STUDIES IN ADHESION OF *CANDIDA ALBICANS* ON HUMAN BUCCAL EPITHELIAL CELLS

Thesis

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I **Pavithra Kumari**, hereby declare that the Research Thesis entitled **"Studies in Adhesion of *Candida albicans* on Human Buccal Epithelial Cells"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** in **Chemical Engineering** is a ***bonafide report of the research work*** carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to certify that the Research Thesis entitled " **Studies in Adhesion of *Candida albicans* on Human Buccal Epithelial Cells "** submitted by **Pavithra Kumari** (Register No: **CH07P02**) as the record of the research work carried out by her is accepted as the *Research Thesis Submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy.**

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DEDICATED

TO

MY FAMILY

ABSTRACT

Adhesion of microbe on host cell is the most important step in establishment of colonization, biofilm formation and infection. In the present study adhesion of *C. albicans* on Human Buccal Epithelial Cells (HBEC) was studied among HIV seropositive individuals in relation to increased candida colonization and formation of Oral Candidiasis (OC). A total of 274 HIV seropositive and 260 healthy HIV seronegative subjects were included in the study. The prevalence of OC in HIV seropositive individuals was 40.8% and 8.57% *C. albicans* strains obtained in HIV seropositive individuals were resistant to fluconazole.

The adhesion was seen more with both HBEC and *C. albicans* isolates obtained from HIV group. Biochemical studies on cell wall protein profiling of candida and HBEC showed importance of lectin-carbohydrate type of mechanisms in candida adhesion to HBEC. Lectin blotting of cell wall proteins using various lectins showed expression of extra bands in *C. albicans* isolates obtained from HIV seropositive group. Thus indicating possible glycosylation of the cell wall proteins and due to which enhancement of host parasite interactions occur.

Physicochemical properties of the cell like cell surface hydrophobicity, cell surface charges, zeta potentials were been analyzed for their role in adhesion. The biochemical functional groups present on candidal cell wall and epithelial cell wall during adhesion and free conditions were analyzed using Fourier Transform Infrared (FT-IR) Spectroscopy. Emergence of newer bands during the cell adhesion was noticed and discussed. Nanotechnological approaches on adhesion research using Atomic Force Microscopy (AFM) also attempted. Adhesion inhibition tried using few surfactants, fluconazole, plant extracts. Neem, tea tree oil, lemon grass extracts, Sodium Dodecyl Sulphate (SDS); are found to be successful candidates in effectively inhibiting the adhesion. A few important findings were also observed here which can help to perceive future research in this field.

Keywords: *C. albicans*; Human Buccal Epithelial Cells; Adhesion; HIV infection; Cell Surface Hydrophobicity; zeta potential; Electrophoretic mobility; antiadhesion.

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NOMENCLATURES

Description	Symbol
Apolar component of the free energy variation between two entities of a given surface (s) immersed in water (w) (mJ m^{-2})	ΔG^{LW}_{AB}
Electron donor component of the liquid	γ^{-1}
Polar surface tension parameter (mJ m^{-2}).	ΔG^{AB}_{S}
Apolar (Lifshitz-vander Waals) surface tension parameter of a surface (mJ m^{-2}).	γ^{LW}_{S}
Apolar (Lifshitz-vander Waals) surface tension parameter of liquid (mJ m^{-2})	γ^{LW}_{l-}
Atmosphere	Atm
Base pairs	bp
Centimeters	cm
Colony forming units	CFU
Daltons	Da
Degree Centigrade	^{O}C
Electron acceptor component of liquid	γ^{+l}
Electron acceptor component of surface	γ^{+S}
Electron donor component of the surface	γ^{-S}
Gram per litre	g/l
Grams	g
Greater than	>
Hour	hr
Lesser than	<
Litre	l
Logarithm	log
Micro gram	µg
Micro litre	µl
Milligrams	mg

Millimolar	mM
Minute	min.
Moles	mol
Nanometer	nm
Optical density	OD
Percentage	%
Polar component of the free energy variation between two entities of a given surface (s) immersed in water (w) (mJm^{-2})	ΔG_{SWS}^{AB}
Revolution per minute	rpm
Time	t
Total free energy variation between two entities of a given surface (s) immersed in water (w) (mJm^{-2})	ΔG_{SWS}^{total}

ABBREVIATIONS

Abbreviation	Description
AIDS	Acquired Immune Deficient Syndrome
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
CFU	Colony Forming Units
Con A	Concanavalin A lectin
DI water	Deionized water
DLVO Theory	Theory of physicochemistry of microbial adhesion by Derjaguin, Landau, Vervey and Overbeek
DTT	Dithiothreitol
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EPM	Electrophoretic Mobility
GPI	Glycosyl Phosphatidyl Inositol linked proteins
HIV	Human Immune Deficiency Virus
NAC	Non Albicans Candida
NBT	Nitro blue Tetrazolium
OC	Oral Candidiasis
OI	Opportunistic Infection
PBS	Phosphate Buffered Saline
PNA	Peanut Agglutinin
RAPD	Randomly amplified polymorphic DNA finger printing
RGD	arginine-glycine-aspartic acid linked
SDS	Sodium Dodecyl Sulphate
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
WGA	Wheat Germ Agglutinin

CHAPTER 1 INTRODUCTION

The management of human health was put into new challenges during the 20th century with the global emergence of Human Immunodeficiency Virus (HIV) and several other types of infections like HINI (SAARS) (Butler 2012).

The majority of the adults with HIV infection of the total HIV seropositive population of the world were concentrated in Eastern, Central and Southern Africa initially. But the infection has been spreading to West and South Africa, India and other countries in South-east Asia, and to a lesser extent to North America (Mertens and Low-Beer 1996)

The first case of HIV infection was documented in India in 1986. India is presently experiencing epidemic of HIV infection in an explosive manner (Bollinger et al.1995). It was estimated that number of HIV seropositive persons in India rose from 1-2 million in 1991, to 3.5 million in 1998 and to 3.9 million in 2000. Women and children constitute more than 50% of infected population. According to a 2009 survey, the prevalence of HIV infection in Indian population is 0.36/1000 among men and 0.25/1000among women (Sontakke et al. 2011). The present rate of transmission shows that, in the near future India will harbor the highest number of HIV seropositive population than all of the countries in the world. Commercial sex workers are the high-risk groups who have been transmitting this infection. Intravenous drug users and commercial blood donors are also at high risk (Bollinger et al.1995).

During HIV infection, once the immunocompromised condition establishes, patients suffer with several types of opportunistic infections (Paniker 2005). Among the Indian HIV seropositive patients, co-infection with tuberculosis appears to be seen predominantly followed by Oral Candidiasis (OC) being other commonest opportunistic infection (Bollinger et al.1995).

1.1 ORAL CANDIDIASIS (OC) IN HIV SEROPOSITIVE INDIVIDUALS

The studies done with Indian HIV seropositive population from various states, showed that, OC was found to be the most common opportunistic infection present in their study subjects (Shobhana et al. 2004; Sontakke et al. 2011; Chopra and Arora 2012; Rao et al. 2012) The appearance of OC in these patients was seen to be further associated with decreased CD4+ count. Patients with CD4+ count less than 200/mm^3 are more prone to develop OC (Sontakke et al. 2011). Thus OC reflects the status of HIV infection entering into phase of declined immunity and terminal stage.

The fungus candida being the etiological agent of OC and *C. albicans* is the most commonly isolated species of candida from OC (Larone 2002). In normal condition, *C. albicans* occurs as normal inhabitant in the oral cavity and known to be isolated from 3-47% of healthy individuals (Samaranayake et al. 1986). However, the asymptomatic carriage rate in HIV population is noted to be more compared to healthy individuals (Ohmit et al. 2003). Oral colonization status of candida in HIV infection further proceeds to the formation of lesions (Vargas and Joly 2002). A few important findings on oral candida carriage studies say that the candida isolates of oral cavity of HIV seropositive patients are found to be more drug resistant and more virulent than the isolates present in normal healthy oral cavity (Antony et al. 2007). The genetic analysis of candida isolates from HIV seropositive individuals revealed that commensal isolates may be retained throughout the HIV infection with minor genetic micro-evolution and later produce episodes of OC (Vargas and Joly 2002).

OC has been treated with different antifungal agents such as polyenes, nystatin, amphotericin, clotrimazole and fluconazole. Fluconazole is more preferable due to its water solubility, excellent tolerance, low toxicity and favorable pharmacokinetics (Enwuru et al. 2008). Use of fluconazole markedly increased with evidence of OC in HIV infection and fluconazole became the most commonly used antifungal agent in the world, resulting in increased prevalence of fluconazole resistant *C. albicans* and the species of candida like *C. glabrata* and *C. krusei*, which are intrinsically less fluconazole-sensitive (Greenspan 1998). Fluconazole resistance is a major problem in the treatment of OC especially in HIV seropositive patients. The fluconazole resistance varies from 5 – 36% (Enwuru et al. 2008). Immunosuppression

and long term use of fluconazole are said to be the factors responsible for fluconazole resistant candidiasis (Nadiger et al. 2008). These findings suggest that knowing candida carriage status, prevalence of OC in HIV seropositive patientsand fluconazole susceptibility pattern of the candida isolates at certain geographical area is of utmost importance.

Emergence of fluconazole resistance as discussed above, suggests the development of a new generation antifungal agents or an agent which can block the virulence of the candida isolate, which is the need of the hour. In this context, developing antiadhesion approach as an alternative method can be thought. Antiadhesion treatment approach is more advantageous asnumber of organisms developing resistance to anti adhesive drug is lesser (Ofek et al. 2003a). Antiadherence therapy is based on the concept of blocking adherence of pathogen to susceptible tissue. The concept of antiadherence therapy has been used in a few viral and bacterial infections (Ofek et al. 2003b). Microbial adhesion has impact on medical, environmental and industrial processes. The ultimate goal of microbial adhesion studies in medical application is to prevent adhesion of pathogenic organisms adhering to human cells and intravascular devices. Adhesion can lead to colonization of the organism and formation of the infection (Hanna et al. 2003). However while implementing these thoughts, antiadherent therapy has many challenges to overcome (Ofek et al. 2003a). Antiadhesion approaches were tried using antiadhesive substances and antiadherent biomaterials (Chandra et al. 2005). Prior knowledge of mechanism and factors affecting the adhesion and specific proteins involved in adhesion which is possessed by the microbe and the host cells could lead to the development of successful compounds as ideal tools for preventing or treating certain infection.

1.2 ADHESION OF *C. ALBICANS* ON HUMAN BUCCAL EPITHELIAL CELLS (HBEC)

Though *C. albicans* exhibits many virulence factors, adhesion to the host cells play an important role. Adherence of the fungus onto host cells is the prerequisite for colonization of the organism in producing infection. The interaction between the organism and the host occurs at the level of cell wall (Marot-Leblond et al. 2000). Candidal adhesion to host cells, inert surfaces and biofilm formation are the major risk factors in developing candida infections in immunodeficient patients and individuals implanted with intravascular devices, like catheters, dentures, contact lenses, heart valves etc (Douglas 2003). The adhesion of this fungus on the host epithelium occurs through the molecules present on cell surface of the candida, which are known to be adhesins. Candida cell wall molecules are acting as receptors for fibrinogen, fibronectin and other components of the extracellular matrix of the host. Candida cell wall glycans are exposed to the surrounding media and are probably the first to interact with components of host cell surfaces. Studies on *C. albicans,* showed that the binding could involve oligosaccharide and lectin-like components (Chaffin 1998). The peptide sequence of lectin-like mannoprotein of the fungal cell wall links to fucosyl or to N-acetyl glucosamine or galactosamine residues belonging to the surface of the epithelial cells (Brassart et al. 1991; Hoffman and Haidaris 1993). Fimbrial adhesins, chitin and integrin like molecules are known to be involved in adhesion (Meitner et al. 1990; Hostetter 1994; Yu et al. 1994). *Candida*-epithelium interaction can provoke reciprocal signaling also (Bailey et al. 1995). Understanding extracellular glycan structure and function could thus provide an important insight into the treatment of fungal diseases.

The interaction between the molecules involves electrostatic forces and repulsion (James 1991). The studies on influence of non-specific or physicochemical factors in interaction of *C. albicans* with HBEC, e.g. Cell Surface Hydrophobicity (CSH), zeta potential, cell topography and type of interaction forces involved can help in exploiting the adhesion mechanism in detail. Blanco et al. (1997) reported that candidal adhesion can be related to surface hydrophobicity.

The transition of candida from a commensal to a pathogen may also be influenced by host environment (Senet 1998). Adherence to HBEC by *C. albicans* from HIV seropositive individuals is seen to be higher, as compared to the isolates from normal subjects (Mane et al. 2012). These changes may be due to the expression of altered receptors both on the surface of candida cells as well as on host buccal epithelial cells. If altered expression of receptors, either on the candida cells or on the host cells or both is observed then the factors which contribute to this phenomenon can be elucidated. The characterization of the carbohydrate groups present on the candida cell wall helps in better understanding of fungal adherence and the mechanism by which the fungus establishes as a pathogen.

In conclusion, the literature available on studies of adhesion of candida onto HBEC reveals that adhesion process is carried out by complex mixture of molecules. However, a few factors on adhesion of candida onto HBEC have not been studied extensively, i.e.,

- Changes occurring in isolates while producing OC infection in HIV seropositive host.
- Mechanisms involved in adhesion process together with biochemical and physicochemical aspects.
- New plant based antifungal agent to control emergence of isolates resistance to fluconazole.

1.3 APPROACHES TO UNDERSTAND ADHESION MECHANISMS INVOLVED

In view of the above discussion, main approaches designed in understanding the mechanism of OC can be: (i). analyzing the mechanisms of adhesion in *C. albicans* isolated from immunocompromised patients by studying the surface molecules, (ii). Studying the role of electrodynamic forces like Cell Surface Hydrophobicity (CSH) and electrostatic forces like zeta potential in adhesion and(iii). Studying the on the antiadhesion approaches.

The extensive literature survey on candidal adhesion on HBEC has revealed that certain aspects of candida adhesion research requires further investigations to bring clarity and clear the conflicting results reported in literature, e.g.,

1. Certain structural changes may occur in cell wall proteins of *C. albicans* isolates from oral cavity of HIV seropositive individuals, which can be studied in detail using panel of lectins. The application of lectin blotting to understand the changes occurring with cell wall proteins has not been reported extensively.
2. Influence of cell surface characters on adhesion were not well documented when considering the adhesion of candida to living objects (host cells).
3. Though Fourier Transform Infrared Spectroscopy (FTIR) was used in studies with candida biofilm formation, yet the involvement of cell surface functional groups in adhesion of *C. albicans*, on HBEC has not been explained. However, FTIR was used to explain adhesion process in other microbes (Parikh and Chrover 2006).
4. The substances which can inhibit the adhesion of candida on the epithelium, have rarely been studied and reported.

1.4 AIM AND OBJECTIVES OF THE STUDY

Considering the above mentioned literature gaps, the hypothesis for the study laid was that, the adhesion of mere commensal candida can lead to colonization and formation of lesions, when the host is immunocompromised. There are several factors in the oral cavity like immune system and flow of saliva which can control candida by inhibiting adhesion to HBEC. Whenever the host factors become weak, candida can develop infection. In such infection process, these changes may be due to enhanced adhesion of this fungus onto HBEC, either through specific and nonspecific mechanisms of adhesion.

Therefore, based on the published reports and the present focus on anti-adhesin research studies, present study was mainly focused on the candida adhesion process leading to the formation of OC in HIV seropositive patients. To achieve this aim following objectives were framed:

1. Comparing the adherence property of oral isolates of *C. albicans* from HIV seropositive and HIV seronegative individuals.
2. Studying the mechanisms of adherence under various conditions, including organism factors and *invitro* experimental conditions.
3. Characterization of adhesins of *C. albicans* and their receptors present on the HBEC.
4. Studying the molecules which block the adherence of candida to respective ligand in the most effective manner.

1.5 ORGANISATION OF THE THESIS

The entire study done is represented in this thesis by arranging the chapters in the following sequence:

- Chapter 2 gives complete information gathered from published reports on *C. albicans*, present scenario of prevalence of OC in HIV seropositive individuals, mechanisms involved in the adherence of candida on to HBEC.
- Chapter 3 provides materials and methods followed to achieve the objectives proposed.
- Chapter 4 presents the results obtained in the present study, discussions and conclusions pertaining to each segment of the work.
- Chapter 5 represents Summary and future research frontiers opened up by the present research work

The appendix, bibliography, publications and presentations carried out using this research work, followed by brief bio-data of the student are given in the proceeding parts of the thesis.

CHAPTER 2. LITERATURE REVIEW

Present chapter deals with the review of published information with respect to factors responsible for infection in human beings in general and HIV seropositive candidates in particular. Further the chapter also reviews the information available in the literature on the oral infections especially with respect to HIV seropositive individuals and factors responsible for OC. These chapters also present the published information with respect to the factors responsible for adhesion of *C. albicans* onto Human Buccal Epithelial Cells (HBEC), and also encompass both specific and non specific mechanisms of adhesion. The salient aspects available in literature with respect to inhibition of adhesion through the use of surfactants, plant extracts and sub inhibitory concentrations of fluconazole have also been presented in the chapter.

2.1 HUMAN INFECTIONS

The infectious diseases are the major hazards for human health in various ways. The infectious diseases result from lodgment and multiplication of microbial agents such as viruses, bacteria, fungi, protozoa, and helminthes in the human body (Presscott et al. 2002). Microorganisms are abundant in nature and most of them are present as saprophytes. Millions of microbes occur as normal inhabitants on skin, gastro intestinal tract, vagina, lower urinary tract etc. Excluding this saprophytic and commensal habitat, only a few thousands of the microbes behave as pathogens. Several pathogenic organisms have a broad range of host and thus their existence can be maintained in the community, without break (Wolfe et al. 2007). The review on the origin of major human infections say that, there can be evolutionary changes occurring in the microbe which lead to the emergence of several infections and which might have happened most commonly within the last 11, 000 years. There are a few exclusive pathogens of human beings which have been noted, in the course of evolution they get confined to only in human body, such as Human Immunodeficiency Virus (HIV) (Wolfe et al. 2007).

2.1.1.1 CLASSIFICATION OF INFECTIOUS DISEASES

Infectious diseases can be classified according clinical presentations, etiological agents involved and type of epidemiology presented etc. The microbiological classification of infections based on the etiological agent involved shows the following groups, i.e., bacterial, viral, fungal, and parasitic and prion induced infections (Paniker 2005).

Infectious diseases can also be classified according to the duration of their appearance, like acute (quick onset), chronic and latent. According to the location, they can be local or systemic infections. The infections can be termed as primary or secondary infections. Secondary infections are produced in humans when their immunity gets weakened by primary infection (Paniker 2005).

2.1.1.2 FUNGAL INFECTIONS

Fungal infections can be further classified as superficial, cutaneous, subcutaneous and deep mycoses depending on the site involved. One more group of fungal infection is opportunistic mycoses where infections commonly seen in patients with debilitated immunity. One such example is Oral Candidiasis (OC) produced in HIV seropositive individuals (Larone 2002).

OC is confined to mucosa of the oral cavity. The infection is produced more commonly as secondary infection or in the presence of pre-disposing factors (Odd 1994).

2.1.2 INTRODUCTION TO HIV INFECTION

As the pathogenic microbe enters the body, immune system gets stimulated and which can further develop a certain type of immune response and by the result of which pathogen can be eliminated. In opposition to the immune response of the host, the pathogens have the virulence factors to fight against the immune system of the host. If the virulence factor of organism is high it can withstand host's immune response and produce infection (Presscott et al. 2002; Joanne et al. 2008).

In case of HIV infection, immunodeficiency is prominently observed in the host. The HIV virus attacks directly one of the components of immune system i.e., CD4+ T cells, and make the host vulnerable even to rare and unusual pathogens and also for developing malignancies. A few of the saprophytic or commensal microbes can easily produce infections in this host, which can be referred as opportunistic infections. Thus immunodeficiency developed due to HIV infection leads to the highest morbidity and mortality in the patient compared to any other infection (Gordon and Alimuddin 2009).

2.1.2.1 HISTORY OF HIV

The first observation of HIV infection was done in five homosexual patients from Los Angeles and New York of United States. Presence of rare types of pneumonia, caused by *Pneumocystis jeroveci* together with other infections and malignancies were observed in these patients. The generalized immunodeficiency and rare type of infections gave first clue about the existence of a new infection, which was named as Acquired Immunodeficiency Syndrome (AIDS). Later by the retrospective analysis, it was observed that the existence of AIDS was thought to be present in Central Africa as early as in 1950's and also the causative viral particles were suspected to be present from 1930 (Gordon and Alimuddin 2009) . The result of extensive research on different AIDS cases, in 1986, the causative agent of the AIDS was defined completely and named by International Committee on Virus Nomenclature as Human Immunodeficiency Virus (HIV) subsequently. Mean while serological tests to detect HIV antibodies in blood of infected patients were also developed (Paniker 2005).

The AIDS infection is caused by the lentivirus HIV, within the virus family retroviridae. Presently HIV-1 virus exhibits 10 sub types, namely, A-J. Sub group C is the predominant type of HIV -1 found in India and Southern Africa. HIV-2 is closely related virus to HIV-1, with some structural difference in reverse transcriptase enzyme of HIV-1 which leads to slower progression to immunodeficiency. HIV-2 is confined commonly to West Africa (Gordon and Alimuddin 2009).

2.1.2.2 SPREAD OF HIV INFECTION

In the present scenario the HIV infection is found worldwide. The 68% of the HIV seropositive population of the entire world, lives in Sub Saharian Africa (UNAIDS 2007). In India, the first case of HIV infection was reported in 1986 from Chennai. Since then AIDS had spread widely in various states of India. It is posing a big challenge to the health of low income group in India. At present India is carrying the second largest population of HIV seropositive individuals in the world. According to National AIDS Control Organization (NACO) HIV sentinel Surveillance report, at the end of 2009, the prevalence of HIV infection in India was, 0.31% with 24.9 lakhs of people infected with HIV throughout the country. The distribution of HIV infection among the states of India is heterogeneous with high prevalence seen mainly in south Indian states and north eastern part. Manipur is having the highest prevalence (1.40%), with Karnataka being fifth highest prevalent state to have HIV infection with the prevalence rate, 0.63%. In Karnataka, prevalence rate of HIV seroreactivity in adult male patients is 0.75% and in adult females is 0.5% (NACO 2011).

HIV spreads mainly through sexual contact and several socioeconomic factors are associated with the spreadingof HIV infection in India. High risk sexual behavior, injectable drug abuse, transmission through infected mother, infected blood and blood products and body fluids are the sources for the spreading of infection. Having other Sexually Transmitted Infections (STI), multiple sex partners, alcohol consumption, migrant life are the factors associated with acquisition of HIV infection. Continuous mobility of men as the long distance truck drivers and urbanization are also a few of the socio-demographic factors that nourish the spreading of HIV infection in India (Pandey et al. 2012).

2.1.2.3 OPPORTUNISTIC INFECTION (OI) SEEN IN HIV INFECTION

During HIV infection as the virus attacks on CD4+ T cells and multiply inside these cells, gradual decrease in CD4+ T cell count can be observed. CD4+ T cells are the main components of cell mediated immunity; therefore it is clearly evident that HIV infection causes damage to the immune system (Gordon and Alimuddin, 2009).

In normal healthy Indian population, CD4:CD8 ratio is approximately 1.2, which has been recorded as a mean value by obtaining results from six laboratory centers from different locations in India (Saxena et al. 2004). Ashwini et al. (2010) also reported that an average CD4+ T cell count in healthy Indian population can be varying from, 703 to 1048 cell/mm^3. During HIV infection, as and when viral load increases the CD4/CD8 ratio reverses. Once the CD4 cell counts fall below 200 cells/mm^3, the patient falls into the phase called Acquired Immunodeficiency Syndrome (AIDS). In AIDS phase, cell mediated immunity is lost and viral load will be rapidly increasing and is associated with the appearance of several Opportunistic Infections (OI).

Since the HIV infection causes overall damage to the immune system of infected person, several infections and sarcoma can occur in the host during HIV infection. HIV-related opportunistic infections commonly enumerated are Oral Candidiasis, Histoplasmosis, Cryptococcosis, Penicillosis, Herpes Simplex virus infections, bacterial infections of periodontal cavity and tuberculosis (Paniker 2005). Among the OIs present in HIV infections, oral manifestations play an important role, which act as earliest indicators of HIV infection (Coogan et al. 2005).

2.1.2.4 ORAL HEALTH AND ORAL LESIONS SEEN IN HIV SEROPOSITIVE PATIENTS

Oral manifestations commonly seen in HIV infection are Oral Candidiasis (OC), hairy leukoplakia, Kaposi sarcoma, linear gingival erythema, necrotizing ulcerative gingivitis, necrotizing ulcerative periodontitis and non-Hodgkins lymphoma. The oral manifestations seen in HIV infection (based on EC-Clearinghouse classification) are given in Table 2.1. Oral lesions in HIV infection appear, inversely to the level of CD4+ T cell count , i.e., most of these infections appear when CD4+ T cells fall to 200/mm^3 or less. The intake of HAART (Highly Active Anti Retro Viral Therapy) can improve CD4+ T cell level, and decrease the presence of oral lesions (Coogan et al. 2005).

Table 2.1 Ec-clearinghouse classification of the oral manifestations seen in HIV seropositive adults (Coogan et al. 2005)

Group 1 lesions strongly associated with HIV infection	**Group 2 lesions less commonly associated with HIV infection**	**Group 3 lesions seen in HIV infection**
Oral Candidiasis • Erythematous • Pseudomembranous **Malignancies** • Hairy leukoplakia • Kaposi's sarcoma • Non-Hodgkin's lymphoma **Periodontal disease** • Linear gingival erythema • Necrotizing gingivitis • Necrotizing	**Bacterial infections commonly due to** • *Mycobacterium avium-intracellulare* • *Mycobacterium tuberculosis* Melanotic hyper pigmentation Necrotizing (ulcerative) stomatitis **Salivary gland diseases** • Dry mouth due to decreased salivary flow rate	**Bacterial infections commonly due to** • *Actinomyces israelii* • *Escherichia coli* • *Klebsiella pneumoniae* • Cat-scratch disease **Drug-reactions** • Ulcerative • Erythema multiforme • Lichenoid • Toxic epydemolysis **Epithelioid (bacillary)**

periodontitis	• Unilateral or Bilateral swelling of major salivary glands • Thrombocytopenic purpura **Ulceration NOS** (not otherwise specified) **Viral infections** • Herpes Simplex Virus infections • Human Papilloma Virus lesions • Condyloma acuminatum • Focal epithelial hyperplasia • Verruca vulgaris • Infections due to Varicella Zoster Virus Herpes zoster and Varicella	**angiomatosis** **Fungal agents causing infections other than Candida** • *Cryptococcus neoformans* • *Geotrichium candium* • *Histoplasma capsulatum* • Mucoraceae (mucurmycosis, zygomycosis) • *Aspergillus flavus* **Neurological disturbances** • Facial palsy • Trigeminal neuralgia **Viral infections due to** • Cytomegalovirus • *Molluscum contagiosum*

According to Ec-clearinghouse classification of the oral manifestations seen in HIV seropositive adults, Oral candidiasis (OC) has been placed in Group I lesions strongly associated with HIV infection (Coogan et al. 2005).

The repeated hospitalization, no access to anti retroviral therapy, poor oral hygiene, and impaired cell mediated immunity in the oral cavity are the precipitating factors for oral manifestations in HIV seropositive individuals (Coogan et al. 2005).

In HIV seropositive individuals, the OC is the most common oral health problem observed (Banerjee 2005). As explained previously the infecting candida

species can be endogenous flora itself, which will increase and colonize and later cause invasion (Vargas and Joly 2002). The oral lesions produced can be easily visualized and diagnosed by both clinical and laboratory methods. Several aspects concerning OC in HIV seropositive patients can be further discussed.

2.1.3 ORAL CANDIDIASIS (OC) IN HIV INFECTION

2.1.3.1 INTRODUCTION

Presence of OC is associated with more frequent progression of HIV infection to AIDS. OC occurs in majority (upto 95%) of HIV seropositive individuals at different stages of their illness (Klein et al. 1984; Dos Santos Pinheiro et al. 2009). OC is mucocutaneous candidiasis infection;it affectsonly oral mucosa in early stages of infection in HIV patients. In advanced stages of HIV, OC infection can spread to esophageal mucosa which is referred as Oropharyngeal Candidiasis (OPC) (Klein et al. 1984).

The incidence of candidiasis increases with progressive cellular immunodeficiency, particularly as CD4+ lymphocytes fall below 200-300/mm^3 (Nadiger et al. 2008).

2.1.3.2 ETIOLOGY OF OC

C. albicans is the most frequently reported causative agent associated with OC in HIV seropositive individuals (Dos Santos Pinheiro et al. 2009). Additionally other candida species including *C. glabrata and C. tropicalis* are also being described as important pathogens isolated from HIV seropositive individuals. Prevalence of Non Albicans Candida (NAC) occurs with following prolonged imidazoles or triazole antifungal therapy (Nadiger et al. 2008; Johnson et al. 1995).

2.1.3.3 SYMPTOMS AND CLASSIFICATION OF OC LESIONS

The patients with OC lesions experience metallic taste and burning sensation in the mouth and loss of appetite. Due to the formation of sore, patient experiences pain, while drinking and eating (Robinson 1997). Oral candida lesion can appear with formation of white, curd like plaques or reddening along the dorsum of the tongue or lesions at the fissures of the mouth (Fotos et al. 1992)

According to the type of lesions and chronicity, OC can be broadly divided into 3 main categories.

a) ACUTE

- Pseudomembranous.
- Erythematous (Atrophic).

b) CHRONIC

- Denture stomatitis.
- Hyperplastic variants.
- Angular cheilitis.

c)MUCOCUTANEOUS CANDIDIASIS

- Localized.
- Familial.
- Syndrome related.

Usually in HIV seropositive patients 4 common types of OC can be observed. Characteristic features of each of these lesions are explained in Table 2.2. The two or more types of lesions are known to occur in the same patient. Pseudomembranous, Erythematous and Angular cheilitis are the commonly reported varieties of OC lesions seen in HIV seropositive individuals (Sarika 2005).

Table 2.2 Clinical presentations of major forms of OC lesions

Clinical type	Clinical appearance	Microscopy
Pseudomembranous	White, curd like plaques covering mucosal erythema that are easily removed, revealing underlying erythema and/or ulceration	Hyphae and spore forms in large numbers at mucosal surface with superficial invasion of epithelium
Atrophic	Mucosal erythema and or papillary atrophy of tongue dorsum	Superficial invasion by few or many hyphae and spores into surface epithelium
Hyperplastic	White plaques that can be only partially removed with light abrasion ("thrush")	Hyperkeratosis of epithelium with superficial invasion by hyphae and spores
Epidermal and perioral	Erythematous, pruritic, scaling patches	Superficial invasion of epidermis by hyphae and spores

2.1.3.4 FACTORS INFLUENCING THE ESTABLISHMENT OF OC

Decreased CD4+ T cells and high viral load in the plasma are the main factors for developing oral lesions in HIV seropositive individuals. Protein energy malnutrition which can cause damage to oral mucosa can be one of the predisposing factors for the development of OC in HIV seropositive individuals. Along with poor oral hygiene, illiteracy, smoking, alcohol consumption and limited access to the dental health facilities in the developing countries enhance the rate of development of oral lesions in HIV seropositive individuals (Nittyanannta et al. 2002; Ranganathan et al. 2004).

In normal healthy, HIV seronegative individuals, OC can be treated with azoles and cured. However in HIV seropositive individuals, the infection can remain chronic due to decrease in cell mediated immunity (Cantorna and Balish 1991).

2.1.3.5 PREVALENCE OF OC IN HIV SEROPOSITIVE PATIENTS

It is seen from the studies that higher prevalence of OC occurs in HIV seropositive patients from developing countries (Campo et al. 2002). OC is the most common lesion to be seen among all the other various OIs present in HIV seropositive individuals in developing countries (Ranganathan and Hemalatha 2006). Certain OIs are commonly reported in patients from particular part of the world, e.g., Kaposi's sarcoma only from Africa and Latin America, Histoplasmosis and Penicilliosis in patients from Thailand and HIV-associated salivary gland disease from Africa and Latin America (Ranganathan and Hemalatha 2006). Most of the reports from India show, OC is the most prevalent OI seen in Indian HIV seropositive patients.

According to the reports of NACO (National AIDS Control Organization 2000) and Giri et al. (1995), OC is found to be the commonest OI seen in Indian scenario. Most of the studies were conducted to understand OC in HIV seropositive patients, included work on CD4+ T cell count, and HAART (Highly Active Anti Retro Viral Therapy) status in these individuals. A few such reports are discussed below.

The report from Eastern India, i.e., from Kolkata shows OC (88%) as most common OIs, followed by tuberculosis (57%) in 125 HIV seropositive patients. Appearance of OIs here found to be inversely proportional to CD4+ T cell count of the HIV seropositive patients (Chakraborty et al. 2008). In a recent study Saha et al (2011) reported that OC was the most common (54.43%) OI seen in the 204 HIV seropositive patients observed in their study. They found that, OIs were appearing when CD4+ count goes below 200 cells/mm^3 in these patients. In a south Indian study, 80% of the HIV seropositive patients reported to be showing oral manifestations with erythamatous type of candida lesions as the more prevalent entity. High viral load with a mean CD4/CD8 ratio of 0.24 was found in the study subjects (Gaurav et al. 2011). The study conducted at Manipal in Karnataka, for a period of 3 yrs i.e. from 1999 to 2001, showed that, out of 100 patients evaluated, 59% had oral thrush or OC. *C. albicans* was the most common pathogen isolated from these OC cases. The next common OI's seen with this group of patients were, tuberculosis,

Cryptosporidiosis and *Pneumocystis carinii*. The male patients constituted huge majority of this group with 87% of the whole population tested (Singh et al. 2003). In a study from North India, Chhattisgarh, OC was seen with 17.5% HIV seropositive patients and there was a highly significant correlation ($P < 0.0001$) with the presence of oral manifestations including OC and decreased CD4+ count (Singh et al. 2009).

There are a few Indian studies which showed that OC is the second most common OI following tuberculosis in HIV seropositive patients in their study (Srirangaraju and Venkatesh 2011). A study from Delhi showed that, the tuberculosis was the commonest predominant OI, followed by parasitic diarrhea and OC (Vajpayee et al. 2003). A study from Pune also showed tuberculosis being the most common OI followed by OC (Ghate et al. 2008). The study conducted by Srirangaraju and Venkatesh (2011), from south India, report that, OC (27.2%) was the second common infection comparing to tuberculosis (54.4%) in HIV seropositive individuals who were not treated with HAART. However after 6 months of HAART, the incidences of OI seen in these study subjects were reduced to 5.56%. A report from Chennai, also showed, OC in 24.8% of the patients and in these patients gingivitis was the most common lesion than OC. Prevalence of oral manifestations was higher in male patients than in females according to this study (Ranganathan et al. 2004).

Irrespective of all the records on prevalence, these authors suggest that regular examination of oral cavity is a must as the presence of OC and weight loss are the important markers of HIV disease progression (Ranganathan et al. 2004).

2.1.4 CANDIDA CARRIAGE AND SPECIES DIVERSITY IN ORAL CAVITY OF HIV SEROPOSITIVE INDIVIDUALS

In HIV seropositive individuals' oral candida carriage rate and density is reported to be increasing prior to the episode of OC (Chien-Ching Hung et al. 2005). An increased incidence of candida colonization is seen with HIV seropositive subjects compared to any other group of individuals with underlying predisposing factors (Felix 1994). Fetter et al. (1993), reported that increased candida colonization occur in HIV seropositive individual even without the presence of

lesions of candidiasis, and these observations were not correlated with CD4+ cell depletion. Candida present initially in oral cavity of HIV seropositive individual as a normal flora can outnumber and produce infection as the CD4+ T cell decreases below 200/mm^3 of blood (Vargas and Joly 2002). Korting et al. (1989), reported that, the oral *C. albicans* carriage rate seen in HIV infection of various severity was as follows, 87.5% with HIV seropositive individuals in CDC stage III, 57.5% with stage I patients showed candida carriage in oral cavity. After the introduction of HAART, prevalence of oral lesions has been decreased, but the rate of asymptomatic oral candida carriage studied in the patients receiving HAART, did not show any reduction (Barchiesi et al. 2002)

During candida carriage in HIV seropositive patient, same strain of *C. albicans* may remain (Vargas and Joly 2002). Vargas and Joly (2002), did the genetic finger printing of *C. albicans* isolates of HIV seropositive patients suggested commensal strains colonizing HIV seropositive individuals, remain throughout the infection process with minor genetic changes, by undergoing alterations prior to producing episode of thrush (Vargas and Joly 2002). They also noted that, asymptomatic oral candida carriage is seen more frequently with the patients having CD4+ T cell count 200 to 300/µl, than other groups with higher CD4+ T cell count. Thus, it is evident that the overgrowth of commensal candida in the oral cavity of HIV seropositive patients is one of the predisposing factors for incidence of OC to occur in these patients.

2.1.5 THERAPEUTIC MANAGEMENT OF OC

OC is treated with antifungal medications, which include polyenes such as nystatin and amphotericin B, imidazoles such as clotrimazole, and triazoles such as fluconazole and itraconazole.

2.1.5.1 FLUCONAZOLE TO TREAT OC

Fluconazole is a triazole which is the most commonly used drug to treat OC in HIV seropositive patients. It has advantages like water solubility with excellent tolerance level, low toxicity, and favorable pharmacokinetics. Fluconazole is

recommended as 200 mg on first day followed by 100 mg for at least 2 weeks, to treat oropharyngeal candidiasis (John 2006).

Fluconazole inhibits cytochrome P-450 and 14 α-demethylase, which function in the synthesis of ergosterol from lanosterol. Since ergosterol is the component of fungal cell membrane, action of azoles can thus terminate cell membrane synthesis. Fluconazole is effective for most of the candida species except, *C. krusei,* which is intrinsically resistant to this drug. Few candida species namely, *C. dubliniensis, C. inconspicua and C. glabrata* show resistance to fluconazole occasionally. Even with *C. albicans,* long term use of fluconazole prophylaxis may lead acquired resistance (Murray et al. 2003).

