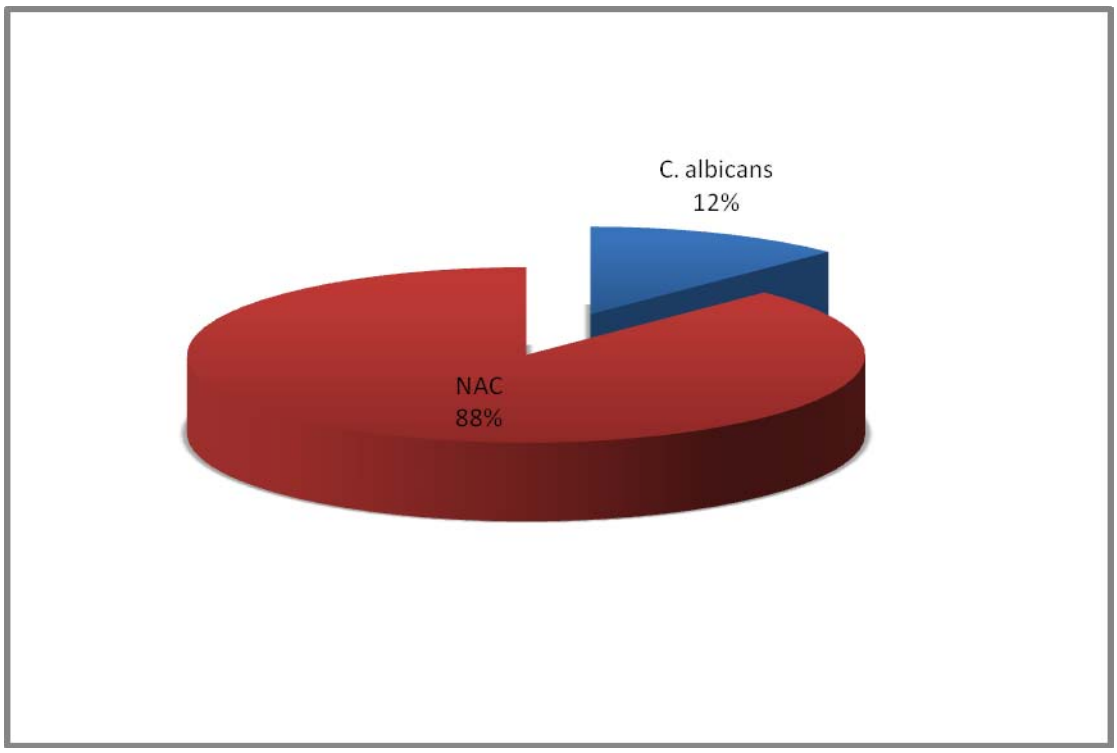


All smear positive budding yeasts cells with or without pseudohyphae were able to show growth on SDA.

Table 17: Distribution of C. albicans and Non albicans Candida

Type of Candida	Number	Percentage
C. albicans	15	12.39 %
NAC	106	87.60 %
Total	121	100 %

Figure 5: Distribution of C. albicans and NAC.

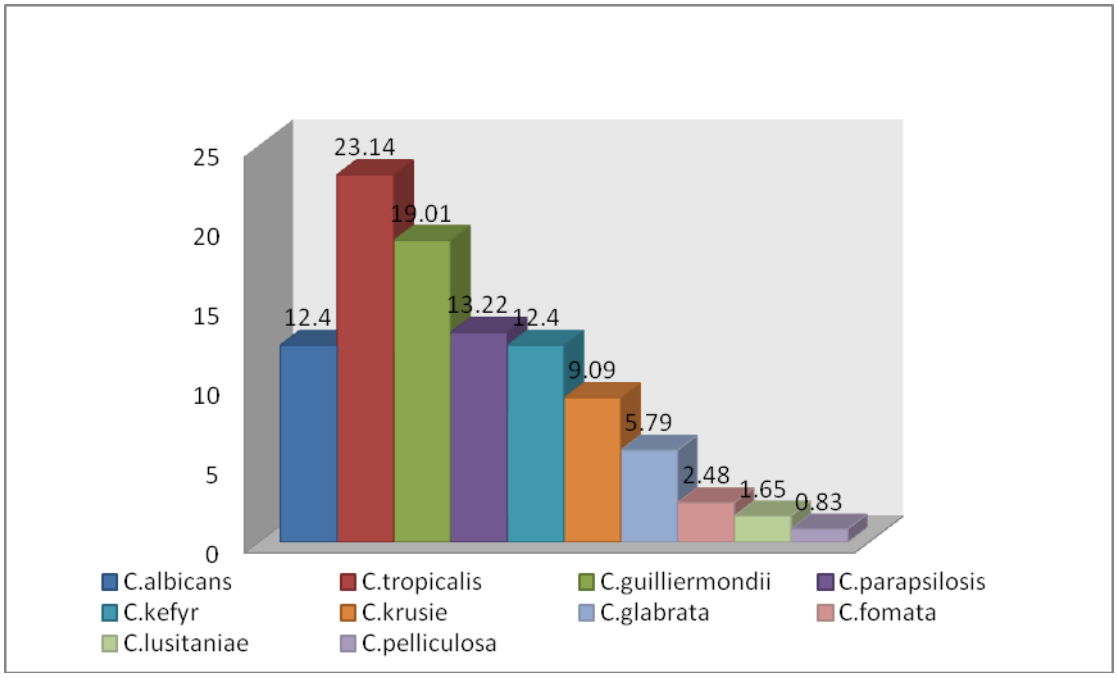


Candida non albicans was the most frequently isolated species accounting for 87.60% and the remaining were Candida albicans accounting for the 12.39%.

Table 18: Species Distribution

Species	Number	Percentage
C. albicans	15	12.39
C. tropicalis	28	23.14
C. guilliermondii	23	19.01
C. parapsilosis	16	13.22
C. kefyr	15	12.40
C.krusei	11	9.09
C. glabrata	7	5.79
C. fomata	3	2.48
C. lusitaniae	2	1.65
C. pelliculosa	1	0.83
Total	121	100

Figure 6: Species Distribution

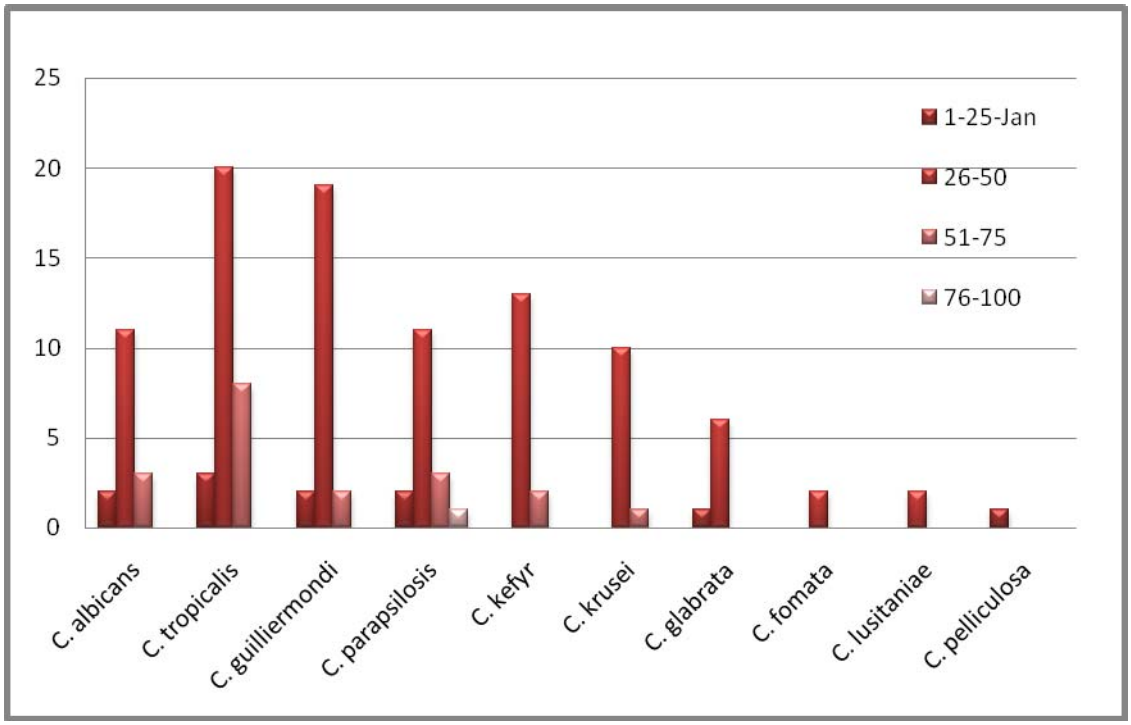


C. tropicalis (23.14%) is the most common species isolated among Non albicans Candida followed by C. guilliermondii (19%), C. parapsilosis (13.22%), C. kefyr (12.4%), C.krusei (9.09%). Rare species like C. glabrata (5.79%), C. fomata (2.48%), C. lusitaniae (1.65%) and C. pelliculosa (0.83%) were also obtained whereas C. albicans were 12.39%.

Table 19: Species distribution by age.

Species	1-25	26-50	51-75	76-100	Total
C. albicans	2	10	3	0	15
C. tropicalis	3	20	5	0	28
C. guilliermondii	2	19	2	0	23
C. parapsilosis	2	11	2	1	16
C. kefyr	0	13	2	0	15
C.krusei	0	10	1	0	11
C. glabrata	1	6	0	0	7
C. fomata	0	2	1	0	3
C. lusitaniae	0	2	0	0	2
C. pelliculosa	1	0	0	0	1
Total	11	93	16	1	121

Figure7: Species distribution by age.