2.1.5.2 *IN VITRO* SENSITIVITY TESTING FOR FLUCONAZOLE

Emergence of fluconazole resistant strains of both *C. albicans* and NAC has been reported from studies done worldwide and also from Karnataka, India (Sanchez-Vargas et al. 2005; Nadiger et al. 2008). Emergence of fluconazole resistance makes the treatment of the patients difficult.

Fluconazole sensitivity testing was done using the standard techniques by estimating the minimum inhibitory concentration (MIC) of the antifungal drug needed to inhibit the growth of candida (CLSI Guidelines 2002). The sensitivity pattern of candida isolates for fluconazole can be tested by using Broth Micro Dilution method as standard method according to CLSI guidelines. In *in vitro* condition, susceptibility pattern of candida can be interpreted based on the MIC required to decrease the growth of the organisms to upto 80% comparing to the turbidity/growth present in fluconazole free medium. Candida isolates sensitive to fluconazole can be inhibited with fluconazole concentration of 8 µg/ml or less. The resistant isolates can grow even at fluconazole concentration of 64 µg/ml or above. Isolates showing growth at fluconazole concentrations 16-32 µg/ml are referred as susceptible dose dependent; in this condition increased dose of fluconazole in *in vivo* conditions can help in treating of candida infection (Murray et al. 2003).

2.1.5.3 PREVALENCE OF FLUCONAZOLE RESISTANCE IN *C. ALBICANS* ISOLATES FROM HIV SEROPOSITIVE PATIENTS

Recent reports from various geographical areas, show altered fluconazole sensitivitypattern in *C. albicans* isolates(Sanchez-Vargas et al. 2005; Nadiger et al. 2008; Maninder and Usha 2008). *C. albicans* from HIV seropositive patients show more resistance to fluconazole, compared to commensal flora from healthy individuals (Mennon et al. 2001). The irregular intake of fluconazole for prior episodes of OC could be the reason for it (Nadiger et al. 2008). Total use of > 10 g of fluconazole has been significantly associated with the development of resistance (Fichtenbaum et al. 1998).

Resistance to fluconazole develops by target alteration, reduced cell permeability, active efflux of the drug etc. Combination of more than one type of mechanisms can be seen in the candida. However, though the isolate shown to be resistant in *in vitro* tests. Yet patients sometimes respond to higher doses of fluconazole in *in vivo* conditions (Fichtenbaum et al. 1998).

After prolonged exposure to fluconazole, resistant strains of *C. albicans* or NAC isolates where getting selected in the lesion environment as observed by (Redding et al. 1997), in the study where treatment with azoles and candida carriage in HIV seropositive patients with OC was frequently monitored.

Since HIV is a complicated condition involving physiological abnormalities also in the patient, the sensitivity pattern has to be checked carefully. Host factors like, medication delivery, reduced absorption of drug due to hypoacidity commonly seen in HIV seropositive patients, drug to drug interactions or metabolism problems can lead to failure of fluconazole in HIV seropositive patients (Fichtenbaum et al. 1998).

2.1.6 *C. ALBICANS*

2.1.6.1 INTRODUCTION TO *C. ALBICANS*

The fungi are commonly considered as saprophytic in nature. They are eukaryotic, chemo heterotrophic organisms; their cell wall contains chitin and or cellulose. Depending on their cell morphology, fungi are divided into four different classes: yeasts, yeast-like fungus; moulds and dimorphic fungus (Paniker 2005). Around 150-200 fungi are identified as primary human fungal pathogens, however list of opportunistic fungal pathogens is increasing, with relation to increase in immunocompromised patients, including cancer patients, HIV seropositive, transplant recipients and debilitated patients (Mandell et al. 1995). Among the human opportunistic fungal pathogens, members of genus candida, (yeast-like fungi) are considered to be important fungi to be reported and studied in various immunocompromised conditions (Rippon 1982 and 1988; Larone 2002).

Candida usually presents itself as normal flora in healthy individuals, throughout the mucus membrane of gastrointestinal tract (except gastric, small intestine and large intestine), female genital tract and on the skin. Around 3-47% of the adult healthy humans carry candida in their oral cavity (Sharon et al. 1977; Arendorf and Walker 1979; Samaranayake et al. 1986).

Genus candida contains oval budding yeast cells, measuring about 6-12 µm in diameter. Morphologically candida is grouped into yeast-like fungus category. Genus candida belongs to the class; Blstomycetes, order; Moniliales and family Cryptococcaceae, within Deuteromycota (Fungi Imperfecti) (Rippon 1982 and 1988; Larone 2002).

There are more than 200 species of candida known and among them *C. albicans; C. krusei,C. parapsilosis, C. glabrata, C. dubliniensis, C. tropicalis, C. lusitaniae, C. guilliermondii* and *C. kefyr* are considered to be the important human pathogens (Rippon 1982 and 1988; Larone 2002).

Though candida appears as normal flora, carriage rate increases under predisposing conditions. The individuals in extremes of age, cancer patients, organ

transplant recipients, patients with indwelling catheters and individuals suffering from immune-compromised conditions will have the risk of developing candida infections (Mandell et al. 1995). The candida affects the mucosa, skin and its appendages and also the internal organs, infections produced by candida, are altogether referred as candidiasis or candidosis (Larone 2002).

2.1.6.2 HISTORY OF *C. ALBICANS*

Existence of candida was first understood in 1846, when Berg suggested that etiology of oral thrush was fungus, based on his experimental observations. After Berg's discovery, it was also found that the same organism found to be causing different types of infections. It was in 1853; Robin renamed this organism as *Oidium albicans*, which was previously named as species of sporotricum by Gruby in 1844. The name candida was first suggested by Berkhout in 1924. The word candida is derived from the Latin word "toga candida", which refers to a special robe worn by candidates in Roman Senate. The oral thrush or the whitish colonies of *C. albicans* may be the reason for the use of this word (Calderone 2002b). Thereafter in 1954, *C. albicans* nomenclature was officially agreed in the Eighth Botanical Congress. The candida genus is now put in Deuteromycetes, which has been described as "taxonomic pit" as yeasts without a known sexual stage or other remarkable phenotypic character are thrown in to this group. Genomic studies proved that *C. albicans* have diploid genome and natural heterozygocity (Odds 1987). Though several species of candida have been now known, yet few phenotypic expressions of *C. albicans* make its identification easy (Rippon 1982 and 1988; Larone 2002).

2.1.6.3 MICROBIOLOGY OF *C. ALBICANS*

2.1.6.3.1 MICROSCOPY

The candida cells can be seen under light microscope either directly or using staining procedures like gram staining. The candida cells are round to oval in shape, measuring about 6-12 µm in diameter. The cells can be seen in single or with budding. Under gram staining they appear as strong gram positive budding yeast cells as shown in Fig. 2.1a. Fig. 2.1b, represents staining of clinical specimens containing

candida, showing the presence of pseudohyphae resembling links of sausage. True hyphae may also be present (Rippon 1982 and 1988; Larone 2002).

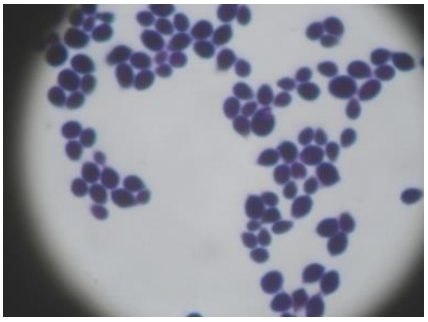


Fig. 2.1a Gram Stain morphology of candida showing gram positive budding yeast cells

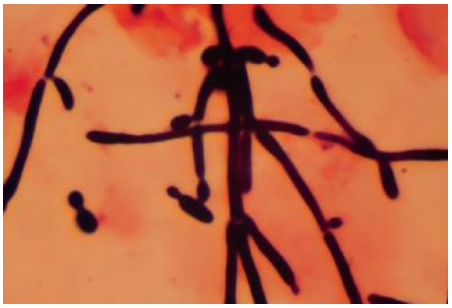


Fig. 2.1b Gram stain from clinical specimen containing *C. albicans*, with pseudohyphae

2.1.6.3.2 CULTURE CHARACTERISTICS

Candida can be cultured on Sabouraud Dextrose Agar (SDA). The colonies develop completely by 48 hrs on incubation at 37 ^{o}C as shown in Fig. 2.2. Colonies of candida are characterized with cream, smooth, moist, pasty with yeasty / sour curd odor. When candida has to be isolated from samples containing other normal flora, SDA with gentamicin, chlorampenicol and cyclohexamide can be used to inhibit the growth of other bacteria and saprophytic fungi. Several additional media are known to be used for isolation of candida e.g., Letman Oxgall agar, Nickerson's Medium Eosin Methylene blue medium etc (Rippon 1982 and 1988; Larone 2002).

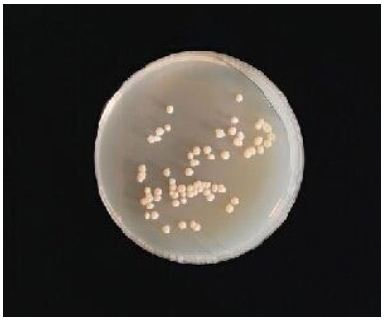


Fig.2.2 Colonies of *C. albicans* grown on SDA after 48 hrs of incubation

2.1.6.3.3 IDENTIFICATION TO SPECIES LEVEL

a. GERM TUBE TEST

To perform this test, candida cells are incubated in pooled human serum at 37^{o}C, for 3 hrs only. On incubation with serum, few species of candida e.g., *C. albicans* and *C. dubliniensis* produce long tube like projections called germ tubes as shown in Fig. 2.3, which can be demonstrated by making the wet mount of the suspension and observing under high power field (Larone 2002).

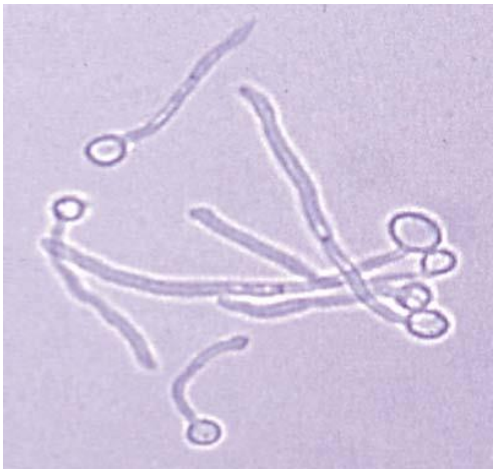


Fig. 2.3 Germ tube formation produced by *C. albicans*

b. CHROMAGAR MORPHOLOGY

A special medium called CHROMagar can be used for presumptive identification of candida into species level. CHROMagar contains chromogenic substance which interacts with β – N- acetyl galactosaminidase produced by *C. albicans*.*C. albicans* produces medium, convex, light green, smooth colonies. Different species of candida produce different colored colonies on this agar as shown in Fig. 2.5 (Koehler et al. 1999).

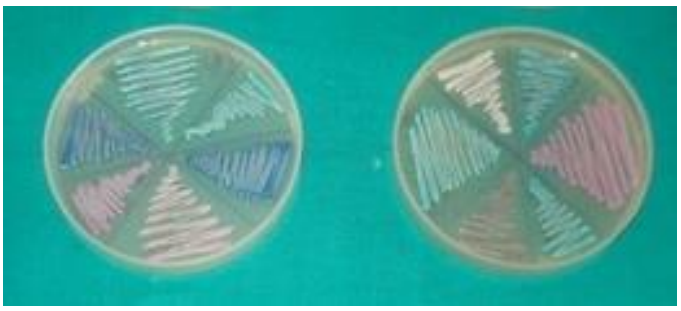
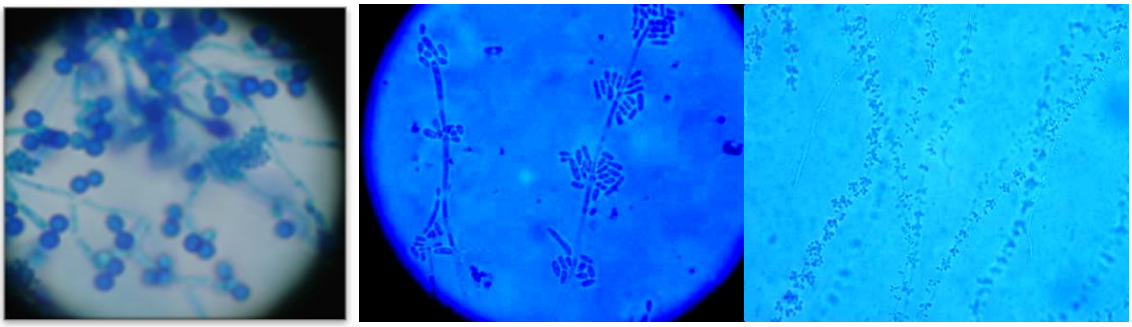


Fig. 2.4Growth of different species of candida on CHROMagar: Different species of candida exhibit specific colony morphology and color

Note: *C. albicans* shows smooth, medium, light green colonies; *C. tropicalis* presents medium, round, dull blue/ pink colonies; *C. krusei* presents large, flat pale pink spreading colonies; C. guillermondii presents purple small colonies.

c. CORN MEAL AGAR (CMA) MORPHOLOGY

Corn meal agar (CMA) contains corn meal as a major content. The candida species grown in this medium are incubated for 72 hrs. The certain species of candida give characteristic type of morphology on CMA upon incubation (Larone 2002). The growth obtained can be directly observed under microscope. The production of chlamydospores, a double walled refractive, round spores is the identifying feature of *C. albicans* which is shown in Fig. 2.5 comparing to other species of candida.



A. B. C.

Fig. 2.5 Morphology of various candida species on Corn Meal Agar in 72 hrs incubation at room temperature, as seen in Lacto Phenol Cotton Blue Preparation under 450x.

Note; A. *C. albicans* produces single terminal chlamydospores on pseudohyphae, bunches of round blastoconidia seen at septa of pseudohyphae. B. *C. krusei* shows pseudohyphae with cross match sticks or tree like blastoconidia. C. *C. tropicalis* shows small oval shaped blastoconidia present anywhere along the graceful pseudohyphae.

d. BIOCHEMICAL IDENTIFICATION TESTS

Sugar assimilation, sugar fermentation test along with few other biochemical tests will help in differentiation of candida species (Larone 2002). Fig. 2.6 represents sugar assimilation test done on few candida isolates. *C. albicans* assimilates glucose, maltose, sucrose, galactose, trehalose and xylose. Similarly, this fungus gives negative results when tested for lactose, cellibiose, raffinose, mellibiose, inositol and dulcitol.

Fig. 2.6 Sugar Assimilation test done using modified Wickerhamm's method (Larone 2002)

Note: Purple color indicates no growth; yellow (to white) indicates presence of assimilation of sugar by candidal growth.

2.2 DEVELOPMENT OF OC IN HIV INFECTION

2.2.1 ESTABLISHMENT OF *C. ALBICANS* AS A PATHOGEN IN ORAL CAVITY

Since candida is a part of the normal flora of oral cavity in healthy conditions; it needs to adhere to the mucosal surfaces and remain unattached against the flow of saliva and other mechanical actions present in the mouth. Candida cells therefore, express various surface structures to mediate the adherence to epithelial cells (Chaffin et al. 1998). The type of interactions carried out between the adhesins and ligand molecules are discussed further.

Adherence of *C. albicans* to host epithelial cells is a first step in the infection process. Adhesion is important both for colonization and to initiate invasion of mucosal surfaces (Marr et al. 2000; Takesue et al. 2004). Some reports say that, increased colonization with *C. albicans* helps this fungus to invade the oral tissues and establish the infection (Senet 1998). Oral candida carriage seems to be increased in immunocompromised conditions of the host as in the case of HIV seropositive patients, even though they do not exhibit any oral candida lesions (Feter et al. 1993).

After the initial adhesion, invasion of HBEC by the candida can be achieved by different mechanisms like induced endocytosis, active penetration into a cell or the candida hyphae moving in the inter junctions of epithelial cells (Marin et al. 2011). A hypothetical set of virulence factors of *C. albicans* is known to help the fungus in the establishment of infection. They are enumerated as, dimorphic transition (morphogenesis), production of hydrolytic enzymes, phenotypic switching, expression of complement receptors, Cell Surface Hydrophobicity (CSH), antigenic variability etc. (Cutler 1991). The various stages of infection establishment in host and the role of virulence factors are given in Fig. 2.7. Adhesins of candida help this fungus in adhering onto target cells. Sap2 and few other proteins also act as adhesins and they help in degrading host proteins, like mucin, immune system molecules, endothelial cell proteins, coagulation and clotting factors etc (Naglik et al. 2003).

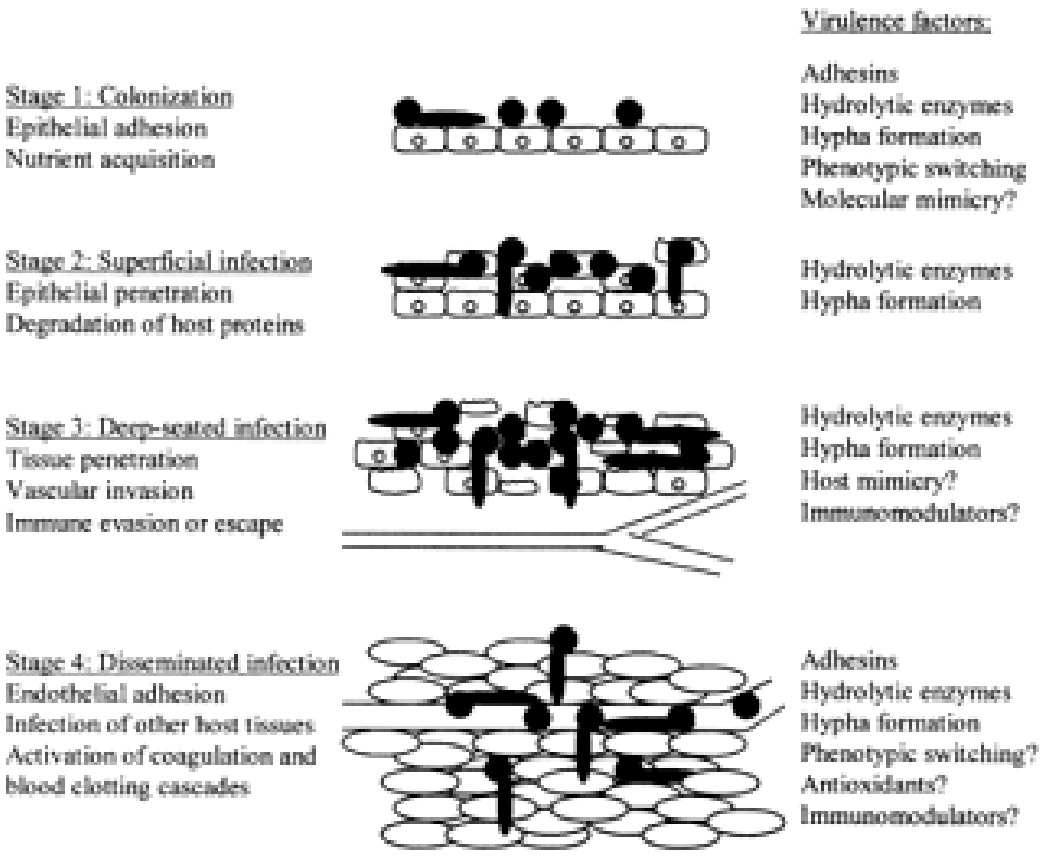


Fig.2.7Schematic diagram showing the role of various virulence factors of *C. albicans* in establishing infection in immunocompromised patients as explained by Naglik et al. (2003)

Among structures of candida, cell wall plays an important role in its virulence. Cell wall of *C. albicans* is the first interface of the organism reacting with the environment. Cell wall not only helps in structural integrity, but also participates in adhesion of the microbe on to host cells which is an initial step in pathogenicity (Chaffin et al. 1998).

2.2.2 HOST FACTORS INFLUENCING THE ESTABLISHMENT OF OC IN HIV INFECTION

Persistence of single strain of *C. albicans* throughout the commensalism and infection is seen in HIV seropositive patients (Vargas and Joly 2002). This process of conversion from commensal to pathogen is unclear but possibilities include factors like, increased adhesiveness and invasion, phenotypic switching by the yeast and acquisition of more virulent strains (Antony et al. 2007).

Although Cell Mediated Immunity (CMI) by CD4 Th1-type cells is considered to be the predominant host defense against mucosal candidiasis, both systemic and local immunity are important for protection against infection. The threshold number of CD4+ T-cells, local immune mechanisms must function exclusively to protect the oral cavity against infection by commensal pathogens. It is also reported that, HIV seropositive individuals show dominant Th2-type salivary cytokine profile (Interleukin-4, Interleukin -10, Interleukin -2, Interferon-γ, Interleukin -12) that seemingly resulted from a lack of Th1-type cytokines rather than enhanced Th2-type cytokines. Further studies showed increase in CD8+ T cells, cytokines associated to this type of cells and chemokines were found increased in persons with OC than with HIV seropositive person having asymptomatic oral candida carriage. The oral epithelial cells also show reduced productions of cytokines and chemokines. Thus decreased in innate and adaptive immune response is seen with HIV seropositive patients. The proteomic studies on epithelial cells from HIV seropositive individuals and HIV seronegative individuals showed that few protective proteins are down regulated in HIV seropositive patients. Finally it appears that several lines of defense may be important for protection against OC, many of which are not evident until CD

4+ T cells are reduced below the protective threshold (Kutin et al. 1983; Cantorna and Balish 1991).

2.2.3 MECHANISMS INVOLVED IN ADHESION OF *C. ALBICANS* ONTO HBECs

2.2.3.1 INTRODUCTION

The mechanisms involved in candidal adhesion onto HBEC can be studied under two categories; specific interactions and non specific interactions. The specific mechanisms involve the ability of yeast to recognize a variety of host cell receptors. Microbial adhesion was previously studied by biochemists and microbiologists to elucidate the role of biomolecules in the adhesion. Whereas physico-chemists were studied the influence of physicochemical characteristics of the cells and the surrounding environment on adhesion and termed this as nonspecific factors of adhesion (Busscher and van de Mei 1997; Leblond et al. 2000).

Specific interactions are established by molecular recognition between ligand and receptor molecules. The various complementary molecules on both the microbe and the host cells are involved in establishing the strong adherence (Leblond et al. 2000).The mechanical interlocking occurs between the cells due to receptor-ligand bonds. These interactions get established between acid (electron accepting) and basic (electron donating) groups or oppositely charged domains close to each other (several nanometers) (Leblond et al. 2000; Emerson et al. 2006).

Recent advances in adhesion studies report that mechanisms involved in microbial adhesion originate from the same, i.e., physicochemical forces originate from all the atoms in the inter connected entities. The sum of the relatively weak pair-wise interaction among the atoms in an adhering microbe and a substratum yields the final interaction force, which is similar to the origin of the gravitational force of the earth. The various long range forces (electrostatic and Van der Waals forces) and short range forces (donor-acceptor, hydrogen, ionic, covalent and co-ordination bonds or stereo chemical molecular recognition interactions) are involved in adhesion (Busscher and Van de Mei 1997). Nonspecific mechanisms include electrostatic

forces, aggregation and cell surface hydrophobicity (Katsikogianni and Missirlis 2004). Both the mechanisms are well explained for their role in candida adhesion.

In candida mannan and mannoproteins are the major adhesins present. Other proteins including complement receptors, chitin and lipids are also known to involve in adhesion. Both mannose moiety and protein portion take part in establishing the attachment of *C. albicans* to the epithelial cells. *In vitro* studies show that host proteins having adhesive properties can be recognized by the yeast, e.g.: iC_{3b} and C_{3b}, fibrinogen and fibronectin, which have been proposed as hostcellreceptors. Acidic pH, sucrose or glucose rich diets and secretion of aspartyl proteinase and cell surface hydrophobicity (CSH) all tend to enhance adhesion (Leblond et al. 2000).

All together, in the attachment of microbes onto cell surfaces, the first step to occur is the approach of the cells through mechanisms of mass transport, e.g., convection, sedimentation, electrophoresis and diffusion followed by the retention of the microbes on the surface by physicochemical interaction and finally colonization of the surface by the microbes with the involvement of biomolecules (adhesins) that strengthen the adhesion (biological factor of adhesion) (Busscher and van de Mei 1997; Leblond et al. 2000). Initial microbial attachment to a substrate is the net result of attractive and repulsive forces and the chemical properties of the microbial surface which is a complex three dimensional structure with a myriad of functional groups (Emerson et al. 2006).

Microbial adhesion mechanisms are well studied in both science and technological fields (Cisar et al. 1979; Buswell et al. 1997; Cookson et al. 1995; Hanna et al. 2003). Surface characteristics become important when organism binds to non biological surface which do not possess specific receptors or binding site as incase of adhesion of organism occurring onto dentures, catheters, other bio invasive devices (Simoes et al. 2008). Adhesion of the organisms on inanimate objects and colonization may lead to formation of biofilms and cause the infection to remain chronically in the host. Therefore, the ultimate goal of adhesion studies of medical application is aimed to prevent adhesion based infections (Chandra et al. 2005). Approaches to prevent microbial adhesion onto host cells and biofilm formation on

biomedical devices have been studied using antiadhesive substances and designing of antiadherent biomaterials respectively (Ofek et al. 2003; Chandra et al. 2005). To develop successful antiadherent agents/approach, understanding both specific and nonspecific factors in adhesion of the microbe to respective targets like host cells or biomaterials is needed (Polaquini et al. 2006).

To explain initial adhesion events, various macroscopic characters are considered. Along with macroscopic characterization, in recent years use of Atomic Force Microscope (AFM) for understanding microscopic characterization of interaction forces involved in microbial adhesion has been reported (Emerson et al. 2006).

2.2.3.2 SPECIFIC MECHANISMS INVOLVED IN CANDIDAL ADHESION ON HBEC

2.2.3.2.1.1 ROLE OF CELL WALL MOLECULES OF *C. ALBICANS* IN ADHESION

Cell wall is important in the interaction with antibiotics, morphogenesis and it also provides hydrophobic character to the cell (Andriole et al. 1962; Chaffin et al. 1998). Cell wall of *C. albicans* has a very dynamic structure and cell surface molecules participate in almost every aspect of the biology and pathogenicity of *C. albicans*. Cell surface molecules act as adhesins and a few of them even mimic host proteins and thus modulate host immune response to candidiasis (Moors et al. 1992).

Major components of the *C. albicans* cell wall are mannoproteins and polymers of glucose and chitin. Mannoproteins appear to be involved in morphogenesis and pathogenicity related aspects i.e., adhesion to host tissues and inert materials and presentation of antigens **(**Cassone et al.1989; Chaffin et al. 1998).

Cell wall of *C. albicans* is mainly composed of 3 components interconnected by covalent bonds, β-1-3 and β-1-6 glucans (50-60%), mannoproteins (30-40%) and a small amount of chitin (0.6-0.9%). An 80-90% of cell wall of *C. albicans* contains carbohydrates. Mannoproteins are the major components of *C. albicans* cell wall,

which are the polymers of mannose covalently associated with proteins forming glycoproteins. These proteins are containing a branched polymers of glucose containing β 1-3 and β 1-6 linkages of chitin. Chitin is unbranched homopolymer of N-acetyl D-glucosamine (β 1-4 Bonds). Proteins (6-25%) and lipids (1-7%) are present as minor components. Cell wall mannoproteins are exposed to outside environment (Marcilla et al. 1998; Chaffin et al. 2008).Thus one can understand that, there is a high degree of complexity associated with protein and mannoprotein composition of *C. albicans* cell wall. The schematic diagram of cell wall of *C. albicans* is presented in Fig. 2.8 at and b.

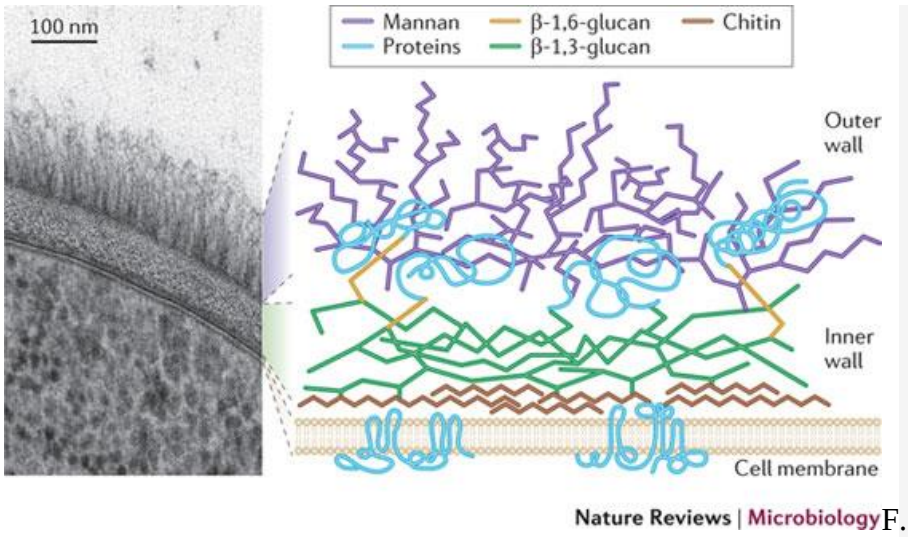


Fig. 2.8a Cell wall of *C. albicans* (Neil et al. 2012)

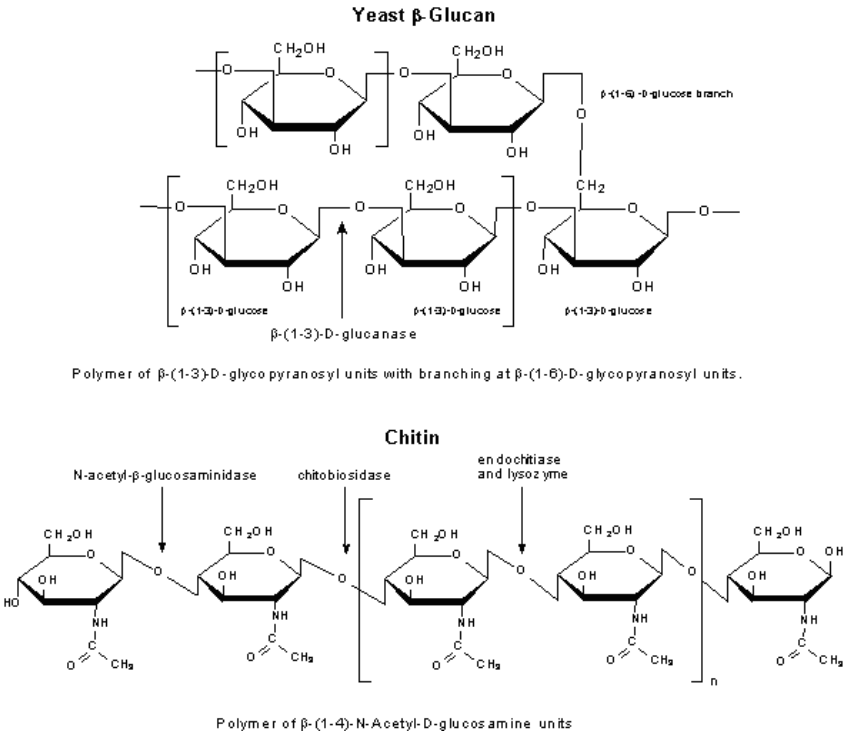


Fig. 2.8 b Structural details of yeast β- Glucan and Chitin (Kapteyn et al.1995)

2.2.3.2.1.2 CELL WALL PROTEINS AND THEIR IMPORTANCE IN ADHESION

Cell wall mannoproteins and other types of proteins appear to be involved in processes like morphogenesis, pathogenicity related aspects (adhesion to epithelial and endothelial cells). Hydrophobic or hydrophilic status of the cell surface isalso determined by these mannoproteins (Chaffin et al. 1998). Cell wall proteins and mannoproteins act as antigens to stimulate immune response in the host. A few studies demonstrated anti-cell wall antibodies in host saliva and serum (Castro et al. 2009).

A variety of cell surface molecules of *C. albicans* are known to be involved in pathogenesis. The receptor MC-1 (CD11b/CD18) on lymphocytes is the surface β2 integrin that mediates adhesion of *C. albicans* onto lymphocyte. Complement fragment iC3b are the receptors for CR3-RP adhesins present in the cell wall of candida, binding of these components results in inhibition of opsonisation. CR3-RP reported to be expressed more in germ tubes and mycelial forms. *C. albicans* are also

able to bind to collagen, laminin or fibronectin via special surface molecules. CR3-RP has been reported to be participating in adhesion of candida to epithelial cells. Adhesion to host cells is important for establishment of candidiasis. CR3-RP is also found in early phases of biofilm formation, thus can lead to medical device related diseases (Sturtevant and Calderone 1997). Jenq and Chen (1994); Angiolella et al. (2002); Bujdakova et al. (2008) proved the role of a major surface antigen 49 kDa of candida in its adhesion to human HBECs. Edwards et al. (1992) reported that cell wall extracts inhibited adherence of *C. albicans* blastospores and germ tubes to endothelium and proved the role of cell wall components in adhesion. Galhan et al. (1998) reported that mannoprotein adhesins responsible for adhesion to epithelial cells will be different from adhesins required for attachment on plastic surfaces.

2.2.3.2.1.3 CELL WALL PROTEIN PROFILES OF DIFFERENT MORPHOLOGICAL FORMS OF *C. ALBICANS*

Angiolella et al. (2002) reported that there are quantitative and qualitative differences in the protein composition of yeast and mycelial cell wall. Molecular structure of cell wall is specific for each morphologic form of *C. albicans*. Approximately 22-40 different polypeptide components are reported from *C. albicans*. They widely differ in their molecular size (15 - 80 kDa range) with different glycosylated forms. Majority of them act like receptors and show adhesion properties (Chaffin et al. 1998; Angiolella et al. 2002).Strongly immunomodulatory mannoprotein complex is expressed in different growth forms of *C. albicans* (Torosantucci et al. 1990). Torosantucci et al. (1990) reported that fractionization of cell wall components of *C. albicans* on a Con A –sepharose affinity column led to identification of two fractions, fraction I and fraction II. Affinity bound mannosylated fraction II blocks the adherence of blastospores to endothelial cells, whereas the fraction I inhibits the adherence of both germ tubes and blastospores to endothelial cells. These findings not only confirm the difference in the protein profiles of different morpholological forms of candida, but more significantly, the differences in the glycan structures of these proteins.

2.2.3.2.1.4 CELL WALL PROTEIN PROFILE OF *C. ALBICANS* ISOLATES FROM HIV INFECTION

Considering the importance of mannoprotein in host-parasite interaction, it is important to understand the nature of these adhesion molecules in candida isolates from HIV seropositive patients. There are very few reports on the nature of cell wall mannoprotein of *C. albicans* isolates from HIV seropositive subjects (Schwab et al. 1997; Lopez-Ribot et al. 1999; Taylor et al. 2000; Shahid et al. 2006).

Cell wall protein profile of *C. albicans* isolates from HIV seropositive individuals in response to their fluconazole sensitivity pattern has been reported by Shahid et al. (2006). The study reports the significant differences in the cell wall protein components of fluconazole resistant and susceptible isolates of *C. albicans* (Shahid et al. 2006). However, further information about the protein profiles of isolates from these two groups and comparison are very sparse.

2.2.3.2.2 TYPES OF INTERACTION SEEN IN ADHESION OF CANDIDA ON HBEC

The interaction of *C. albicans* with HBEC occur by 3 different recognition systems to date, but existence of additional type cannot be ruled out. Protein-protein interactions, lectin-carbohydrate type of interactions explained by various authors. In the third type, mannan polysaccharide adhesin molecules of candida to buccal epithelial cells are explained but the ligand for the same on oral epithelial cells is unidentified (Hostetter 1994; Calderone 1993).

2.2.3.2.2.1 PROTEIN–PROTEIN INTERACTIONS

In this type of interaction, the proteins of candida cell surface bind to peptide or protein present on host cell surface. Integrin analogues present on candidal cell surface carry out this type of interaction. The candidal proteins resemble complement receptors 3 and 4 (CR3 and CR4).These proteins resemble CR3 and CR4 of mammalian cells structurally and functionally and thus referred as integrin analogues (Hostetter 1994). Candidal CR3 and CR4 are assumed to be binding to RGD

containing ligand in extracellular matrix as proven with studies done using integrin mAbs (Bendel and Hostetter 1993).

Fibronectin receptors are another example for this kind of interactions. It was first demonstrated by Skerl et al. (1984) by showing the adherence of *C. albicans* to fibronectin coated microtitre wells. Preincubation of epithelial cells with rabbit anti-fibronectin antibodies and treatment of *C. albicans* with fibronectin reduced adhesion of candida on both HBEC and vaginal epithelial cells (Skerl et al. 1984; Kalo et al. 1988). However, complete elucidation of mechanisms involved in this type of interaction is not yet solved.

2.2.3.2.2.2 LECTIN-CARBOHYDRATE LIKE INTERACTIONS

In this type of interaction mannoprotein of candida recognizes carbohydrate containing ligands on the host cell wall. Douglas and Cameron (1998) proved the adhesion of candida cell wall protein with fucose containing moieties on epithelial cells. The host with blood group O, will bear H Ag on their RBCs. H Ags can also be expressed in few individuals on their oral epithelial cells. H Ag contains D-galactose, N-acetyl D-glucosamine, N-acetyl D-galactosamine and α-1-2 fucose moieties. It is reported by Douglas and Cameron (1998), that individuals bearing H Ags on their oral epithelium can be more prone to oral colonization with candida. Douglas and Cameron (1998) also reported that the adhesive component of candida responsible for this type of adhesion further proved to be protein in nature, with the experiments where the crude extracts of candida treated with proteolytic enzymes, heat and papain altered the adhesion reaction.

2.2.3.2.2.3 INCOMPLETELY DEFINED INTERACTIONS

Here a surface component of *C. albicans* attaches to an epithelial cell surface by an unidentified ligand. Binding of secretory aspartyl proteinase or factor 6 are associated with this kind of interactions. Factor 6 is an epitope present in outer chain of mannoprotein in *C. albicans* serotype A isolates and this mannoprotein has terminal side chains of mannoprotein residues, bound via β linkage to inner branched structures. The isolate lacking factor 6 showed decreased adhesion with epithelial cell lines of human squamous cell carcinoma (Hostetter 1994; Calderone 1993).

2.2.3.2.3 ADHESINS OF *C. ALBICANS* PARTICIPATE IN BINDING ONTO HBEC

Adhesins are defined as biomolecules that promote adhesion of microbial cell to host cell or host cell ligands. The outer most layer of the *C. albicans* cell wall plays an important role in pathogenesis mainly because it possesses macromolecules that adhere to host tissues (Fonzi and Trwin 1993). The peptide sequence of lectin-like mannoprotein of the fungal cell-wall links to fucosyl or to N-acetyl glucosamine residues present on the surface of the epithelial cells (Brassart et al. 1991; Hoffman and Haidaris1993). The fungal binding structures have also been described as fimbrial adhesins, chitin or integrin-like molecules (Meitner et al. 1990; Yu et al. 1994). A few examples for adhesins are described below.

2.2.3.2.3.1 AGGLUTININ LIKE SEQUENCE (ALS) PROTEINS

There are eight glycosyl phosphatidyl inositol (GPI) linked cell surface protein adhesins which are encoded by the ALS (agglutinin-like sequence) gene family and these proteins are required for cell-cell recognition during mating (Hoyer 1998; Calderone and Fonzi 2001). Hoyer et al. (2001) demonstrated that, ALS genes can vary in their regulation depending on growing stages and morphological stage of growth.ALS protein contain substrate binding region in their N-terminal domain. Other than this, they contain central domain and C- terminal domain consisting of serine and threonine and GPI anchorage sequence (Loza et al. 2004; Sheppard et al. 2004; Rauceo et al. 2006). ALS proteins are heterologus in their binding specificity (Gaur and Klotz 1997; Hoyer 2001 Sheppard et al. 2004; Zhao et al. 2004; Braun et al. 2005; Zhao et al. 2005). ALS 1, ALS 3 and ALS 5 mediate adherence to multiple host constituents including oral epithelial cells, whereas ALS 6 and ALS 9 do not mediate adherence to epithelial cells. ALS 7 does not bind to any host substrates tested to date (Sheppard et al. 2004). ALS2, ALS3 and ALS4 are known to bind to HBEC (Zhao et al. 2004; Zhao et al. 2005).

2.2.3.2.3.2 HWp1

These are GPI proteins, expressed on hyphal forms of *C. albicans*, highly essential for virulence of candida in producing mucosal candidiasis. These proteins functionally mimic small proline rich proteins of the host which are the substrate for epithelial cell associated transglutaminases. N-terminal of this protein act as substrates to epithelial cell associated transglutaminases (Staab et al. 1999; Ponniah et al. 2007). HWP1 also adheres to ALS proteins of the candida itself and thus known to be involved in biofilm formation (Nobile et al. 2008).

2.2.3.2.3.3 Eap1

This protein is structurally similar to ALS protein and contains 3 domains.Substrate binding occurs through N domain. The protein helps in adherence of the candida to plastic and kidney epithelial cell line (Li and Palecek 2003).

2.2.3.2.3.4 Iff4

Iff4 is a member of a 12 protein family. Iff4 is involved in adhesion of *C. albicans* to an oral epithelial cell. However Iff2, Iff3, and HYR1 in *C. albicans*not show anyto play any role in adhesion to oral epithelial cells in *in vitro* conditions (Fu et al. 2008).

2.2.3.2.3.5 SECRETED ASPARTYL PROTEINASES (SAP)

These proteins were first described by Staib in 1965; there are at least nine isoenzymes of SAPs (Morrison et al. 1993; Miyasaki et al. 1994; Monod et al. 1998). They have unique role in interactions with host; their secretion is controlled by growth conditions, phenotypic switching, etc. These are proteolytic enzymes, which help in invading the tissue, and facilitate adherence and colonization of host environment (de Bernardis 1990; White et al. 1993; Morrison et al. 1993; Schaller 1999).

2.2.3.2.3.6 OTHER KNOWN ADHESINS

A mannoprotein MP 65, a GPI protein present on the candidal cell surface known is to act as adhesin during attachment of candida on epithelial cells. ECM33 is another protein involved in adhesion. An ECM 33 mutant reduced adhesion of candida on oral epithelial cell lines. Martinez-Lopez et al. (2004) reported that, ECM 33 is also required for the normal function and localization of other adhesins. Several other adhesins like Utr 2, fimbriae, Csh 1, Ywp 1, Pra 1, known to play role in adhesion to oral epithelial cells (Chaffin 2008).

2.2.3.2.4 DIFFERENTIAL EXPRESSION OF ADHESINS

Candida is a polymorphic fungus, exhibiting different morphology under various environmental conditions. Usually candida appears in blastoconidial form in culture and when present as normal commensal flora. Pseudohyphae formation by candida cells are seen when the specimens are collected from infected tissues. In *C. albicans*, a few adhesins are expressed only in specific morphological forms. For e.g., ALS3 and HWP1 proteins are expressed only by hyphal forms and ALS1 expressed only in yeast cells. EAP1 is expressed by both yeast and hyphal forms, thus mediating binding of different forms of candida on epithelial cells (Chaffin 2008). The presence of pseudohyphae evidenced to be more virulent and more adherent form in*in vitro* conditions. Germ tube of *C. albicans* were also shown to possess different cell wall protein varieties which were absent in cell wall of blastoconidial forms (Oller and Calderone 1990; Klein and Tebbets 2007).

Gene regulation of adhesins also is studied in *C. albicans*. Candida-epithelium interaction provokes reciprocal signaling (Bailey et al. 1995). Few developments regulated genes like HWP1encode for candida cell wall mannoprotein present in germ tube and hyphal forms and interact with epithelial trans-glutaminase (Staib et al. 1999). All these studies reveal the eclectic behavior of candida in providing adhesive components for epithelial cells.

2.2.3.2.5 MECHANISMS INVOLVED IN INVASION OF ORAL EPITHELIAL CELL BY *C. ALBICANS*

The candida cells colonize on oral mucosa by initial step consisting adhesion which further leads to invasion and damage of the epithelial cells. Epithelial cells also produce various factors to combat the infection, which again depends on the immune status of the host and thus determines whether the organism can remain as colonizer or an invader (Dongari-Bagtzoglou et al. 2004; Feng et al. 2005;Villar et al. 2005; Yano et al. 2005; Steubesand et al. 2009).

2.2.3.2.5.1 PROCESS OF INVASION OF HBEC BY *C. ALBICANS*

Electron microscopy studies on invasion of oropharyngeal epithelial cells by candida were noted by various researchers through electron microscopes which revealed the following important observations. The invasion of candida occurs mainly in hyphal forms and usually intracellular organisms are exhibited in hyphal forms; yeasts forms are seen located mainly on the surface of the epithelial cells (Garcia-Tamayo et al. 1982; Scherwitz 1982; Rajasingham et al. 1989). Invasion occurs by 2 mechanisms, for e.g., endocytosis, where invasins like proteins are involved. Other mechanism is the active penetration of the host cells by converting to hyphal forms. Both the mechanisms are involved in invasion of HBEC, but when invading epithelial cells of gastrointestinal layer only active penetration was observed (Park et al. 2005; Phan et al. 2007; Dalle et al. 2009).

The schematic representation of process of candidal infection development is given in Fig. 2.9.

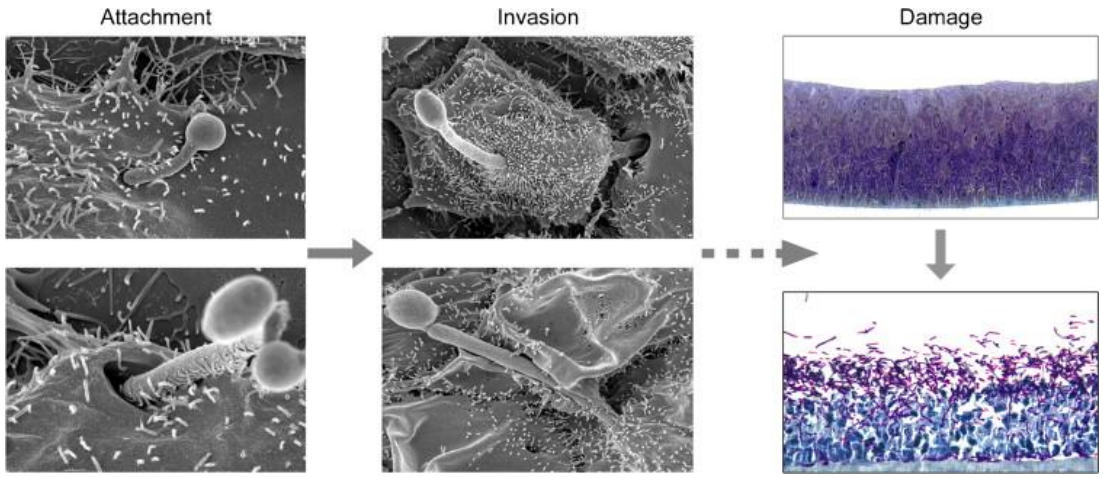


Fig. 2.9 Interactions of *C. albicans* with epithelial cells (Marin et al. 2011).

A. INVASION INDUCED BY ENDOCYTOSIS

In this process organism does not have any active role as proven by endocytosis of even killed hyphae (Park et al. 2005). It has been reported that ALS3 protein functions here as an invasin and induces epithelial cell endocytosis, as ALS3 null mutant of *C. albicans* has markedly impaired capacity to invade epithelial cells (Phan et al. 2007). ALS3 plays a major role in endocytosis, as it induces the process by binding to E-cadherin and other proteins on the epithelial cell surface, by stimulating clathrin-dependent endocytosis pathway (Phan et al. 2007). Similarly ALS1 also is reported to induce epithelial cell endocytosis, but with lower efficiency than ALS3 (Sheppard et al. 2004; Phan et al. 2007). Few components of Candida cell surface mediate both adhesion and invasion and are named to be Rim101 (transcription factor of *C. albicans*), ZRT1 (a zinc transporter) etc.

B. ACTIVE PENETRATION

C. albicans can be converted to hyphal forms and actively either invade into an epithelial cell without inducing the formation of epithelial cell pseudopods or pass through the intercellular junction between epithelial cells, however mechanisms involved here is not completely understood (Dalle et al. 2009). In a study by Ray and Payne(1988), it is shown that Pepstatin A, an inhibitor of aspartyl proteinase, has been reported to inhibit the invasion of *C. albicans* into corneocytes in mice with cutaneous

candidiasis, thus proving the role of SAP in invasion. Several other reports also showed that SAP is important in active penetration of oral and vaginal epithelial cells by candida (Schaller et al. 1999; Schaller et al. 2003; Naglik et al. 2008).

Proteolytic degradation of E-cadherin concentrated at intracellular junctions between epithelial cells is shown to be caused by *C. albicans* in *in vitro*conditions (Frank and Hostetter 2007; Rollenhagen et al. 2009). In support of these *in vitro* findings, E- cadherin antigen is reduced in the oral epithelium of HIV seropositive patients who have oropharyngeal candidiasis compared to those without this disease (McNulty et al., 2005).

2.2.3.2.6 EPITHELIAL CELL FACTORS INVOLVED IN ADHESION

2.2.3.2.6.1 FIBRONECTIN

Fibronectin which is a plasma interstitial glycoprotein ranging from MW 37 to 120 kDa, was the first molecule to be proved as ligand involved in adhesion of *C. albicans* on HBEC (Skerl et al. 1984).

2.2.3.2.6.2 INTEGRINS

Integrin receptor is an integral membrane protein present in plasma membrane of epithelial cells, adheres to candida proteins having functional similarities to human complement receptors 3 and 4 (CR3 and CR4) (Hostetter 1994). Calderone et al. (1988), reported that C3 receptors on candida species bind to C3D fragment of complement component 3 (C3) and the presence of C3 receptor in candida species is highly suggestive of their high pathogenicity.

2.2.3.3 INFLUENCE OF PHYSICOCHEMICAL FACTORS OF *C. ALBICANS* CELL ON ITS ADHESION TO HBEC

2.2.3.3.1 MACROSCOPIC CELL SURFACE PROPERTIES

The behavior of an adhering cell is governed by the physical and chemical interactions of the macromolecules in the interfacial region as explained above. Macroscopic approaches such as zeta potential, chemical composition of the cell surface and CSH measurements play important role in the understanding of microbial physicochemical properties. Non specific forces involved in adhesion are electrostatic interaction (ion-ion interactions), electro dynamic interactions (Van der Waals interactions) and dispersion forces (Marshall 1991).

2.2.3.3.2 MICROSCOPIC CHARACTERISTICS OF CELL SURFACE

Examination of macroscopic expression of interactions in a microscopic level, concerned to entire surface of the cell can be done. Atomic Force Microscope (AFM) is used in analyzing microscopic characteristics of the cell. The cells which are interacting in adhesion will be present in a suspension medium. While measuring microscopic characteristic of the cell surface, between microbe and medium a third component also occurs i.e., AFM tip, which can be moved along the surface and adhesion forces exerted at different sites of the cells can be studied (Rodriguez et al. 2004).

2.2.3.3.3 THEORIES OF ADHESION

The theoretical analysis of microbial adhesion was done using Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, thermodynamic approaches and the extended DLVO theory. DLVO theory predicts that the total interaction energy between the particle and the collector surface as the sum of Van der Waals and electrostatic double layer interactions. Knowledge of cell surface free energies and Electrophoretic mobilities makes possible to calculate contributions of electrostatic and Lifshitz – Van der Waals forces occurring between two entities. In extended DLVO theory, acid-base interaction forces are accounted in addition to Lifshitz-

Vander walls and electrostatic forces (Katsikogianni and Missirlis 2004). However, as biological cells are living systems and they perform metabolism, secretion of various biomolecules and carry extracellular appendages they may not always be considered as mere colloidal particle in explaining their adhesion mechanisms (Polaquini et al. 2006).

2.2.3.3.4 ELECTROSTATIC FORCES AND ADHESION OF CANDIDA CELLS TO HBEC

Different ways to obtain information on electrostatic interactions are present. Electrical potential on the surface can be measured by Electrophoretic mobility as measurement of electrostatic state of microbe. Electrostatic forces are known to be repulsive. Both hydrophobicity and electrostatic charges of adhering cells seem to be the most important factors in adhesion of candida to inert surface but its role in adhesion to HBEC has to be evaluated (Henrique 2005). Van Loosdrecht et al. (1990) reported that electrostatic forces do not have role in adhesion as they are stronger and act over longer ranges (>10nm).

2.2.3.3.5 CELL SURFACE CHARGES

The cell surface of most microbes bear negative charges due to phosphate, carboxylate and sulphate groups in the cell walls. Carboxyl and phosphate groups may present as uniformly distributed layer or alternate on a patch work pattern with positive charge from amino groups (Katsikogianni and Missirlis 2004). Few adhesins of the cell wall of microbe forms bridge between microbe and cell surfaces. The cell to cell adhesion occurs with net result of repulsion effects (electrostatic, steric ones) and attractive effects (specific bonding) (James 1991).

2.2.3.3.6 CELL SURFACE HYDROPHOBICITY (CSH)

2.2.3.3.6.1 INFLUENCE OF CSH OF *C. ALBICANS* ON ITS ADHERENCE TO HBEC

Hydrophobic interaction is a reaction between two or more compounds in an aqueous phase with the subsequent elimination of water molecules associated with interfacing particles (Jones et al.1995). Changes in interfacial free energy occur when the mibrobe attaches to a hydrophobic surfaces and that is corresponding to the process of attachment (Gerson 1982). When microbe is moving towards substrata through air-liquid interfaces, the adhesion strongly depends on hydrodynamics of the system studied. Greater CSH can enhance attractive forces both in adhering cells and substrata and will be favorable for adhesion. Hydrophobicity is widely accepted as a determinant of microbial adhesion (Katsikogianni and Missirlis 2004). Specific molecular components that have been implicated in contributing to surface hydrophobicity include, surface associated proteins such as fimbriae, in case of bacteria. The adhesiveness and the biofilm production in uropathogenic *E. coli* isolates from indwelling catheters from pregnant and non pregnant patients, was reduced after getting exposed to sub-inhibitory concentrations of cefotaxime. Reduction in adherence seen here was due to decreased exopolysaccharide production and decreased CSH of *E.coli* strains (Balague et al. 2011).

In case of *C. albicans*, role of CSH in disease progression, morphogenesis and phagocytosis had been proved. One of the most significant events in the pathogenesis of candidiasis is the ability of commensal *C. albicans* to switch from hydrophilic to a hydrophobic state (Hazen and Hazen 1992).

Hydrophobic *C. albicans* were more resistant to killing after phagocytosis (Antley and Hazen 1988). Cell wall mannosylation in *C. albicans* can bring changes in CSH (Masouka and Hazen 2004). Regulation of β-1- 2 mannosyl tranferase activity that particularly involved β- 1-2 mannan synthesis can be one of the mechanisms to determine CSH and pathogenesis of serotype A and B strains in immunocompromised patients (Masouka and Hazen 2004).

2.2.3.3.6.2 FACTORS INFLUENCING THE CSH OF *C. ALBICANS*

CSH is demonstrated to be the factor involved in the initial stages of adhesion. There are reports saying that CSH changes according to growth phases, morphological forms of the organisms. External factor like temperature, nutrition and host fibronectins can influence the CSH of candida cells (Fukazawa and Kagaya 1997).

2.2.3.3.6.2.1 ROLE OF MORPHOLOGICAL FORMS OF THE CANDIDA ON CSH

Germ tube formation from blastoconidia also results in increased CSH (Rodrigues et al. 1999). Germ tubes of *C. albicans* show higher levels of CSH in both *in vivo* and *in vitro* conditions. The authors also suggest that due to this increased hydrophobic interactions, the candida can easily invade the tissue in filamentous form. CSH of germ tubes is independent of the mother cells from which it originates (Rodrigues et al. 1999).

Silva et al. (1995) reported that germ tubes of *C. albicans* are able to adhere to fibronectin, fibrinogen, and complement factors via cell surface receptors, which is shown again due to elevated CSH. The increased adherence seen by germ tubes continued to be true with adhesion to plastic surfaces also. Increased CSH can impair the phagocytosis also; there by can slow down the clearance of candida hyphal forms in the blood (Rodrigues et al. 1999).

2.2.3.3.6.2.2 INFLUENCE OF GROWTH CONDITIONS

C. albicans grown at 25^{o}C are seen to be more hydrophobic than the one grown at 37^{o}C (Hazen and Hazen1987). In the growth phases, stationary phase cells are shown to be more hydrophobic than exponential phase cells (Hazen et al. 1986).

2.2.3.3.6.2.3 SPECIES OF CANDIDA AND CSH

Rodrigues et al. (1999) reported that Non Albicans Candida (NAC) isshown to be more hydrophobic than *C. albicans* and this finding correlated with the increased involvement of NAC in disseminated infections. They also reported that, candida recovered from mucosal surfaces are hydrophilic, and suggested that in binding of organism onto epithelial cells, CSH does not have much importance.

2.2.3.3.6.3 PROTEINS INVOLVED IN DETERMINING CSH OF *C. ALBICANS*

Changes in surface proteins of candida cells are seem to influence CSH and changes in CSH in turn influences the adhesion as shown by Masouka et al. (1999) and Glee et al. (2001).These studies were confined to adhesion of candida onto endothelial cells, i.e., in the vascular modeling systems. Masouka et al. (1999), studied 6C5 protein of MW 38 kDa and another protein of MW 37 kDa reported that pretreatment of the hydrophobic *C. albicans* cells with MAb 6C5 decreased the adherence of candida on endothelial cells. But the adhesion inhibition results were not shown with treating candida cells with MAb produce to 37 kDa protein.

Glee et al. (2001) also reported that, the 6C5, a 38 kDa protein of candida is directly involved in determining CSH and adhesion of *C. albicans* to endothelial cells. The MAb 6C5 in their study inhibited the adhesion by reacting with this 38 kDa hydrophobic protein, in *in vitro* conditions. Here the authors suggest that blocking effect of this MAb could be resulted from direct blocking of an adhesin function, by steric hindrance of adhesin, or by changing the hydrophobic interactive capacity of the yeast cell surface.

2.2.3.3.6.4 CSH IN ADHESION OF *C. ALBICANS* ONTO EPITHELIAL CELLS

Klotz and Penn (1987) reported that adhesin-receptor interactions largely involve dipole-dipole and hydrogen bonds and electrostatic forces. Studies by Henrique (2002), suggested that, there is no relation found between CSH and adhesion of *C. albicans* to epithelial cells. Previous to this report Hazen et al. (1989), demonstrated that adhesion of candida on epithelial cells is not highly dependent on CSH. Blanco et al. (2010), reported CSH as a variable characteristic of candida cell surface. They reported that CSH was influence directly the adherence of the candida to plastic and in biofilm formation, with highly hydrophobic cells adhering more significantly to the plastic surfaces. But this study also showed adherence to HBEC was not significantly associated with CSH of the candida cells. However, Macura (1987) and Reinhart et al. (1985) reported that CSH can influence adherence of candida cells to buccal epithelial cells as well as intestinal epithelial cells.

The variations in the results may be due to the use of single strain, as single isolate is not representative of the species (Kennedy et al. 1987). The other problems associated may be the type of method used to study CSH and cell growth conditions involved etc. When multiple isolates used, auto aggregation of the isolate during the assay may occur. With the above described disagreement as explained by Hazen et al. (1989), CSH is proven to be a highly variable condition of the cells. Hazen et al. (1989) reported that the hydrophilic cells can get converted into hydrophobic in 30 minutes, under influence of growth environment. Yoshijima et al. (2010) reported that, hydrophilic coating of the artificial denture surfaces prevents the adhesion of candida. Blanco et al. (1997) reported that, production of extracellular polysaccharides under starvation condition induced decrease in CSH and decrease in adherence to HBEC. However, the references discussed so far with endothelial cells and epithelial cells separately, indicates that adhesion mechanism is dependent on the target host tissue type also.

2.2.3.3.7 INFLUENCE OF ZETA POTENTIAL ON THE ADHESION OF CANDIDA ON HBEC

Zeta potential is the electric potential located at the electro kinetic plane of shear and can be calculated from Electrophoretic mobility (EPM) measurements (James 1991). Studies on influence of zeta potential and acandidal adhesion are sparse. However, the role of zeta potential on adhesion was explained in case of other microorganisms is worth mentioning here.

Microorganisms as well as most of the surfaces to which they adhere usually have a negative zeta potential under physiological conditions (Rad et al. 2000). Hodden et al. (1995) reported that, decrease in zeta potential correlates with nitrogen to carbon ratio to phosphorous ratio of microbial surfaces as measured by X-ray photoelectron spectroscopy (XPS). A reduction of zeta potential of *E. coli* following trypsin treatment which suggested that, surface proteins possibly associated with fimbriae are determining surface potential of those bacteria (Hodden et al. 1995).

In a study by Jones et al. (1997), who worked on the influence of surface properties on the adherence of the respiratory isolates, *Staphylococcus aureus* and *Pseudomonas aeruginosa* to PVC reported that decreased adherence to PVC was observed when bacteria were grown in CO_2. When these CO_2-grown bacteria were treated with saliva their adherence to PVC significantly increased; and also, their adherence was significantly reduced to saliva-treated PVC. Treatment of both bacterial isolates with saliva decreased their negative zetapotential. These authors reported that reduced negative zeta potential may directly contribute to microbial (saliva pretreated) adherence to PVC. CSH did not appear to be a dominant factor here as the CSH of *S. aureus* was decreased by saliva treatment but was unchanged for *Ps. aeruginosa* (Jones et al. 1997).

Zeta potential of the microbe is measured by calculating the Electrophoretic Mobility (EPM), by micro electrophoresis method. EPM values obtained were converted to zeta potential using Smoulouski's equation (James 1991). Rad et al.

(2000) reported that zeta potential of *C. albicans* strains obtained were -21.0 and -22.0 mV, as reported in their study.

2.2.3.3.8 MICROSCOPIC CHARACTERISTICS OF CANDIDAL CELL SURFACE USING ATOMIC FORCE MICROSCOPE (AFM)

2.2.3.3.8.1 INTRODUCTION

AFM was used to study biological samples in late eighties. It had come out to be an excellent tool in providing three dimensional images of biomolecules, lipid membranes, 2D protein crystals. Living cells in aqueous solutions can also be observed in AFM with unprecedented (nm) resolution (Binnig et al.1986).

AFM helps in revealing the surface topography, measures adhesion forces and elasticity of the cell with nanometer lateral resolution.Vander walls and electrostatic forces expressed on microbial cell surface can be studied by AFM which can map the adhesion forces on microbial cell surfaces (Dufrene et al. 2001). AFM probes, can be functionalized with confluent microbial lawns, proteins or lectins, biomaterial spheres and can be used in characterizing microbial – reciprocal substances interactions. Chemically immobilized AFM probes with lectins used to study lectin-carbohydrate reactions. (Razatos et al. 1998; Touhami et al. 2003). OH^- and CH_3^- terminate probes of AFM are used to quantitatively map hydrophobicity of microbial spores (Ahimou et al. 2002). Growth of the organisms and germination of spores can cause dramatic changes in the surface structure, which can be studied by AFM (Dufrene et al. 1999).

2.2.2.8.2 WORKING PRINCIPLE OF AFM

The advantage of AFM in force measurement has been acquired, due to the mechanism of cantilever deflection. The cantilever deflection is recorded as the tip moves on the sample. When there is an attraction, the tip is pushed towards the sample and when there is retraction tip is pushed away from the sample. This movement connected to laser beam and approach and retraction curve is plotted, by taking log of force exerted and distance of separation between tip and the sample (Dufrene 1999). The example of this force-distance curve plot is given in Fig. 2.10.

Thus AFM can be used to probe quantitatively physical properties such as local elasticity, surface forces, surface charges and hydrophobicity. AFM is used to study adhesion between two moieties.

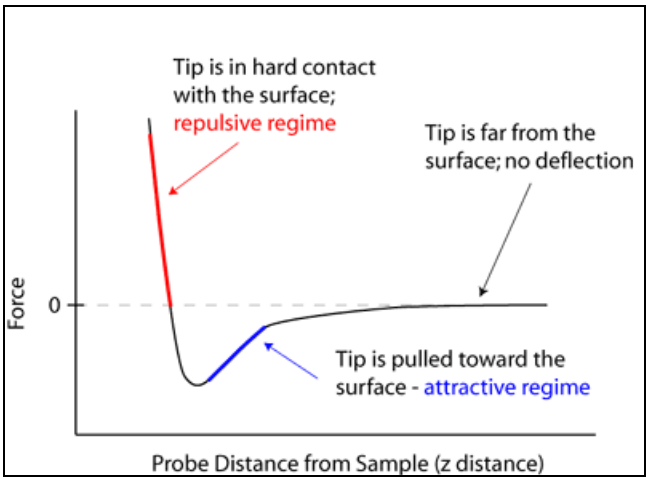


Fig. 2.10 Force distance curve obtained in AFM: Force is measured here using Hook's law

Note: Hook's law gives F = -kz, where F is the force, k is the stiffness of the lever, and z is the distance the lever is bent (http: //www.nanoscience.com/education/afm.html., Atomic Force Microscopy)

2.2.2.8.3 USE OF AFM IN CANDIDAL STUDIES

Braga and Ricci (2011) used to AFM images to know the thymol induced alterations in *C. albicans*. Thymol affected the envelope of *C. albicans* cells and caused major structural deformities like holes and ghost cell formation. Similarly, interaction of candida with macrophage was studied by Sofiane and Dufrene (2012). However, information on AFM studies on interaction of *C. albicans* with HBEC are not available.

2.3 STUDIES ON ADHESION OF *C. ALBICANS* ON HBEC IN *IN VITRO* CONDITIONS

2.3.1 ADHERENCE BEHAVIOUR OF *C. ALBICANS* ISOLATES FROM HIV SEROPOSITIVE INDIVIDUALS COMPARING TO ADHESION BEHAVIOUR OF ISOLATES FROM HEALTHY INDIVIDUALS

Few researchers have reported that, during HIV infection more virulent strains of *C. albicans* were selected in the oral cavity (Vargas and Joly 2002; Mane et al. 2012). Mane et al. (2012) also showed *C. albicans* from HIV seropositive subjects had greater ability to adhere to HBEC and virulence factors like increased expression of proteinase, phospholipase and hemolytic activities were observed in these isolates. However no significant difference in adhesion ability of *C. albicans* within HIV seropositive individuals who are having asymptomatic candida carriage and the one with OC were observed. *C. albicans* from HIV seropositive individuals showed significantly higher number of isolates adhering to HBEC comparing to isolates from HIV seronegative individuals in other studies also (Sweet et al. 1995; Macura and Bort 2001).The *C. albicans* isolates obtained from patients with HIV -1 infection also showed increased adhesion to fibronectin (Pugliese et al. 2000).

However, Liu et al. (2005), showed that, the virulence factors like aspartyl proteinase production and adhesion to HBEC in candida isolates from HIV seropositive individuals was found to be lower than in isolates from HIV seronegative subjects. They found no difference in virulence factors shown by pathogenic and commensal isolates from HIV seropositive patients. Tsang and Samaranayake (1999) reported that, the adhesion of candida depends mainly on receptivity of HBECs to candida. Variables like use of antiviral agents, antibiotics, hemophilia, increased age, decreased CD4+T cell count and several other host factors associated with HIV infection influences adhesion of candida. Similarly, another report from Imbert-Bernard et al. (1994) reported that, the *in vivo* virulence behavior of *C. albicans* exhibited in mouse experiments did not correlate with capacity of each yeast in its adherence onto HBEC in *in vitro*. The results discussed are not in the same trend as observed in earlier researches.

2.3.2 *IN VITRO* EXPERIMENTS TO DEMONSTRATE *C. ALBICANS* ADHESION ONTO HBEC

Various *in vitro* adhesion assays were developed to study the adhesion of *C. albicans* onto HBEC, with each method having its own limitations. Kimura and Pearsall (1978) who demonstrated *in vitro* adherence assay used the HBEC taken from oral mucosa of the volunteers. The sterile cotton swab was used to swab oral mucosa and the swab was then immersed into PBS to release the HBEC. HBEC thus collected were centrifuged and washed with PBS before the experiment. The original method of Kimura and Pearsall (1978) is still being used by many researchers (Antony et al. 2007). Donor type, time of collection, number of nonviable cells and enzymatic modification of cell surface can lead to variation in the results (Cotter and Kavanagh 2000). However, in a study by Gorman et al. (1996), it is showed that the adhesion pattern of *C. albicans* do not change between viable or non viable epithelial cells. The HBECs obtained in the method of Kimura and Pearsall (1978) are directly from the natural oral cavity environment and can be the relevant candidates for the test.

After Kimura and Pearsall (1978), a few modifications were made to the original procedure by other researchers to carry adhesion tests in *in vitro* conditions; e g: monolayer cultures with combination of stromal equivalent and epithelial cells commonly called organotrophic cultures were used (Papaioannoun 1998). The drawbacks found in this system were, mixture of viable and non-viable cells, donor type, time of sampling, colonization with normal flora and degree of exposure to various enzymatic secretions (Henriques et al. 2006).

To avoid contamination with normal flora and to maintain uniformity in the viability and other features use of cell lines were recommended. The cell lines like HeLa (the cell line derived from cervical cancer cell) and KD (derived from epidermal carcinoma of the mouth) were used for adhesion studies by a few researchers (Henriques et al. 2002).

Recently, Reconstituted Human Oral Epithelium (RHOE) was used as a successful model for experimental candidiasis (Schaller et al. 1998; Jayatilake et al. 2005). This is a multilayer structure, with close match to oral epithelium. The pathological changes occurring in experimental candidiasis using this model exhibited close similarity with the human infections.

Visual, radiometric methods and recently adopted ATP measurement methods were used to quantify the adhesion of candida onto HBEC. The visual method utilizing use of light microscopy is demonstrated by many authors (Kimura and Pearsall 1978; Henriques et al. 2006). Gram staining or simple Methylene blue staining was used here. Number of candida cells adhering to HBEC enumerated directly on microscopic observation. However this method is time consuming. In the visual methods, fluorescent and electron microscope can also be utilized instead of routine light microscopes. The radio labeling method were tried to have rapid quantification. Disadvantage of radiometric method is leachable isotopes, which can produce misleading results (Henriques et al. 2006). More recently, ATP measurement based method was tried by Nikawa et al. (2002). they developed *in vitro* quantitative evaluations of the adhesion of candida onto monolayer of epithelial cells by extracting host cellular and fungal ATP separately.

2.3.3 ULTRA STRUCTURAL EVALUATION OF *C. ALBICANS* ON HBEC USING SCANNING ELECTRON MICROSCOPY (SEM)

Scanning electron microscopy (SEM) was utilized for ultra structural visualization of the *C. albicans* and its adhesion on HBEC. SEM and other high resolution microscopy techniques have been used to study biofilm formation, antimicrobial effects of various substances, adhesion on other metals and host tissues/cells since a long time (Guibaud et al. 2005; Reith et al. 2006; Milani et al. 2012).

Application of SEM is highly popularized, when used in the combination of energy dispersed X-ray diffractive spectroscopy (EDX). Use of EDX reveals nature and amount of different elements (K, Ca, C, N, Si, Mg, Al) at different parts of the

material's surface. Studies of nanobiology and bioremediation using microbes utilize this facility thoroughly (Guibaud et al. 2005).

Pope and Cole (1981) used SEM in candida adhesion study in 1981. They had used SEM to reveal the location of the yeast and their structural association with the surface of tissues of the gut after intragastric inoculation of infant mice. Microenvironment of GI segment of each animal was preserved by freezing samples in liquid nitrogen. SEM analysis done showed that the yeasts were associated with the mucus layer and the epithelial surface throughout the GI tract. Segal et al. (1997) reported that, nikkomycin treated *C. albicans* showed less affinity to Wheat Germ Agglutinin (WGA), which was demonstrated in SEM studies. Nikkomycin is a chitin synthase inhibitor. Variation in WGA binding pattern indirectly proved the involvement of chitin in the adhesion of *C. albicans* to epithelial cells. The ultra structural study on adhesion of *C. albicans* on human oral epithelium using SEM done by Jayatilake et al.(2005) revealed that cavitations on the surface of epithelial cells particularly pronounced at the sites of hyphal invasion and these invasions were seen both at cell surfaces and intracellular cell junctions of the epithelium. Invasions at intercellular cell junctions of the epithelium resembled here thigmotropic behavior of the yeast during adhesion.

2.3.3.1 SAMPLE PREPARATION FOR SEM STUDIES

In conventional SEM studies samples used must be completely dry (Goldstein et al. 2003). The sample preparation include, isolating the organism from the required condition, fixation of organism either alone or with its microenvironment surrounding like agar or tissue along with. For any kind of samples, dehydrating using ethanol or any other suitable method is required. After these steps before mounting on an SEM stub, sputter-coating with gold has to be done to maximum of 30 s. Sputter coating can prevent the charging of electrons at the sample. Lohnes and Demirel (1978) and Goldstein et al. (2003) had reported that sputter-coating done more than 30 scan cause of artificial topography (small blobs of coating material) on a sample. Once the sputter coating process is over, the images will be recorded at a required accelerating voltage and magnification.

2.3.4 GENOTYPING OF *C. ALBICANS*ISOLATES AND THEIR RELATION WITH ADHESION BEHAVIOUR

2.3.4.1 BACK GROUND

When looked for changes in virulence factors in isolates from different groups of subjects, genetic microevolution was demonstrated among the isolates (Samaranayake et al. 2003). During conversion of mere commensal candida to pathogenic strain, the possible genetic difference can be occurring and which can be evaluated by genotyping methods (Diz Dios et al. 2001).

During HIV infection upto 90% of the infected individuals experience OC, as their immune status keep on waning (Klein et al.1984; Torssander et al. 1987; Feigal et al. 1991). Though candida species occur as commensal in oral cavity and other mucosal surfaces, under certain predisposing conditions, this organism can cause severe infections (Odds 1994). The frequency of isolation of *C. albicans* increases in HIV seropositive patients as the infection advances (Vargas and Joly 2002).Soll et al. (1988) reported that, during colonization, variable genotypes may start evolving. However, role of each genotype, towards infection process is uncertain or difficult to determine. Diz Dios et al. (2001) reported that the micro evolution in the DNA of commensal *C. albicans*, may aid in the process of developing it as an aggressive pathogen, which can colonize and invade host tissue and cause infections.

High frequency switching is observed in 21 *C. albicans* harboring oral cavity of HIV seropositive individuals (Antony et al. 2007). Bio typing and serotyping methods were used to understand the difference among the closely related strains. However strain typing methods can be done more reliably using molecular typing methods. Molecular typing methods can measure genetic relatedness between the isolates. Clonal variations in *C. albicans* during asymptomatic oral candida carriage or recurrent OC have been rarely studied. The clonal variation can also have impact in antifungal susceptibility pattern as few clones may be highly resistant (Pfaller et al. 1994; Samaranayake et al. 2001)

Studies on relationship between virulence factors in oral *C. albicans* isolates of HIV seropositive subjects with genetic relatednesshave been carried out by few researchers. Several virulence factors, like adherence to host tissues and medical biomaterial, secretion of hydrolytic enzymes (extracellular proteinases and phospholipases) and production of hemolytic factor, dimorphism attributes to pathogenicity of *C. albicans* are influenced by genetic variations (Gannoum 2000). Noumi et al. (2011) compared genetic relatedness to the adherence behavior of oral *C. albicans* isolates of HIV seropositive individuals.

2.3.4.2 METHODS FOLLOWED FOR GENOTYPING OF *C. ALBICANS*

To assess genetic relatedness of strains of microbes different methods of analysis can be used. In any method the conditions of experiments should be stable towards changes in the environment to reveal similarity distance among the strains of a chosen population in an effective manner as explained by Soll (2000). Along with varying sequences, few sequences of DNA are remaining stable over a period of time. Typing methods should assess these stable sequences in a microbe to store and form permanent data. These data can be retrieved rapidly and enabled results of different studies to be compared with (Soll 2000). According to the study by Soll (1992), high frequency switching is observed in *C. albicans* harboring oral cavity of HIV seropositive individuals.