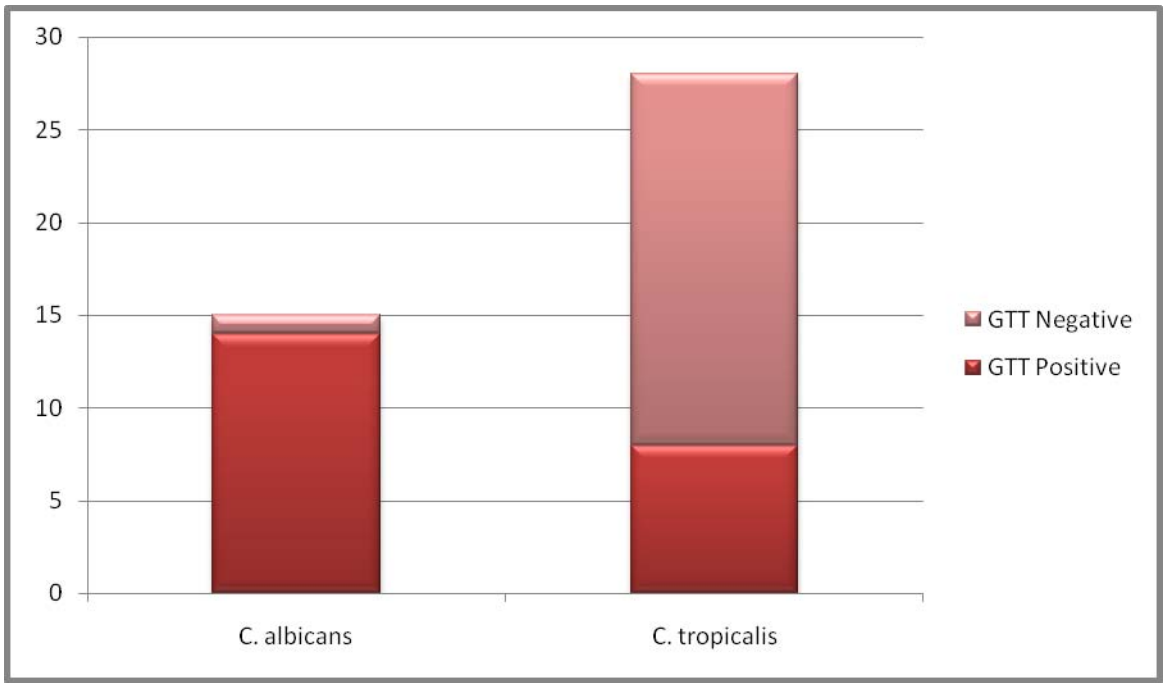


Majority of the Candida species were isolated between the age group 26-50 years.

Table 20- Characterization of species based on GTT.

Test	C. albicans		C. tropicalis	
	No of isolates	**Percentage**	**No of isolates**	**Percentage**
GTT Positive	14	93.33%	8	28.58%
GTT Negative	1	6.67%	20	71.42%
Total	15	100%	28	100%

Figure 8: Characterization of species based on GTT.

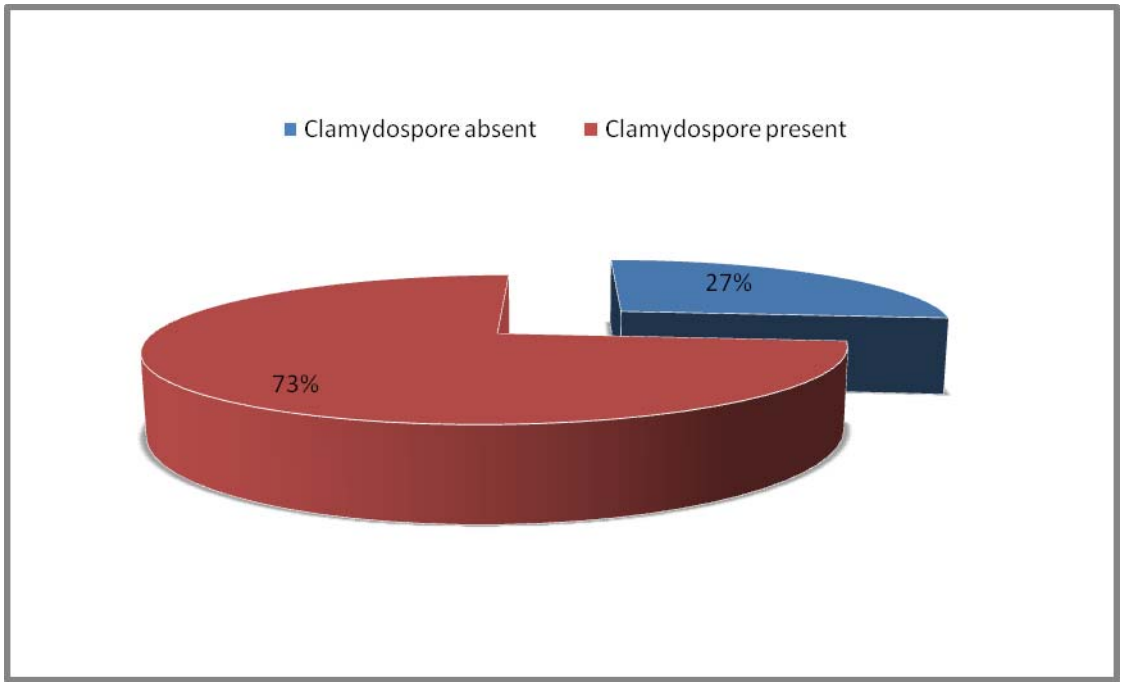


C. albicans showed 93.33% of Germ tube formation and C. tropicalis showed 28.58% Germ tube formation.

Table 21: Characterization of species based on Chlamydospores formation.

Test	C. albicans (n)	C. albicans (%)
Chlamydospores present	11	73.33%
Chlamydospores absent	4	26.66%

Figure 9: Characterization of species based on Chlamydospores formation.

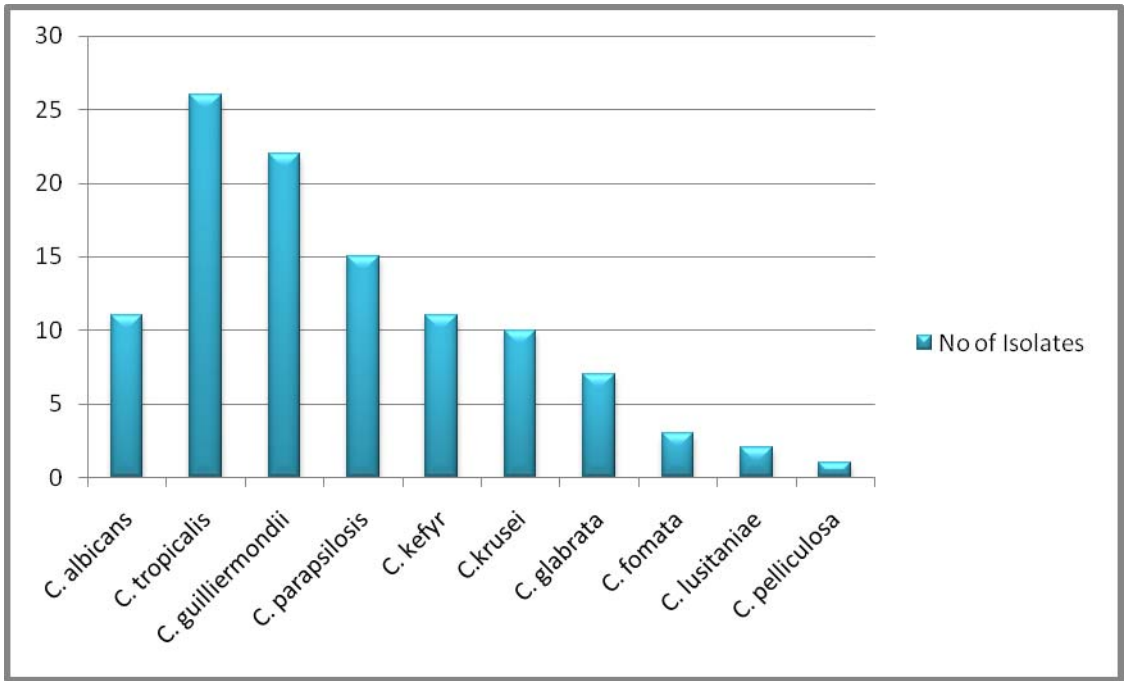


Chlamydospores formation was seen with 73.33% of C. albicans and 26.66% of C. albicans did not show Chlamydospore formation

Table 22: Characteristic morphology of Candida species based on Dalmau plate culture.

Species	Number	Dalmau plate culture	
		Number	Percentage
C. albicans	15	11	73.33%
C. tropicalis	28	26	92.86%
C. guilliermondii	23	22	95.65%
C. parapsilosis	16	15	93.73%
C. kefyr	15	11	73.33%
C.krusei	11	10	90.90%
C. glabrata	7	7	100%
C. fomata	3	3	100%
C. lusitaniae	2	2	100%
C. pelliculosa	1	1	100%
Total	121	108	

Figure10: Characteristic morphology of Candida species based on Dalmau plate culture.