Genetic relatedness among strains of *C. albicans* from HIV seropositive patients at different intervals has been studied by various workers (Pfaller et al. 1994; Barchiesi et al. 1995; Dromer et al. 1997; Pizzo et al. 2005). The genetic evaluation studies were done by various methodologies like, Electrophoretic karyotyping (Clemons et al. 1997; Dassanayake et al. 2002; Rho et al.2004; Shin et al. 2004), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) (Boldo et al. 2003; Lian et al. 2004; Pinto et al. 2004), Multilocus Enzyme Electrophoresis (MLEE) (Rosa et al. 2000), Microsatellites (Lott et al. 1999; Botterel et al. 2001), Multilocus Sequence Typing (MLST) (Tavanti et al. 2003; Odds et al. 2007), and DNA fingerprinting with Ca3 probes (Blignaut et al. 2002; Pujol et al. 2002). Among all the above mentioned genotyping methods, RAPD

finger printing proved to be the easy and rapid method to perform (Boldo et al. 2003). If technically considered, RAPD is easy to conduct, less time consuming with high discriminatory power. While doing large number of samples, RAPD turns out to be cost effective and also matches the resolving power of Electrophoretic karyotyping (Robert et al. 1995; Soll 2000).

2.3.4.3 RAPD FINGER PRINTING METHOD TO STUDY GENOTYPIC RELATEDNESS IN CANDIDA

RAPD utilizes short synthetic oligonucleotide (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperature by PCR. Though random primers are used in RAPD, they are selected after many possible permutations of bases composing of oligomers and are tested for useful selection of DNA fragment in PCR. No prior knowledge of the genome subjected to analysis is required for RAPD analysis (Williams et al. 1990; Welsh and McClelland 1990).

One of the most commonly used probes has been the repetitive element or complex probe, which is a DNA fragment containing sequences that are dispersed throughout the genome of the organism. RAPD method has been described for *A. fumigatus*, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. dubliniensis* (Soll, 2000). These probes are useful because they provide fingerprints of sufficient complexity so that genetic variability can be analyzed at multiple levels. These fingerprint patterns contain bands that arise as a result of microevolution (most variable), as well as bands of moderate variability, and low or no variability. However, a number of problems have been identified in obtaining intra and inter laboratory reproducibility of this method (Meunier and Grimont 1993).

A study by Samaranayake et al. (2003) revealed that there were prevalence upto 14 different genotypes per individual during their study with 16 HIV seropositive ethnic Chinese individuals. In their study, the oral samples were collected from both HIV seropositive patients with and without OC, to understand genotypes distribution

of oral *C. albicans* isolates during progression of HIV disease over a period 12 months.

2.3.4.4 DENDROGRAM CONSTRUCTION BASED ON RESULTS OBTAINED FROM GENOTYPING

Results obtained from any of these DNA finger printing technique can be analyzed using dendrogram program available (Clemons et al. 1997; Gyanachandani et al. 1998; Soll 2000; Samaranayake et al. 2003). Gyanachandani et al. (1998) showed in their work that dendrogram construction based on results of genotyping, groups the isolates tested according to their distance of similarity or similarity coefficient among their genetic profiles. Different techniques were implemented for DNA finger printing to categorize*C. albicans* strains into different clades according to the genetic evaluation occurring in their DNA. Clades are isolates originated from single ancestor.One can categorize *C. albicans* strains into different clade according to the genetic evaluation occurring in their DNA (Samaranayake et al. 2003). Clemons et al. (1997) placed the isolates according to their genetic similarities by dendrogram construction and showed high degree of genetic diversity present between *C. albicans* isolates from south East Asia and those from United States and Europe. Thus with RAPD analysis and dendrogram construction the genetic similarity between strains from different groups can be explained. The pathogenicity of the isolates can be further correlated between the genomic clades and thus microevolution can be explained.

2.3.5 STUDIES ON MECHANISMS OF ADHESION

2.3.5.1 SPECIFIC BIOMOLECULES INVOLVED IN ADHESION OF *C. ALBICANS* TO HBEC

2.3.5.1.1 CELL WALL PROTEIN EXTRACTION METHODS

Cell wall protein studies have been done using intact cells; or after cell wall protein extraction using detergents or chaotropic agents, or by using, secretary products of developing cells from the culture filtrate (Farhanezad et al. 2004; Feiz et al. 2006; Casanova and Chaffin 1991). In few studies along with detergents, enzymes like glucanases were used to extract cell wall proteins of candida (Pitarch et al.2002). There are reports about the presence of cytosolic protein contaminating the cell wall protein preparation in the methods using detergents as reported by Feiz et al. (2006). However Pitarch et al. (2002) further confirms theabsence of cytosolic contamination. Due to the disagreement between various methods followed, proper evaluation and comparison of extraction procedures is required and best method for extraction to be developed.

2.3.5.1.2 LECTINS TO STUDY THE CELL WALL PROTEINS *C. ALBICANS*

Lectins are glycoproteins, which bind to specific carbohydrate moieties as represented in Fig. 2.11(Sharon and Lis 1972).

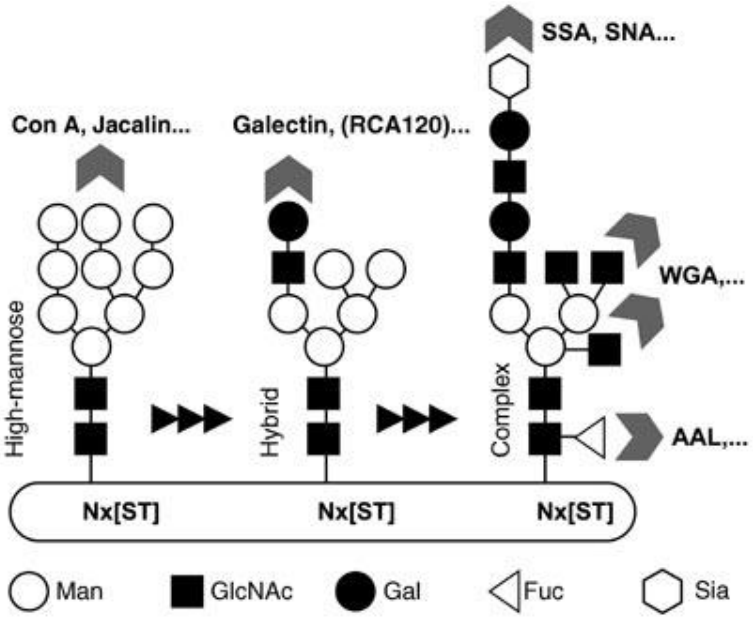


Fig.2.11Binding of various lectins to specific carbohydrates

Note: Con A lectin binds to mannose moieties. Galectin binds to galactose moieties. Whereas, Lectin SSA (Sambucus sieboldiana lectin), SNA (Sanbucus nigra Lectin) binds to sialic acid moieties, WGA(wheat germ agglutinin) binds to N-Acetyl glucosamine moieties.AAL (Aleuria aurantia lectin) binds to fucose moieties.

Protein fractions separated on SDS PAGE transferred to Polyvinylidene Difluoride (PVDF) membranes and were localized using a battery of biotinylated lectins. Lectin interacting protein bands were localized using Extravidin alkaline phosphatase reaction. This procedure is called lectin blotting (Walker 2009). Lectin blotting is an important and specific tool to identify post translational modifications such as glycosylation in the proteins (Walker 2009). Glycosylation is a fundamental process in biopolymers found in the cells, where carbohydrate will be added on to protein or lipid and results in structural and functional changes in that molecule. Glycosylation of membrane proteins play a role in cell-cell adhesion via carbohydrate binding proteins called lectins (Goldstein and Hayes 1978).

Lectin is known to detect candidal cell surface molecules in *in-vivo* conditions as reported by Joseph et al. (2006). Their study results suggest that Mannose binding-

lectin (MBL) is an important component of innate resistance to candidiasis. MBL found in human plasma binds to mannose, N-acetyl glucosamine and fucose present in candidal cell surface. MBL deficiency leads to recurrent vaginal candidiasis in women as shown by these authors.

In *in vitro* conditions lectins were used in candidal studies, with different approaches. Lectins were used in *in vitro* conditions to type the *C. albicans* from HIV seropositive individuals by Korting and Abeck (1992).Fifty *C. albicans* strains were typed with 14 different lectins by lectin agglutination reactions. The authors reported the existence of 16 different lectin types, with most frequently seen lectin type was occupying 22% of the total isolates tested. This study reveals that lectin typing is an easy and efficient tool for epidemiological typing of *C. albicans*. Lectin blotting technique was used in candida research to evaluate the glycosylation occurring during cell wall formation in spheroplasts (Kapteyn et al. 1995). Kapteyn et al. (1995) reported the detection of β-1,6-glucosylated proteins in the cell wall of the spheroplast as and when it becomes osmotically stable, these proteins were absent in the medium previously. However, *in-vitro* studies to demonstrate changes occurring with candidal surface proteins pertaining to their carbohydrate moities expressed during commensalism and pathogenic status using lectin blotting are sparse.

2.3.5.2 EXPERIMENTAL METHODS TO ILLUSTRATE PHYSICOCHEMICAL FACTORS

2.3.5.2.1 CELL SURFACE HYDROPHOBICITY (CSH)

Salt aggregation, microcele formation, aqueous hydrocarbon partitioning and hydrophobic interaction chromatography, sessile drop method to detect contact angle and co-aggregation with *Fusarium nucleatum* are the different methods used to measure CSH. In *C. albicans,* CSH was measured using liquid partition method (using hydrocarbon solutions) and contact angle methods by Rosen berg et al. (1980) and Minagi et al. (1985). The authors showed that both methods gave similar results. They demonstrated less adherent cells had smaller contact angles, than more adherent species. The electrostatic forces will influence adhesion behavior along with surface hydrophobicity, thus CSH may get masked as with microsphere assays, co-aggregation and microbial adhesion to hydrocarbon tests (Doyle 2000). Since contact angle method is not involving the use of any reagents, do not cause changes in cell surface properties. Therefore contact angle measurement or sessile drop method has been used in candida studies extensively (Van der Mei et al.1998). Doyle (2000) also preferred contact angle technique as it gives average value of hydrophobicity and does not take cell cycle variations into account or individual cell contributions.

2.3.5.2.2 SESSILE DROP METHOD USED FOR DETERMINATION OF CONTACT ANGLE (θ)

Here, to determine CSH, microbial lawns were prepared either on filter discs (0.45 μm pore size) or clean glass slides. The microbial cell suspension can be prepared by growing the cells in required conditions. The cells were washed thoroughly to remove all the media contents and then suspended in deionized (DI) water. The lawn prepared on the solid substrate has to be dried carefully in air as suggested by Van der Mei et al. (1998). Water and formamide are the Polar components used and
L bromonaphthalene and di- iodomethane are the apolar components used according to references (Henriques 2005). The instrument used for the measurement is having

aholding plate to carry the slide/filter paper disc. The contact angles formed on the microbial lawn with particular liquid can be captured immediately using a camera which is connected to the computer.

Contact angle (θ) formed by a liquid over a solid surface and the components of the surface tension (of the liquid L and surface S) polar and a polar can be established by the young Good Giricalco–Fowkes equation (van Oss et al.1987).

$$(1-\cos\theta)\,Yl = 2(\sqrt{Ys}^{LW}.\,Yl^{LW} + \sqrt{Ys}^{+}.\,Yl^{-} + \sqrt{Ys}^{-}.\,Yl^{+})$$

$$\cos\theta = \frac{[(0.15\gamma^{MV}-2.0)(\gamma^{MV}\gamma^{LV})]^{1/2}+\gamma^{LV}}{\gamma^{L}\,[0.15\,(\gamma^{MV}\gamma^{LV})^{1/2}-1]}$$

This equation has 3 unknown surface parameters of 3 different liquids that are 2 polar and one apolar in nature are needed for calculating the surface tension ($Ytotal = Y^{AB} + Y^{LW}$).

2.3.5.2.3 CALCULATION OF CELL SURFACE HYDROPHOBICITY

Free energy comprises a polar (AB) and an apolar (LW) component and the variation of the total energy. Measurement of CSH can be done according to Van Oss and Giese (1995). CSH can be measured as variation of the free energy of interaction between two moieties of that material immersed in water. The free energy can be calculated as

$\Delta G_{SWS}^{total} = \Delta G_{SWS}^{LW} + \Delta G_{SWS}^{AB}$ AB is a polar component, LW is an apolar component.

Hydrophilic cells give total free energy (ΔG_{SWS}^{total}) >0, here free energy of interaction between molecules is attractive and cell will have less affinity for water, than themselves. Hydrophobic cells will be $\Delta G_{SWS}^{total} < 0$, i.e., (Van Oss and Giese 1995).

2.3.5.3 PROCEDURES FOLLOWED FOR AFM WORK IN BIOLOGICAL CELLS

2.3.5.3.1 PREPARATION OF MICROBIAL CELLS

Sample preparation is the crucial step for AFM analysis. The cells should be attached to smooth solid substrate, since microbes suspended in liquid media may exert lateral force on the tip of the AFM (Doktycz et al. 2003). Substrates like mica and mica surface modified with silacanes, glass or glass surfaces like modified cover slips with silicane molecules had been used for sample preparation (Lyubehenko et al. 1992; Karrasch et al. 1993). Other than these commonly used surfaces, Highly Oriented Pyrolytic Graphite (HOPG) (Cullen and Lowe 1994). Spin coating of polymers (Polystyrene, poly (methyl) methacrylate were used as the hydrophobic substrates for the study of biological samples (Dupont – Gillain et al. 1999). Since gold is chemically an inert substance, gold has been used with few modifications to obtain very smooth surfaces (with maintaining the roughness of 01.1 nm) for the attachment of biomolecules (Wagner et al. 1995).

Tremendous research carried out in this field had finally made sample preparation task easy. Simple preparation method is used to exploit the ability of cells to spread and adhere to solid surfaces (Radmacher et al. 1992). Coating with collagen was an improved method which has been explained by Henderson et al (1992). Chemical fixation using glutaraldehyde can prevent all damages or detachment by scanning tip (Le Grimellec et al. 2002). Since some of the procedures may not be suitable for microbial cells, pretreatment of substrate by polycations or by bonding the cells covalently to the substrate is suggested for establishing stronger attachments (Gad and Ikai 1995; Camesano et al. 2000; Schaer-Zammeretti and Ubbink 2003).

2.3.5.3.1.1 TRAPPING THROUGH MEMBRANE FILTERS

A mechanical method used for sample preparation is trapping the organism through pores of the filter membrane. A concentrated solution of organism is gently sucked through a membrane filter with pore size slightly smaller than the size of the microbe. The filter membrane is quickly dried on a sheet of tissue. And then the specimen is immediately attached to the sample holder using small piece of adhesive tape. This procedure does not include chemical treatment, thus do not cause denaturation of surface molecules. This procedure has been tried for fungus *Asperigillus oriyzae* spores, bacterium *Lactococcus lactis*, parasite *Cryptosporidium parvum* (Touhami et al. 2004). The cells can be imaged repeatedly in all aqueous solutions, without cell detachment or damage. Trapping the organism in membrane filter or on the agar gel is used as soft deformable immobilization matrix. In agar gel, direct visualization of growth processes can also be admired (Gad and Ikai 1995). In membrane trapping method organism will be made to sit in pore which is appropriate to the cell size. Thus it can allow repeated imaging without cell detachment or cell damage (Kasas and lkai 1995; Dufrene 2004). This method is popularized due to its high reliability in studying different types of microbes (Vadillo-Rodriguez et al. 2004).

2.3.5.3.1.2 PREPARATIONS OF BIOMOLECULES ON SOLID GLASS SLIDES

Immobilization of proteins can be done by simple methods. The slides were prepared by pouring the sample over the cleaned glass slide and allowing it to dry in liquid nitrogen or immersing slide in solution of biomolecules and allowing the biomolecules to get adsorbed (Bustamanate et al.1992).

The biomolecules adsorbed can be imaged in air, under alcohols (Bustamanate et al.1992), or in aqueous solutions (Hasma et al. 1992). However, sample adsorption onto mica can improve the resolution (Rebke et al. 1994).

Surface of substrate modified by coating with polycation such Poly – L-Lysine can enhance the adsorption of negatively charged samples (Muller et al. 1997).

2.3.5.3.2 PERFECTION OF RESOLUTION

To achieve high resolution, few cautions to be taken, e.g. tip-specimen interactions can be reduced by using tapping mode AFM with an active resonance control (Tamayo et al. 2001). Use of photonic force microscope where AFM cantilever is replaced by the 3D trapping potential of a laser focus can allow 3D imaging of biological structures with extremely small loading forces (Pralle et al. 2000).

2.3.6 USE OFFOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY ANALYSIS TO STUDY ADHESION OF *C. ALBICANS* ONTO HUMAN BUCCAL EPITHELIAL CELLS (HBEC)

Fourier Transform Infrared (FTIR) spectroscopy is based on the vibrational excitation of molecular bonds by absorption of infrared light energy. The technique gives rapid, inexpensive and highly specific results (Naumann et al. 1991). In recent years, FTIR is used for characterization of microorganisms by taking the sum vibration spectra for cells macromolecule content (nucleic acids, proteins, lipids, polysaccharides, etc.) as a spectral "fingerprint" for that organism.FTIR spectra between 4000–400 /cm include stretching vibrations and deformation of C–H, C=O, N–H, P=O, and C–O–C functional groups. The information on these functional groups reflect the differences in the biochemical composition of microbial cells i.e., DNA, proteins, polysaccharides and fatty acids (Naumann et al. 1991; Hong et al.1999; Kansiz et al.1999; Oberreuter et al.2002; Winder et al. 2004). FTIR has been used for identification of different bacteria and candida species from different clinical specimens and reported to be giving promising and reproducible results (Goodacre et al. 1996; Maquelin et al. 2003; Essendoubi et al. 2005; Elsayed et al. 2010; Filip and Hermann 2010).

Sample preparation methods for FTIR are non destructive and hence this technique has been used for understanding a few sophisticated information in microbial research also. Microbial adhesion and biofilm formation other than mere

identification of the microbes were studied using FTIR. ATR/FTIR has been widely used in many adsorption studies as in studies of adhesion of bacteria onto metals, biofilm formation etc (Sandt et al. 2006). FTIR has been recorded to be the useful tool for characterizing Candida from clinical specimens. FTIR is used to understand morphological changes occurring during growth phases of *C. albicans* (Niven et al. 1993; Choo- Smith et al. 2001). FTIR was used here to study structural modifications during the morphogenesis of *C. albicans* in detail, thus polymorphism nature of this fungus was explained in relation to the functional groups presented. Sandt et al. (2006) used FTIR to study biofilm formation in *C. albicans*. In the study by Sandt et al. (2006), candida was observed at various growth forms and quantitative evaluation of functional groups found on components of the cell wall was done. Through this earlier literature survey, it is evident that one can also use FTIR for understanding candidal adhesion on HBEC which was not attempted by researchers yet.

2.3.7 STUDYING THE MOLECULES WHICH BLOCK THE ADHERENCE OF THE ORGANISM TO RESPECTIVE LIGANDS IN MOST EFFECTIVE MANNER

Antiadherence approach is mainly developed due to the alarming increase in drug resistance. Microbial adhesion analogues, dietary constituents, sub lethal concentrations of antibiotics and adhesion based vaccines have been used as antiadhesive approaches. With antiadhesive substances organisms developing resistance also have been less compared to actual antimicrobial agents (Ofek et al. 2003a; Ofek et al. 2003b)

Microbes can express more than one type of adhesin molecules; therefore multiple anti-adhesin agents inhibiting each type of adhesin of the pathogen has to be developed to combat the adhesion (Kahane and Ofek 1996; Sharon and Ofek 2001). This is the major drawback of success of antiadhesion therapy.

2.3.7.1 BACKGROUND OF ANTIADHESIVE APPROACHES IN PREVENTING INFECTIONS

2.3.7.1.1 NATURAL ANTIADHESIVE PHENOMENON IN HUMAN IMMUNE SYSTEM

In humans, there are large number of inhibitors for microbial adhesins found in body fluids and mucosal surfaces. These prevent colonization of organisms by inhibiting their adhesion on host cell. Sphinganine in sphingolipid is a hydrophobic molecule which decreases adhesion of *Streptococcus mitis* to HBEC and *Staphylococcus aureus* to nasal mucosal cells (Bibel et al.1992). Secreted mucins have great ability in preventing adhesion of microbes on mucosal surfaces (Ofek et al. 2003).

Similarly, human milk rich in fucosyl 2' – lactose can prevent *Campylobacter species* mediated diarrhea in breast fed children (Ruiz-Palacios et al. 2003). Lactoferrin, mucin, fat globules, and lipid contents of the human milk inhibited various anaerobic bacteria and *E. coli* binding to HeLa cells and RBCs (Longhi et al. 1993). Sulphated components of gastric mucosa are inhibitory to *H. pylori*. Individuals who show presence of blood group determinants in the fluid bathing mucosal surfaces are known as secretors. These blood group determinants are glycoconjugates, which can bind to the pathogens and inhibit the adhesion as shown in candida vaginal carriage study by Kulkarni and Venkatesh et al. (2004); they reported non secretor being more prone to vaginal candidiasis. Blood group antigens, i.e., H blood group antigen types, act as epithelial receptor for *C. albicans* and shown to react with candida adhesins (Cameron and Douglas 1996).

2.3.7.1.2 ADHESION BASED IMMUNIZATION

Immunization based onantiadhesion concept holds good both in active and passive mediated immunity. Antiadhesive antibodies form passive means of immunity. Adhesins of pathogen can be used for vaccine preparation which contains receptor binding domains (Langermann et al. 1997; Wizemann et al. 1999). The K88 fimbriae and related adhesins of farm animal pathogens were used to vaccinate

mother pigs so that suckling piglet get antibodies through mother's milk. This prevents infection by the pathogens in piglets (Moon and Bunn 1993). In near future consumption of milk from immunized cows may become useful to target many such infections in human beings (Ofek et al. 2003).

Antiadhesion antibodies can be explained with the example of Bordetella vaccine also, which contains the hemagglutinin adhesins. Vaccinated individuals develop anti-hem-agglutinin antibodies, which can inhibit adhesion of Bordetella to epithelial cells (Relman et al. 1989). All these reports indicate that studies with antiadhesive drugs are promising; however one should solve the problems of transient effect of these drugs and colonization of affected area with other microbes (Kelly and Younson 2000).

2.3.7.1.3 DIFFERENT APPROACHES TO ESTABLISH ANTIADHESION THERAPY

Receptor analogues, adhesion analogues, dietary components, surfactants, plant extracts, sub lethal concentrations of antibiotics are the different groups of agents which have been studied for their efficiency in preventing adhesion of the organisms on to the host cells (Ofek et al. 2003).

2.3.7.1.3.1 SACCHARIDES USED AS RECEPTOR ANALOGUES TO BRING ANTIADHESIVE EFFECT

This approach was tried for the infectious agents, binding to the host cells via carbohydrate specific adhesins. Use of saccharides for antiadhesion has been tried from 2 decades ago for *in vitro* conditions and commonly in animal studies. Clinical trials also were done, as in one of the studies where Sialyl – 3' – lacto-N-neotetrose was given to treat colonization of upper respiratory tract bacteria and to treat otitis media. The results obtained were not promising in this trial (Ukkonen et al. 2000). Other similar kind of approaches also was documented with *H. pylori* and *E. coli* (Mulvey et al. 2001). However, these *in vitro* tests showed high concentration of sugars needed to bring antiadhesion effect. Instead of increasing concentrations of the carbohydrates while using for *in vitro* conditions, affinity of these substances towards

bacterial lectins can be increased by several magnitudes by covalently linking a hydrophobic residue such as phenyl or methyl belliferyl to the saccharide (Firon et al. 1987). Another approach was carried out by attaching many copies of saccharide to a suitable carrier and converting them into multivalent adhesion inhibitors (Mulvey et al. 2001).

2.3.7.1.3.2 ADHESIN ANALOGUES AS ANTIADHESIVE AGENTS

Lectins, synthetic peptides and a few enzymes were used as adhesin analogues in preventing adhesion process.

i) Lectins as antiadhesive agents: Food grains, cereals and millets are proved to contain lectins. Some food lectins have deleterious effects on humans, e.g.: wheat germ agglutinin (WGA) stuck to HBEC of individuals who had consumed raw wheat and showed increased numbers of *Streptococcus sanguis* adheres to the WGA, and colonizes (Gibbons and Denkers 1983). Similarly certain dietary lectins may reach intestine and enhance the binding of bacteria to different parts of intestine. Many lectins have been isolated and characterized from plants. In animal studies these plant lectins have been proven to be good in enhancing the clearance of bacteria (Slifkin and Doyle 1990).

ii). Synthetic peptides: Relatively small peptides can be employed for adhesion therapy. Synthetic 20 residue mimicking sequence of *Streptococcus mutans* cell surface adhesin which mediates binding of bacteria to salivary protein on a dental surface was successful in inhibiting the adhesion of *Streptococcus mutans* in *in vitro* studies using an artificial tooth pellicle (Kelly et al. 1999). One more study reported that, lipoteichoic acid (LTA) mediated adhesion of group A and group B streptococci was inhibited by using synthetic LTA. However these studies were conducted on animals. As LTA is toxic for humans, use of it for treatment of human infections is not possible (Dale et al. 1994).

iii) Enzymes: Enzymes such as lysozyme, lactoferrin and lactoperoxidase are been proposed for the use in patients with Xerostomia to prevent Xerostomia related diseases (Tenovuo et al. 1991; Hahnel et al. 2010).

2.3.7.2 PLANT EXTRACTS SCREENED FOR ANTIADHESION APPROACH

Since plant extracts exhibit less toxicity, it is easier to perform clinical trials. Extracts of neem stick, green tea and lemon grass have been used by various workers for antiadhesion activities (Polaquini et al. 2006; Tyagi and Malik 2010). Cranberry extracts have shown promising results in preventing *E. coli* mediated urinary tract infections by inhibiting the adhesion of these bacteria onto uroepithelial cells (Papas et al. 1968; Liu et al. 2006). Several such plant extracts are used in inhibition of adhesion of candida onto host cells. Aloe vera, Lemon Grass, Neem extract, tea tree oil is the few examples of plant extracts used for studying antimicrobial effect.

Aloe vera (*Aloe barbadensis*) has got versatile medicinal properties with 400 different species known. It has hypoglycemic activity with decreasing fasting glucose levels. Aloe vera intake decreases hepatic transaminases, plasma and liver cholesterol, triglycerides, fatty acids and phospholipids and also improves plasma insulin level. Aloe vera diminishes degenerative changes observed in kidney tissues (Yagi et al. 2009; Sudha et al. 2011). In antimicrobial studies, different materials from Aloe vera, i.e., leaf, gel, secretions from the cut base of the leaf are studied. Results are not similar in all the cases. Gel has proved to have more antimicrobial effect on *Staphylococcus aureus* and *Pseudomonas aeruginosa*and fungus *Trichophyton metagrophyte* than leaf extracts of Aloe vera. However, gel did not show any inhibitory effect on *C. albicans* in the study by Kareru et al. (2010).

Aloe vera gel contains 99.3% water, 0.7% solids with glucose and mannose constituting large part. Gel from Aloe vera also contains aloctin A and acemannan as immunomodulatory components (Agarry et al. 2005). Agarry et al. (2005) reported in *in vivo* studies, these Aloe vera components injected via intra-peritoneal and intramuscular route to a streptozotocin induced diabetic mice, caused reduced growth of candida pathogen in their body. These components also increased ovalbumin (OVA), specific cytotoxic T lymphocyte (CTL) generation in high fat diet induced mice and also purified fractions of Aloe vera gels also significantly increased viability of macrophages infected with candida.

Azardirachta indica (Neem), also known to be used in antiadhesion studies. Polaquini et al. (2006) reported that Neem used in 0.01g/ml concentration, significantly increased surface hydrophobicity of light cured resins, thus decreased candidal adhesion and biofilm formation.

Cymbopogan citratus (Lemon grass) is yet another plant used frequently in antimicrobial studies. Lemon grass contains 500 described spp. Phyto constituents found in this plant are saponin, tannins, alkaloids and flavonoids (Anonymous 2005). Lemon grass has been used in many antimicrobial studies. One of the zone inhibition tests showed both ethanol and aqueous *C. citrias* inhibited candida growth (Sharifa 2008). Lemon grass oil vapors and Lemon grass oil (LGO) studied together showed, LGO in vapor phase causes complete degradation of cells than in liquid phase, as studied under Transmission Electron Microscopy (TEM). AFM studies showed untreated candida cells had roughness of 211.97 nm; the candida cells treated with LGO gave roughness of 143 nm and with LGO vapors, the cell roughness found to be 5.981 nm. Authors say that, treating with LGO leads to rupture of the cells and LGO vapors cause complete destruction of the cells (Tyagi and Malik 2010). In a study of antifungal assay of oils from plants on various *Candida species* tested, lemongrass oil, clove oil and coconut oils were showed maximum anticandidal activity, almond oil showed least activity against *C. albicans* compared to other species (Kumar et al. 2012).

A few other plant extracts, which exerted antiadhesive action on *C. albicans* adhesion explained here. *C. albicans* isolates from HIV seropositive and HIV seronegative individuals showed decreased adherence after treating with *Dodonaea viscosae* (Hop bush) (Patel et al. 2009). But on the other hand, the production of proteinase and phospholipase was not affected in candida after treating with these plant extracts.

Abu-Elteen (2000) had tested date extract for their anti- adhesion activity. In this study HBEC collected immediately and 5-20 minutes after the mouth rinsing with 10% date extract. Other set of HBEC were preincubated with date extract in *in vitro* condition. In all the three conditions, HBEC showed significant inhibition in candida

adhesion. Pre-incubation of both HBEC and candida done with date extract showed higher magnitude of inhibition (Abu-Elteen 2000).

Phyllanthus emblica Linn (Indian Gooseberry), ethanolic extracts reduced adherence of *C. albicans* to HBEC and acrylic strips at the concentrations of 75-300 µg/ml (Thaweboon and Thaweboon 2011). *Streblus asper leaf*, ethanolic extracts (SAE) which is commonly known to be Siamese rough bush tree/tooth brush tree, proved to inhibit candida adhesion onto acrylic surface in a concentration of 6.25 mg/ml with minimum exposing time to the extract, i.e., 1 minute. Whereas pretreatment of acrylic sheets with SAE did not yield any reduction in the adherence (Taweechaisupapong et al. 2006). A 30 kDa protein isolated from seeds of *Streblus asper* also caused ultra structural changes in *C. albicans* cells as shown in TEM. This protein at the concentrations of 300 µg/ml reduced adherence of candida onto cover glass. The protein didn't exert any toxicity on Hep 2 cells at the concentration of 1000 µg/ml (Mincoff et al. 2005). *Allium sativum* (Garlic) reduced adherence of pretreated candida onto HBEC. The reduction was also seen when HBEC were pretreated with *Allium sativum* extracts. Germ tube formation in *C. albicans* was also suppressed on exposure to these extracts. HBEC collected from the individuals just after 15 minutes of the garlic mouth wash rinse also showed inhibiting the adhesion of candida on HBEC (Ghannoum 1990).

An interesting study explains the role of plants, where, plants used as sources for the production of antiadhesive antibodies. *Nicotiana benthamiana* (wild tobacco species endemic Australia) plants were used for transient expression on 2 chimeric mouse human Ab derivatives from mAb2G8. These antibodies recognize ß 1-3 linked glucan on cell surface of *C. albicans* and most of the pathogenic fungi. These antibodies also inhibited adhesion of *C. albicans* on HBEC in *in vitro* conditions. The antibodies produced here, retained their β- glucan binding specificity, antifungal activities and also expressed with high yield, i.e., approximately 50 mg Ab/kg of plant tissue (Capodicasa 2011).

2.3.7.2.1 PROCEDURES USED FOR PREPARATION OF PLANT EXTRACTS

The extraction procedures followed in most of the antimicrobial experiments were used certain measured quantity of plant materials. The required plant materials were cleaned, dried, ground, then subjected to extraction using solvents. The solvents used for extraction were generally water and ethanol for varying time intervals e.g.: 12, 24, 72 hrs etc. After the extraction, the solvent was subjected to evaporation (Okigboand Mmeka 2008). Lemongrass extraction was done using acetone, dichloromethane, methane, hexane, and water as solvents in a study by Hamza et al. (2009), while demonstrating antimicrobial activity of this plant material. Thus extraction methodology and solvents used vary according to various workers. After extraction, the solvent removed completely, either by evaporating in desiccators, according to the above said studies. In case of Aloe vera, different materials were used. Only leaf without gel and only gel were used to know antimicrobial activity separately for bacteria and fungus. In the study by Agarry et al. (2005), gel removed from leaves was dried, ground and soaked in ethanol for entire extraction period and then filtered. Filtrate was evaporated and extract was dissolved in distilled water and used for sensitivity testing. Pure gel of Aloe vera in the study by Sudha et al. (2011), subjected to extraction in distilled water with 2 and 4% concentrations. Few workers used oil forms of the plant for both antimicrobial activity and antiadhesion test (Tyagi and Malik 2010; Kumar et al. 2012).

2.3.7.3 SURFACTANTS USED AS ANTIADHESIVE AGENTS

Surfactants or "surface active agents" are the substances which exhibit some superficial or interfacial activity and which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids. Thus they increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds. Due to their use as emulsifyable and wettable agents, surfactants are used in different industries for e.g. the production of soaps, cosmetic powders, petroleum processing, food and agro processing, alcohol and ester and amide derivative production and also in biomedical fields (Sing et al. 2007).

Surfactants are tested for antiadhesion approaches also. Surfactants which usually increase the hydrophobicity of the surfaces were demonstrated using surfactants coated nanoparticles by McCarron et al. (2007). The authors reported that surfactants coated nanoparticles were resistant to adhesion with candida.

2.3.7.3.1 USE OF SURFACTANTS IN INHIBITION OF CANDIDAL ADHESION

Lyon et al. (2011) had tested Cetyl-trimethyl-ammonium chloride (CTAC), sodium dodecyl sulfate (SDS), N-hexadecyl-N-N'-dimethyl-3-ammonio-1-propane-sulfonate (HPS) and octyl-phenoxy-poly-ethoxyethanol (Triton X-100) for their effect on viability, adhesion ability and exoenzyme production by *Candida species*. All surfactants demonstrated are capable in inhibiting the adhesion of candida species to HBEC and the proteinase production in candida. The phospholipase production remained unaltered and growth was also less inhibited by the action of these surfactants.

Chlorhexidine is commonly used in mouth washes and also used as a disinfectant in dental procedures. This surfactant is known to induce cell surface alteration. Thus it causes osmotic imbalance and cytoplasmic precipitations (Delilbasi et al. 2002). Pizzo et al. (2001) reported that *in vitro* exposure of HBEC to mouth rinse products, containing chlorhexidine (CHX) 0.2% andCetyl Pyridinium Chloride

(CPC) 0.05%; reduced the candida adhesion in significant manner. The HBEC collected from both adults and children showed decrease in adhesion when their HBEC were collected immediately after chlorhexidine rinse and with certain time line of exposure. Tobgi et al. (1987) reported that *in vitro* exposure of HBEC from both children and the adult to 0.2%, 0.02%, and 0.002% chlorhexidine, also showed inhibition to candidal adhesion. Faria et al. (2011) reported that the chlorhexidine digluconate (0.12%) containing mouth washes tested in patients who were undergone extraction of unerrupted third molar, showed inhibited adhesion of microorganism including candida species, onto suture materials in very effective manner.

However, the use of chlorhexidine has disadvantages like, staining of teeth and dentures, dysgeusia etc. (Groppo et al.2002). Chlorhexidine can bind on both hard and soft surfaces and get released slowly thus its action can be sustained. The compound is successfully used in different mouth washes. Listerine, Triclosan, quaternary ammonium compounds and sanguinarine compounds, which are used in mouth washes with proven antimicrobial action. New products containing various fluorides and oxygenating agents have also been studied for anti plaque formation (Mandel et al. 1994).

A nonionic surfactant, lauryl glucose found to be effective in preventing biofilm formation and adherence of *C. albicans* and *C. lipolytica* and other bacterial strains, on polystyrene and glass surfaces (Dusane et al. 2008). Non-drug-loaded poly (ethylcyanoacrylate) nanoparticles (NP) coated with cationic, anionic and non-ionic surfactants are used to study their effect on candidal blastospores adhesion onto HBEC by McCarron et al. (2007). They reported that, Cetrimide and Pluronic P123 (cationic surfactant) reduced percentage of adhesion. This study showed that preparation using anionic surfactants was not successful with low yield and wide particle size distribution. Non-ionic surfactant-coated NP was intermediate in microbiocidal action. This study proved that, NP coated with cationic surfactants can form the good prophylactic formulations to target candida adhesion on HBEC compared to other types. However the use of this surfactant did not show significant influence on cell surface hydrophobicity.

In a study by Goldberg et al. (1990), Cetyl Pyridinium Chloride (CPC) enhanced adhesion of *C. albicans* along with few bacteria onto hexadecane. The enhanced action is explained here is due to, CPC diminishing cell surface charges of the treated cells and increasing cell surface hydrophobicity. They reported that, this phenomenon may help in preparing oral formulations and these hydrocarbon droplets may be used for cell immobilization.

2.3.7.3.2 BIOSURFACTANTS USED FOR ANTIADHESION STUDIES OF CANDIDA

Pseudofactin II, a biosurfactant produced by Arctic bacterium, *Pseudomonas fluorescens* BD5 had reduced adherence of few bacterial pathogens and *C. albicans* strains on glass, polystyrene and silicone surfaces and also dislodged pre existing biofilm effectively. The pretrement of polystyrene surfaces with 0.5 mg/ml of Psedofactin, inhibited adhesion of *C. albicans* from 92 to 99%, and also known to change the CSH of the surfaces treated (Janek et al. 2010). Biosurfactants of *Streptococcus thermophilus*, showed antiadhesionactivity on C. *albicans* and inhibited its adhesion onto silicone rubber effectively (Busscher et al. 1997). Similarly, rhamnolipid a biosurfactant released by *Pseudomonas aeruginosa*, inhibited adhesion of *C. tropicalis* and other bacteria onto voice prostheses and silicone rubber (Rodrigues et al. 2006).