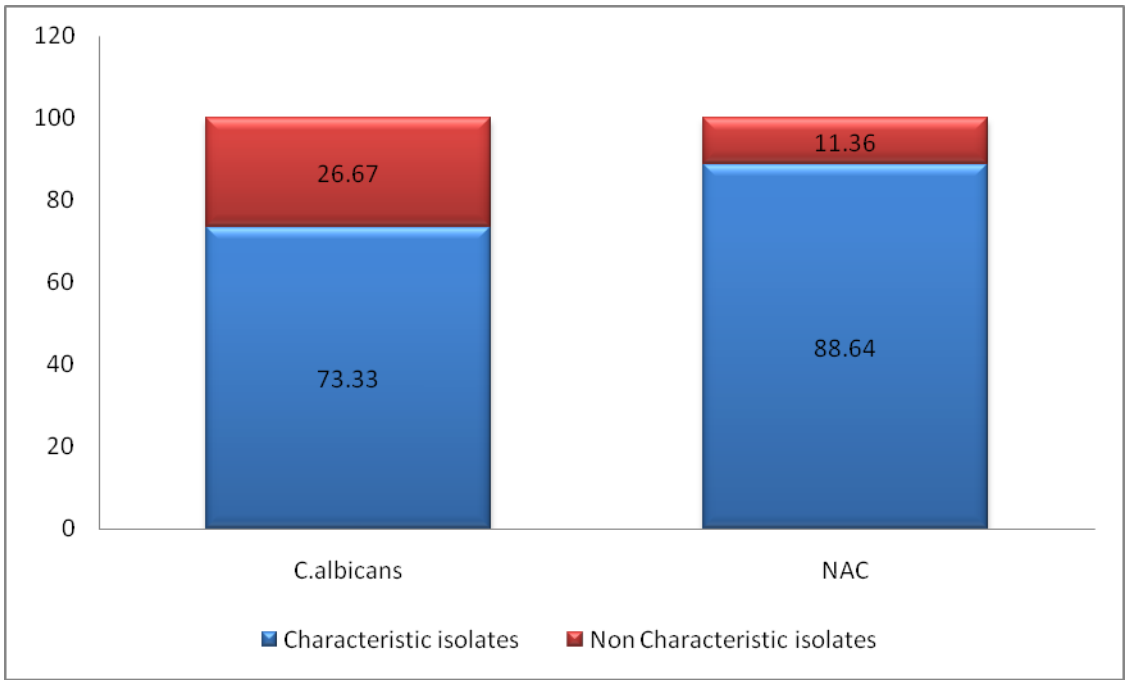
In our study 92.86% of C. tropicalis showed characteristic morphology and C. guilliermondii, C. parapsilosis, C. kefyr and C. krusei showed 95.65%, 93.73%,

73.33% and 90.9% characteristic morphology on CMA respectively. All rare Candida species showed characteristic morphology on CMA.

Table 23: Characteristic morphology of Candida species based on Auxonographic sugar assimilation tests.

	Total isolates	Characteristic isolates	Percentage
C. albicans	15	11	73.33%
NAC	106	94	88.64%

Figure 11: Characteristic morphology of Candida species based on Auxonographic sugar assimilation tests.

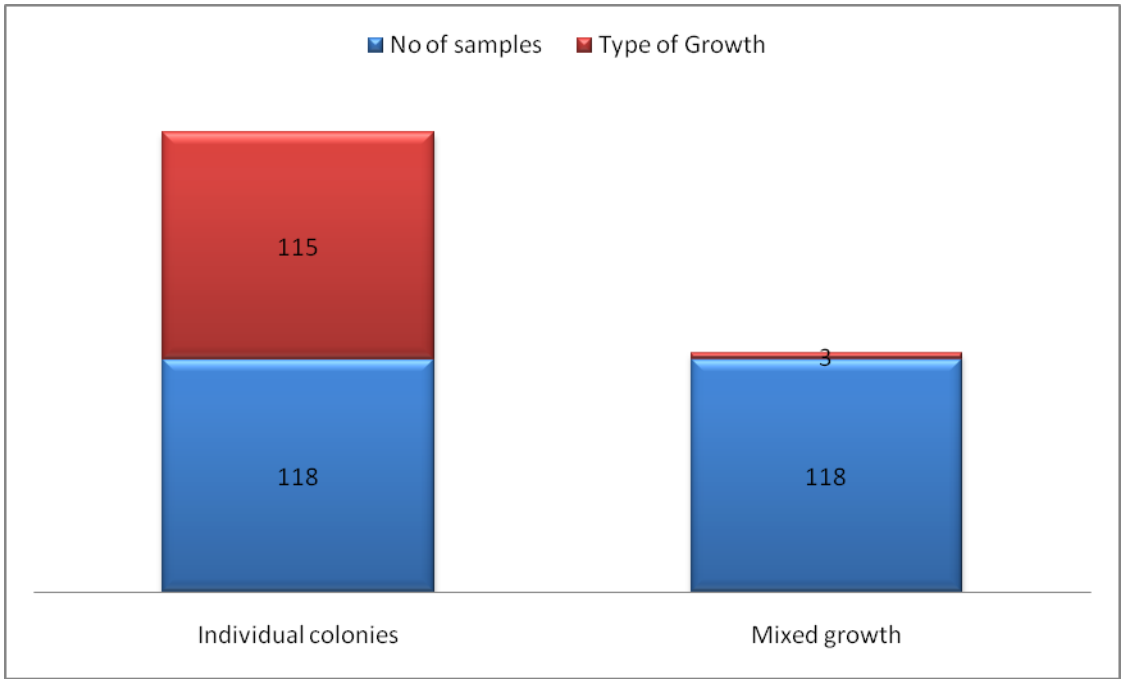


In the present study 73.33% C. albicans and 88.64% of NAC showed characteristic assimilation character.

Table 24: Number of mixed growth

Growth on SDA	Positive	Percentage
Individual colonies	115	97.5%
Mixed colonies	3	2.5%
Total	118	100%

Figure 12: Number of mixed growth

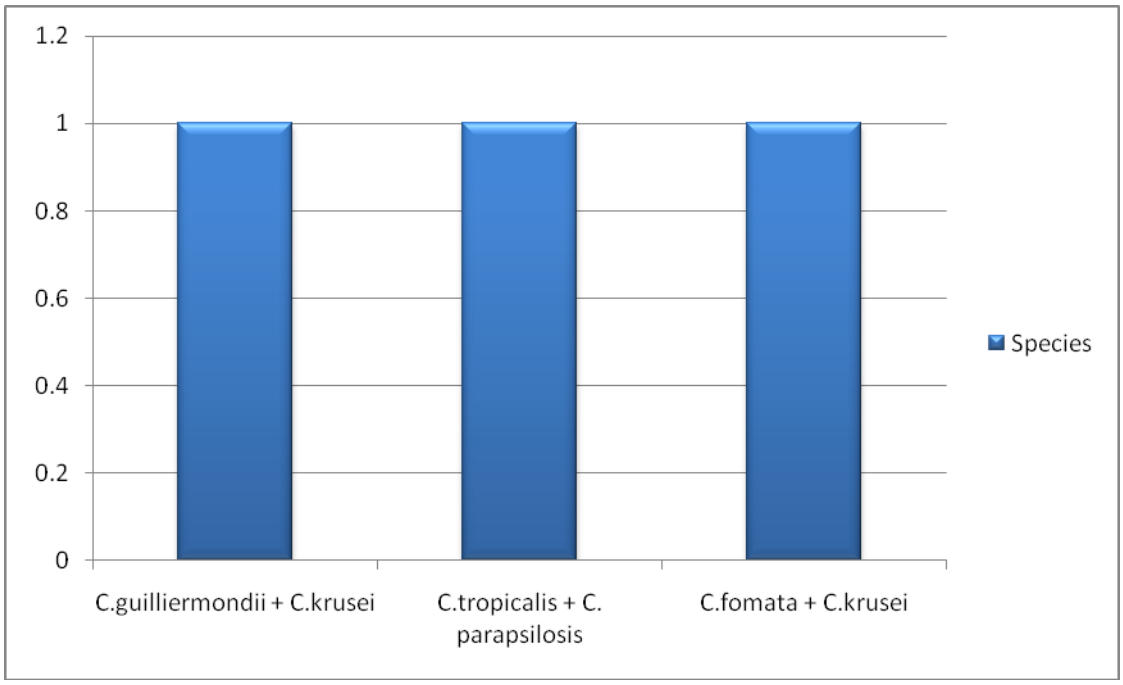


The present study showed 6 mixed colonies and 115 pure colonies on SDA.

Table 25: Species of mixed growth

SI No	Spices combination	Number of species
1	C. guilliermondii + C.krusei	1
2	C. tropicalis + C. parapsilosis	1
3	C. fomata + C.krusei	1

Figure 13: Species occurring in mixed isolates.