2.3.7.4 SUB LETHAL CONCENTRATIONS OF ANTIBIOTICS IN ANTIADHESION APROACH

By referring to earlier literatures on the use of sub lethal concentrations of antibiotics, results obtained are contrasting. Ben-Redjeb et al. (1982) reported that enhanced percentage of adhesion of *E. coli* onto uro-epithelial cells was seen after pretreating with sub MIC doses of cefotaxime. Commonly the low doses of fluconazole was also tried by many researchers to inhibit the adhesion of *C. albicans* onto HBEC and which was shown to be effective in combating the adhesion (Ellepola et al. 1998; Egusa et al. 2000; Lyon et al. 2006; Blanco et al. 2006)

2.4 CONCLUSION OBTAINED FROM THE ENTIRE LITERATURE SURVEY

It is clearly shown through the above published informations that, in present situation HIV infection is the burden to Indian population. The patients with this infection also suffer with various opportunistic infections. In Indian scenario one of the most common opportunistic infections found in HIV seropositive individuals is Oral Candidiasis (OC) apart from tuberculosis. OC was previously treated successfully with fluconazole and other antifungal agents. In recent years, since the causative agents of OC, i.e., *C. albicans* and other candida species are showing resistance to these antifungal agents, risk of HIV seropositive patients for developing chronic lesions of OC is been enhanced.

In this context an alternative approach can be tried, which has been discussed in the literature survey, i.e., using various antiadhesive agents. Indian reports, on antiadhesion approach for candida from HIV infection are again rarely present.

To develop any kind of antiadhesion approach, the mechanisms involved in adhesion has to be studied and understood clearly. The present literature on adhesion pattern and enhancement of other associated enzymes involved in adhesion of candida from HIV seropositive individuals on HBEC have contradictory results. The involvements of certain proteins or carbohydrates on adhesion have not been explained or again have contradictory reports.

While studying the mechanisms of adhesion, involvement of specific and non specific mechanisms are reported. Regarding involvement of non specific factors like cell surface hydrophobicity (CSH), zeta potential etc, extensive data are not available mainly when adherence onto another biological surfaces i.e., when adhesion on host was considered. The approaches in this regard will help in developing natural remedies to cure OC in HIV seropositive patients.

Natural remedies like medicinal plants as antiadhesive agents have not been extensively studied and reported. The use of natural remedies has been a time tested way which can give slow but complete cure without any side effects, as per the Indian

traditional medicine. Organisms developing resistance to these kinds of substances are also rare. Therefore, these approaches can be novel and excellent targets to combat OC. As it has been explained earlier with regard to the OC in HIV, many gaps are present in the literature. The current study is aimed at extensive understanding of adhesion process in *C. albicans* onto HBEC with special emphasis on development of antiadhesion approach.

CHAPTER 3. MATERIALS AND METHODS

Present chapter explains the experimental methods followed to carry out the present study to generate experimental data to meet the stated objectives presented in Chapter 1.

Isolation and identification of candida from the selected population using standard microbiological methods, details on experiments followed to study adhesion behavior of *C. albicans* isolates from HIV seropositive individuals on HBEC, various factors effecting and mechanisms involved in the adhesion along with screening of different substances for inhibition of adhesion are explained in the present chapter. Supporting details on preparations of the required culture media, reagents and buffers are given in the appendix.

3.1 SAMPLE COLLECTION TO DETERMINE ORAL CANDIDA COLONIZATION STATUS AND ISOLATION OF *C. ALBICANS*

3.1.1 STUDY POPULATION AND DATA COLLECTION

The present research work was carried out in SDM College of Medical Sciences and Hospital, Dharwad after obtaining permission from the Institutional Ethical Committee. The work was done from January 2008 to August 2011. The study group comprised of 274 HIVseropositive subjects and 260 HIV seronegative subjects. HIV serostatus of the individuals was tested according to the NACO guidelines using three rapid tests based on different principles (Tridot-Biomed Industries, COMB Elisa - Span Diagnostic Ltd, HIV Capillus-Trinity Biotech Plc.). All the individuals included in the study were clinically examined for the presence of OC. Oral samples were collected from the study population to know the prevalence and colonization pattern exhibited by candida in their oral cavity. While collecting the samples from HIV seronegative subjects, only those who were not having any complaints of oral lesions or any other infections had been selected.

The HIV seropositive group was further subdivided into,

Sub Group 1: This group contains HIV seropositive subjects with clinically proven OC and the number of subjects included was 110.

Sub Group 2: This group contains HIV seropositive subjects without OC and the number of subjects included was 164.

The individuals with the history of treatment with antifungal or antibiotics within three months prior to the study and with diabetes mellitus or other predisposing conditions for OC were excluded from the study.

3.1.1.1 COLLECTION OF SAMPLES

The oral rinse samples were collected after obtaining the informed consent signed by the study subjects. Concentrated oral rinse method was used to collect the specimen, which gives the semi quantitative measure of the oral candida carriage (Samaranayake et al. 1986). In brief, the subject was instructed to rinse the mouth with 10 ml sterile Phosphate-buffered Saline (PBS) (pH 7.2) given in a sterile container for one minute and then spit it into the same container.

3.1.1.2 CULTURE AND IDENTIFICATION

The oral rinse specimen thus collected was immediately transported to the laboratory. The sample was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the sediment was suspended in one ml of sterile PBS (pH 7.2) and vortexed for one minute. With a sterile micropipette, 100 µl of this preparation was transferred on to Sabouraud Dextrose Agar (SDA) (HiMedia) and was spread evenly using sterile L-spreader. The plates were incubated at 37 ^{o}C for 48 hrs. The colonies of candida were counted and Colony Forming Units (CFU) per ml for candida positive cultureswas calculated. The candida colonies were identified on the basis of Gram's stain, germ tube test, corn meal agar morphology, CHROM-agar morphology, carbohydrate assimilation and temperature sensitivity tests (Larone 2002; Rippon 1982). *C. albicans* isolates were stored in yeast long storage media at -20 ^{o}C till further use.

3.1.2 FLUCONAZOLE SUSCEPTIBILITY TESTING

A total of 153 *C. albicans* and 83 NAC isolates obtained both from HIV seropositive and HIV seronegative groups were subjected for fluconazole sensitivity testing. Fluconazole susceptibility was tested by a broth micro dilution method according to the guidelines given in CLSI-M27 A2 document (CLSI 2005). Briefly, the susceptibility testing was performed in microtitre plates. Candida inoculum was prepared to obtain a final turbidity of $5x10^2$ to $2.5x10^3$ candida cells/ml in RPMI 1640 buffered to pH 7.0 using 0.165 mM Morpholino Sulphonylic Acid (MOPS)/l of RPMI. The candida cells were inoculated to the microtitre wells containing fluconazole concentrations ranging from 64 to 0.125 µg/ml. Results were read visually after 48 hrs of incubation. *C. albicans* MTCC 3017 was used as a control strain.

3.1.3 STATISTICAL ANALYSIS

The rate of candida carriage and prevalence of *Candida spp.* were statistically analyzed using Chi-square test. Mean density of candida carriage in each group was statistically analyzed using unpaired‘t’ test. Prevalence of *C. albicans* and NAC and their fluconazole sensitivity pattern in study population were also analyzed using Chi-square test.

3.2 SCREENING THE ISOLATES FOR THEIR ADHERENCE PROPERTY ON HUMAN BUCCAL EPITHELIAL CELLS (HBEC)

The adherence assay method described by Kimura and Pearsall was used (Kimura and Pearsall 1978). The ultra structural examination of adherence of candida on HBEC was done using SEM. The genetic similarity between the strains and their adhesion pattern was also evaluated.

3.2.1 ADHERENCE ASSAY CARRIED OUT BETWEEN *C. ALBICANS* ISOLATES FROM HIV SEROPOSITIVE AND HIV SERONEGATIVE INDIVIDUALS

3.2.1.1 PREPARATION OF CELLS FOR ADHERENCE ASSAY

3.2.1.1.1 COLLECTION AND PREPARATION OF POOLED HUMAN BUCCAL EPITHELIAL CELLS (HBECs) FROM NORMAL HEALTHY INDIVIDUALS

HBECs were obtained from five healthy volunteers after obtaining the informed written consent. The cells were collected by scrapping the inner side of the cheeks using sterile wooden sticks and released to the tube containing PBS (pH 7.2). The HBEC suspension was prepared in the PBS (pH 7.2) was washed as follows; the suspension was mixed with 5 ml of PBS, centrifuged for 10 min at 5000 rpm. The supernatant was discarded; sediment retained was resuspended in 5 ml of PBS, vortexed for 30 s. Two additional washings were done in PBS. The HBECs were then adjusted to a concentration of $1X10^5$cells/ml using the hemocytometer (Neubauer counting chamber).

3.2.1.1.2 PREPARATION OF *C. ALBICANS* CELLS

A total of 26 *C. albicans* isolates were screened for their adherence pattern. Among them 19 isolates were from HIV seropositive group and 6 isolates were from HIV seronegative group. A standard strain, *C. albicans*, MTCC 3017 was also used.

Candida isolates to be tested were sub cultured from the stock vials on SDA, incubated for 48 hrs at 37 ^{o}C to check purity and viability of the cells. The colonies were then inoculated into 50 ml of Yeast Nitrogen Broth (YNB) containing 500 mM galactose and incubated for 24 hrs at 37 ^{o}C and 150 rpm. The cells in the stationary phase were selected for the study. The candida cells were washed using PBS (pH 7.2). The final suspension was prepared in PBS (pH 7.2) and adjusted to a concentration approximately equal to $1x10^{7}$ cells/ml using a turbidometry.

3.2.1.2 ADHERENCE TEST

A 200 µl of the PBS buffer containing candida cells were mixed with 200 µl of the PBS containing HBECs, where ratio of approximately 5:1 of candida to HBECs was maintained. The mixture was incubated for 1 hr at 37 ^{o}C. After incubation, the mixture of cells passed through a membrane filter (Sartorius) of pore size 8 µm, to remove unattached candida cells. The retenant side of the filter disc were washed and collected in a sterile tube. The tubes were then centrifuged; a gram stained smear was made from the sediment and observed under 1000x. Minimum of 100 epithelial cells were screened for each smear. The number of epithelial cells showing candida cells adhered to them was noted and the percentage of adherence was calculated. The average number of candida cells adhered per HBEC was also calculated. The experiment was repeated four times and the average of the four readings was taken.

3.2.1.3 ADHERENCE ASSAY OF CANDIDA ISOLATES ONTO HBEC COLLECTED FROM HIV SEROPOSITIVE INDIVIDUALS

The entire experiment was carried out using HBEC collected from HIV seropositive individuals and HBEC collected from normal healthy individuals and results were compared. HBEC were collected from HIV seropositive patients without showing any candida colonization or infection. The method explained in 3.2.1 and 3.2.2 was followed for preparation of cells and carry out to adhesion assay. Four isolates numbered RL-112, RL-24 CN-102, CN-192were included in this experiment.

3.2.2 SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS OF CELLS

3.2.2.1 SAMPLE PREPARATION USING HIGH VAPOUR PRESSURE SOLVENTS

C. albicans isolates obtained from two different HIV seropositive individuals, numbered RL-112 and RL-24 were selected for SEM analysis.SEM analysis of plain candida cells, plain HBEC and HBEC adhered with candida was carried out. The samples were prepared by taking pellets of HBEC and candida cells as explained in previous sections (3.2.1.1.1 and 3.2.1.1.2). Water content present in the pellets was replaced by treating the cells with high vapor pressure solvents such as ethanol and acetone (Folk 1993). The cells were treated with gradually increasing concentrations of acetone and alcohol; this process accelerated the drying of the pellets. The procedure followed is explained briefly as follows. The PBS washed candida cells were first treated with 2.5% glutaraldehyde for 30 min at room temperature, followed by overnight incubation at 37 ^{o}C. To remove the water contents present in the cell deposits, pellet obtained was subsequently incubated at increasing concentrations of ethanol, i.e., 10%, 30%, 50%, 70%, 90% each for 10 min and centrifuged for 10 min after incubating with each concentration. Final incubation of the pellet was done for 1 hr in absolute alcohol (99.99% Ethyl Alcohol) and centrifuged for 10 min. After the alcohol washing, the washing procedure was continued with increasing concentrations of acetone, in exactly similar manner. The concentrations of acetone taken were 10%, 30%, 50%, 70%, 90% and 100%. The micro centrifuge tube containing washed cells obtained after final acetone washing was kept in the hot air oven at the 50 ^{o}C for overnight to achieve complete removal of water. The HBEC attached with candida was prepared by first subjecting both the cells for adhesion reaction as explained in section 3.2.1.1.3. The plain HBEC and HBEC adhered with candida were then prepared for SEM analysis in similar manner, as explained for preparation of candida cells.

3.2.2.2 SPUTTER COATING OF THE SAMPLE

Sputter coating was done using JEOL JFC-1600-Autofine coater. The procedure was done for less than 30 s to minimize the production of artificial topography (small blobs of coating material) on a sample as suggested elsewhere (Folk 1993; Folk 1994).

3.2.2.3 SEM IMAGING

Images were taken using JEOL JSM-6380LA, Analytical Scanning Electron Microscope at the magnification of 15,000x. EDX (Energy-Dispersive X-ray spectroscopy) was used for all the types of samples analyzed.

3.2.3 GENETIC ANALYSIS OF THE ORAL CANDIDA ISOLATES BY RANDOMLY AMPLIFIED POLYMERASE DNA (RAPD) AND COMPARISON OF THE GENETICALLY CONSTRUCTED GROUPS WITH THEIR ADHESION BEHAVIOR

3.2.3.1 DNA EXTRACTION

A total of 48 *C. albicans* isolates were used for the test, details are presented in Table 3.1. For DNA extraction, the candida cells were obtained from their stationary phase of growth in similar culture conditions as explained in section **3.2.1.1.2**.

Table 3.1 Details of *C. albicans* isolates tested for RAPD Analysis

Sl.No	*C. albicans* isolates from HIV seropositive group	*C. albicans* isolates from HIV seronegative group
1	RL-02	CL-01
2	RL-03	CN-7/1
3	RL-04	CL-09
4	RL-06	CN—28
5	RL-08	CN-31
6	RL-09	CN-65
7	RL-11	CN-67
8	RL-13	CN-69
9	RL-14	CN-102
10	RL-17	CN-111
11	RL-18	CN-136
12	RL-20	CN-147
13	RL-23	CN-149
14	RL-24	CN-162
15	RL-26	CN-163
16	RL-27	CN-172
17	RL-29	CN-173
18	RL-29G	CN-176
19	RL-39	CN-181
20	RL-40	CN-188
21	RL-41	CN-192
22	RL-47	CN-194
23	RL-51	
24	RL-53	

25	RL-61B	
26	RL-112	

The candida cells were washed thoroughly in sterile normal saline for complete removal of culture media and polluted down. DNA was extracted from these washed candida cells using the method explained by Sambrook and Russell (2001).

Candida cells were centrifuged at 10,000 g for 1min at room temperature. Cultures were suspended in a tube filled with 200 µl of extraction buffer and 0.30 to 0.35 µg of acid washed 0.4 mm glass beads. Extraction buffer known to be STET buffer, which contains, 10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM Ethylene Diamine Tetra Acetic acid (EDTA), 5% (v/v) Triton X-100. After putting all the ingredients, pH was measured and set to 8.0. A 60 µl of phenol: chloroform (1:1 ratio followed) was added to these tubes and capped. The tubes were vortexed for 1min to mix organic and aqueous phases. Centrifugation was done at 14000 g for 5 min at room temperature. Upper aqueous phase was transferred to a fresh micro centrifuge tube. Phenol: chloroform extraction was repeated in similar way until no white precipitate was visible at the interphase.

DNA was collected by standard precipitation with ethanol for 15 min at 0 ^{o}C. Precipitate of nucleic acids was removed by centrifuging at 14000 g for 10 min at 4 ^{o}C. Supernatant was aspirated and pellets rinsed with 100 µl of 70% ethanol in water. Tubes were again centrifuged at 14000 g for 1 min at room temperature and supernatant was removed by aspiration. Pellets were allowed to dry in air for 15 min, then redissolved in 40 µl of Tris-EDTA buffer (100 mM Tris HCL, 1 mM Na_2EDTA) (pH 7.6) .

Final DNA preparation measured in a spectroscopy at 260 and 280 nm and ratio obtained as 1.8:2.1 at each wavelength respectively was taken as acceptable value. The presence of nucleic acid bands was confirmed by agarose gel electrophoresis run using 1.5% agarose.

3.2.3.2 RAPD-DNA PCR ANALYSIS

RAPD analysis was performed according to the method explained by Del Castillo-Agudo et al. (1995). Briefly, every reaction mixture for RAPD analysis contained 10 µl of genomic DNA extracted. The amplification was performed in an automated thermo cycler (Applied biosystems) with a final reaction volume of 25 µl containing the OPA-18 (5'-GGACTGCAGA-3') and OPE-18 (5'-AGCTGACCGT-3') primers at a concentration of 0.4 mM. The dATP, dCTP, dGTP, and dTTP (Chromous Biotech), were added each at a concentration of 200 µM. Other contents added were, 2 mM $MgCl_2$, and 1.2 U of *Taq* DNA polymerase (Ecotaq) along with 2.5 µl of 10x PCR buffer provided by the manufacturer (Chromous Biotech). Amplification consisted of 1 cycle at 94 ^{o}C for 5 min, then 38 cycles as follows: 1 min of denaturation at 94 ^{o}C, 1 min of annealing at 36 ^{o}C, and 2 min of primer extension at 72 ^{o}C. At the final cycle, an additional 5 min of incubation at 72 ^{o}C was done followed by complete polymerization.

The resultant fragments of amplified DNA sample was subjected to agarose gel electrophoresis (Bio bee: electrophoresis unit) containing 1.5% of agarose (Chromous biotech) in Tris acetate EDTA (TAE) buffer at 20 V for 4 h. Gels stained with ethidium bromide and then visualized on an ultraviolet transilluminator (Bo-imaging systems). A 100-bp ladder (Chromous Biotech) was used as a size marker. Photograph of each gel was taken under UV light scanner and saved. DNA fragment patterns obtained for OPA-18 and OPE-18 were read and compared with the standard 100 DNA markers (Chromous Biotech). The base pair bands obtained for each isolate with a particular primer was noted and a dendrogram was constructed, using IBM-SPSS-version 20.

3.3 EVALUATION OF ADHESION OF CANDIDA AT VARIOUS EXPERIMENTAL CONDITIONS

3.3.1 ADHESION KINETIC STUDY AT DIFFERENT TIME INTERVALS

Two oral *C. albicans* isolates from HIV seropositive patients namely RL-112 and RL-24 were randomly selected and used for the study. The period of incubation required for the adhesion of candida on HBEC to happen was determined by conducting an adhesion kinetic study. The number of candida cells adhered to HBEC

under various time intervals i.e., from 10 to 200 min was calculated along with percentage of HBEC containing candida cells attached on them. The graph was plotted, where X axis indicates time of exposure in min and no. of. HBEC adhered with candida plotted in Y axis. No. of cells adhered to HBEC become constant after 60 min of exposure. Therefore, for further experiments time of exposure of HBEC to candida cells was fixed to 60 min only.

3.3.2 ANALYSIS OF ADHESION BEHAVIOR OF *C. ALBICANS* ALONG WITH ITS GROWTH PHASES

3.3.2.1 DETERMINATION OF GROWTH CURVE

C. albicans isolates obtained from two HIV seropositive individuals (RL-24 and RL-112) were used for the present research work. A 5ml was taken from the 18 hrs broth culture of *C. albicans* was adjusted to 0.5 McFarland standard using sterile saline and was inoculated aseptically into 250 ml of YNB containing 500 mM galactose/l in a conical flask. These flasks were incubated at 35 ^{o}C at 150 rpm in a shaking incubator (Rotek, India) and 1000 µl from each conical flask was taken aseptically at predetermined intervals of growth.

3.3.2.2 GROWTH CURVE EXPERIMENT

To determine the growth curve of candida, sampling was done at every 2 hrs interval and continued up to 100 hrs of incubation. For RL-24 sampling done at different intervals was numbered as A1, A2, and A3…. up to A29. Similarly for isolate RL-112, samples were numbered as B1, B2…up to B29. Growth curve was obtained by calculating the viable count (pour plate method) and total count (using Neubauer chamber) as per standard protocols given by Brown and Poxton (2006).

The total count of candida cells at each phase of growth was determined using Neubauer chamber under high power objective (x 450). Dilution of the broth was done, whenever number of cells found to be very high. Average of 3 counts was taken to have final count of cells. Viable count was measured using pour plate method in

SDA. The plates were then incubated at 37 ^{o}C for 48 hrs. Colony Forming Units (CFU) was counted carefully in each plate and CFU within the range of 30-300/petridish only were considered (Brown and Poxton 2006).

3.3.2.3 ADHESION STUDIES ALONG THE GROWTH PHASES OF *C. ALBICANS*

Candida cells from the broth were tested at specific intervals of growth for their adhesion behavior on to HBEC. HBEC was collected from healthy, HIV seronegative volunteers. Kimura and Pearsall (1978) method of adhesion assay was used in the present study. Both candida and HBEC were washed separately in PBS (pH 7.2) for 3 times. The adhesion assay was carried out as explained in section **3.2.1.1.3.** Smears were prepared and stained by gram staining and methylene blue staining method. The stained smears were observed under oil immersion field. Percentage of HBEC adhered with candida cells and average number of candida per HBEC was calculated. The graph of adhesion behavior at various time intervals of growth in both the strains was plotted.

3.3.3 ADHESION IN DIFFERENT SUSPENDING SOLUTIONS

3.3.3.1 ADHESION IN ARTIFICIAL SALIVA MEDIUM (ASM)

A total of 17 isolates randomly selected from HIV seropositive and HIV seronegative groups and used in this part of study, details are given in Table 3.2.

Table 3.2 *C. albicans* isolates tested for adhesion under ASM Vs PBS

	Isolates tested	
Sl. No.	**Oral *C. albicans* isolates from HIV seropositive group**	**Oral *C. albicans* isolates from HIV seronegative group**
1	RL-24	CN-31
2	RL-23	CN-67
3	RL-26	CN-102
4	RL-28	CN-111
5	RL-27	CN-173
6	RL-29	CN-176
7	RL-112	CN-181
8		CN-188
9		CN-192
10		CN-194

Candida and HBEC were suspended in the ASM (pH 6.8) instead of PBS (pH 7.2) for adhesion reaction to occur. Artificial saliva was preparedby mixing NaCl: 125.6 mg, KCl: 963.9 mg, KSCN: 189.2 mg, KH_2PO_4: 654.5 mg, Urea : 200 mg, $Na_2SO_410H_2O$: 763.2 mg, NH_4Cl : 178 mg, $CaCl_2.H_2O$: 887.8 mg, $NaHCO_3$: 630.8 mg in 1 litre of distilled water and pH was adjusted to 6.8 (Gal et al. 2001). To make artificial saliva as growth medium, glucose (20 g/l), yeast extract (2 g/l) and peptone (5g/l) were added to the medium, as shown by Henriques (2005a). All the other conditions maintained were similar to the previous adhesion experiments.

3.3.3.2 ADHESION IN PBS WITH VARIOUS IONIC STRENGTHS OF NaCl

C. albicans isolate, RL-112 and HBEC were suspended in the PBS with carrying concentrations of NaCl. PBS with varying ionic strengths of NaCl from 0.001 M till 0.934 M was prepared. The ionic strengths of NaCl used in the PBS were, 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.154, 0.318, 0.472, 0.626, 0.780 and 0.934 mM. The adhesion behavior of the candida cells suspended at different ionic concentration of NaCl containing PBS was tested.

3.4 MECHANISMS OF ADHESION

3.4.1 TESTING FOR PRESENCE OF LECTIN CARBOHYDRATE MECHANISM INVOLVED IN THE ADHESION PROCESS

During adhesion of candida onto HBEC, it has been reported in literatures that lectin- carbohydrate type of interaction occurs between adhesins and the receptor molecules (Chaffin 1998). To check the lectin activity exhibited by candida and role of lectin-carbohydrate interactions in adhesion of candida onto HBEC extensive studies were carried out.

3.4.1.1 LECTINACTIVITY TESTING IN *C. ALBICANS* AND GROWTH MEDIUM

Both the cells and secreted proteins of *C. albicans* isolates numbered RL-112 and RL-24 were checked for presence of lectin activity.

3.4.1.1.1 PREPARATION OF CANDIDA CELLS AND CULTURE SUPERNATANT FROM THE GROWTH MEDIUM FOR THE TEST

C. albicans isolates selected for the study were sub cultured on SDA at 37 ^{o}C for 48 hrs. From this growth, 2-3 colonies were selected and inoculated to flasks containing 25 ml of YNB with 500 mM galactose. The flasks were incubated at 37 ^{o}C

in the shaking incubator at 150 rpm. Around 5 ml of sample was taken i.e., once at 30 hrs, another at 64 hrs of incubation. Both the samples were centrifuged at 5,000 rpm for 10 min and supernatant was collected separately in sterile tubes. Candida cells were pelleted down, washed thrice using PBS (pH 7.2), and again suspended in PBS (pH 7.2). The PBS containing washed candida cells was adjusted to the O.D of 1.5 at 600 nm. A 250 µl of this suspension was taken in a micro centrifuge tube kept in an ice pack and subjected for sonication for 5 minutes with 30 sec on/off at 20 kHz, using sonicator (VCX 130; Sonic Vibra Cells). After sonication, 250 µl PBS (pH 7.2) was added to the same tube and the tube was centrifuged at 6,000 rpm for 10 min to pellet down the sonicated cells. The supernatant obtained here too was stored separately for lectin activity testing by hemagglutination assay.

Finally, washed intact candida cells, supernatant from direct candida culture broth, sonicated pellets from sonicated cells and supernatant obtained from the sonicated cell suspensions were the preparations tested for lectin activity using RBCs from O^+, A^+, B^+ blood groups. The samples were prepared both from 30 and 64 hrs of incubation.

Lectin activity testing was done by hemagglutination method and the procedure is explained is explained as follows.

3.4.1.1.2 LECTIN ACTIVITY TESTINGBY HEMAGGLUTINATION ASSAY

3.4.1.1.2.1 PREPARATION OF TRYPSINISED ERYTHROCYTES

Trypsinised erythrocytes were prepared for hemagglutination assay by the method described by Sharon and Lis (1972). Whole blood (2 ml) collected from required blood group person, put in an equal volume of Alsever's solution (2.05 g glucose, 0.8 g sodium citrate and 0.42 g NaCl dissolved in 100 ml distilled water and pH adjusted to 6.1 by the addition of citric acid) and was centrifuged at 1000 rpm for 5 min at room temperature.

The plasma was separated and erythrocytes were repeatedly washed with normal saline (0.90% w/v NaCl) by centrifugation. Washed RBCs were suspended in

PBS (pH 7.2) and the absorbance of the cell suspension was adjusted to 3.5 at 620 nm by diluting suitably with PBS.

Resulting cell suspension was incubated with 0.025% (w/v) trypsin at 37 ^{o}C. After incubating for one hr, erythrocytes were repeatedly washed by centrifugation in saline to remove traces of trypsin. Trypsinised erythrocytes were finally suspended in saline and diluted to O.D. 2.5 at 620 nm and used for hemagglutination assay.

3.4.1.1.2.2 HEMAGGULATINATION ASSAY

The samples were tested using hemagglutination assay by the serial two fold dilution techniques of Liener and Hill (1953). Hemagglutination was done in U bottomed microtitre assay plates. At first, 50 µl of saline was pipetted individually into all the wells of a row (12 wells). Followed by this; 50 µl of the desired sample was added to the first well of the assay plate. The contents in the first well (now containing a total of 100 µl solution) mixed thoroughly and 50 µl of it was transferred to the second well, thus resulting in doubling dilution of the test solution, the procedure was carried out similarly for the remaining wells. The last well in the row left without adding the sample and considered as control. As the next procedure to all these wells 50 µl of trypsinised erythrocytes suspension was added and gently mixed on a rotary shaker.

After incubation for one hr at 37 °C, the plates were visually examined for hemagglutination. Here, the hemagglutination indicates the presence of lectin activity in the test solution. The 'titer' is the maximum dilution showing hemagglutination activity. It is otherwise referred to as MCA (minimum concentration of protein required for hemagglutination), which was noted for each set of reaction.

3.4.1.2 ADHESION EXPERIMENTS WITH CARBOHYDRATES AND CON A

3.4.1.2.1 PREPARATION OF SUGAR SOLUTIONS

The carbohydrates and Con A used here were procured from Sigma-Aldrich. The sugar solution prepared for adhesion inhibition test were, D-mannose, D-galactose, Dextrose, Sucrose, D-fucose, D-N-acetyl galactosamine and D-N-acetyl

Glucosamine. All solutions were taken in the concentration of 200 mM. The *C. albicans* isolates tested were RL-112 and RL-24.

Table 3.3 Details on various carbohydrates used in adhesion inhibition studies

Sl. No	Carbohydrates used for the test	MW	Chemical formula
1	D- mannose	180.16	$C_6H_{12}O_6$
2	D- Galactose	180.16	$C_6H_{12}O_6$
3	Dextrose	180.16	$C_6H_{12}O_6$
4	Sucrose	342.30	$C_{12}H_{22}O_{11}$
5	D-Fucose	164.16	$C_6H_{12}O_5$
6	D- Gal NAc	221.21	$C_6H_{12}NO_6$
7	D- Gluc NAc	221.21	$C_8H_{15}NO_5$

3.4.1.2.2 ADHESION TEST

C. albicans isolates were mixed with 500 µl sugar solution and incubated at 37 ^oC and 150 rpm for 2 hrs with individual sugars. After the incubation, candida cells were thoroughly washed in PBS (pH 7.2), to remove unattached sugars. The cells then made to react with HBEC in the adhesion assay. Adhesion reactions were also carried out using plain untreated HBEC and plain untreated candida cells and these set of experiments were taken as control condition. The smears were prepared after the adhesion reaction to know the percentage of HBEC adhered with candida and average number of candida cells per HBEC, after exposure to particular sugar in each set of experiment. The results were compared to control condition and effect of the specific carbohydrate on adhesion was evaluated.

3.4.1.2.3 ADHESION EXPERIMENTS WITH CON A

Con A (Concanavalin A) is the lectin isolated from Canavalia ensiformis (Jack Bean) and has binding affinity to α-mannose and α- glucose moieties (Sharon and Lis 1972). Con A was used in the concentration of 1 µg/ml of was used to treat candida

cells. A total of 8 *C. albicans* isolates; i.e., 4 each from HIV seropositive individuals (RL-112, RL-24, RL-29 and RL-23) and HIV seronegative subjects (CN-69, CN-01, CN-12 and CN-63) were used in the study. Candida cells were incubated with Con A for 1 hr at 37 ^{o}C and 150 rpm. After treating with Con A, the candida cells were washed thrice in PBS. The cells were then subjected to adhesion reaction with HBEC as per standard method (Kimura and Pearsall, 1978). The results were compared to the control slides and inhibition effect seen in treated candida cells was calculated in percentages.

3.4.1.3 CELL WALL PROTEIN PROFILING AND LECTIN BLOTTING OF CANDIDA ISOLATES

For the detailed comparison of protein profiling and lectin blotting a total of 34 *C. albicans* were selected and studied, the details are given in Table 3.4.

Table 3.4 Details of isolates tested for SDS PAGE and lectin blotting

Sl. No	Isolates obtained from HIV seropositive individuals	Isolates obtained from HIV seronegative healthy individuals
1	RL-02	CL-01
2	RL-03	CN-07
3	RL-04	CN-28
4	RL-06	CN-31
5	RL-07	CN-62
6	RL-08	CN-65
7	RL-09	CN-67
8	RL-10	CN-69
9	RL-12	CN-102
10	RL-13	CN-111
11	RL-17	CN-173
12	RL-18	CN-176
13	RL-23	CN-181
14	RL-24	CN-188

15	RL-40	CN-192
16	RL-41	CN-194
17	RL-47	
18	RL-112	

3.4.1.3.1 PREPARATION OF CELL WALL PROTEIN EXTRACTS AND PROFILING

3.4.1.3.1A *C. ALBICANS* GROWTH CONDITIONS

C. albicans was grown in YNB containing 500 mM galactose (HiMedia) /litre for 30 hrs at 35 ^{o}C in a shaking incubator (Rotek, BOD Cooling Incubator Shaker ROSI-1) at 150 rpm, to limit the growth to stationary phase. The culture was centrifuged and candida cells were washed 3 times with DW in a cold centrifuge (Eppendorf India Centrifuge 5415 R) at 14,000 rpm at 4 ^{o}C. The washed cells were processed for cell wall protein extraction by two different methods followed by selection and modification of best Method.

3.4.1.3.1B EXPERIMENTS CARRIED TO ACHIEVE THE BEST YEILD OF PROTEINS

Two different cell wall protein extraction procedures were tested before finalizing the method to be used. Method 1 is reported by Pitarch et al. (2002) where cell wall protein extraction was done using detergents and Method 2 reported by Feiz et al. (2006), where extraction was done using $CaCl_2$ and LiCl salts. As the salts used in Method 2 (Feiz et al. 2006) had lead to poor quality of bands in SDS gels and because of better performance of method 1 (Pitarch et al. 2002), the method 1 was followed for further experiments. However, for finer results, mechanical disruption procedure carried out in method 1 was further subjected to certain modifications as explained below.

I. MECHANICAL DISTRUPTION OF CANDIDA CELLS BY GLASS BEAD HOMOGENIZATION

Glass bead homogenization is the mechanical disruption procedure followed in Pitarch et al. (2002), during the extraction of cell wall proteins. Using only two isolates of candida namely, RL-112 and CN-192 the procedures of protein extraction was studied in detail. For standardization of homogenization with glass beads, one ml of washed pellet was again suspended in lysis buffer and adjusted to required turbidity for candida. Ten ml screw capped sterile plastic tubes, were prefilled with beads up to roughly 10 mm height. To this 1 ml of the pellet adjusted to the required turbidity were added and tubes were cooled at -20 ºC for 30 min before being subjected to disruption.

Two types of beads were used, a 0.5 mm glass beads (HiMedia) and 3-5 mm diameter glass beads (HiMedia), separately. Proper head space was left for the beads and pellet to move freely during vortexing. The tubes were labeled as 0 min, 3 min, 6 min, 9 min, 12 min, 15 min and 18 min; accordingly bead beating was done for 3, 6, 9, 12, 15 and 18 min for respective tubes. The experiments were carried out separately for both 3-5 mm and 0.5 mm glass beads. The first tube labeled as 0 min, was not subjected to glass bead homogenization. Second tube onwards, the tubes were vortexed continually for 3 min then kept in the ice box to cool for 2 min before next vortexing. This was to minimize warming up of the suspension during homogenization, so as to avoid thermal disruption of proteins in the suspension. A 100 µl of extracted sample from each tube of bead homogenization experiment were diluted 1:10 times using sterile normal saline. A 10 µl quantity of the diluted sample was cultured on to SDA by semi quantitative streak culture method. The plates were incubated at 37 ^{o}C for 48 hrs. After incubation, number of colonies was carefully counted and candida colony count was estimated for 1 ml of direct sample. The results of both the techniques of bead beating were compared.

Representative samples were homogenized with glass beads for various time intervals and processed by Pitarch et al. (2002) method was analyzed for the best condition required. Evaluation was done by doing protein estimation by Folin Lowry method (Lowry et al. 1951) and checking the quality of extracted proteins by SDS-PAGE (10-17%), (MONOKIN, Techno Source, and Mumbai). Along with these tests, cell disruption occurred was observed by microscopy. For microscopic observation,

extracted samples from each set of experiments were smeared, stained by gram stain and observed under 100x objective. Disrupted yeasts appeared as dark "ghost" cells while intact yeasts were stained uniformly. The percentage of disruption of cells was calculated by counting ratio of disrupted cells to total no. of cells in 25 oil immersion fields. All the tests were conducted in duplicate. The final outcome obtained by these experiments indicated that, in the method described by Pitarch et al. (2002), the glass homogenization process can be done using 0.5 mm small beads for the duration of 18 min, which brings the best yield. Therefore, same was followed for further isolates also.

II. CELL WALL PROTEIN EXTRACTION (FINAL PROCEDURE FLLOWED)

Almost all the chemicals used for the SDS PAGE and lectin blotting were of analytical grade and were procured from Sigma-Aldrich. However a few chemicals were procured from other companies which have been mentioned in the text wherever they appear. Cells were washed 5 times initially with lysis buffer (10 mM Tris HCl, pH 7.4, 1 mM PMSF). Homogenization was done with 0.5 mm glass beads for 18 min and washed again with DW. The pellet, was washed in 5%, 2% and 1% NaCl solutions and then with cold DW 5 times each and boiled twice in extraction buffer (50 mM Tris HCl, pH 8.0, 0.1 M EDTA (Ethylenediamine Tetra Acetic acid), 2% SDS (Sodium dodecyl sulfate), 10 mM DTT(Dithiothreitol). The preparation was cooled to room temperature and washed with 0.1 mM sodium acetate buffer (pH 5.5). The resulting pellet was then suspended in extraction buffer and treated with chitinase (2 units in 0.2 ml) (Sigma, Aldrich) for overnight at 35 ^{o}C. The suspension was pelleted down by washing five times in cold DW followed by washing ten times in 0.1 mM sodium acetate buffer (pH 5.5). Finally, resulting proteins in the pellets were precipitated by Tri-chloro acetic acid (Tri-chloro acetic acid with final concentration not exceeding 6%) method.

III. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

The protein concentration of the extracts was estimated by Folin Lowry's methodusing Bovine Serum Albumin (BSA) as a standard (Lowry et al. 1951). Proteins were then separated on SDS PAGE, according to the method explained previously with a few modifications (Laemmli 1970). Samples were suspended and vortexed for 1 minute in a SDS sample buffer containing tris-HCl pH 6.8, glyceine, β-Mercaptoethanol and Bromophenol blue. Then it was kept in a water bath at 95 ^{o}C for 5 min and cooled to room temperature. Since, candida cell wall proteins are of broad range molecular weight, SDS gradient gels (5-20% and 10-17% separately) was used throughout the study instead of single percentage gels. During whole SDS PAGE run, constant voltage was maintained, as 120 V till the sample reached stacking gel thereafter 150 V was maintained for resolvation. After completion of electrophoresis, gel was stained with silver staining method and bands were compared with molecular weight marker run in the same gel. However during standardization Commassie Brilliant Blue (CBB) was also used for gel staining.