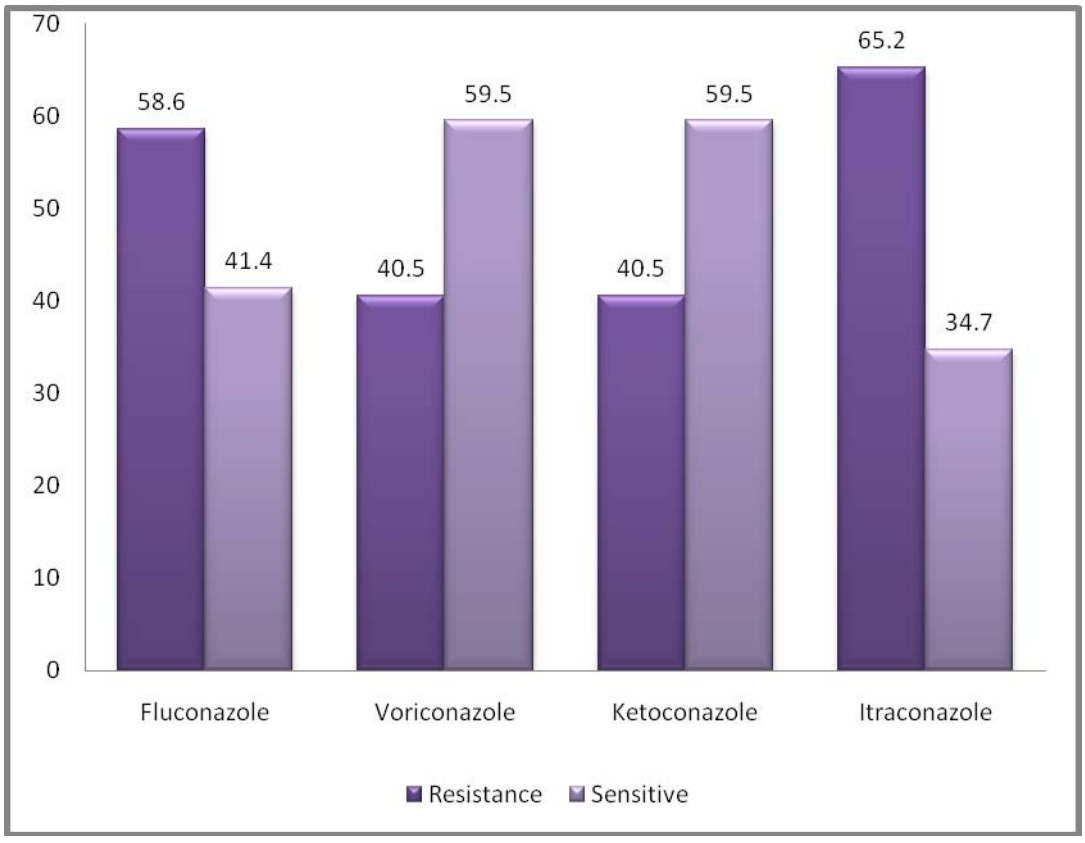


Three mixed isolates were obtained with two species each in the present study.

Table 26: Distribution of the susceptibility pattern among the Candida isolates by disk diffusion method.

AFST	Resistance	Percentage	Sensitive	Percentage
Fluconazole	71	58.6%	50	41.4%
Voriconazole	49	40.5%	72	59.5%
Ketoconazole	49	40.5%	72	59.5%
Itraconazole	79	65.2%	42	34.7%

Figure 14: Distribution of the susceptibility pattern among the Candida isolates by disk diffusion method.



In the present study Fluconazole has resistance of 58.6% followed by Voriconazole and Ketoconazole of 40.5 % respectively. Itraconazole has maximum resistance of 65.2 %.

Table 27: AFST pattern seen with respect to species

Species	Fluconazole		Voriconazole		Ketoconazole		Itraconazole	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**
C. albicans	7 46.6%	8 53.4%	5 33.4%	10 66.6%	4 26.6%	11 73.6%	10 66.6%	5 53.4%
C. tropicalis	17 60.7%	11 39.3%	11 39.3%	17 60.7%	11 39.3%	17 60.7%	16 57.2 %	12 42.8%
C. guilliermondii	14 60.8%	9 39.2%	10 43.8%	13 56.2%	9 39.2%	14 60.8%	16 69.6%	7 30.4%
C. parapsilosis	12 75%	4 25%	8 50%	8 50%	6 37.5%	10 62.5%	10 62.5%	6 37.5%
C. kefyr	9 60%	6 40%	8 53.4%	7 46.6%	7 46.6%	8 53.4%	10 66.6%	5 33.4%
C.krusei	6 54.5%	5 45.5%	3 27.2%	8 72.3%	3 27.2%	8 72.3%	6 54.5%	5 45.5%
C. glabrata	4 57.1%	3 42.8%	2 28.5%	5 71.5%	5 71.5%	2 28.5%	7 100%	0
C. fomata	2 66.6%	1 33.4%	1 33.4%	2 66.6%	3 100%	0	3 100%	0
C. lusitaniae	1 50%	1 50%	1 50%	1 50%	1 50%	1 50%	1 50%	1 50%
C. pelliculosa	0	1 100%	0	1 100%	0	1 100%	1 100%	0
Total	**71%**	**50%**	**49%**	**72%**	**49%**	**72%**	**79%**	**42%**

In the present study C. albicans showed 53.4%, 66.6%, 73.6% and 53.4% sensitivity to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. C. tropicalis showed 39.3%, 60.7%, 60.7% and 42.8% sensitivity to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. C. guilliermondii was 39.2%, 56.2%, 60.7% and 42.8% sensitive to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. C. parapsilosis was 25%, 50%, 62.5% and 37.5% sensitive to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. C. kefyr showed sensitivity of 40%, 46.6%, 53.4% and 33.4% to

Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. C. krusei was 45.5%, 72.3%, 72.3% and 45.5% sensitive to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively.

C. glabrata showed sensitivity of 42.8%, 71.5%, and 28.5% to Fluconazole, Voriconazole, and Ketoconazole. C. fomata was 33.43% and 66.6% sensitive to Fluconazole, Voriconazole. None of the C. fomata isolates were susceptible to Ketoconazole and Itraconazole. 50% isolates of C. lusitaniae were sensitive to Fluconazole, Voriconazole, Ketoconazole and Itraconazole respectively. Single isolate of C. pelliculosa were sensitive to Fluconazole, Voriconazole and Ketoconazole while all isolates were resistant to Itraconazole.

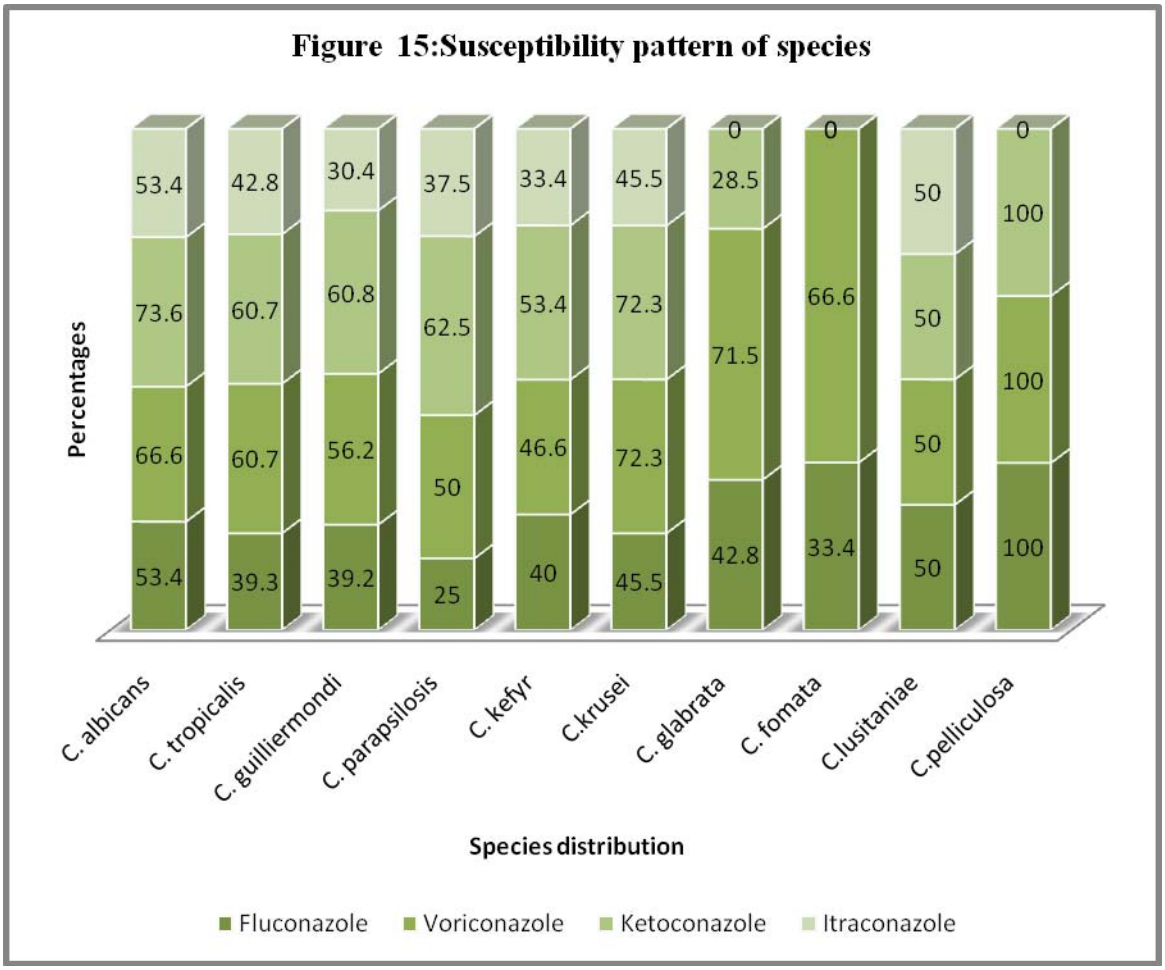
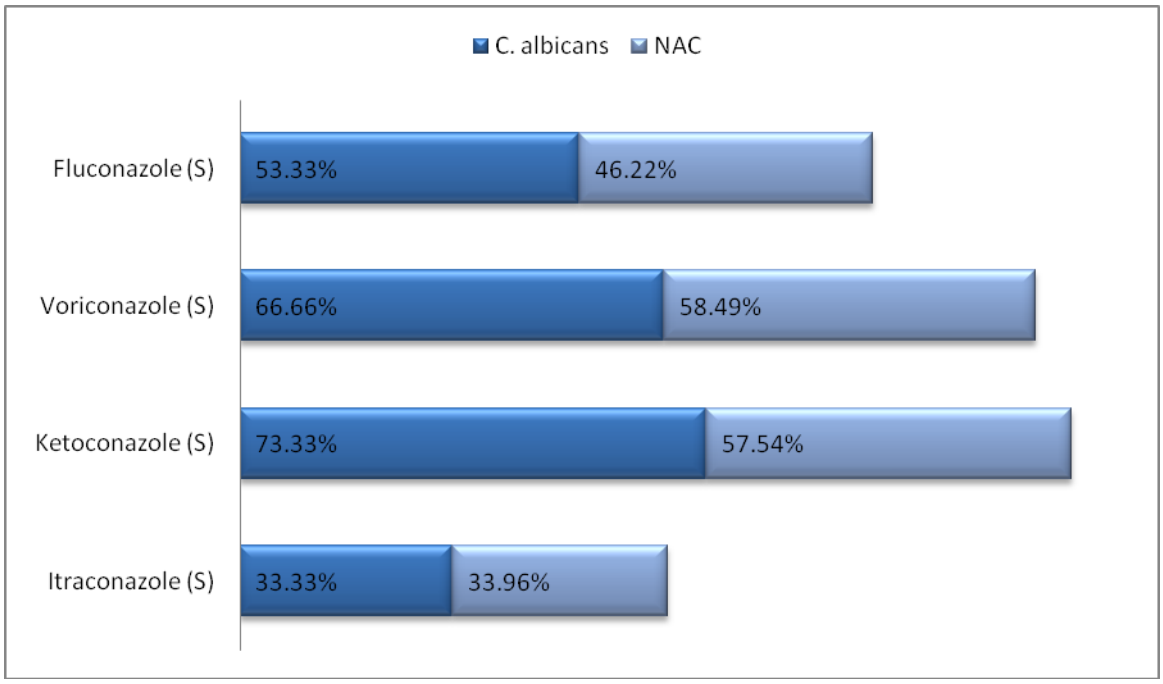