IV. DETERMINATION OF MOLECULAR WEIGHT OF THE PROTEIN BANDS

Since SDS PAGE here was done using gradient gel system, the calculation of molecular weight was done as shown in other references (https://www.nationaldiagnostics.com/electrophoresis/article/measuring-molecular-weight-sds-page).

A graph of log (MW) versus log (P) was jotted down, where MW is the molecular weight and P is the concentration of acrylamide at the protein band position. A graph of log (MW) vs. log (P) is first plotted for all the bands obtained in the standard protein marker lane, where MW of each component will be known. The graph obtained here will be linear in nature. By referring to this line, determination of MW's of other protein bands separated in test sample was calculated.

For further comparison protein profiles were analyzed using GEL Doc System. All the tests were repeated thrice to ensure the reproducibility.

3.4.1.4 LECTIN BLOTTING

The proteins separated on the polyacrylamide gel were transferred to PVDF membrane. The proteins were then made to react with panel of lectins and thus their glycan specificity was determined.

3.4.1.4A ELECTROTRANSFER OF PROTEINS FROM GEL TO THE PVDF MEMBRANE

Protein fractions separated in the gel during SDS PAGE were transferred to Polyvinylidene Difluoride (PVDF) membranes using an elecctroblotting instrument (TechnoPhage from Techno Source India) by wet electro transfer method. Gel and PVDF membrane were arranged according the instruction given in the manual. Preparation of buffers, and trouble shootings were done by referring to Walker (2009). The electrotransfer was carried out at 120 mA for 5 hrs, for efficient transfer of proteins. Prestained standard protein markers were used to ensure the transfer of proteins from gel to the membrane.

3.4.1.4B DETECTION OF GLYCOPROTEINS BY LECTIN BLOTTING METHOD

After Electrophoretic transfer, PVDF membrane was stained by Ponceau red staining method. The membrane was placed in a container having Ponceau red stain for 3-5 min with gentle agitation. The entire membrane gets stained red. The PVDF membrane was further washed for 2-3 times in sterile DW to destain the membrane. Only protein bands retain red color as shown in Fig. 3.1, and the back ground becomes clear. Once the transfer of proteins on the membrane was confirmed, as a prerequisite for lectin blotting, Ponceau red stain was removed completely from the membrane by rinsing in DW until the membrane becomes clear.

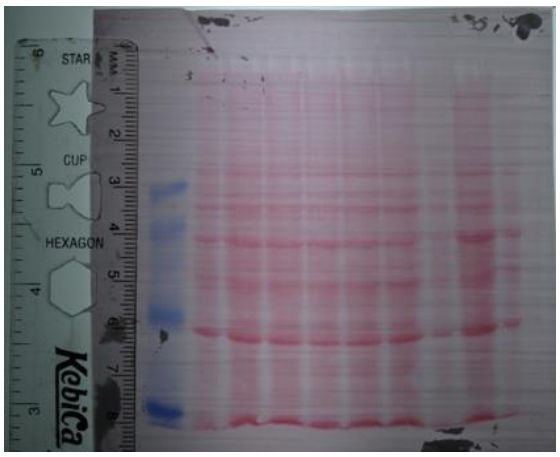


Fig. 3.1 Ponceau staining of PVDF membrane indicating protein bands transferred from gel. The first left corner lane represents prestained standard protein marker

During the entire procedure of lectin blotting, to avoid contamination of the membrane blunt ended forceps and latex free gloves were used. PVDF membrane with the transferred protein was washed thrice with DW. The membrane was then subjected for lectin blotting procedure according to the standard method (Walker 2009). The procedure followed is briefly explained as follows. The PVDF membranes were incubated with biotinylated lectin (1 µg/ml) in the blocking solution with continuous agitation for two hours at room temperature. Biotinylated lectin localizes the specific carbohydrate containing proteins and binds to that protein during this incubation period. The proteins bound with lectins were further detected by colorimetric reaction. The membranes were incubated with Extravidin- alkaline peroxidase (Sigma-Aldrich) (diluted to 1:2000) for one hour. This step was followed by incubation with 156µl BCIP (5-bromo-4-chloro-3'-indolyphosphate) (SRL Chemicals) stock solution and 312 µl NBT (Nitro-blue Tetrazolium) (SRL Chemicals) stock solution in 50 ml of alkaline phosphatase buffer for 10-20 min. The proteins bound with biotinylated lectins, was represented by purple hue, as shown in Fig. 3.2.

The panel of biotinylated lectins used in the study are i) Lectin C*oncanavalin A* (Con A) from *Canavalia ensiformis*; Jack-bean, ii) Lectin PNA (Peanut Agglutinin) obtained from *Arachis hypogaea* (Peanut), iii) Lectin from *Dolichos biflorus* (Horse gram) and iv) Lectin WGA (Wheat Germ Agglutinin) from *Triticum vulgaris* (Wheat). All the lectins were procured from Sigma-Aldrich.

Fig. 3.2 Lectin blotting of PVDF membrane

3.4.1.5 PRELIMINARY STUDY ON SECRETED PROTEINS OF *C. ALBICANS*

Culture supernatant of four randomly selected *C. albicans* isolates (RL-24, RL-112, CN-111, and CN-192) were extracted by growing the cells till stationary phase. Here also similar growth condition as explained for cell wall protein extraction (3.4.1.3.1A) was followed. A 50 ml of culture broth was centrifuged to remove candida cells. Supernatant was lyophilized for 10 hrs (Christ Alpha 1-2 LD *Plus* lyophiliser) (Fig. 3.4). The lyophilized protein was dissolved in 1-2 ml of Tris-HCl buffer (pH 6.8) and subjected to dialysis at 4 ^{o}C, in 10 kDa dialysis bag (HiMedia), against DW for 3 days. A total of 12 water changes carried out as first 4 changes were done in every 4th hr, thereafter for every 8 hrs water changes was carried out. Protein concentration of the dialyzed samples was measured by Folin–Lowry method (Lowry et al. 1951). Secreted Proteins, was then subjected to SDS PAGE analysis and lectin blotting, according to the procedure explained in the section 3.4.1.3.1.B, II part and section 3.4.1.4.

Fig. 3.3 Lyophilization of candida culture broth secreted proteins

3.4.1.6 STATISTICAL PROCEDURES FOLLOWED TO ANALYZE THE RESULTS OBTAINED FROM SDS PAGE ANALYSIS AND LECTIN BLOTTING

For the comparison between the protein profiling and lectin blotting pattern, statistical analysis was done. After assessment of molecular weight, the average number of bands in each sample was calculated. Various statistical tests were used to analyze the results. To compare cell wall protein profile pattern between the HIV seropositive and HIV seronegative groups unpaired‘t’ test was used. Two ways Anova with interaction effect was used to compare the significant differences in the main effects of groups and four different lectins, also for finding the significant difference between interaction effect among the groups and lectins. Least square difference (LSD) post hoc procedure was used for pair wise comparison among the interaction effects of groups and lectins. Karl Pearson’s correlation coefficient was used to assess the relationship among 4 different lectins interactions with proteins of different samples in HIV seronegative and HIV seropositive samples separately. Multiple logistic regression analysis was used to find out the significant influence of independent variables on dependent binary variables (groups). In all the above

statistical procedures a statistical significance was set at 5% level of significance ($p < 0.05$).

3.4.1.7 ADHESION ASSAY OF *C. ALBICANS* ON HBEC TREATED WITH CELL WALL PROTEIN EXTRACTS AND SECRETED PROTEINS OF *C. ALBICANS*

Effect of both cell wall proteins and secreted proteins on adhesion pattern of *C. albicans* was tested by performing adhesion experiment. HBEC were pretreated with cell wall proteins and culture supernatant separately.Adhesion results with treated HBEC, was compared with the adhesion reaction occurred with untreated cells. The isolates tested for adhesion experiments with cell wall proteins were CL-01 and CL-09. For experiments with supernatant proteins, isolates RL-112, RL-24, CN-192 and CN-112 were used.

The procedure followed for adhesion assay is explained as follows. The HBEC were pretreated with cell wall protein extracts and secreted proteins separately, then exposed to adhesion experiments. To expose HBEC to proteins, the protein solutions containing 250 µg of protein/ml, was used. Similarly, with the secreted proteins of candida, protein concentration of 250µg/ml was preferred. PBS containing 100-150 cells/10 µl HBEC was taken for both of the experiment. Before the actual experiments, a few trials have been done using combinations of different concentrations of HBEC and proteins along with varying the time of incubation.

After the pretreatment with proteins, HBECs were washed thrice using PBS, and subjected to adhesion reaction with untreated *C. albicans* cells, by Kimura Pearsall method (Kimura and Pearsall 1978). The results were analyzed by comparing to the adherence reaction shown by untreated HBEC and *C. albicans* cells.

In the final experiment, 500 µl PBS containing HBEC was treated with equal volume of protein solution. The HBEC were incubated with respective proteins for 2 complete hrs, in a shaking incubator at 37 ^{o}C and 150 rpm. Treated cells were then washed thrice in PBS (pH 7.2) at 5000 rpm. The turbidity of HBEC was again adjusted to 100-150 cells/10 µl, as done with plain HBEC. In case of testing for effect

of cell wall proteins on adhesion, the HBEC was treated with a particular cell wall protein from a particular isolate and adhesion was carried out with the same isolate. At another set, the HBEC were treated with proteins of one isolate and adhesion reaction was carried out with different isolate. This kind of experiment was done here mainly to know whether the action of these cell wall proteins is strain specific. The HBEC were treated with supernatant of the same isolate with which adhesion test was going to be performed.

3.4.1.8 DETECTION OF POSSIBLE PROTEIN BANDS INVOLVED IN ADHESION BY ELECTROPHORESIS AND LECTIN BLOTTING METHODS

Cell wall proteins of *C. albicans* (RL-112 and RL-24) were first incubated with epithelial cell membrane proteins individually and an attempt was made to find out the proteins involved in the adhesion process. Required proteins from both the cells were extracted using the method of Pitarch et al. (2002). The concentrations of proteins used of candida cell wall and HBEC cell membrane were 500 µg/ml and 250 µg/ml respectively. Both the protein solutions were mixed in a conical flask and incubated for 1 hr at 37 ^{o}C and 150 rpm. After the incubation period, the mixture was washed thrice in PBS buffer to remove unadhered proteins. Both the pellet and supernatant obtained by these washings were subjected to SDS PAGE (10-17%) along with candida cell wall proteins and cell membrane proteins of HBEC. Lectin blotting was carried out with the proteins separated here using 4 lectins, named i) Lectin C*oncanavalin A* (Con A) from Jack-bean, ii) Lectin PNA from *Arachis hypogaea* (Peanut), iii) Lectin from *Dolichos biflorus* (Horse gram), iv) Lectin WGA (Wheat Germ Agglutinin) from *Triticum vulgaris* (wheat). Plain candida cell wall protein, epithelial cell membrane proteins along with protein molecular weight marker were also run in the same SDS PAGE (10-17%). Lectin blotting trials also were carried out and comparisons of results were done.

3.4.2 PHYSICOCHEMICAL FACTORS OF *C. ALBICANS* AND THEIR INFLUENCE ON ADHESION

3.4.2.1 ANALYSIS OF MACROSCOPIC CHARECTEISTICS OF CELL SURFACE

3.4.2.1.1 MEASUREMENT OF CELL SURFACE HYDROPHOBICITY USING CONTACT ANGLE METHOD

Same 17 isolates mentioned in Table 3.2, were used for studies on CSH and its influence on adhesion.

3.4.2.1.1. 1 GROWTH OF *C. ALBICANS*

The *C. albicans* was grown on SDA for 48 hrs at 35 ^{o}C. Pure discrete colonies were selected and inoculated into YNB with 2% Dextrose and 500 mM D-galactose incubated for 30 hrs at stationary phase. Cells were pelleted down by centrifugation at 5000 rpm for 10 min. Pellet was washed twice with sterile PBS, pH 7.2 followed by washing twice in sterile DW.

3.4.2.1.1.2 CANDIDA LAWN PREPARATION

For the preparation of candida lawn; clean (75 x 25mm) glass slides precasted with 10% glycerol and air dried were made ready. On these slides around 1 ml of DW containing candida cells (10^9 cells/ml) were slowly dispensed using bunt ended tip, to form uniform layer (Henriques et al. 2002). The slides were then dried in the incubator at 45 ^{o}C for 2-3 hours. Care was taken not to over dry the sample. These slides were kept on projector slot for contact angle measurement. Contact angles were measured by sessile drop technique using an in-house apparatus in Physics department of Karnataka University, Dharwad.

Experiment was done at room temperature.
A drop of the desired liquid was carefully put on this lawn and pictures were taken immediately through the camera connected to the computer. The images were further evaluated and contact angle was measured using GIMP software. For contact angle

measurement water and formamide were used as polar components. Apolar component used in the study was α di-iodomethane. Contact angle was measured around six times with each liquid for all the samples and the average was taken. This was compared with adhesion assay results obtained with the same isolates.

3.4.2.1.1.3 CONTACT ANGLE MEASUREMENT IN ARTIFICIAL SALIVA

Artificial saliva medium (ASM) was prepared as per the details given in section 3.3.3.1 (Henriques et al. 2002).Candida cells were grown in ASM for 24 hrs at 35^{o}C and 150 rpm. Cells were pelleted down and suspended in artificial saliva again and lawn of cells was prepared as explained in section 3.4.2.1.1. 2., andContact angle measurement was also done. In this condition also the results obtained in contact angle measurements were compared with the adhesion assay results of the isolates.

3.4.2.1.1.4 CALCULATION OF SURFACE FREE ENERGY USING CONTACT ANGLE MEASUREMENT DATA

The available data on contact angle was used to determine surface free energy of the organism using the formula suggested by Minagi et al. (1985).

3.4.2.1.2 ELECTROPHORETIC MOBILITY (EPM) AND MEASUREMENT OF ZETA POTENTIAL CANDIDA

3.4.2.1.2.1 MEASUREMENT OF ELECTROPHORETIC MOBILITY (EPM) OVER THE RANGE OF KCL IONIC CONCENTRATIONS

EPM was measured as a function of ionic strength by micro electrophoresismethod, using the instrument Zeta Pals(Brookhaven Instruments Corporation). ThePBS containing KCl at a range of ionic strengths varying from 0 to 100 mM KCl at pH 7.4 and 22 °C were used. The isolates RL-112 and RL-24 were used in the study. Human erythrocytes which give constant EPM of 1.32 μm/s/v um were used as standard for setting the trials. Human RBC diluted to a final concentration of 10^{4}-10^{5} cells/ml in PBS (pH 7.475.6 mM) was used. Five replicates of the setting were studied and most accurate measurements were obtained. To allow the polarization of electrode at the studied ionic strength and to obtain stable

conductance readings, prior to each fresh sample, 2 or 3 conductance readings of buffer solution were taken.

3.4.2.1.2.2 PREPARATION OF CANDIDA CELLS FOR EPM

Around 10^9cells/ml candida cells were suspended in 10 mM KCl from which 5 µl of the inoculum was taken to 5 ml solution of desired ionic strength with attaining thousand times dilution of original inoculum. Sample preparation was freshly done, just before taking the reading. Average of 10 successive readings was taken for each sample by keeping the settings as 2.0 Hz frequency and voltage 2.5. Between each EPM measurements, the electrode set was, thoroughly rinsed; first with 75% ethanol followed by de-ionized (DI) water. EPM measurements of HBEC also were done similarly in these experiments. For every candida strain 4 different cultures of the same strain were used.

3.4.2.1.2.3 EPM MEASUREMENT DONE FOR THE CANDIDA ISOLATES SUSPENDED IN PBS (pH 7.2) AND ASM SEPERATELY

Both candida and HBEC were diluted in PBS (pH 7.2) and artificial saliva to an absorbance of approximately 0.1 (measured at 600 nm). The EPM of the specific suspension was measured in Zeta Analyzer, based on micro electrophoresis method. EPM values were then converted to zeta potentials using Smoluchowslki equation (Minagi et al., 1985).

3.4.2.1.2.4 ADHESION EXPERIMENTS

Adhesion experiment was conducted using various ionic concentrations of KCl solutions (0 to 100 mM) as suspension media. Candida adhesion pattern at different ionic strengths of KCl was compared with its Electrophoretic mobility and Zeta Potential values. Similarly, adhesion experiments were carried out with the candida isolates suspended in ASM Vs PBS (pH 7.2). ASM was prepared according to Gal et al. (2001), with the preparation methods as given in section 3.3.3.1. Adhesion pattern was compared with their Electrophoretic mobility and zeta potentials calculated.

3.4.2.2 MICROSCOPIC CHARACTERIZATION OF CANDIDAL CELL SURFACE BY ATOMIC FORCE MICROSCOPY (AFM)

3.4.2.2.1 GROWTH CONDITIONS MAINTAINED FOR *C. ALBICANS* STRAINS

C. albicans isolates RL-112 and RL-24 were first sub cultured on SDA plate and incubated for 48 hrs at 37 ^{o}C, till discrete colonies of 1 mm diameter appeared. Four to five colonies of *C. albicans* grown on SDA agar was inoculated to YNB with 500 mM galactose. The inoculated YNB was kept at 37 ^{o}C at 150 rpm for 30 hrs, till the cells attained mid stationary phase. The cells thus obtained were washed in PBS (pH 7.2) at 5000 rpm for 10 min. After centrifugation, washing with DI water was carried out twice.

3.4.2.2.2 SMEAR PREPARATION FOR AFM STUDIES BY GLASS COVERSLIP IMMOBILIZATION METHOD

3.4.2.2.2.1 GLASS COVER SLIP CLEANING

Micro cover glass slips (Alpha-Chem) were cleaned using the mixture of H_2SO_4 and H_2O_2 (4:1 ratio) for 25 min followed by rinsing in ultrapure water. The cover slips were then stored in the refrigerator in a sterile box containing ultrapure water until the use.

3.4.2.2.2.2 PREPARATION OF GLASS COVER SLIPS PRIOR TO THE ATTACHMENT OF *C. ALBICANS*

The cleaned cover slips were treated first with 100% ethanol for 5 min followed by treatment with 100% methanol for 5 min. The cover slips were allowed to stay in the aminosilane solution for 15 min. The aminosilane solution was prepared by adding 1 ml of 3-aminopropyl dimethoxysilane (Sigma) to 9 ml of methanol. Finally, the cover slips were rinsed with at least 50 ml of methanol followed by 25 ml of ultrapure water, and kept in methanol until the candidal smear preparation. Immediately before candida lawn preparation, cover slips were coated with 1 ml of poly-L-Lysine (0.1% w/v) and dried in a laminar flow hood for 10 min.

3.4.2.2.2.3 IMMOBILISATION OF CANDIDA ON GLASS COVER SLIP

Washed candida cells were subjected to further centrifugation in 0.1 M 2-(*N*-Morpholino) Ethane Sulfonic acid (MES) buffer (Sigma-Aldrich; pH 7.1) at 1,350 *g*, 15 min and vortexed for 1 minute. The suspension was adjusted to turbidity of $1x10^6$ using McFarland standard 0.5 (Brown and Poxton 2006). A 40 µl of this suspension was poured on cover slips prepared as explained earlier. The cover slips were then kept in the clean and sterile petri dish and placed on a shaker table for 20 min to allow attachment of candida and form thin and uniform smear. Similar, procedure was followed for attachment of HBEC on the cover slip.

3.4.2.2.4 AFM IMAGING

A Dimension 3100 AFM (Bruker) with Nanoscope IIIa controller (Veeco Metrology Group, Santa Barbara, CA and NanoWizard® 3-AFM from JPK instruments) was used for the experiments. Mikromasch NSC36-C and Mikromasch CSC38-B type cantilevers were used for imaging. Cantilevers were cleaned by exposure to UV light for 5 min to remove adsorbed water and/or hydrocarbons. The unit was configured for tapping mode as per the manufacturer's instructions.

3.4.2.2.5 SPRING CONSTANT MEASUREMENTS

Spring constant was determined before and after the AFM experiments by thermal methods (Dufrene et al. 2001). The values should be constant and close to the manufacturer's nominal value, 0.03 $Nm^{-1.}$ However, the deviation ranged from 10% to 65% was taken as permissible range.

3.4.2.2.6 AFM SCANNING MODE

Zoom in method is adopted to screen and find the area that contains a candida on the slide. Scan size had been reduced to1 µm x 1 µm and candida/HBEC was centered within the centre of the AFM scan size. AFM images were captured for each cell found and five force cycles per cell were recorded with drive amplitude set to zero (approximating contact mode). All force curves were recorded approximately at

the same point on the cell, defined as the highest point midway down the length of the cell.

3.5 CELL SURFACE FUNCTIONAL GROUP ANALYSIS USING FOURIER-TRANSFORM INFRARED (FTIR) SPECTROSCOPY TO UNDERSTAND THEIR ROLE IN ADHESION PROCESS

FTIR analysis elucidates the details of different functional groups present on cell surface and in turn gives biochemical finger printing of the cell surface (Naumann et al. 1991; Hong et al.1999). Therefore, in the present study, FTIR was used with various set of experiments to understand adhesion.

3.5.1 PREPARATION OF CANDIDA CELLS FOR FTIR SPECTROSCOPY, ALONG THEIR GROWTH PHASES

3.5.1.1 GROWTH OF CANDIDA

To perform the experiment, *C. albicans* were cultured on SDA, incubated for 48 hrs at 37 ^{o}C. Around 5-6 similar colonies selected and inoculated to 250 ml of YNB containing 500 mM galactose and 2% dextrose. Incubation was done at 35 ^{o}C in the shaking incubator at 150 rpm. This part of the study was performed with 20 isolates, which are mentioned in Table 3.5.

Table 3.5 Details of isolates tested for FTIR spectroscopy analysis

	Isolates tested	
Sl. No.	**Oral *C. albicans* isolates from HIV seropositive group**	**Oral *C. albicans* isolates from HIV seronegative group**
1	RL-24	CN-194
2	RL-23	CN-176
3	RL-26	CN-67
4	RL-28	CN-102
5	RL-27	CN-181
6	RL-29	CN-188
7	RL-09	CN-173
8	RL-18	CN-31
9	RL-40	CN-111
10	RL-112	CN-192

3.5.1.2 SAMPLING

The growth phases of *C. albicans* were previously studied in section 3.3.2.2 and exactly similar conditions were followed here to collect the cells at four different phases, namely lag, log, stationary and phase of decline. For analysis of the lag phase cells, sample was taken after 4 hrs of incubation. Since fewer amounts of cells will be present in the broth at this stage, 20 ml of broth was taken from growth flasks for FTIR analysis and adhesion assay. Sterile solutions and centrifuge tubes were used throughout the sample preparation. After sterilization, the tubes were rinsed with sterile DI water and made completely dry, so as to avoid contamination with other organisms as well as chemicals. Same procedure was followed for broth samples which were taken from log phase after 8 hrs, stationary phase cells at 30 hrs, phase of decline after 10 days of incubation, with the exception of volume of broth taken to pellet down the cells in all these phases was 10 ml.

3.5.1.3 PREPARATION OF SAMPLES FOR FTIR

The cells were washed at 5000 rpm for 10 min to remove the culture medium. Further washing was done twice using sterile PBS (pH 7.2), followed by two washes with sterile DI water. The pellets were suspended in 300 µl of DI water and homogenized in vortex mixer for 30 s to1min, then put into clean sterile glass petridish and kept at 50 ^{o}C for 6-7 hrs for complete evaporation of water. Once all the water contents dried up, the dried cells were scraped using a blunt end of a sterile scalpel. The fine powder obtained was stored in sterile pre weighed and labeled storage vials. The whole procedure was carried out in a Biosafety cabinet. To have uniform quantity of materials, the weight of the organisms containing scraping powder was maintained to be minimum 1.3 g to maximum 1.5 g. All the experiments were carried out in duplicates.

3.5.2 PREPARATION OF CANDIDA CELLS GROWN IN ARTIFICIAL SALIVA FOR FTIR ANALYSIS

This part of the study was done with *C. albicans*isolates, as shown in Table 3.1. Candida cells were taken from stationary phase of their growth and washed twice in normal saline and DI water to remove the culture medium. The cells were then suspended in artificial saliva and kept for incubation at 37 ^{o}C for 4 hrs in a shaking incubator at 150 rpm. The cells were then washed and prepared for FTIR analysis as explained above in section 3.5.1.3.

3.5.3 SAMPLE PREPARATION TO ANALYZE FUNCTIONAL GROUPS INVOLVED IN ADHESION PATTERN OF *C. ALBICANS* ONTO HBEC USING FTIR

In a separate experiment, washed candida cells (isolates numbered RL-112 and RL-28) grown up to 30 hrs in YNB broth were subjected to adhesion reaction with HBEC. Few modifications were done here, e.g., 2 ml each of candida and HBEC cells used in this experiment to meet the minimum quantity (1.3 g) required for the FTIR analysis. The HBEC and candida suspension were washed through 8 µm membrane filter (Sartorius) to remove unattached candida cells. The retenant side of

the filter was washed using PBS into a sterile container. The washings were centrifuged three times with DI water to remove PBS, under low speed (3000 rpm). The washed sediment was tested now with gram stain to confirm the presence of HBEC adhered with candida cells. The sediment was mixed with 1 ml of DI water. After vortexing for 1min it was poured in to a sterile petridish, and dried at 50 ^{o}C for 5-6 hrs, to evaporate the water. The sample was further prepared for FTIR analysis according to the procedures mentioned in section 3.5.1.3. Candida cells (plain) and HBEC (plain) were also prepared and subjected to FTIR analysis.

3.5.4 FTIRSTUDIES ON CELL WALL PROTEINS OF *C. ALBICANS* AND HBEC

The cell wall proteins of *C. albicans* isolate (RL-112) and epithelial cell membrane proteins were extracted separately and subjected to FTIR analysis. Extraction method of Pitarch et al. (2002) was followed for isolation of cell wall proteins. The proteins isolated were combined in an optimum proportion and kept for incubation in a shaking incubator at 37 ^{o}C for 2 hours. The solution was washed 2 times in PBS (pH 7.2) followed by washing with sterile DI water twice. The pellets obtained were dried and prepared for FTIR analysis.

3.5.5 FTIR SPECTROSCOPY AND MEASUREMENT

FTIR candida spectra were collected using Thermo Nicolet Avatar 330 FTIR spectrometer (Thermo Electron Corp, San Jose, CA). The sample compartment in the FTIR spectrometer was continuously purged with dry air to prevent water vapor. The dried powders of the organisms were positioned in direct contact with an infrared attenuated total reflection zinc selenide crystal. FTIR spectra were recorded from 400–4000/cm at resolution of 2/cm with scan rate of 64 s. Data obtained were interpret manually.

Thirty spectra were acquired for each sample at room temperature (each spectrum composed of an average of 128 separate scans). FTIR spectra were mean centered and the baseline was corrected. The system was operated by using the OMNIC 5.3 software and the experiments were replicated three times.

3.6 INHIBITION OF ADHESION OF *C. ALBICANS* USING VARIOUS AGENTS- A PRELIMINARY STUDY

This experiment was done by testing various plant extracts, surfactants and fluconazole for their effect on candidal adhesion with HBEC. Two oral *C. albicans* isolates obtained from HIV seropositive individuals, numbered RL-112 and RL-24 were used in the entire adhesion inhibition studies.

3.6.1 EXPOSURE OF CANDIDA AND HBEC TO PLANT EXTRACTS

3.6.1.1 PREPARATION OF THE PLANT EXTRACTS

Plant variety used, and material preferred is given in Table 3.6.A set of 5 g and 10 g each of plant material in 50 ml of extraction solute was used leading to a final concentration of 10% and 20% respectively.

Table 3.6 Details of plant materials used for antiadherence study

Sl. No.	Plant name	Scientific Name	Material used from the plant
1	**Neem**	*Azadirachta indica*	Leaf
2	**Aloe Vera**	*Aloe barbadensis*	Gel present inside the leaf
3	**Tea tree**	*Melaleuca alternifolia*	Tea Tree Oil (TTO)
4	**Lemon Grass**	*Cymbopogon citrates*	Grass
5	**Singapore Cherries**	*Muntingia calabura*	Ripened fruits

The required fresh plant material was washed in autoclaved tap water, dried under shadow and ground in sterile mortar and pestle to a fine paste. The paste was transferred to two sterile conical flasks containing 50 ml of DW and ethanol separately. The extraction was done overnight at room temperature in a shaking incubator at 150 rpm. After extraction, to remove all the visible plant artifacts, the solution was filtered through sterile muslin cloth into a sterile conical flask. Further, the solution was filter sterilized by passing through membrane filter of pore size 0.1 µm (Sartorius). The filtrate was lyophilized (Christ Alpha 1-2 LD *Plus* lyophiliser) and stored for future use. One gram of lyophilized powder was dissolved in 5 ml of sterile Phosphate Buffered Saline (PBS), pH 7.2, to attain a final concentration of 200 mg/ml. The same procedure was followed for all the plant varieties used in this study. Each extraction was carried out in two sets. Due to the unavailability of tea tree in the locality of the present study, tea tree oil (Falcon, Bangalore. India) was procured and used.

3.6.1.2 EXPOSURE OF CELLS TO THE PLANT EXTRACTS

A. TREATING CANDIDA CELLS WITH PLANT EXTRACTS

The experiments were carried out using two isolates of *C. albicans*, RL-24 and RL-112 which were isolated from OC lesions in HIV seropositive individuals and maintained in the laboratory. For performing the test, *C. albicans* cells were grown in YNB with 500 mM galactose up to stationary phase. The cells were then washed thrice in PBS (pH 7.2) and turbidity was adjusted to McFarland standard 0.5 (10^5 cells/ml). One ml of candida cells were incubated with 1ml of concentrated extracts for 2 hours in a shaking incubator at 37 ^oC at 150 rpm. The candida cells pre-exposed to plant extracts were washed thrice in PBS (pH 7.2) to remove excess extract. Candida cells were again adjusted to McFarland standard 0.5 using PBS (pH 7.2) before subjecting to adhesion.

In addition, to know the probable variation in morphology and viability of candida cells treated with plant extracts, gram staining and sub culturing on SDA plates were done.

B. TREATING OF HBEC WITH PLANT EXTRACTS

The HBECs were collected from healthy volunteers, by scraping inner side of the cheeks using sterile wooden sticks. Cells attached to the wooden sticks were released and suspended in PBS, pH 7.2. This cell suspension was washed thrice with PBS (pH 7.2). Concentrations of HBEC was adjusted to around 150-200 cells/10 µl of the suspension using Neubauer counting chamber. The aliquots of 500 µl each of HBEC were prepared and incubated with 500 µl of concentrated plant extracts for 2 hours at 37 ^oC at 150 rpm. The treated HBEC were then washed thrice in PBS (pH 7.2) and were re-adjusted to required turbidity.

3.6.1.3 ADHESION EXPERIMENT

Adhesion experiment was carried out in *in vitro* condition, according to the method followed by Kimura and Pearsall (1978). Four different adhesion conditions were tested as explained below.

CONDITION A

Candida cells treated with plant extracts were exposed to untreated (plain) HBEC.

CONDITION B

Untreated candida cells exposed to HBEC pretreated with plant extract.

CONDITION C

Both candida and HBEC were pretreated with the plant extracts and adhesion was carried out also in different plant extracts separately, instead of PBS (pH 7.2).

CONDITION D

Untreated candida cells were exposed to untreated HBEC. This condition served as control.

In all the different types of conditions, after pretreating, cells were washed in PBS (pH 7.2) and adjusted to required turbidity and the final ratio of 5:1 of Candida to HBEC was maintained in the reaction mixture. In brief, adherence test was carried out by mixing 200 µl of PBS containing the candida cells with 200 µl PBS containing of HBEC. However in condition C, the suspensions of candida and HBEC were prepared in specific plant extracts instead of PBS (pH 7.2). The reaction mixture was incubated at 37 ^{o}C for one hour in shaking incubator with 100 rpm. The mixture was then passed through a membrane filter of pore size 8 µm and the retenant was washed off into a sterile tube, using PBS (pH 7.2), and centrifuged. The sediment was subjected to gram stain. The Gram stained smear was observed under oil immersion objective (100x) and a minimum of 100 epithelial cells were screened. The number of epithelial cells showing yeast cells adhered on them was noted and percentage of adherence was calculated. The adhesion reaction was carried out for all the different sets of plant extract conditions thrice, and average of all the readings was calculated. The percentage of HBEC with adhered *C. albicans* cells in test slides were compared with the results of control slide and there by the percentage of variation either reduction or enhancement in adhesion was calculated.

3.6.1.3 STATISTICAL ANALYSIS

Statistical analysis was done using two way Anova, using IBM- SPSS-version 20. The significance of conditions followed and effects of both aqueous and ethanol plant extracts were evaluated separately for their effect on the adhesion.

3.6.2 ADHESION EXPERIMENT WITH SURFACTANTS

Candida cells were treated with certain surfactant solutions (Table 3.7) with final concentration of 0.75% for 2 hrs at 150 rpm and 37 ^{o}C. Candida cells were then washed thrice in PBS (pH 7.2) and allowed to react with plain HBEC to perform the adhesion assay. The adhesion assay was performed as explained in section **3.2.1.2.**

Table 3.7 Details of surfactants used in the experiment

Sl. No	Name of the surfactant	Type of surfactant	Abbreviation used	Final concentration used
1	Sodium Dodecyl Sulphate	Anionic	SDS	0.75 g in 100ml
2	Polysorbate 80	Nonionic	Tween 20	0.75 g in 100ml
3	Cetyl Pyridinium Chloride	Cationic	CPC	0.75 g in 100ml
4	Ethylenediamine tetra acetic acid	Chelating agent	EDTA	0.75 g in 100ml

3.6.3 ADHESION EXPERIMENT DONE WITH SUB-INHIBITORY CONCENTRATION OF FLUCONAZOLE

Fluconazole solution was prepared in sterile DW to the final concentration of 0.125 mg/ml. Candida cells were first treated with fluconazole suspension for 1 hr at 150 rpm and 37 ^{o}C, then washed thrice in PBS (pH 7.2). Adhesion assay was further carried out as explained in section **3.2.1.2.**

CHAPTER 4. RESULTS AND DISCUSSIONS

The Present chapter presents the details of the results obtained from the experiments conducted with respect to isolation and evaluation of oral candida carriage along with extensive study on adhesion mechanism of *C. albicans* on HBEC, as per methodologies and procedures explained in Chapter 3, to meet the stated objectives presented in Chapter 1. The results obtained are presented in the form of tables and figures and discussed with relevant literatures. The results obtained are the average of minimum three readings in all the cases.

4.1 EVALUATION OF ORAL CANDIDA CARRIAGE, ISOLATION OF *C. ALBICANS* AND FLUCONAZOLE SUSCEPTIBILITY PATTERN

In the present study, a total of 534 individuals (274 HIV seropositive individuals and 260 HIV seronegative healthy subjects) were evaluated for oral candida carriage, density of candida population and species diversity. This part of the study is aimed at evaluating oral candida carriage in both HIV seropositive individuals, who are having the Oral Candidiasis (OC) and the one without any OC lesions. These results were compared with true commensalism which was shown in healthy HIV seronegative healthy individuals. It is shown from the various publications that, carriage rate for candida will be increasing in the presence of HIV infection, though the person is not exhibiting any OC lesions (Felix 1994; Korting et al.1988). Evaluating the status of candida commensalism in HIV seropositive patients who are not having OC and comparing this pattern with healthy individuals, therefore can prove the role of oral candida flora itself leading to infection in these patients. The prevalence of OC, oral candida carriage, species distribution in the subjects of entire study is explained below with comparison on published reports.

4.1.1.1 OCCURRENCE OF OC IN HIV SEROPOSITIVE INDIVIDUALS

While collecting the samples, proper inclusion and exclusion criteria were followed as explained in chapter 3. In addition to the age, sex, type of OC lesions present, education, oral hygiene and other relevant information of every individual

were recorded and these details are shown in Table 4.1 (HIV seropositive individuals) and Table 4.2 (HIV seronegative individuals).