Table 28: Sensitivity pattern to antifungal drugs with respect to C. albicans and Non albicans Candida.

Species	Fluconazole		Voriconazole		Ketoconazole		Itraconazole	
	R	***S***	***R***	***S***	***R***	***S***	***R***	***S***
C. albicans	7 46.6%	8 53.33%	5 33.33%	10 66.66%	4 26.66%	11 73.33%	10 66.66%	5 33.33%
NAC	65 61.3%	41 46.22%	44 41.50%	62 58.49%	45 42.45%	61 57.54%	70 66.03%	36 33.96%

Figure 16: Sensitivity pattern to antifungal drugs with respect to C. albicans and Non albicans Candida.



In the present study Candida albicans were more sensitive to antifungal drugs than Non albicans Candida.

DISCUSSION

In the present study of 118 HIV seropositive patients with clinically diagnosed oral candidiasis attending BLDEU'S Shri B M Patil medical college and Research Center, Bijapur were included. Samples showing budding yeast cells with/without pseudohyphae were inoculated on SDA. Later Candida isolates were subjected for germ tube test, Dalmau plate culture and for sugar assimilation tests. Antifungal susceptibility testing was done by Disk- diffusion method according to CLSI guidelines M44-A2.

Comparison of present study with other studies.

Table: Age distribution

Studies	Range (years)	Mean age
Sharon Walmsley et al (2001)[82]	-	39.1
Vargas KG (2002)[2]	30-45	38
Anupriya et al (2008)[86]	21-40	34.9
Present study	**26-50**	**44.25**

In the present study majority of the patients belong to age group 26-50 years with mean age of 44.25 years, this correlated with the study of Vargas KG (2002)[2] and Anupriya et al (2008)[86]. In the present study, males belonged to a wider age spectrum and the females were a considerably of younger population.

Table: Sex wise distribution

Studies	Percentage male	Female
Vaishali Wabale et al (2008)[38]	76%	24%
Vargas KG (2002)[4]	79.1%	20.8%
VP Baradkar et al (2009)[87]	69%	31%
Present study	**70%**	**30%**

There was a male preponderance accounting for 70% in this study. Vaishali Wabale et al (2008),[38] Vargas KG (2002)[4] and VP Baradkar et al (2009),[87] also reported similar results in their studies. Most of the females acquired infection from their spouses, reflecting the male dominance in society and emphasizing an increased need for awareness and counseling of both the spouses.

Table: Types of lesions

Studies	Pseudomembranous	Angular cheilitis	Erythematous
Omar JM Hamza et al (2008)[4]	66.4%	4.1%	1.4%
Ranganathan et al (2008)[88]	72%	13%	15%
Present study	**92.3%**	**1.69%**	**5.93%**

In the present study, Pseudomembranous candidiasis was the predominant type of Oral candidiasis 92.3% followed by Erythematous lesions 5.93% and Angular cheilitis 1.69%. This was similar to the study of Omar et al (2008)[4], in which 66.4% patients had pseudomebranous type of lesion followed by 4.1% patients had combined lesions with pseudomembranous and erythematous type of lesion whereas Ranganathan et al (2008)[88] reported 72% pseudomembranous lesion as the most common type followed by erythematous type 15% and Angular cheilitis 13%.

Table: Growth on SDA

Studies	Number of isolates	% of growth on SDA
Schmidt AM et al (2004)[89]	121	100%
Shobha ND et al (2008)[37]	132	100%
Present study	118	100%

Growth of Candida was obtained from all samples 100%, similar to other studies by Schmidt AM et al (2004)[89] and Shobha ND et al (2008)[37]. This indicates that SDA can be effectively used for isolation of Candida species.

Table: Comparison of C. albicans and Non albicans Candida

Studies	Percentage	
	C. albicans	**NAC**
Patel M et al (2006)[35]	78.6%	21.4%
Vargas et al (2005)[90]	66.7%	33.3%
Anupriya et al (2007)[86]	50% (11/22)	50% (11/22)
Shobha N et al (2008)[37]	66.6%	33.3%
Enwuru et al (2008)[91]	40.5%	59.5%
Present study	**12.39%**	**87.6%**

There is changing trend in occurrence of the C. albicans and NAC species in the recent past in HIV seropositive patients. It has been suggested in context of HIV related Oral Candidiasis, isolates of NAC species are commensal rather than pathogens and their isolation is of no clinical relevance, whereas JD Cartledg et al[80] showed clinical correlation and significance of NAC in his study with 52 patients with symptomatic pseudomembranous oral candidiasis isolating 97 NAC species. In our study it showed gross rise in NACs as compared to other studies, which correlates with previous studies[24] suggesting repeated exposure to antifungal agents and recurrent infections might predispose to a shift to non-albicans Candida species.

Table: Comparison of different species of Candida

Studies	C. albicans	C. tropicalis	C. guilliermondii	C. parapsilosis	C. kefyr	C. krusei	C. glabrata	C. fomata	C. lusitaniae	C. pelliculosa
Walmsley et al (2001)[82]	79.4%	2%	-	3.1%	-	-	6.2%	-	2%	-
Carolina R C et al (2006)[4]	50%	20.9%	4.8%	19.3%	1.61%	1.61%	-	-	1.61%	-
Mrudula P et al (2006)[35]	78%	0.6%	-	1.7%	0.6%	0.6%	5.2%	-	1.7%	0.6%
Anupriya et al (2007)[86]	59.3%	4.5%	4.5%	11.8%	-	4.5%	14.8%	-	-	-
Omar JM et al (2008)[4]	84.5%	2.2%	-	-	1%	3.4%	6.8%	-	-	-
Shobha ND et al (2008)[37]	66.6%	8.9%	4.9%	11%	-	20%	-	-	-	-
Ranganathan et al (2008)[88]	86%	23%	6%	-	-	5%	-	-	-	-
Present	**12.4%**	**23.14%**	**19.1%**	**13.22%**	**12.4%**	**9.0%**	**5.7%**	**2.48%**	**1.63%**	**0.83%**

Among NAC it is the C. tropicalis was the predominant isolate accounting for 23.14% which correlated with Carolina R C et al (2006)[4] and Ranganathan et al (2008).[88] Isolation of C. guilliermondii in other studies is relatively less as compared to our study which is 19.1%. 13.22% C. parapsilosis were isolated in our study which goes in correlation with Anupriya et al (2007)[86] and Shobha N D et al (2008)[37]. Secondary Candida species has shown variable isolation rate depicting that these species are emerging and there is need for their accurate identification. This variability in isolation may be caused by geographic differences; time of sampling or the different methods used for yeast recovery.

Table: Characterization of species based on GTT for C. albicans.

Studies	Germ tube test Positive	Germ tube test Negative
Salkin et al (1987)[92]	95%	5%
Present study	**93.33%**	**6.67%**

In our study 93.33% of C. albicans showed Germ tube formation which correlated well with Salkin et al (1987)[92] who reported 95% of Germ tube formation for C. albicans. 6.67% GTT negative C. albicans showed Chlamydospores formation and characteristic sugar assimilation tests therefore all isolates of C. albicans were identified by combining these two methods.

Table: Characterization of species based on Chlamydospores formation.

Studies	Chlamydospores present	Chlamydospores absent
Simpanya FM et al (1995)[60]	90%	10%
Koehler et al (1999)[62]	95%	5%
Singh K et al (1999)[15]	100%	-
Present study	**73.33%**	**26.64%**

In our study 73.33% of C. albicans showed Chlamydospores which is much lower than other studies[15, 60, 62] this may be due to atypical strains of C. albicans. This method is helpful in identification of GTT negative C. albicans. Some strains of C. albicans may not form Chlamydospores, those strains has to identified by using Rice extract agar, Potato Dextrose agar or Husk seed agar.[24,54]

Table: Characteristic morphology of Candida species based on Auxonographic sugar assimilation tests.

Studies	Sugar assimilation tests
Sood et al (2000)[65]	72%
Present study	**73.33%**

In the present study characteristic assimilation reaction is seen with 73.39% of C. albicans which correlated with Sood et al (2000)[65] and other previous studies.[24]Higer efficacy of characterization of NAC by sugar assimilation tests is observed of about 88.64% in our study indicating sugar assimilation tests can be effectively used for NACs whereas all isolates of C. albicans can be accurately identified by combining GTT and Dalmau plate culture. Though it is time consuming of about 7-10 days, it gives accurate results for species identification.

Table : Multiple species isolated

Studies	Number	Percentage
Jabra Risk et al (2001)[93]	3 of 11 samples	27%
Vargas KG et al (2002)[2]	3 of 44 samples	6.8%
White PL et al (2004)[94]	10 of 85 samples	11.76%
Shobha ND et al (2008)[37]	3 of 132 isolates	2.2%
Present study	**3 of 118 samples**	**2.5%**

In the present study 2.5% of samples showed multiple isolates which correlated well with Shobha ND et al (2008)[37] whereas White PL et al (2004)[94] reported 11.76%. Multiple isolates were identified on SDA by their colony size, mycelia formation and duration of growth. Several courses of short or long term suppressive therapies in

these patients, reduced susceptibility to antifungals with greater duration of HIV infection and severe immunosuppression has led to multiple species infection.

Table: Antifungal drug resistance

AFST	Fluconazole	Voriconazole	Ketoconazole	Itraconazole
Tumbarello et al (1996)[77]	45%	-	3%	38%
Walmsley et al (2001)[82]	58%	-	25%	17%
Shobha ND et al (2008)[37]	43.3%	-	21%	-
Present study	**58.6%**	**40.5%**	**40.5%**	**65.2%**

Our study also confirms the increasing problem of azole resistance in the course of HIV infection as shown by other authors. The role of previous use of azole drugs in selecting Fluconazole resistant strains of Candida has been reported previously by Bailey et al(1994)[95]. Moreover, analogous effect of Itraconazole had also been reported. Our study revealed even higher resistance to Itraconazole 65.2% than Fluconazole 58.6%.This may be perhaps reflecting recent extensive use of Itraconazole in the treatment of fungal infections in patients with HIV. Relatively low resistance is seen for Voriconazole and Ketoconazole in our study, this could be due to reduced use of this drug in our area. Furthermore, a change in the biochemical and physical properties of cell membranes has greater effect on the uptake of Fluconazole than of Ketoconazole which is a hydrophobic drug and this may explain the absence of cross resistance between Fluconazole and Ketoconazole.

Table: Antifungal drug resistance among species to Fluconazole

Studies	C. albicans	C. tropicalis	C.guilliermondii	C. parapsilosis	C. kefyr	C. krusei	C. glabrata	C. fomata	C. lusitaniae	C. pelliculosa
Shobha N D et al (2008)[37]	12.2%			60%		44.4%				
Arati Mane et al (2010)[39]	14%	10%	-	-	-	40%	50%	-	20%	-
Present study	**46.6%**	**60.7%**	**60.8%**	**75%**	**60%**	**54.5%**	**57.1%**	**66.6%**	**50%**	-

The present study showed overall higher resistance to Fluconazole as compared to previous studies. Testing of Fluconazole depends on several factors, including culture medium composition, pH, inoculum concentration, method employed in addition to the level of resistance of the isolate. Disk diffusion method was used in our study which may be unable to differentiate fully resistant strains from those with dose-dependent susceptibilities, which may have been considered as resistant strains in our study. Further study is needed on resistant strains to give their breakpoints and SDD by more precise procedures such as NCCLS dilution tests.

Thus our study highlights the fact that there is potential risk of emergence and selection of azole resistant strains of Candida isolated from AIDS patients. This warrants careful selection of an antifungal drug for therapy of mild fungal infections after evaluation of in-vitro sensitivity of the isolated strains.

SUMMARY

The study was cross sectional one and was carried out from December 2010 to May 2012 at the Department of Microbiology of BLDEU'S Shri B M Patil medical College, Bijapur. Hundred and eighteen clinically diagnosed cases of oral candidiasis from HIV seropositive patients attending OPD and inpatients at various wards of BLDEU'S Shri B M Patil medical College, Hospital and Medical Research Center, Bijapur; regardless of their age and sex were included. Isolation and identification of Candida species were carried out as per standard mycological methods.

Following observations were made in our study:

- In the present study, majority of the study population belonged to the age group of 26 – 50 years (78.31%).
- There was a male preponderance accounting for 70%, with Male to female ratio 2.6:1.
- Majority of the HIV patients with oropharyngeal Candidiasis presented with pseudomembranous type of lesions constituting for 92.3%.
- 118 samples from HIV patients with oral Candidiasis were studied and Candida species were isolated from all the 118 samples yielding a culture positivity of 100%.
- Out of the 118 patients, three patients showed two Candida species each resulting in a total of 121 Candida isolates.
- Majority of the isolates were Non albicans Candida (87.6%) and remaining were Candida albicans (12.39%).

- Among the non albicans, Candida tropicalis (23.4%) is the most common isolate followed Candida guilliermondii (19.01%), Candida parapsilosis (13.22%), Candida kefyr (12.4%), Candida krusei (9.09%), Candida glabrata (5.79%), Candida fomata (2.48%), Candida lusitaniae (1.65%) and Candida pelliculosa (0.83%).
- Majority of the Candida isolates were obtained from population between 26-50 years followed by 51-75 years.
- Rare Candida species were obtained from younger age group 1-25years.
- Single isolates were seen among 97.8% and mixed growth among 2.5% of sample.
- Three mixed growths of two species each; C. guilliermondii + C.krusei, C. tropicalis + C. parapsilosis and C. fomata + C.krusei were isolated.
- By Disk Diffusion Method, 41.4% of the Candida isolates were sensitive to Fluconazole, 59.5% were sensitive to Voriconazole, 59.5% sensitive to Ketoconazole and 34.7% sensitive to Itraconazole.
- Non albicans Candida showed much higher resistance pattern to antifungals than Candida albicans.
- Among Non albicans Candida, it is the C. parapsilosis; C. glabrata and C. tropicalis were more resistant to Fluconazole. C. kefyr and C. parapsilosis showed more resistance to Voriconazole. C. glabrata and C. kefyr were more resistant to Ketoconazole. All Non albicans Candida species showed relatively higher resistance to Itraconazole.

CONCLUSION

Oropharyngeal candidiasis or oral thrush is a common and localized infection typically caused by the yeast Candida albicans, a normal component of the human gastrointestinal microflora. While the incidence of OPC has declined for HIV-infected persons with access to antiretroviral therapy, it remains a significant problem for those in resource-limited settings. Effective control of infection is achieved by the judicious use of topical and systemic antifungal agents.

Deterioration of host immunity and alteration in virulence factors of commensal Candida in HIV seropositive individuals led to emergence of non albicans Candida species and these pathogens warrant discussion due to their potential to harbor antifungal resistance mechanisms, some of them are intrinsically resistant to azoles. These organisms may exhibit decreased susceptibility or frank resistance to Fluconazole so that recognition of these important causes of OPC may aid the clinician in cases recalcitrant to Fluconazole therapy.

It is noted in our study, higher prevalence of non albicans Candida causing oral candidiasis and also posing problem of drug resistance to azoles among these species. In view of the potential risk for the emergence and selection of azole resistant strains of Candida in patients with AIDS, it is important to select an appropriate antifungal drug for the therapy of fungal infections after evaluation of the in-vitro sensitivity of the isolated strains. Therefore the isolation, identification of Candida species and performing antifungal susceptibility from clinical specimens are very essential in deciding the antifungal of choice.

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ANNEXURES
PROFORMA

SCHEME OF CASE TAKING:

1. NAME	:	CASE NO:
2.AGE/SEX	:	IP NO:
3.OCCUPATION	:	DOA:
		DOS:
		DOD:
		LAB NO:
4. RESIDENCE	:	

5. CHIEF COMPLAINTS :

6. PAST HISTORY :

7.CLINICAL EXAMINATION:

A . General Physical Examination

Pallor : Icterus : Pulse: Temperature : BP :

B. Systemic Examination

CVS :
RS :
PA :
CNS :

C. Oral Examination : Type of lesion -

D. Lymph node Examination :

8. Investigations : Hb %
RBC count

WBC count
Urine analysis
CD4 T cell count

9. Microbiological study :

I. Microscopy - Gram staining

II. Culture- On Emmons's modified SDA medium

III. Biochemical tests -

Germ Tube Test -

Growth on Corn meal-Tween 80 agar –

Sugar assimilation tests.

Sucrose	Trehalose	Xylose	Lactose	Maltose	Cellibose	Dulcitol

10. Antifungal susceptibility testing using Kirby Bauer disc diffusion method.

Fluconazole	Voriconazole	Ketoconazole	Itraconazole

INFORMED CONSENT FORM

TITLE OF TOPIC : IDENTIFICATION, CHARACTERIZATION & ANTIFUNGAL SUSCEPTIBILITY TESTING OF CANDIDA SPECIES USING CONVENTIONAL METHODS FROM ORAL THRUSH IN HIV SEROPOSITIVE PATIENTS.

PRINCIPAL INVESTIGATOR : DR SHYAMALA.R

PG GUIDE NAME : DR.PRASHANT. K. PARANDEKAR.

PURPOSE OF RESEARCH:

I have been informed that this study will detect species of Candida from oral lesions and will further tested for antifungal susceptibility pattern.

PROCEDURE:

I am aware that in addition to routine care received & I will be asked series of questions by the investigator. I understand that my oral swabs will be subjected to various investigations needed for research purpose.

RISK AND DISCOMFORTS:

I understand that I may experience some pain & discomfort during collection of oral swabs from oral lesions. This is mainly the result of my condition & the procedure of the study is not expected to exaggerate these feelings which are associated with the usual course of the treatment.

BENEFITS:

I understand that my participation in the study as one of the study subject will help the researcher to identify the species of organism & its antifungal

susceptibility pattern. Study will not have direct benefits to me other than the potential benefits of the study for choosing appropriate antifungals.