Table 4.1 Master chart representing details of HIV seropositive individuals

Sl.No	DATE	Lab No/PID	SEX	AGE (yrs)	Symptoms	O.C	Organism	CFU/ml	Abs
1	7/1/08	RL-1	M	40	Generalized weakness	No	**NO CANDIDA**	0	HIV-1
2	7/1/08	RL-2	M	58	Cough	No	*C. albicans*	>100000	HIV-1
3	7/1/08	RL-3	F	7	Omitting and diarrhea	No	*C. albicans*	800	HIV-1
4	7/1/08	RL-4	M	70	Inguinal hernia	Ps.M	*C. albicans*	>100000	HIV-1
5	7/1/08	RL-5	F	45	Loose motion omitting	No	*C.albicans*	650	HIV-1
6	11/1/08	RL-6	M	44	Nil	Ps.M	*C. albicans*	>100000	HIV-1
7	12/1/08	RL-7	M	34	Nil	No	***NO CANDIDA***	345	HIV-1
8	14/1/08	RL-8	M	28	Cryptococcal meningitis	Ps.M	*C.albicans*	>100000	HIV-1
9	14/1/08	RL-9	F	40	Nil	No	*C. albicans*	7000	HIV-1
10	14/1/08	RL-10	M	35	Milliary TB	Ps.M	*C.albicans*	>100000	HIV-1
11	14/1/08	RL-11	F	30	Fever 4 months	No	***NO CANDIDA***	0	HIV-1
12	17/1/08	RL-12	M	68	Cardiac problem	No	***NO CANDIDA***	0	HIV-1
13	18/1/08	RL-13	F	39	Herpes Zoster	No	*C.albicans*	5000	HIV-1
14	19/1/08	RL-14	M	57	Rt LL Consolidation	Ps.M	*C.albicans*	>100000	HIV-1
15	19/1/08	RL-15	F	15	Nil	No	***NO CANDIDA***	0	HIV-1
16	19/1/08	RL-16	M	44	Severe anemia IDS with fever	No	***NO CANDIDA***	0	HIV-1
17	19/1/08	RL-17	M	36	fever, cough, vomiting	Ps.M	*C. albicans*	>100000	HIV-1
18	23/1/08	RL-18	M	49	Fever	No	*C. albicans*	80	HIV-1
19	23/1/08	RL-19	M	24	good health	No	***NO CANDIDA***	0	HIV-1
20	23/1/08	RL-20	M	36	TB	No	*C. albicans*	560	HIV-1
21	28/1/08	RL-21	M	52	Fever, TB	No	***NO CANDIDA***	0	HIV-1
22	28/1/08	RL-22	F	35	Nil	No	***NO CANDIDA***	0	HIV-1
23	28/1/08	RL-23	M	49	Fever, Meningoencephalitis	Ps.M	*C.albicans*	1000	HIV-1
24	28/1/08	RL-24	M	43	Fever and chills	No	*C.albicans*	526	HIV-1
25	13//2/08	RL-25	M	28	Fever	No	*C.guilliermondii*	206	HIV-1
26	13/2/08	RL-26	M	64	fever, omitting	No	*C.albicans*	64	HIV-1
27	15/2/08	RL-27	M	28	Fever, chills, stomach pain	No	*C.albicans*	1532	HIV-1

28	15/2/08	RL-28	M	32	Liver cirrhosis	No	*C.albicans*	700	HIV-1
29	17/2/08	RL-29	F	35	fever, cough	No	*C.albicans*	204	HIV-1
30	17/2/08	RL-30	M	38	Gastric pain	No	*C.albicans*	6212	HIV-1
31	19/2/08	RL-31	M	45	Fever	Ps.M	*C.albicans*	88	HIV-1
32	19/2/08	RL-32	F	24	Mental disorder	No	*C.albicans*	806	HIV-1
33	19/2/08	RL-33	M	47	Pneumonia	No	*C.albicans*	5812	HIV-1
34	21/2/08	RL-34	M	52	Fever	No	***NO CANDIDA***	0	HIV-1
35	21/2/08	RL-35	F	32	Fever with cold	No	***NO CANDIDA***	0	HIV-1
36	21/2/08	RL-36	F	53	Fever	No	***NO CANDIDA***	0	HIV-1
37	25/2/08	RL-37	F	20	Fever	No	*C. guillermondii*	0	HIV-1
38	25/2/08	RL-38	F	38	Fever	No	***NO CANDIDA***	0	HIV-1
39	28/2/08	RL-39	F	34	TB	No	*C. albicans*	1292	HIV-1
40	29/2/08	RL-40	M	45	Fever, Head ache	No	*C.albicans*	5424	HIV-1
41	9/3/2008	RL-41	M	44	Fever, back pain	No	*C.albicans*	8	HIV-1
42	9/4/2008	RL-42	M	65	Fever, cough, head ache	No	*C.albicans*	532	HIV-1
43	9/5/2008	RL-43	M	48	fever,UTI	No	*C. guilliermondii+C. tropicalis*	673	HIV-1
44	9/6/2008	RL-44	M	50	Stomach pain	No	*C.albicans*	2278	HIV-1
45	9/6/2008	RL-45	M	42	Fever, cough, head ache	No	***NO CANDIDA***	0	HIV-1
46	14/6/08	RL-46	F	69	Leg pain	No	*C.lusitanae*	14	HIV-1
47	14/6/08	RL-47	M	42	Fever, PTB	Ps.M	*C.albIcans+C.krusei*	3216+50	HIV-1
48	21/6/08	RL-48	M	40	Gynac problem	No	***NO CANDIDA***	0	HIV-1
49	23/6/08	RL-49	M	28	Fever	No	***NO CANDIDA***	0	HIV-1
50	23/6/08	RL-50	M	29	Weakness	Ps.M	*C. albicans*	130	HIV-1
51	23/6/08	RL-51	M	55	Fever	no	*C.albicans*	52	HIV-1
52	23/6/08	RL-52	M	37	Dehydration	No	*C.albicans*	2	HIV-1
53	23/6/08	RL-53	M	35	Fever	No	*C. albicans*	200	HIV-1
54	25/6/08	RL-54	M	24	Chicken pox	Ps.M	*C. lusitanae*	6	HIV-1
55	25/6/08	RL-55	M	40	Left uterine calculi	No	*C. guillermondii*	220	HIV-1
56	25/6/08	RL-56	M	51	Fever	No	*C.tropicalis*	4	HIV-1
57	25/6/08	RL-57	M	38	Diarrhea, fever	Ang.che	*C. krusei*	364	HIV-1

58	25/6/08	RL-58	F	26	good health	No	***NO CANDIDA***	0	HIV-1
59	25/6/08	RL-59	F	25	Fever	No	*C.albicans*	22	HIV-1
60	25/6/08	RL-60	F	35	good health	No	*C.albicans*	>100000	HIV-1
61	25/6/08	RL-61	M	44	Fever and Hepatitis B	Ps.M	*C.albicans*	45	HIV-1
62	25/6/08	RL-62	F	45	Fever	no	*C. tropicalis*	>100000	HIV-1
63	25/6/08	RL-63	M	41	Fever, cervical nodes	No	*C. glbrata+ C.krusei*	298+13	HIV-1
64	26/6/08	RL-64	M	35	Cough, fever	No	*C.albicans*	>100000	HIV-1
65	26/6/08	RL-65	M	56	Fever, giddiness, gen weakness	No	*C. guillermondii*	630	HIV-1
66	27/6/08	RL-66	M	44	Loss of appetite	No	***NO CANDIDA***	0	HIV-1
67	27/6/08	RL-67	F	39	good health	No	***NO CANDIDA***	0	HIV-1
68	27/6/08	RL-68	M	40	exanthemic viral infection	Ps.M	*C.albicans*	>100000	HIV-1
69	27/6/08	RL-69	F	46	Nil	Ang.che	*C.albicans*	2176	HIV-1
70	27/6/08	RL-70	F	33	good health	No	*No candida*	0	HIV-1
71	4/7/08	RL-71	F	35	Fever	No	***NO CANDIDA***	0	HIV-1
72	4/7/08	RL-72	F	32	Head ache	No	***NO CANDIDA***	0	HIV-1
73	8/1/09	RL-73	F	28	Nil	No	*C. tropicalis*	2	HIV-1
74	8/1/09	RL-74	M	55	Loss of appetite	Ps.M	*C. albicans*	62	HIV-1
75	8/1/09	RL-75	F	13	Fever	No	***NO CANDIDA***	0	HIV-1
76	8/1/09	RL-76	M	28	TB	No	*C. albicans*	142	HIV-1
77	10/1/09	RL-77	M	42	Fever	No	*No candida*	0	HIV-1
78	17/1/09	RL-78	M	42	good health	No	*C.albicans*	184	HIV-1
79	17/1/09	RL-79	M	43	Chest pain	No	*C.albicans*	122	HIV-1
80	20/1/09	RL-80	F	27	Nil	No	*C.albicans*	320	HIV-1
81	22/1/09	RL-81	F	81	Etching	No	***NO CANDIDA***	0	HIV-1
82	27/1/09	RL-82	F	32	Fever	No	***NO CANDIDA***	0	HIV-1
83	27/1/09	RL-83	M	13	Head achae,Fever	No	*C. albicans*	18	HIV-1
84	25/1/09	RL-84	M	40	Fever	No	*C. albicans*	1252	HIV-1
85	1/2/09	RL-85	M	43	Fever	No	***NO CANDIDA***	0	HIV-1
86	3/2/09	RL-86	M	64	good health	No	*C. albicans*	20	HIV-1
87	3/2/09	RL-87	M	48	Piles	No	*C. albicans*	2	HIV-1

88	7/2/09	RL-88	F	36	Head ache	No	***NO CANDIDA***	0	HIV-1
89	13/2/09	RL-89	F	60	Head achae,systemic illness	Ps.M	*C. albicans*	>100000	HIV-1
90	14/2/09	RL-90	F	30	Anaemia,weakness,fever	Ps.M	*C. albicans*	200	HIV-1
91	14/2/09	RL-91	F	40	Weakness	No	*C. albicans*	4	HIV-1
92	14/2/09	RL-92	F	37	good health	No	*C. albicans*	2	HIV-1
93	20/2/09	RL-93	F	30	Anemic, weakness	No	*C. tropicalis*	1984	HIV-1
94	21/2/09	RL-94	F	30	good health	No	*C. parapsilosis*	2	HIV-1
95	21/2/09	RL-95	F	32	good health	No	*C. albicans*	80	HIV-1
96	21/2/09	RL-96	M	45	good health	No	*C. albicans*	44	HIV-1
97	21/2/09	RL-97	M	28	good health	No	*C. albicans*	22	HIV-1
98	21/2/09	RL-98	M	37	Fever Head ache	No	*C.albicans*	2	HIV-1
99	21/2/09	RL-99	F	38	Stomach pain, TB	no	*C.albicans*	>100000	HIV-1
100	26/2/09	RL-100	F	33	Skin rashes	No	***NO CANDIDA***	0	HIV-1
101	26/2/09	RL-101	M	45	Fever	No	*C.albicans. guillermondii*	90+60	HIV-1
102	26/2/09	RL-102	F	25	Weakness	No	*C.albicans*	52	HIV-1
103	28/2/09	RL-103	F	40	RTI	No	***NO CANDIDA***	0	HIV-1
104	28/2/09	RL-104	M	41	Epilepsy, skin rashes	No	*C. guilliermondii*	2	HIV-1
105	28/2/09	RL-105	M	43	PTB	No	*C.albicans*	220	HIV-1
106	28/2/09	RL-106	F	45	Blood Pressure	No	*C. parapsilosis*	20	HIV-1
107	28/2/09	RL-107	M	35	good health	No	*C.albicans*	60	HIV-1
108	2/3/09	RL-108	F	32	good health	No	*C.albicans +C.krusei*	180+50	HIV-1
109	2/3/09	RL-109	F	9	good health	No	*C. albicans*	44	HIV-1
110	2/3/09	RL-110	F	32	PTB	No	*C.albicans*	16	HIV-1
111	2/3/09	RL-111	F	32	Fever loss of appetite	No	***NO CANDIDA***	0	HIV-1
112	2/3/09	RL-112	F	24	No	No	*C. krusei+C. albicans*	>100000	HIV-1
113	2/3/09	RL-113	F	42	Yes	No	*C. krusei+C. albicans*	80	HIV-1
114	2/3/09	RL-114	M	26	No	No	***NO CANDIDA***	**0**	HIV-1
115	1/5/09	RL-115	F	30	Yes	No	*C. albicans*	96	HIV-1
116	1/6/09	RL-116	M	55	Yes	No	*C. albicans*	80	HIV-1
117	8/7/09	RL-117	M	25	Yes	Ps.M	*C. albicans*	81	HIV-1
118	8/7/09	RL-118	F	11	Yes	ER	*C. guilliermondii*	2	HIV-1

119	9/7/09	RL-119	F	32	No	No	*C. albicans*	77	HIV-1
120	9/7/09	RL-120	M	35	Yes	Ps.M	*C. guilliermondii*	>100000	HIV-1
121	11/7/09	RL-121	F	28	No	No	***NO CANDIDA***	**0**	HIV-1
122	11/7/09	RL-122	M	26	Yes	Ps.M	*C. kefyr*	>100000	HIV-1
123	13/7/09	RL-123	M	25	No	No	***NO CANDIDA***	**0**	HIV-1
124	13/7/09	RL-124	F	48	Yes	Ps.M	*C. albicans*	1390	HIV-1
125	13/7/09	RL-125	F	21	Yes	ER	*C. albicans*	18	HIV-1
126	14/7/09	RL-126	F	35	Yes	ER	*C. albicans*	>100000	HIV-1
127	14/7/09	RL-127	M	42	Yes	No	*C. guilliermondii*	1000	HIV-1
128	14/7/09	RL-128	F	30	No	No	*C. albicans*	4	HIV-1
129	14/7/09	RL-129	F	30	Yes	Ps.M	*C. albicans*	44	HIV-1
130	15/3/10	RL-130	M	64	Yes	ER	*C. albicans*	>100000	HIV-1
131	15/3/10	RL-131	F	30	No	No	*C. guilliermondii*	4	HIV-1
132	15/3/10	RL-132	M	38	Yes	ER	*C. albicans*	>100000	HIV-1
133	21/3/10	RL-133	M	35	Yes	ER	*C. albicans*	>100000	HIV-1
134	24/3/10	RL-134	M	41	Yes	No	*C. guilliermondii*	2	HIV-1
135	25/3/10	RL-135	M	7	Yes	Ps.M	*C. albicans*	2000	HIV-1
136	25/3/10	RL-136	F	30	Yes	ER	*C. albicans*	1000	HIV-1
137	24/3/10	RL-137	M	32	Yes	ER	*C. albicans*	1000	HIV-1
138	24/3/10	RL-138	M	32	Yes	No	*C. lusitaniae*	40	HIV-1
139	24/3/10	RL-139	F	20	Yes	Ps.M	*C. guilliermondii*	81	HIV-1
140	24/3/10	RL-140	F	22	Yes	Ps.M	*C. albicans*	1000	HIV-1
141	1/7/2010	RL-141	M	45	Yes	No	*C. albicans*	83	HIV-1
142	1/7/2010	RL-142	F	26	Yes	ER	*C. guilliermondii*	>100000	HIV-1
143	3/7/2010	RL-143	F	38	Yes	Ps.M	*C. albicans*	>100000	HIV-1
144	3/7/2010	RL-144	M	45	Yes	No	*C. albicans+C. tropicalis*	>100000	HIV-I
145	3/7/2010	RL-145	M	40	Yes	ER	*C. albicans*	>100000	HIV-1
146	3/7/2010	RL-146	M	30	Yes	ER	*C. albicans*	>100000	HIV-1
147	3/7/2010	RL-147	F	38	No	No	*C. guilliermondii*	60	HIV-1
148	3/7/2010	RL-148	M	52	Yes	ER	*C. tropicalis*	>100000	HIV-1
149	3/7/2010	RL-149	M	32	Yes	Ps.M	*C. albicans*	>100000	HIV-1

150	5/7/2010	RL-150	M	46	Yes	Ps.M	*C. albicans*	>100000	HIV-1
151	19/7/10	RL-151	M	45	Yes	ER	*C. albicans*	>100000	HIV-1
152	19/7/10	RL-152	M	65	Yes	Ps.M	*C. albicans+C. tropicalis*	>100000	HIV-1
153	19/7/10	RL-153	F	22	No	No	***NO CANDIDA***	**0**	HIV-1
154	20/7/10	RL-154	M	36	Yes	Ps.M	*C. albicans*	100	HIV-1
155	20/7/10	RL-155	M	58	Yes	ER	***NO CANDIDA***	**0**	HIV-1
156	21/7/10	RL-156	F	45	Yes	ER	*C. guilliermondii*	62	HIV-1
157	21/7/10	RL-157	F	40	Yes	ER	*C. albicans*	2000	**HIV-2**
158	21/7/10	RL-158	M	35	Yes	Ps.M	*C. albicans*	1000	HIV-1
159	29/7/10	RL-159	M	31	Yes	Ps.M	*C. albicans,C. dubliniensis*	2000	HIV-1
160	29/7/10	RL-160	M	55	Yes	Ps.M	*C. albicans*	356	HIV-1
161	29/7/10	RL-161	M	37	No	No	*C. albicans*	100	HIV-1
162	29/7/10	RL-162	M	37	Yes	No	*C. krusei*	100	HIV-1
163	29/7/10	RL-163	F	22	No	No	***NO CANDIDA***	**0**	HIV-1
164	20/12/10	RL-164	F	30	Yes	ER	*C. albicans*	>100000	HIV-1
165	20/12/10	RL-165	M	32	Yes	Ps.M	*C. kefyr*	>100000	HIV-1
166	21/12/10	RL-166	F	37	Yes	Ps.M	*C. albicans*	>100000	HIV-1
167	22/12/10	RL-167	F	25	Yes	Ang.che	*C. albicans*	>100000	HIV-1
168	24/12/10	RL-168	M	38	Yes	No	***NO CANDIDA***	**0**	HIV-1
169	24/12/10	RL-169	M	38	Yes	No	*C. albicans*	4	HIV-1
170	24/12/10	RL-170	F	40	Yes	ER	*C. guilliermondii+C. tropicalis*	2000	HIV-1
171	24/12/10	RL-171	F	25	Yes	No	*C. albicans*	20	HIV-1
172	28/12/11	RL-172	M	45	Yes	ER	*C. lusitaniae+C. krusei*	>100000	HIV-1
173	28/12/10	RL-173	M	44	Yes	ER	*C. albicans*	>100000	HIV-1
174	28/12/10+B213	RL-174	F	26	Yes	No	*C. albicans*	20	HIV-1
175	29/12/10	RL-175	M	26	Yes	ER	*C. albicans*	23	HIV-1
176	29/12/10	RL-176	F	26	Yes	No	***NO CANDIDA***	**0**	HIV-1
177	29/12/11	RL-177	M	42	Yes	No	***NO CANDIDA***	**0**	HIV-1
178	31/12/11	RL-178	F	33	No	No	*C. albicans*	30	HIV-1
179	4/1/2011	RL-179	F	29	Yes	Ps.M	*C. albicans*	>100000	HIV-1

180	6/1/2011	RL-180	M	28	Yes	Ps.M	*C. albicans*	>100000	HIV-1
181	7/1/2011	RL-181	F	33	No	No	*C. parapsilosis*	4	HIV-1
182	15/1/11	RL-182	F	24	Yes	No	*C. lusitaniae+C. krusei*	65	HIV-1
183	17/1/11	RL-183	F	30	Yes	No	*C. albicans*	118	HIV-1
184	23/5/11	RL-184	M	48	No	No	*C. tropicalis*	43	HIV-1
185	28/5/11	RL-185	M	50	No	No	*C. albicans*	2	HIV-1
186	17/6/11	RL-186	F	26	Yes	Ps.M	*C. albicans*	1140	HIV-1
187	18/6/11	RL-187	F	43	Yes	ER	*C. albicans*	>100000	HIV-1
188	21/6/11	RL-188	M	28	Yes	ER	*C. dubliniensis*	>100000	HIV-1
189	21/6/11	RL-189	M	28	No	No	*C. albicans*	8	HIV-1
190	23/6/11	RL-190	F	34	No	No	*C. tropicalis*	>100000	HIV-1
191	23/6/11	RL-191	F	30	No	No	*C. krusei*	80	HIV-1
192	23/6/11	RL-192	M	35	Yes	No	*C. albicans+C. tropicalis*	50	HIV-1
193	24/6/11	RL-193	M	58	Yes	No	***NO CANDIDA***	**0**	HIV-1
194	24/6/11	RL-194	M	37	Yes	ER	*C. albicans*	>100000	HIV-1
195	28/6/11	RL-195	F	58	No	No	***NO CANDIDA***	**0**	HIV-1
196	30/6/11	RL-196	M	46	Yes	Ps.M	*C. guilliermondii*	>100000	HIV-1
197	1/7/2011	RL-197	M	40	Yes	ER	***NO CANDIDA***	**0**	HIV-1
198	5/7/2011	RL-198	M	59	Yes	Ps.M	*C. albicans*	>100000	HIV-1
199	5/7/2011	RL-199	F	35	Yes	ER	*C. guilliermondii*	>100000	HIV-1
200	7/7/2011	RL-200	M	36	Yes	ER	*C. albicans*	>100000	HIV-1
201	12/7/2011	RL-201	F	45	Yes	ER	*C. albicans*	>100000	HIV-1
202	14/7/11	RL-202	F	23	No	No	***NO CANDIDA***	**0**	HIV-1
203	14/7/11	RL-203	F	40	Yes	Ps.M	*C. tropicalis*	>100000	HIV-1
204	15/7/11	RL-204	M	32	Yes	ER	*C. kefyr*	>100000	HIV-1
205	15/7/11	RL-205	M	45	Yes	ER	*C. tropicalis*	>100000	HIV-1
206	16/7/11	RL-206	M	52	Yes	ER	*C. tropicalis+C. albicans*	>100000	**HIV-2**
207	18/7/11	RL-207	M	55	Yes	ER	*C. albicans*	50	HIV-1
208	19/7/11	RL-208	F	18	Yes	ER	*C. albicans*	600	HIV-1
209	20/7/11	RL-209	M	30	Yes	ER	*C. albicans*	5500	HIV-1
210	20/7/11	RL-210	F	34	Yes	ER	*C. albicans*	>100000	HIV-1

211	21/7/11	RL-211	M	55	Yes	ER	*C. albicans*	>100000	HIV-1
212	22/7/11	RL-212	M	42	Yes	ER	*C. dubliniensis*	>100000	HIV-1
213	22/7/11	RL-213	M	42	Yes	ER	***NO CANDIDA***	**0**	HIV-1
214	23/7/11	RL-214	M	35	Yes	No	***NO CANDIDA***	**0**	HIV-1
215	25/7/11	RL-215	M	33	Yes	Ps.M	***NO CANDIDA***	**0**	HIV-1
216	26/7/11	RL-216	M	30	Yes	Ps.M	*C. albicans*	900	HIV-1
217	27/7/11	RL-217	M	52	Yes	ER	*C. albicans*	>100000	HIV-1
218	29/7/11	RL-218	F	38	Yes	ER	*C. albicans*	670	HIV-1
219	29/7/11	RL-219	M	30	Yes	No	*C. albicans*	480	HIV-1
220	1/8/11	RL-220	M	32	Yes	ER	*C. albicans*	>100000	HIV-1
221	1/8/11	RL-221	F	68	Yes	Ps.M	*C. albicans*	>100000	HIV-1
222	1/8/11	RL-222	M	40	Yes	ER	*C. albicans*	>100000	HIV-1
223	1/8/11	RL-223	F	25	Yes	Ps.M	*C. albicans*	>100000	HIV-1
224	2/8/11	RL-224	F	30	Yes	Ps.M	*C. albicans*	>100000	HIV-1
225	3/8/11	RL-225	M	45	Yes	ER	*C. albicans*	>100000	HIV-1
226	3/8/11	RL-226	M	12	NO	No	***NO CANDIDA***	**0**	HIV-1
227	5/8/11	RL-227	M	25	NO	No	***NO CANDIDA***	**0**	HIV-1
228	6/8/11	RL-228	F	41	NO	No	***NO CANDIDA***	**0**	HIV-1
229	8/8/11	RL-229	M	55	Yes	Ps.M	*C. albicans*	3000	HIV-1
230	8/8/11	RL-230	M	10	Yes	No	*C. guilliermondii*	700	HIV-1
231	8/8/11	RL-231	F	37	Yes	No	*C. albicans*	480	HIV-1
232	8/8/11	RL-232	F	40	No	No	***NO CANDIDA***	**0**	HIV-1
233	8/8/11	RL-233	M	35	No	No	***NO CANDIDA***	**0**	HIV-1
234	8/8/11	RL-234	F	40	Yes	ER	*C. dubliniensis*	>100000	HIV-1
235	8/8/11	RL-235	F	18	Yes	ER	*C. albicans*	>100000	HIV-1
236	8/8/11	RL-236	M	35	Yes	Ps.M	*C. albicans*	2400	HIV-1
237	8/8/11	RL-237	M	22	Yes	No	*C. albicans*	423	HIV-1
238	8/8/11	RL-238	F	35	Yes	No	*C. albicans*	680	HIV-1
239	8/8/11	RL-239	M	1.5	Yes	Ps.M	*C. dubliniensis*	730	HIV-1
240	8/8/11	RL-240	M	35	Yes	No	***NO CANDIDA***	**0**	HIV-1

241	8/8/11	RL-241	F	25	Yes	Ps.M	*C. albicans+C. tropicalis*	2000	HIV-1
242	8/8/11	RL-242	M	28	No	No	*C. tropicalis+C. albicans*	>100000	HIV-1
243	8/8/11	RL-243	M	58	Yes	ER	*C. albicans*	>100000	HIV-1
244	8/8/11	RL-244	M	33	Yes	Ps.M	*C. albicans*	>100000	HIV-1
245	8/8/11	RL-245	M	38	Yes	ER	*C. albicans*	>100000	HIV-1
246	8/8/11	RL-246	F	58	Yes	ER	*C. guilliermondii*	>100000	HIV-1
247	9/8/11	RL-247	F	27	Yes	Ps.M	*C. albicans*	>100000	HIV-1
248	9/8/11	RL-248	F	35	No	No	***NO CANDIDA***	**0**	HIV-1
249	9/8/11	RL-249	F	29	No	No	***NO CANDIDA***	**0**	HIV-1
250	9/8/11	RL-250	M	38	Yes	Ang.che	*C. tropicalis+C. albicans*	>100000	HIV-1
251	9/8/11	RL-251	M	32	Yes	Ps.M	*C. albicans*	840	HIV-1
252	9/8/11	RL-252	F	30	Yes	Ps.M	*C. lusitaniae*	>100000	HIV-1
253	9/8/11	RL-253	M	35	No	No	***NO CANDIDA***	**0**	HIV-1
254	9/8/11	RL-254	F	36	No	No	***NO CANDIDA***	**0**	HIV-1
255	9/8/11	RL-255	M	55	No	No	***NO CANDIDA***	**0**	HIV-1
256	9/8/11	RL-256	M	55	No	No	***NO CANDIDA***	**0**	HIV-1
257	9/8/11	RL-257	M	60	No	No	***NO CANDIDA***	**0**	HIV-1
258	9/8/11	RL-258	M	40	Yes	ER	*C. tropicalis+C. albicans*	>100000	HIV-1
259	9/8/11	RL-259	F	30	Yes	ER	*C. albicans*	>100000	HIV-1
260	9/8/11	RL-260	M	35	Yes	ER	*C. dubliniensis*	>100000	HIV-1
261	9/8/11	RL-261	F	32	Yes	ER	*C. albicans+C. tropicalis*	>100000	HIV-1
262	9/8/11	RL-262	F	28	No	No	***NO CANDIDA***	**0**	**HIV-2**
263	9/8/11	RL-263	M	40	No	No	*C. albicans*	600	HIV-1
264	9/8/11	RL-264	M	33	Yes	Ps.M	*C.zeylanoides*	680	HIV-1
265	9/8/11	RL-265	M	48	No	No	*C. albicans*	>100000	HIV-1
266	9/8/11	RL-266	F	26	No	No	*C. albicans+C.parapsilosis*	1210+250	HIV-1
267	9/8/11	RL-267	M	25	No	No	*C. albicans+C,krusei*	360+4	HIV-1
268	9/8/11	RL-268	M	34	No	No	*C. albicans*	900	HIV-1
269	9/8/11	RL-269	M	60	No	No	*C. albicans*	3995	HIV-1
270	9/8/11	RL-270	F	36	No	No	*C. albicans*	209	HIV-1

271	9/8/11	RL-271	M	28	No	No	*C. albicans*	1738	HIV-1
272	9/8/11	RL-272	M	42	No	No	*C. albicans*	24	HIV-1
273	9/8/11	RL-273	F	35	No	No	*C. albicans*	>100000	HIV-1
274	11/8/11	RL-274	F	37	No	No	*C.kefyr*	210	HIV-1

Note;M=Male, F=Female, UTI= Urinary Tract Infection, RTI= Respiratory Tract Infection, LL= Left Lung, PS. M=Pseudomemranous, ER=Erythamatous, ANG.CHEL=Angular Cheilitis, PTB=Pulmonary Tuberculosis

Table 4.2 Master chart representing details of HIV seronegative individuals

SL.NO	Date	LAB.NO	AGE (yrs)	SEX	OC	GROWTH	CFU/ml	IDENTIFICATION
1	4/1/08	CN-1	22	M	N	N	NG	-
2	4/1/08	CN-2	35	M	N	N	NG	-
3	4/1/08	CN-3	27	M	N	N	NG	-
4	4/1/08	CN-4	21	M	N	N	NG	-
5	4/1/08	CN-5	39	M	N	N	NG	-
6	4/1/08	CN-6	27	M	N	Y	120	*C. tropicalis*
7	5/1/08	CN-7	28	M	N	Y	140	*C. albicans*
8	5/1/08	CN-8	21	M	N	Y	280	*C. albicans*
9	5/1/08	CN-9	28	M	N	Y	800	*C. albicans*
10	5/1/08	CN-10	20	M	N	N	NG	-
11	5/1/08	CN-11	40	F	N	N	NG	-
12	5/1/08	CN-12	24	M	N	N	NG	-

13	5/1/08	CN-13	38	M	N	N	NG	-
14	5/1/08	CN-14	22	F	N	N	NG	-
15	5/1/08	CN-15	34	F	N	N	NG	-
16	5/1/08	CN-16	29	M	N	Y	40	*C. albicans*
17	5/1/08	CN-17	31	M	N	N	NG	-
18	5/1/08	CN-18/1	29	F	N	Y	40,70	*C.lusitanae*
19	5/1/08	CN-18/2	33	M	N	Y	78	*C. albicans*
20	5/1/08	CN-19	28	M	N	N	NG	-
21	5/1/08	CN-20	38	M	N	N	NG	-
22	7/1/08	CN-21	35	M	N	N	NG	-
23	6/1/08	CN-22	25	M	N	Y	80	*C. albicans*
24	7/1/08	CN-23	35	M	N	N	NG	-
25	7/1/08	CN-24	30	F	N	N	NG	-
26	7/1/08	CN-25	21	F	N	N	NG	-
27	11/1/08	CN-26	58	M	N	Y	50	*C. albicans*
28	13/1/08	CN-27	21	F	N	N	NG	-
29	14/1/08	CN-28	48	M	N	Y	30	*C. albicans*
30	14/1/08	CN-29	60	M	N	Y	40	*C. glabrata*
31	28/1/08	CN-30	29	M	N	N	NG	-
32	28/1/08	CN-31	30	M	N	Y	40	*C. albicans*

33	28/1/08	CN-32	22	F	N	N	NG	*C. albicans*
34	28/1/08	CN-33	35	F	N	Y	1000	*C. albicans*
35	17/108	CN-34	27	F	N	Y	1000	*C. albicans*
36	17/1/08	CN-35	21	F	N	Y	1000	*C. tropicalis*
37	17/1/08	CN-36	39	F	N	N	NG	-
38	18/1/08	CN-37	27	F	N	N	NG	*C. albicans*
39	19/1/08	CN-38	28	M	N	N	NG	-
40	19/1/08	CN-39	62	M	N	N	NG	-
41	19/1/08	CN-40	35	M	N	Y	>100000	*C. albicans*
42	19/1/08	CN-41	37	M	N	Y	>100000	*C. albicans,C.tropicalis*
43	23/1/08	CN-42	31	F	N	NG	NG	-
44	23/1/08	CN-43	25	M	N	NG	NG	-
45	23/1/08	CN-44	28	F	N	NG	NG	-
46	14//2/08	CN-45	31	M	N	NG	NG	-
47	14/2/08	CN-46	27	F	N	NG	NG	-
48	15/2/08	CN-47	32	F	N	NG	NG	-
49	15/2/08	CN-48	32	F	N	Y	NG	*C. albicans*
50	17/2/08	CN-49	29	F	N	NG	NG	-
51	17/2/08	CN-50	26	F	N	NG	NG	-
52	19/2/08	CN-51	40	F	N	NG	NG	-
53	19/2/08	CN-52	33	M	N	NG	NG	-

54	19/2/08	CN-53	28	M	N	NG	NG	-
55	21/2/08	CN-54	26	M	N	NG	NG	-
56	21/2/08	CN-55	25	F	N	NG	NG	-
57	21/2/08	CN-56	23	F	N	NG	NG	-
58	25/2/08	CN-57	25	M	N	NG	NG	-
59	25/2/08	CN-58	35	M	N	NG	NG	-
60	28/2/08	CN-59	32	M	N	NG	NG	-
61	29/2/08	CN-60	48	F	N	NG	NG	-
62	9/3/08	CN-61	26	M	N	Y	200	*C. tropicalis*
63	9/4/08	CN-62	40	F	N	NG	NG	*C. albicans*
64	9/5/08	CN-63	35	F	N	NG	NG	-
65	9/6/08	CN-64	29	F	N	Y	100	*C. tropicalis*
66	9/6/08	CN-65	40	M	N	Y	700	*C. albicans*
67	14/6/08	CN-66	22	F	N	N	NG	-
68	14/6/08	CN-67	26	M	N	N	NG	*C. albicans*
69	21/6/08	CN-68	28	F	N	N	NG	-
70	23/6/08	CN-69	22	F	N	Y	80	*C. albicans*
71	23/6/08	RC-70	24	M	N	NG	NG	-
72	23/6/08	RC-71	40	M	N	NG	NG	*C. albicans*
73	23/6/08	RC-72	34	F	N	NG	NG	-
74	23/6/08	RC-73	32	M	N	NG	NG	-

75	25/6/08	RC-74	28	M	N	NG	NG	-
76	25/6/08	RC-75	20	M	N	NG	NG	-
77	25/6/08	RC-76	21	M	N	NG	NG	-
78	25/6/08	RC-77	19	F	N	NG	NG	-
79	25/6/08	RC-78	45	M	N	NG	NG	-
80	25/6/08	RC-79	45	M	N	Y	100000	*C. albicans*
81	25/6/08	RC-80	45	F	N	NG	NG	-
82	25/6/08	RC-81	37	M	N	NG	NG	-
83	25/6/08	RC-82	55	M	N	NG	NG	-
84	25/6/08	RC-83	30	F	N	NG	NG	-
85	26/6/08	RC-84	24	F	N	NG	NG	-
86	26/6/08	RC-85	18	M	N	Y	60	*C. tropicalis*
87	27/6/08	RC-86	25	F	N	Y	60	*C. tropicalis*
88	27/6/08	RC-87	35	F	N	NG	NG	-
89	27/6/08	RC-88	36	F	N	NG	NG	-
90	27/6/08	RC-89	35	F	N	NG	NG	-
91	27/6/08	RC-90	35	F	N	NG	NG	-
92	4/7/08	RC-91	25	F	N	NG	NG	-
93	4/7/08	RC-92	40	F	N	Y	150	*C. albicans*
94	8/1/09	RC-93	24	F	N	NG	NG	-
95	8/1/09	RC-94	20	F	N	NG	NG	-

96	8/1/09	RC-95	24	F	N	Y	1000	*C. krusei*
97	8/1/09	RC-96	45	F	N	Y	30	*C. guilliermondii*
98	10/1/09	RC-97	31	F	N	NG	NG	-
99	18/1/09	RC-98	30	F	N	NG	NG	-
100	17/1/09	RC-99	59	M	N	NG	NG	-
101	20/1/09	RC-100	35	F	N	NG	NG	-
102	22/1/09	RC-101	29	F	N	NG	NG	-
103	27/1/09	RC-102	82	M	N	NG	NG	*C. albicans*
104	27/1/09	RC-103	34	M	N	NG	NG	-
105	25/1/09	RC-104	22	F	N	NG	NG	-
106	1/2/09	RC-105	30	F	N	NG	NG	-
107	3/2/09	RC-106	30	F	N	NG	NG	-
108	3/2/09	RC-107	26	F	N	NG	NG	-
109	7/2/09	RC-108	45	F	N	Y	10	*C. guilliermondii*
110	13/2/09	RC-109	35	M	N	NG	NG	-
111	14/2/09	RC-110	22	F	N	NG	NG	-
112	14/2/09	RC-111	58	F	N	Y	50	*C. albicans*
113	14/2/09	RC-112	50	F	N	Y	500	*C. albicans*
114	20/2/09	RC-113	30	F	N	Y	1000	*C. guilliermondii*
115	21/2/09	RC-114	43	F	N	NG	NG	-
116	21/2/09	RC-115	24	F	N	NG	NG	*C. albicans*

117	21/2/09	RC-116	30	F	N	NG	NG	-
118	21/2/09	RC-117	53	M	N	Y	100	*C. guilliermondii*
119	21/2/09	RC-118	24	F	N	NG	NG	-
120	21/2/09	RC-119	65	F	N	NG	NG	-
121	26/2/09	RC-120	70	F	N	NG	NG	-
122	26/2/09	RC-121	48	M	N	NG	NG	-
123	26/2/09	RC-122	14	M	N	NG	NG	-
124	28/2/09	RC-123	34	F	N	NG	NG	-
125	28/2/09	RC-124	40	M	N	Y	100	*C. guilliermondii*
126	28/2/09	RC-125	9	F	N	Y	100	*C. parapsilosis*
127	28/2/09	RC-126	24	F	N	NG	NG	-
128	28/2/09	RC-127	38	F	N	NG	NG	*C. albicans*
129	1/3/09	RC-128	44	M	N	Y	10	*Candida glabrata*
130	1/3/09	RC-129	24	F	N	Y	1000	*C. albicans*
131	1/3/09	RC-130	30	F	N	NG	NG	-
132	1/3/09	RC-131	31	M	N	NG	NG	-
133	1/3/09	RC-132	58	F	N	NG	NG	-
134	2/3/09	RC-133	19	F	N	NG	NG	-
135	2/3/09	RC-134	28	M	N	NG	NG	-
136	1/5/09	RC-135	48	F	N	NG	NG	-
137	1/6/09	RC-136	50	M	N	Y	50	*C. albicans*

138	8/7/09	RC-137	47	M	N	NG	NG	-
139	8/7/09	RC-138	17	F	N	NG	NG	*C. albicans*
140	9/7/09	RC-139	22	F	N	NG	NG	-
141	9/7/09	RC-140	30	F	N	NG	NG	-
142	12/7/09	RC-141	28	F	N	NG	NG	-
143	12/7/09	RC-142	23	M	N	NG	NG	-
144	13/7/09	RC-143	32	F	N	NG	NG	-
145	13/7/09	RC-144	30	M	N	Y	700	*C. albicans*
146	13/7/09	RC-145	23	M	N	NG	NG	-
147	14/7/09	RC-146	33	F	N	NG	NG	-
148	14/7/09	RC-147	29	F	N	Y	1000	*C. albicans*
149	14/7/09	RC-148	29	M	N	NG	NG	-
150	14/7/09	RC-149	29	F	N	Y	2800	*C. albicans*
151	14/3/10	RC-150	26	F	N	Y	200	*C. albicans*
152	14/3/10	RC-151	65	M	N	NG	NG	-
153	14/3/10	RC-152	20	M	N	NG	NG	-
154	21/3/10	RC-153	41	F	N	Y	30	*C. albicans*
155	24/3/10	RC-154	55	M	N	NG	NG	-
156	25/3/10	RC-155	40	M	N	NG	NG	-
157	25/3/10	RC-156	22	m	N	NG	NG	-
158	24/3/10	RC-157	68	F	N	Y	1000	*C. albicans*