CONFIDENTIALITY:

I understand that the medical information produced by this study will become a part of hospital records & will be subject to confidentiality. Information of sensitive personal nature will not be part of the medical record, but will be stored in the investigators research file & identified only by code number; the code key connecting name to numbers will be kept in separate secure location.

If the data are used for publication in the medical literature or for teaching purpose, no name will be used.

I understand that the relevant designated authorities are permitted to have access to my medical record & to the data produced by the study for audit purpose, however they are required to maintain confidentiality.

REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at any time. Dr. Shyamala R at the department of Microbiology is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of the study, which might influence my continued participation. A copy of this consent form will be given to me to keep for careful reading.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw consent and discontinue participation in the study at any

time without prejudice. I also understand that Dr Shyamala R may terminate my participation in the study at any time after she has explained the reasons for doing so.

INJURY STATEMENT

I understand that in the unlikely event of injury to me resulting directly from my participation in this study, if such injury were reported promptly, the appropriate treatment would be available to me. But no further compensation would be provided by the hospital. I understand that by my agreements to participate in this study I am not waiving any of my legal rights.

I have explained to Mr. /Ms_____ the purpose of the research, procedures required & the possible risks to the best of my ability.

_____

Dr Shyamala R Date:
(Investigator sign)

STUDY SUBJECT CONSENT STATEMENT:

I confirm that Dr. Shyamala has explained to me the purpose of research, the study procedure that I can undergo & the possible discomfort as well as benefits that I may experience in my own language. I agree with full conscious to give consent to participate as subject in the research project.

_____ Date:
(Participant signature)

_____ Date:
(Witness signature)

TEST PROCEDURES

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)

Alcohol - Decolourising agent

Dilute Carbol fuchsin (1%) - Secondary stain

SABOURAUD DEXTROSE AGAR WITH ANTIBIOTICS

Peptone - 10 gm

Dextrose - 40 gm

Agar - 20 gm

Distilled water - 1000ml

Chloramphenicol - 50 mg

Gentamicin - 10mg

Final pH was adjusted to 5.6

CORNMEAL- TWEEN 80 AGAR

Composition

Corn meal - 50 gm

Agar - 15 gm

Distilled Water - 1000ml

Tween 80 (1%) -1 ml

Suspend the ingredients in 1000ml distilled water. Boil to dissolve completely. Add Tween80 to the above medium. Sterilize by autoclaving at 121^0C for 15 minutes.

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

YNB MEDIA- 6.7 g

Agar – 20 g

Distilled water-1000 ml

Dissolve 6.7g YNB base in distilled water. Prepare molten agar, autoclave at 121^0c for 15 minutes. Pour 2ml yeast –YNB suspension into 90cms petriplates. Add 18ml molten agar after cooling it to 40-50^0c into petriplates, mix evenly and allow media to solidify.

MUELLER HINTON AGAR (MH-GM agar)

Beef infusion - 300ml

Casein hydrolysate - 17.5 gm

Starch - 1.5 gm

Agar - 10gm

Distilled Water - 1000ml

pH – 7.4

Sterilize by autoclaving at 121^0C for 20 minutes.

10g Glucose is added to above molten media. Add 5µg/ml of Methylene blue to it, shake vigoursly, and pour it petriplates.

Mc Farland 0.5 Turbidity standard

Prepare this turbidity standard by adding 0.05ml of 1.175% BaCl2 to 9.95ml of 1% 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 standards.

Distribute 4 to 6ml into screw capped tubes and tightly seal these tubes and store them in the dark at room temperature.

Vigorously agitate this turbidity standard on a mechanical vortex just before use.

McFarland 4 Turbidity standard

Prepare this turbidity standard by adding 0.4ml of 1.175% BaCl2 to 9.6ml of 1% 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 0.10 for the 4 McFarland standards.

Distribute 4 to 6ml into screw capped tubes and tightly seal these tubes and store them in the dark at room temperature.

Vigorously agitate this turbidity standard on a mechanical vortex just before use.

STATISTICAL METHODS APPLIED

1. SAMPLING

With Prevalence rate of Oral Candidiasis 58.7% (≈59%) .

With 95% confidence interval, 15% of marginal error the sample size (n) is

$$n = \frac{(1.96)^2 \; p \; (1-p)}{l^2}$$

n=118

p = prevalence rate - ≈59 %.

l = marginal error.

2. The **Arithmetic mean** is calculated by the formula

X = AM = a + cd

Where a = Arbitrary mean
c = Width of Class interval
d = Mean of the deviated value

SAMPLING ANALYSIS

Diagramatic presentation.

Mean ± SD.

Percentages.

MASTER CHART

SI NO	Name	Age	Sex	IP/OP NO	Types of lesion	Gram stain	Growth on SDA	Germ Tube Test	Clamydaspore Forms	Sugar assimilation Test							AFST				Species	
										Sucrose	Maltose	Trehalose	Xylose	Lactose	Cellibose	Dulcitol	Fluconazole	voriconazole	Ketoconazole	Itraconazole	NO Species	Species identified
1	Ranjabi	70	F	1431	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	s	s	s	r	1	C. parapsilosis
2	Ramanna	42	M	27138	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	r	s	1	C. parapsilosis
3	Rukmini	38	F	162	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	s	s	s	1	C. parapsilosis
4	Shanthawwa	45	F	216	Pseudomemb	+	+	-	-	-	+	+	+	+	-	-	r	s	s	s	1	C.tropicalis
5	Sushilabai	60	M	103	Pseudomemb	+	+	+	-	+	+	+	+	-	+	-	r	s	s	r	1	C. tropicalis
6	Ashok	30	M	1421	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	s	s	s	s	1	C.albicans
7	Sheeshail	38	M	1582	Erythematous	+	+	-	-	+	-	+	+	-	+	-	s	s	s	s	1	C.tropicalis
8	Neelamma	32	M	6513	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	s	r	1	C.guilliermondii
9	Lakshman	40	M	1923	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	s	s	s	s	1	C.krusei
10	Adevappa	38	M	40937	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	s	s	1	C.guilliermondii
11	Sidamalla	48	M	40895	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	s	1	C.albicans
12	Chandabai	30	F	50478	Erythematous	+	+	-	-	+	+	+	+	-	-	-	s	s	s	s	1	C.lusitaniae
13	Prema	32	F	47073	Pseudomemb	+	+	-	-	+	-	-	+	+	-	-	r	r	r	r	1	C.lusitaniae

14	Shantappa	56	M	3858	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	s	s	s	r	1	C.kefyr
15	Nagappa	43	M	4276	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	s	s	s	r	1	C.krusei
16	Rajusindhe	38	M	4159	Pseudomemb	+	+	-	-	+	+	+	+	+	+	+	r	s	r	r	1	C.glabrata
17	Nagappa Hiregowda	43	M	4276	Angular chelitis	+	+	-	-	-	+	-	+	+	+	-	r	r	s	r	1	C.kefyr
18	Deepa	36	F	11423	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	r	s	s	r	1	C.albicans
19	Shivu	51	M	11389	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	s	s	s	r	1	C.albicans
20	Somaiah gultar	60	M	10484	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	s	s	s	r	1	C.albicans
21	Hanumanth	54	M	10484	Pseudomemb	+	+	+	-	+	+	+	+	-	+	-	r	r	r	r	1	C.tropicalis
22	Basavraj	30	M	9302	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	s	s	s	r	1	C.glabrata
23	Allabksha Tikotakar	32	M	10284	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	r	r	1	C.glabrata
24	Dullappa	25	M	11451	Pseudomemb	+	+	-	-	+	-	+	+	+	-	+	s	s	s	r	1	C.glabrata
25	Sanganna Patrimath	39	M	11454	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	r	r	r	r	1	C.krusei
26	Namadev Agasar	50	M	11460	Pseudomemb	+	+	-	-	+	-	+	+	+	+	-	r	s	r	r	1	C.fomata
27	Shivanand	40	M	134238	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	r	s	s	1	C.kefyr
28	Savithri	38	F	11383	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	r	1	C.tropicalis
29	Shankar	42	M	22861	Pseudomemb	+	+	-	-	+	+	+	+	+	-	-	r	r	s	s	1	C.guilliermondii
30	Sugappa Hamapur	48	M	12750	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	s	s	r	r	1	C.fomata
31	Muniyappa	59	M	12763	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	s	s	1	C.guilliermondii
32	Bassappa Choudaki	35	M	151083	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	r	r	r	r	1	C.guilliermondii

33	Ningappa Neelappa	26	M	13000	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	r	s	r	r	1	C.krusei
34	Siddappa Kanse	55	M	11381	Pseudomemb	+	+	-	-	-	+	+	+	-	+	+	r	r	r	r	1	C.guilliermondii
35	Yankawwa	35	F	163893	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	r	r	r	1	C.kefyr
36	Ballappa Gowda	42	M	16489	Pseudomemb	+	+	-	-	-	-	+	+	-	-	-	r	s	s	r	1	C.krusei
37	Mallamma Talwar	35	F	13973	Pseudomemb	+	+	-	-	+	-	+	+	-	+	+	s	s	s	r	1	C.guilliermondii
38	Nandabassappa	38	M	13303	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	s	r	1	C.guilliermondii
39	Ruchita	6	F	14182	Pseudomemb	+	+	-	-	+	-	+	+	+	+	-	s	s	s	r	1	C.pelliculosa
40	Laxmi	28	F	14080	Pseudomemb	+	+	-	-	-	+	+	+	-	+	-	r	r	s	r	1	C.tropicalis
41	Sangappa	40	M	15309	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	s	1	C.guilliermondii
42	Borawwa	35	F	15402	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	r	r	1	C.guilliermondii
43	Sheshikala	22	F	15707	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	r	r	r	r	1	C.tropicalis
44	Yamanappa	30	M	15801	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	s	r	s	s	1	C. parapsilosis
45	Shankarappa	48	M	14744	Pseudomemb	+	+	-	-	+	+	+	+	+	+	-	r	s	s	r	1	C.tropicalis
46	Lakshmibai	45	F	15866	Pseudomemb	+	+	-	-	-	-	+	+	-	-	-	r	s	s	r	1	C. parapsilosis
47	Madiwallamma	40	F	15343	Pseudomemb	+	+	-	-	-	-	+	+	+	-	-	r	r	r	r	1	C.glabrata
48	Nagappa	35	M	15903	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	r	s	2	C.guilliermondii
								-	-	+	+	-	+	-	-	-	s	s	r	s		C.krusei
49	Channanna	36	F	16020	Pseudomemb	+	+	-	-	+	+	+	+	+	+	+	r	r	r	r	1	C.kefyr
50	Mantayya	30	M	16011	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	s	r	1	C. parapsilosis

51	Sadashiv	30	M	160701	Pseudomemb	+	+	-	-	+	+	+	+	+	-	-	s	s	s	s	1	C. parapsilosis
52	Gallalappa	40	M	185142	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	s	s	r	1	C.guilliermondii
53	Basavraj	20	M	16077	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	s	1	C.tropicalis
54	Mahadevi	48	F	16277	Pseudomemb	+	+	+	-	+	+	+	-	-	+	-	s	s	s	s	1	C.tropicalis
55	Siddanagowda	30	M	17096	Pseudomemb	+	+	-	-	+	+	+	+	+	-	-	r	r	r	r	1	C. parapsilosis
56	Ramangowda	38	M	17669	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	r	r	r	1	C.guilliermondii
57	Sangappa Biradar	30	M	17776	Pseudomemb	+	+	-	-	-	+	-	+	+	-	-	s	s	s	s	1	C.kefyr
58	Abdul Kaumsab	45	M	17355	Pseudomemb	+	+	+	-	+	+	+	+	+	-	-	s	s	s	s	1	C.albicans
59	Sidheswar	48	M	14096	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	s	s	s	s	1	C.krusei
60	Sumithra	40	F	214788	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	s	1	C. tropicalis
61	Chandrakanth	35	M	18434	Pseudomemb	+	+	+	-	+	+	+	+	-	+	-	r	r	r	r	1	C.tropicalis
62	Paresh	34	M	18828	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	r	r	r	r	1	C.albicans
63	Mallappa	30	M	18877	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	r	r	r	r	1	C.albicans
64	Sanjay Pawar	35	M	18885	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	r	s	r	r	1	C.albicans
65	Siddappa	25	M	18318	Pseudomemb	+	+	+	+	+	+	+	+	+	+	-	s	r	r	r	1	C.albicans
66	Basavraj Konnur	58	M	15967	Pseudomemb	+	+	-	-	+	+	+	+	+	+	+	s	s	s	r	1	C.kefyr
67	Basappa	45	M	19455	Pseudomemb	+	+	+	-	+	+	+	+	-	+	-	s	s	s	r	1	C.tropicalis
68	Barathi	35	F	22610	Pseudomemb	+	+	-	-	+	+	+	+	+	+	+	r	s	r	r	1	C.glabrata
69	Siddangowda	30	M	21906	Pseudomemb	+	+	-	-	+	+	+	+	+	+	+	r	r	r	r	1	C.glabrata

70	Parmesh	42	M	22900	Pseudomemb	+	+	+	+	+	+	+	+	+	-	+	r	s	s	r	1	C.albicans
71	Chanappa Sinae	33	M	246879	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	r	r	s	r	1	C. krusei
72	Basavraj Belagali	28	M	25350	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	s	s	r	1	C.guilliermondii
73	Janubai Kambale	48	F	23704	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	s	s	r	1	C.tropicalis
74	Dalawwa	20	F	23279	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	r	s	r	1	C.guilliermondii
75	Sharanappa	45	M	21813	Pseudomemb	+	+	-	-	-	+	+	+	-	+	-	r	r	r	r	1	C.tropicalis
76	Deepak	38	M	23490	Pseudomemb	+	+	-	-	+	+	+	+	+	-	+	r	r	r	r	1	C.guilliermondii
77	Muttappa	40	M	23481	Erythematous	+	+	-	-	-	+	-	+	+	+	-	r	s	s	r	1	C.kefyr
78	Chandrshekar	45	M	23582	Pseudomemb	+	+	+	-	+	-	+	+	-	+	-	r	r	s	r	1	C.tropicalis
79	Sukadevi	21	F	24277	Pseudomemb	+	+	+	-	+	+	+	+	-	+	-	r	s	s	r	1	C.tropicalis
80	Shivagangamma	72	F	24348	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	s	s	r	1	C.guilliermondii
81	Suvarna Pujari	26	F	24170	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	s	s	r	1	C.guilliermondii
82	Jettappa	48	M	24003	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	r	r	r	1	C.kefyr
83	Ramesh Khatikar	32	M	24935	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	r	r	r	1	C.kefyr
84	Gowrawwa	60	F	24983	Pseudomemb	+	+	+	-	+	+	+	+	+	-	+	s	s	s	s	1	C.albicans
85	Pavithra	42	F	25611	Pseudomemb	+	+	+	+	+	-	+	+	+	+	+	r	r	s	r	1	C.albicans
86	Charu patil	32	M	24786	Pseudomemb	+	+	+	-	+	+	+	+	+	+	+	r	s	r	r	1	C.tropicalis
87	Gangabai	45	F	25349	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	s	s	s	s	1	C. kefyr
88	K B Baradd	89	M	290812	Angular chelitis	+	+	-	-	+	+	+	+	-	-	-	r	r	s	r	1	C. parapsilosis

89	Suresh Shanthappa	38	M	25509	Pseudomemb	+	+	-	-	-	+	+	+	-	+	+	r	r	r	r	1	C.guilliermondii
90	Shanthaveri	25	F	2771	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	r	r	r	r	1	C.guilliermondii
91	Naveen B	35	M	3240	Pseudomemb	+	+	-	-	+	+	-	+	-	+	+	s	s	s	s	1	C.guilliermondii
92	Janatha bai	55	F	25602	Pseudomemb	+	+	-	-	+	+	+	+	+	-	+	s	s	s	s	1	C.tropicalis
93	Hanumanth Bagli	60	M	37724	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	s	s	1	C. parapsilosis
94	Kadappa	45	M	25690	Pseudomemb	+	+	-	-	+	+	+	+	-	+	+	s	s	s	r	1	C.guilliermondii
95	Somappa	40	M	26189	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	s	r	r	r	1	C.kefyr
96	Umesh Kabadi	38	M	24567	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	r	r	1	C. parapsilosis
97	Channappa	35	M	26242	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	s	s	s	1	C.tropicalis
98	B Chinnappa	17	M	22088	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	s	s	s	r	1	C. parapsilosis
99	Chanagondi Jettappa	40	M	26310	Pseudomemb	+	+	-	-	+	+	-	+	-	-	-	s	s	s	s	1	C.krusei
100	Mallikarjun Hosmani	40	M	26525	Pseudomemb	+	+	+	-	+	+	+	+	+	-	+	s	s	s	s	1	C.albicans
101	Umesh Maruddi	41	M	26504	Erythematous	+	+	-	-	+	+	-	+	-	-	-	r	r	s	s	1	C.krusei
102	Kavyashree	33	F	276	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	r	r	r	1	C.kefyr
103	Devanna	44	M	22408	Pseudomemb	+	+	-	-	+	+	+	+	+	+	-	r	s	r	s	1	C.tropicalis
104	Praveen Aloor	47	M	2361	Erythematous	+	+	-	-	+	+	+	+	-	+	-	r	r	r	r	2	C.tropicalis
								-	-	+	+	+	+	-	-	-	r	s	r	r		C. parapsilosis
105	Rajesh Prabu	58	M	2466	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	r	r	r	s	1	C.tropicalis
106	Sneha T	29	F	237	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	r	s	s	s	1	C.kefyr

107	Prabu Gayak	32	M	2468	Pseudomemb	+	+	-	-	+	+	-	+	-	+	+	r	r	r	s	1	C.guilliermondii
108	Anirudh Koyala	22	M	213	Erythematous	+	+	-	-	+	+	+	+	-	-	-	r	s	r	r	1	C. parapsilosis
109	Bhagyalakshmi	34	F	22467	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	r	s	r	1	C.tropicalis
110	Mahadevi	35	F	113	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	r	r	r	1	C. parapsilosis
111	Mallamma	42	F	467	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	s	r	r	s	1	C.tropicalis
112	Hanmanth Doddamani	40	M	192	Pseudomemb	+	+	-	-	+	+	+	+	-	-	-	r	s	s	s	1	C. parapsilosis
113	Basavraj	30	M	476	Pseudomemb	+	+	-	-	+	+	+	+	+	-	-	s	s	s	s	1	C.tropicalis
114	Shankarrappa Tumba	60	M	107	Pseudomemb	+	+	-	-	+	+	+	+	+	+	-	r	r	r	r	2	C.fomata
								-	-	-	+	+	+	-	-	-	r	s	s	r		C.krusei
115	Paramanand	53	M	109	Pseudomemb	+	+	-	-	+	+	-	+	-	+	-	r	r	r	r	1	C.tropicalis
116	Nanasaheb	30	M	27878	Pseudomemb	+	+	-	-	-	+	-	+	+	+	-	s	s	r	s	1	C.kefyr
117	Prakash Pujari	30	M	27594	Pseudomemb	+	+	-	-	+	+	+	+	-	+	-	r	s	r	s	1	C.tropicalis
118	Savitha	25	F	27700	Erythematous	+	+	+	+	+	+	+	+	+	+	-	r	r	s	r	1	C.albicans