159	24/3/10	RC-158	86	F	N	Y	158	*C. albicans*
160	24/3/10	RC-159	37	F	N	NG	NG	-
161	24/3/10	RC-160	35	M	N	NG	NG	-
162	1/7/10	RC-161	46	M	N	Y	500	*C. albicans*
163	1/7/10	RC-162	38	M	N	Y	200	*C. albicans*
164	3/7/10	RC-163	58	M	N	Y	400	*C. albicans*
165	3/7/10	RC-164	28	F	N	NG	NG	*C. guilliermondii*
166	3/7/10	RC-165	26	M	N	NG	NG	-
167	4/7/10	RC-166	64	M	N	NG	NG	-
168	4/7/10	RC-167	42	M	N	NG	NG	*C. albicans*
169	4/7/10	RC-168	25	F	N	Y	50	*C. albicans*
170	4/7/10	RC-169	45	F	N	Y	500	*C. albicans*
171	5/7/10	RC-170	46	F	N	NG	NG	-
172	20/12/10	RC-171	24	F	N	NG	NG	-
173	20/12/10	RC-172	22	F	N	Y	20	*C. albicans*
174	21/12/10	RC-173	23	F	N	Y	260	*C. albicans*
175	22/12/10	RC-174	24	F	N	Y	10	*C. albicans*
176	24/12/10	RC-175	24	F	N	NG	NG	-
177	24/12/10	RC-176	42	M	N	Y	NG	*C. albicans*
178	24/12/10	RC-177	26	F	N	NG	NG	-
179	24/12/10	RC-178	38	F	N	NG	NG	-

180	28/12/10	RC-179	33	F	N	NG	NG	-
181	28/12/10	RC-180	49	F	N	NG	NG	-
182	28/12/10	RC-181	25	F	N	Y	140	*C. albicans*
183	29/12/10	RC-182	26	F	N	NG	NG	-
184	29/12/10	RC-183	21	F	N	NG	NG	-
185	29/12/10	RC-184	38	M	N	NG	NG	-
186	31/12/10	RC-185	26	F	N	NG	NG	-
187	4/1/11	RC-186	30	M	N	Y	76	*C. albicans*
188	6/1/11	RC-187	30	M	N	NG	0	NO CANDIDA
189	7/1/11	RC-188	35	F	N	NG	0	-
190	15/1/11	RC-189	28	F	N	NG	0	-
191	17/1/11	RC-190	67	M	N	Y	34	*C. albicans*
192	23/5/11	RC-191	45	F	N	Y	500	*C. lusitaniae*
193	28/5/11	RC-192	35	M	N	Y	1	*C. albicans*
194	17/6/11	RC-193	30	F	N	NG	0	-
195	18/6/11	RC-194	30	M	N	Y	64	*C. albicans*
196	21/6/11	RC-195	56	F	N	Y	>100000	*C. albicans*
197	21/6/11	RC-196	45	F	N	NG	0	-
198	23/6/11	RC-197	4	M	N	NG	0	-
199	23/6/11	RC-198	50	M	N	NG	0	-
200	23/6/11	RC-199	43	M	N	Y	2	*C. albicans*

201	24/6/11	RC-200	26	M	N	Y	4	-
202	24/6/11	RC-201	27	M	N	NG	0	-
203	28/6/11	RC-202	34	M	N	Y	98	*C. tropicalis*
204	30/6/11	RC-203	33	M	N	Y	55	*C. albicans*
205	1/7/11	RC-204	20	F	N	Y	>100000	*C. tropicalis +C. albicans*
206	1/7/11	RC-205	35	F	N	NG	0	-
207	1/7/11	RC-206	32	M	N	Y	100	*C. albicans*
208	1/7/11	RC-207	50	M	N	Y	54	*C. albicans*
209	1/7/11	RC-208	35	M	N	NG	0	-
210	1/7/11	RC-209	54	M	N	Y	2	*C. albicans*
211	1/7/11	RC-210	25	M	N	NG	0	-
212	1/7/11	RC-211	40	M	N	NG	0	-
213	2/7/11	RC-212	25	M	N	NG	0	-
214	2/7/11	RC-213	40	M	N	NG	0	-
215	2/7/11	RC-214	46	M	N	NG	0	-
216	2/7/11	RC-215	61	M	N	Y	4	*C. albicans*
217	2/7/11	RC-216	50	M	N	Y	1	-
218	2/7/11	RC-217	38	M	N	NG	0	-
219	2/7/11	RC-218	55	M	N	Y	300	*C. albicans*
220	2/7/11	RC-219	45	M	N	NG	0	-
221	2/7/11	RC-220	21	F	N	Y	5	*C. albicans*

222	26/7/11	RC-221	35	M	N	NG	0	-
223	26/7/11	RC-222	51	M	N	Y	>100000	*C. albicans*
224	26/7/11	RC-223	22	M	N	NG	0	-
225	26/7/11	RC-224	38	F	N	Y	89	*C. albicans*
226	28/7/11	RC-225	30	M	N	Y	80	*C. albicans*
227	27/7/11	RC-226	48	M	N	Y	36	*C. albicans*
228	26/7/11	RC-227	21	M	N	NG	0	-
229	27/7/11	RC-228	42	M	N	Y	440	*C. albicans*
230	27/7/11	RC-229	48	M	N	Y	86	*C. albicans*
231	26/7/11	RC-230	46	M	N	Y	4260	*C. albicans*
232	28/7/11	RC-231	44	M	N	Y	480	*C. albicans*
233	26/7/11	RC-232	43	M	N	NG	0	-
234	26/7/11	RC-233	30	F	N	NG	0	-
235	26/7/11	RC-234	32	M	N	NG	0	-
236	26/7/11	RC-235	26	M	N	NG	0	-
237	26/7/11	RC-236	36	M	N	Y	120	*C. albicans*
238	26/7/11	RC-237	40	M	N	NG	0	NO CANDIDA
239	26/7/11	RC-238	41	M	N	NG	0	-
240	27/7/11	RC-239	32	M	N	NG	0	-
241	26/7/11	RC-240	26	M	N	NG	0	-
242	26/7/11	RC-241	29	M	N	NG	0	-

243	8/8/11	RC-242	29	M	N	NG	0	-
244	17/1/11	RC-243	37	M	N	Y	100	*C. albicans*
245	15/12/11	RC-244	39	M	N	NG	0	-
246	17/1/11	RC-245	29	M	N	Y	60	*C. tropicalis*
247	17/1/11	RC-246	28	M	N	NG	0	-
248	17/1/11	RC-247	30	M	N	Y	600	*C. albicans*
249	17/1/11	RC-248	45	M	N	Y	120	*C. albicans*
250	17/1/11	RC-249	28	M	N	NG	0	-
251	17/1/11	RC-250	39	M	N	NG	0	-
252	17/1/11	RC-251	33	M	N	NG	0	-
253	17/1/11	RC-252	35	M	N	NG	0	-
254	17/1/11	RC-253	28	M	N	NG	0	-
255	17/1/11	RC-254	36	M	N	NG	0	-
256	17/1/11	RC-255	32	M	N	NG	0	-
257	17/1/11	RC-256	30	M	N	NG	0	-
258	17/1/11	RC-257	39	M	N	NG	0	-
259	17/1/11	RC-258	31	M	N	Y	680	*C. albicans*
260	17/1/11	RC-259	35	M	N	Y	234	*C. albicans*

Note: M= MALE, F= FEMALE NR=NORMAL N=NO TB=Tuberculosis, G=GOOD NG =NO GROWTH Y= Yes

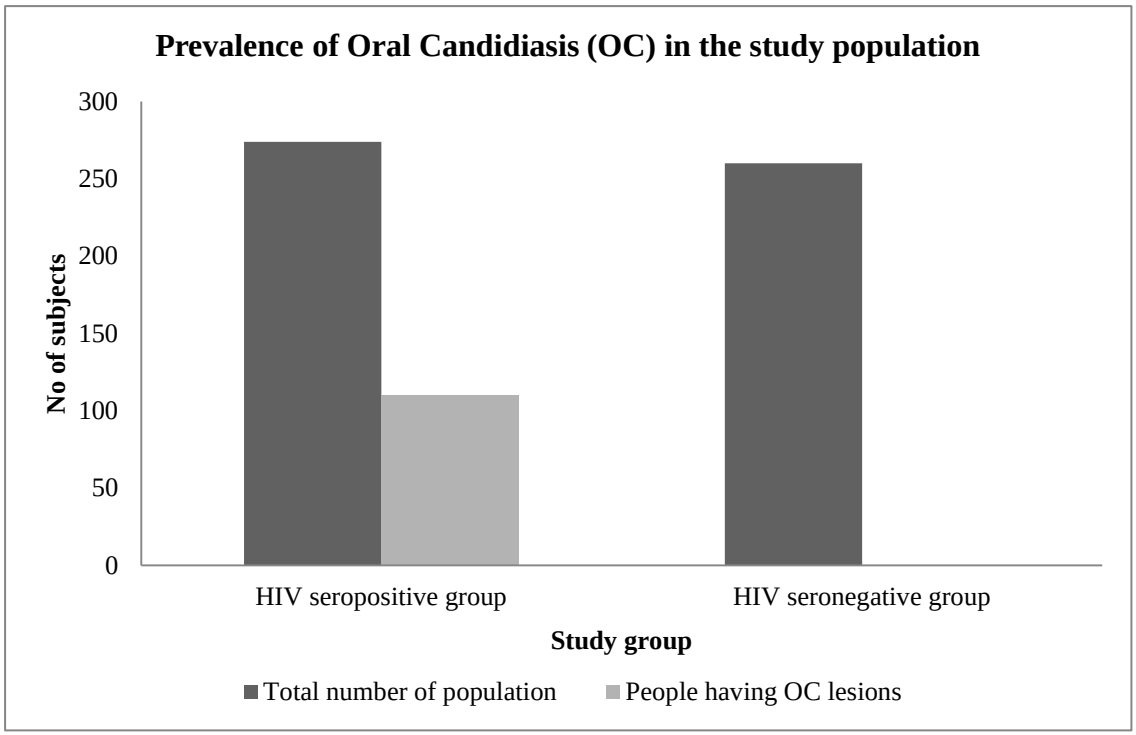


Fig. 4.1 Prevalence of OC in study population

OC is one of the most common oral lesions seen in HIV seropositive individuals and the presence of OC indicates progression to AIDS (Banerjee 2005). Therefore presence of any kind of lesions and type of the lesion was carefully studied in HIV seropositive individuals. The OC lesions were present in 110 out of 274 HIV seropositive individuals, i.e., 40.14% of HIV population had this infection. The details are shown in Fig. 4.1.

According to the presence of OC, the HIV population was divided into two sub groups, i.e., sub group 1; HIV seropositive individuals with OC and sub group 2; HIV seropositive individuals without OC. The oral candida carriage, prevalence of *C. albicans* and fluconazole sensitivity pattern were studied in these sub groups, separately and comparison was done. This will help to know the status of candida in oral cavity of immunocompromised host, both in pathogenesis and when only colonization is present.

The present study showed that 40.14% of the HIV seropositive subjects had clinically apparent OC which is in accordance with the observations of Nadiger et al. (2008), who reported 38.8% clinically proven OC cases in HIV seropositive patients from Hubli-Dharwad region itself. There are many other reports also supporting the observation as OC being the most common opportunistic infection seen in HIV seropositive individuals (Scully et al. 1994; Vargas and Joly 2002; Coogan et al.2005).

Pseudomembranous, erythamatous and angular cheilitis are the common types of OC lesions present in HIV seropositive patients (Fotos et al. 1992). The OC lesions present in the study subjects were characterized into Pseudomembranous, erythamatous and angular cheilitis type of lesions. The number of patients who present in each type of lesion is shown in Table 4.3 and Fig. 4.2. Pseudomembranous type of OC was the most prevalent type of lesion present in this study group, however Pseudomembranous (49.09%) and erythamatous type of OC lesions (47.27%) were occurring in almost similar proportions. Schoidt et al. (1990) also reported that, 23% of HIV seropositive individuals with OC in their study had Pseudomembranous lesions and 21% had erythamatous candidiasis. In the present study, angular cheilitis type of lesions was presented in only four HIV seropositive patients.

Table 4.3 Type of Oral Candidiasis lesions presented in HIV seropositive patients

Type of OC lesions present	**Number of patients**
Pseudomembranous candidiasis	54 (49.09%)
Erythamatous candidiasis	52 (47.27%)
Angular cheilitis	4 (3.63%)
Total	110

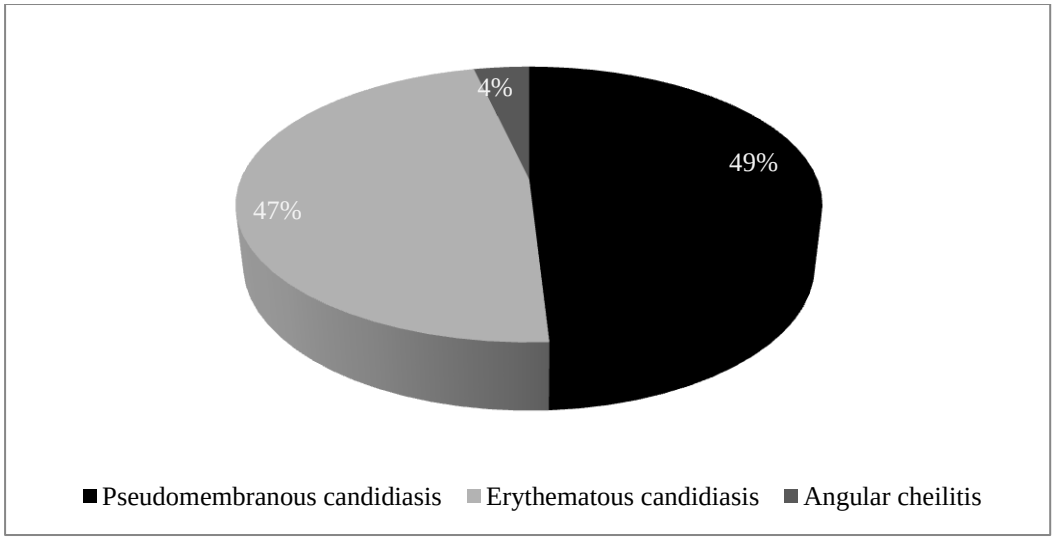


Fig. 4.2 Types of OC lesions found in HIV seropositive individual

4.1.1.2 AGE AND GENDER WISE DISTRIBUTION OF OC

In HIV seropositive patients of the study group, total population of males were 58.25% (159/ 274) and females were 41.97% (115/274). In the case of seropositive male patients, over all 44.65% (71/159) had OC lesions, whereas in females, 39 out of 115 (33.91%) had OC.

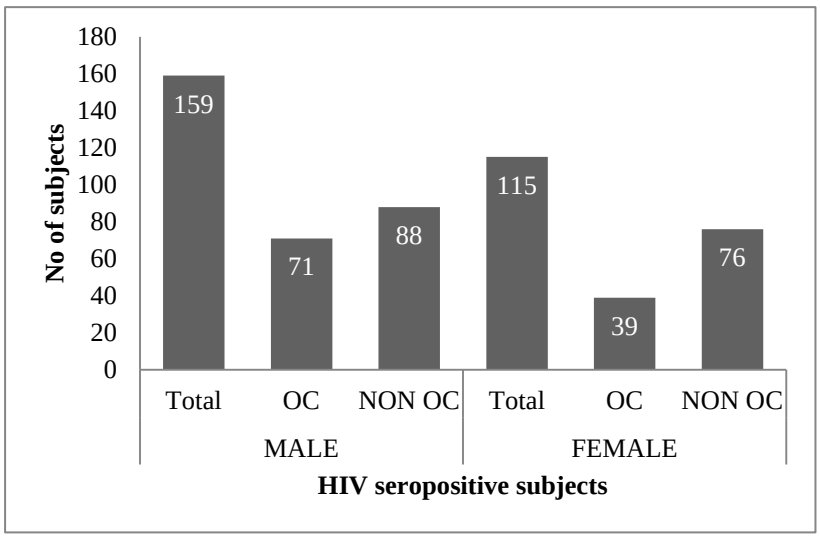


Fig. 4.3 Gender wise Prevalence of OC in HIV seropositive individuals

The prevalence of OC among HIV seropositive group was seen more in the age group of 30-39 yrs as shown in Table 4.4 and Fig 4.4.

Table 4.4 Age-wise distribution of OC in HIV seropositive individuals

Age group (in Yrs)	Total number of patients showing OC	Percentage of prevalence
1 to 4	1	0.91
5 to 14	2	1.82
15-19	2	1.82
20-24	4	3.64
25-29	14	12.73
30-34	22	20.00
35-39	19	17.27
40-44	15	13.64
45-49	12	10.91
50-54	3	2.73
55-59	10	9.09
60-64	2	1.82
65-69	2	1.82
70-74	1	0.91
75-79	0	0.00
80-84	1	0.91
85-89	0	0.00
Total	110	

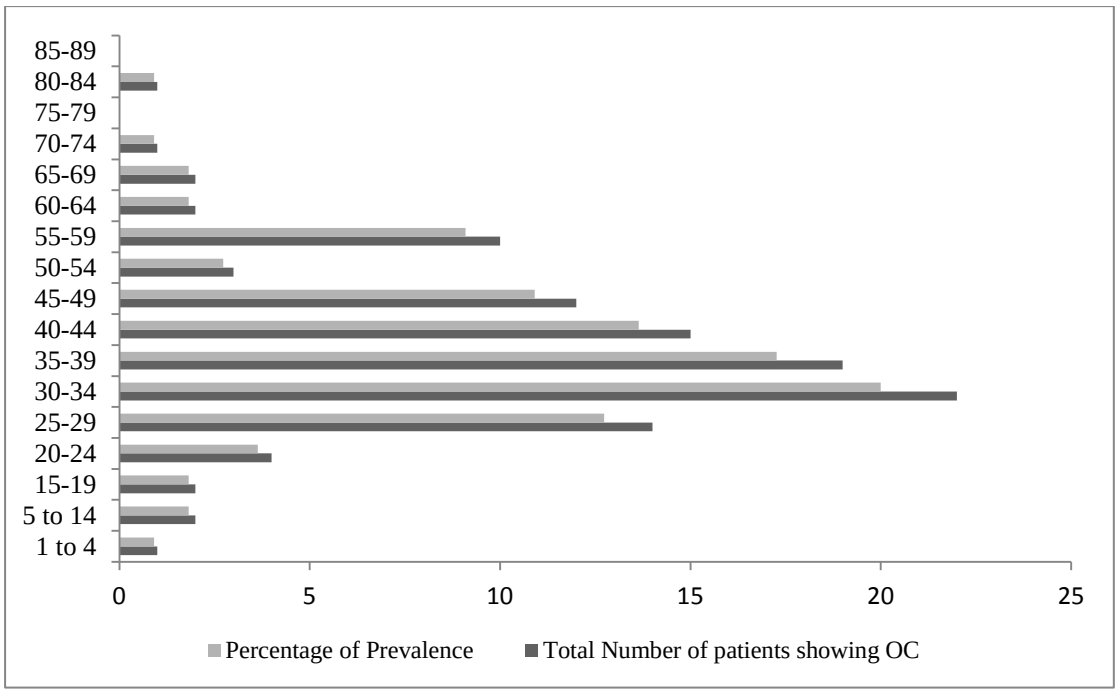


Fig. 4.4 Age-wise distribution of OC in HIV seropositive individuals

Note: Age represented in years

Table 4.5 and Fig. 4.5 shows age wise distribution of OC in HIV seropositive male individuals. The number of patients from age group 25 till 50 was high comparing to other age groups. If the prevalence of OC among these age range was considered, the age range 30-34 had shown highest percentage of incidence of OC in male patients. In females, more number of patients were present in the age range of 20 to 44 and among them OC was seen in the highest percentage in the age group of 40-44 yrs. The details are shown in Table 4.6 and presented in the Fig. 4.6.

Table 4.5 Prevalence of OC among male HIV seropositive individuals, in different age group

HIV seropositive males				
Age group (in yrs)	**OC**	**NON OC**	**Total**	**Percentage of patients showing OC**
1 to 4	1	0	1	100
5 to 14	1	3	4	25
15-19	0	0	0	0
20-24	1	2	3	33.33
25-29	8	12	20	40
30-34	13	4	17	76.47
35-39	14	16	31	48.38
40-44	11	21	32	34.37
45-49	8	11	19	42.10
50-54	3	5	8	37.5
55-59	8	7	15	53.33
60-64	1	4	5	20
65-69	0	2	2	0
70-74 and above	1	0	1	100
Total	72	87	159	45.28

Age group 30-34 yrs showed more percentage of patients having OC than any other age groups.

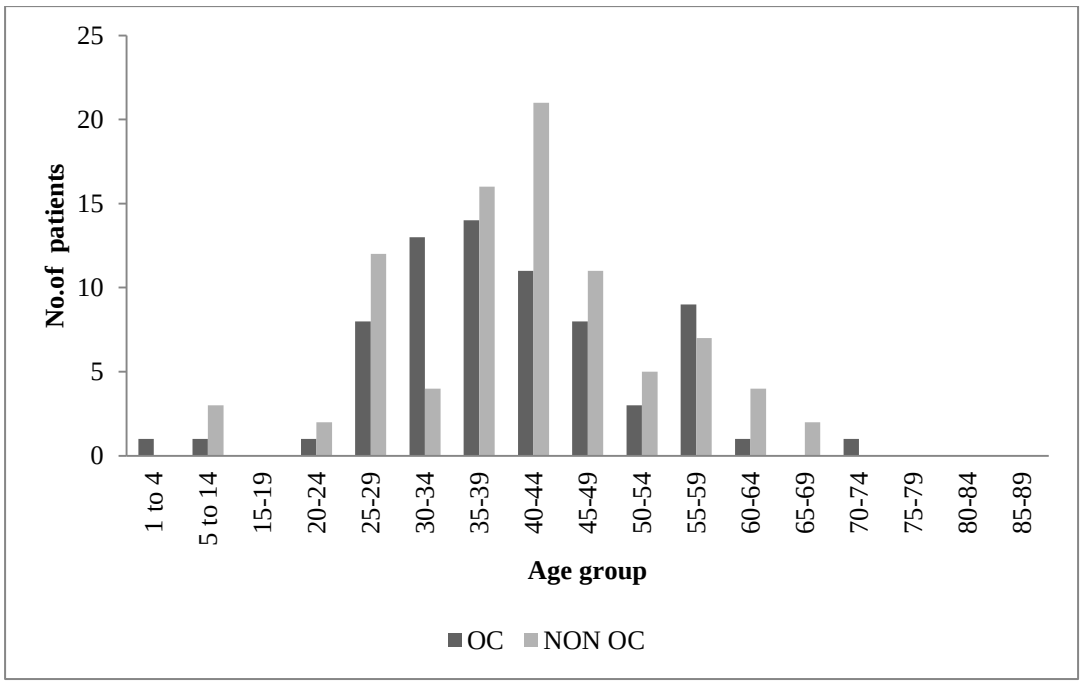


Fig. 4.5 Prevalence OC in HIV seropositive male patients

Note: Age values are represented in years

Table 4.6 Incidence of OC in Female patients along their age

HIV seropositive female patients				
Age group (in Yrs)	**OC**	**NON OC**	**Total**	**Percentage of patients having OC**
1 to 4	0	0	0	0
5 to 14	1	6	7	14.28
15-19	2	1	3	66.66
20-24	3	7	10	30
25-29	7	13	20	35
30-34	9	22	31	29.03
35-39	5	18	23	21.73
40-44	5	6	11	45.45
45-49	3	3	6	50
50-54	0	1	1	0
55-59	1	1	1	50
60-64	1	0	1	100
65-69	1	1	2	50
70-74	0	0	0	0
75-79	0	0	0	0
80-84	1	1	2	50
85-89	0	0	0	0
Total	39	76	115	32.77

In the case of female patients, of 30-34 age groups showed 29.03% having OC. Since sufficient number patients of 40-44 yrs of age group were available, prevalence of OC (45.45%) presented in this group was considered as the highest.

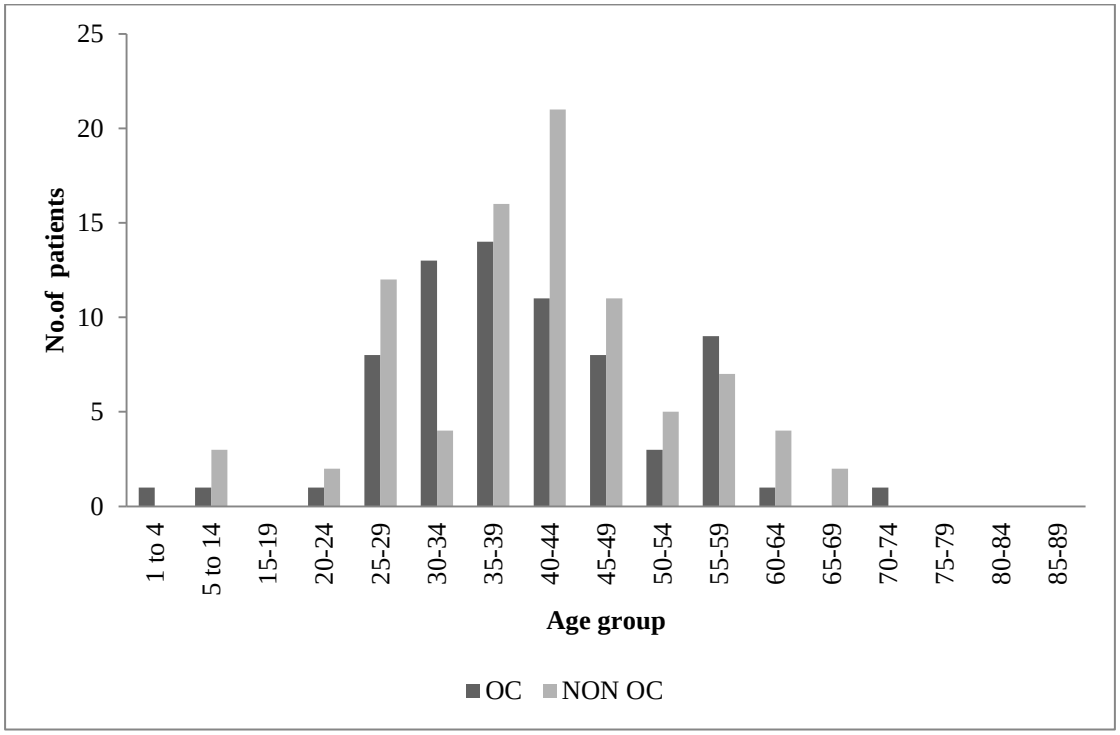


Fig. 4.6 Incidence of OC in HIV seropositive female patients along their age

Note: Age values are represented in years.

In both male and female groups, majority of HIV seropositive patients, belong to 20 to 44 yrs of age group (70.80%). Among females, subjects from 25 to 39 yrs of age group were more in number. In the total population the overall number of patients showing OC lesions were present in the age group of 25 to 44 (66.60%). Results obtained here finally give detailed picture on the prevalence of OC in HIV seropositive patients, in accordance to their age and gender. In the study by Sarika (2005) similar observation was reported, where 81% of the HIV seropositive patients who had OC were belonging to the age group of 21-40 years.

4.1.1.3 ORAL CANDIDA CARRIAGE AND SPECIES DISTRIBUTION

The details on candida carriage, *C. albicans* isolation pattern along with details on Non Albicans Candida (NAC) isolated from different study groups are presented in Table 4.7. The HIV seropositive group showed highest candida isolation of 71.53% (196/274) as compared to HIV seronegative group with 34.07% (86/260). In sub group 1 (HIV seropositive individuals with OC), 97.32% (107/110) of candida isolation was seen. Sub group 2 (HIV seropositive individuals without OC), showed 53.70% of candida carriage. The candida isolation rate in sub group 2 was significantly higher compared to HIV seronegative group; p value being < 0.001. Isolation of more than one (multiple) type of candida species was observed in a few samples. Out of 282 candida positive samples, 29 (10.28%) samples gave multiple candida isolates. Most commonly seen combination was *C. albicans* with *C. tropicalis* (20 out of 29).

Table 4.7 Candida isolation rate in the subgroups of HIV seropositive patients and HIV seronegative healthy population

Study Group (*n*)	Candida Carriage *n* (%)	*C. albicans* isolation n (%) *	NAC Isolation n (%) *
HIV seropositive group (274)	196 (71.53)×	153 (153/216,70.83%) §a	63(63/216, 29.17%) §b
Sub group 1 (110)	107 (97.32)**	94 (94/119,78.99%)¶ a	25 (25/119, 21.01%)¶ b
Sub group 2 (164)	89 (53.70)*	59 (59/97,60.82%)¶c	38 (38/97, 39.18%)¶d
HIV seronegative group (260)	87 (33.46)××	77 (77/97, 79.38%)§c	20 (20/97, 20.62%)§ b

Note:* Percentage of isolation was calculated by considering total number of isolates

1. HIV seropositive Group v/s HIV seronegative group, Candida carriage. ×, ×× $\chi 2$ =77.62, DF=1, p<0.001 Very highly significant.

2.Sub-group2 v/s HIV seronegative group, Candida carriage *,** $\chi 2$ = 16.9, DF=1, p<0.001 Very highly significant.

3. HIV seropositive group v/s HIV seronegative group, *C albicans* v/s NAC. §a,b,c,d $\chi 2$ = 2.51, DF=1, p=0.11 Not significant.

4.Sub-group 2 v/s Sub-group 1, NAC v/s *C albicans* Isolation ¶ a,b,c,d $\chi 2$ = 8.54, DF= 1, p< 0.01Highly significant.

4.1.1.4 AGE AND GENDER WISE ORAL CANDIDA CARRIAGE INCIDENCE IN HIV SEROPOSITIVE MALES VS FEMALE SUBJECTS

As patients were selected randomly, there was no equal number of patients available in each age group. The total presentation of oral candida carriage among male Vs female HIV seropositive study subjects is given in Table 4.8., and Fig. 4.7. Table 4.9 and Fig. 4.8 explain oral candida carriage for HIV seronegative group. The oral candida carriage in male subjects of sub group 1 is 93.05%, and in the case of females of sub group 1 was 97.36%. In sub group 2 oral candida carriage was 70.11% in males and 67.10% in females. Overall candida carriage in HIV seropositive male subjects was more i.e., 80.50% which is slightly more than female subjects who showed 77.39%, of carriage. The oral candida carriage in entire group of HIV seronegative males and females are exhibiting similar pattern, i.e., 38.62% and 39.13% respectively.

Table 4.8 Male Vs Female and gender wise distribution of incidence of oral candida carriage in HIV seropositive individuals

HIV Seropositive Individuals								
Age group (in Yrs)	**Sub group 1 (with OC)**				**Sub group 2 (Non-OC)**			
	Males		**Females**		**Males**		**Females**	
	Total subjects	**Oral candida carriage**	**Total subjects**	**Oral candida carriage**	**Total subjects**	**Oral candida carriage**	**Total subjects**	**Oral candida carriage**
1 to 4	1	1	0	0	0	0	0	0
5 to 14	1	1	1	1	3	2	6	4
15-19	0	0	2	2	0	0	1	0
20-24	1	1	3	3	2	1	7	3
25-29	8	7	7	7	12	8	13	7
30-34	13	12	9	9	4	3	22	16
35-39	15	15	5	5	16	11	18	13
40-44	11	11	5	5	21	14	6	3
45-49	8	8	3	3	11	11	3	3
50-54	3	3	0	0	5	3	1	1
55-59	9	8	1	1	7	4	1	0
60-64	1	1	1	1	4	3	0	0
65-69	0	0	1	1	2	1	1	1
70-74	1	0	0	0	0	0	0	0
75-79	0	0	0	0	0	0	0	0
80-84	0	0	1	0	0	0	1	0
85-89	0	0	0	0	0	0	0	0
Total	**72**	**67 (93.05%)**	**39**	**38 (97.36%)**	**87**	**61 (70.11%)**	**76**	**51 (67.10%)**

Table 4.9 Prevalence of oral candida carriage according to the age and gender, in HIV seronegative individuals

HIV Seronegative Individuals				
	Males		Females	
Age group (in yrs)	Numbers of subjects	Oral candida carriage	Number of subjects	Oral candida carriage
1 – 4	0	0	0	0
5- 14		0	1	1
15-19	1	1	3	1
20-24	14	1	27	11
25-29	31	8	25	12
30-34	24	11	21	2
35-39	26	7	14	3
40-44	15	9	6	3
45-49	12	7	10	4
50-54	7	5	1	1
55-59	6	3	3	2
60-64	4	2	2	1
65-69	2	1	3	2
70-74	0	0	1	1
75-79	0	0	0	0
80-84	1	1	0	0
85-89	0	0	1	1
Total	**145**	**56 (38.62%)**	**115**	**45 (39.13%)**

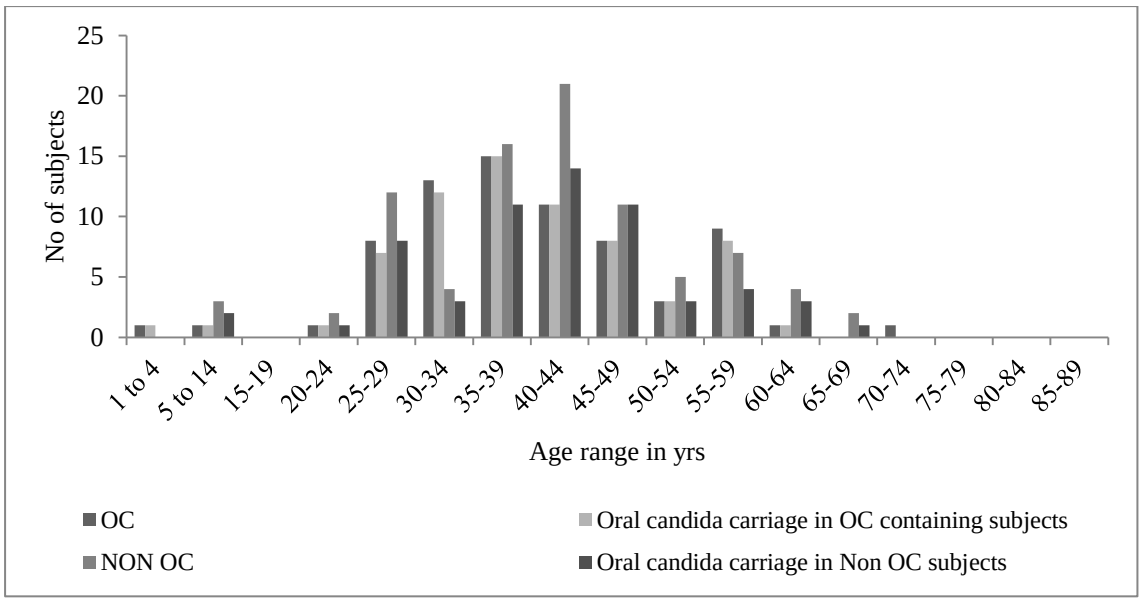


Fig. 4.7 Age and Gender wise prevalence of oral candida carriage in HIV seropositive male patients

Note: Age range wasrepresented in yrs.

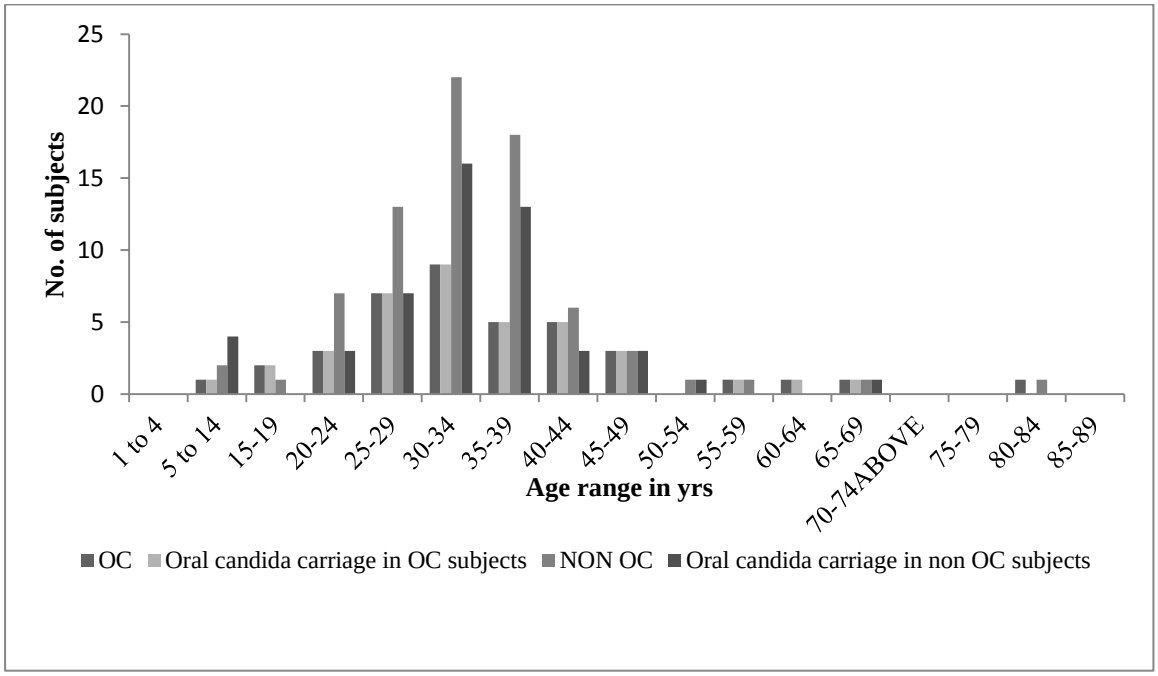


Fig. 4.8 Presence of OC and oral candida carriage in HIV seropositive female patients

Note: Agerange wasrepresented in yrs.

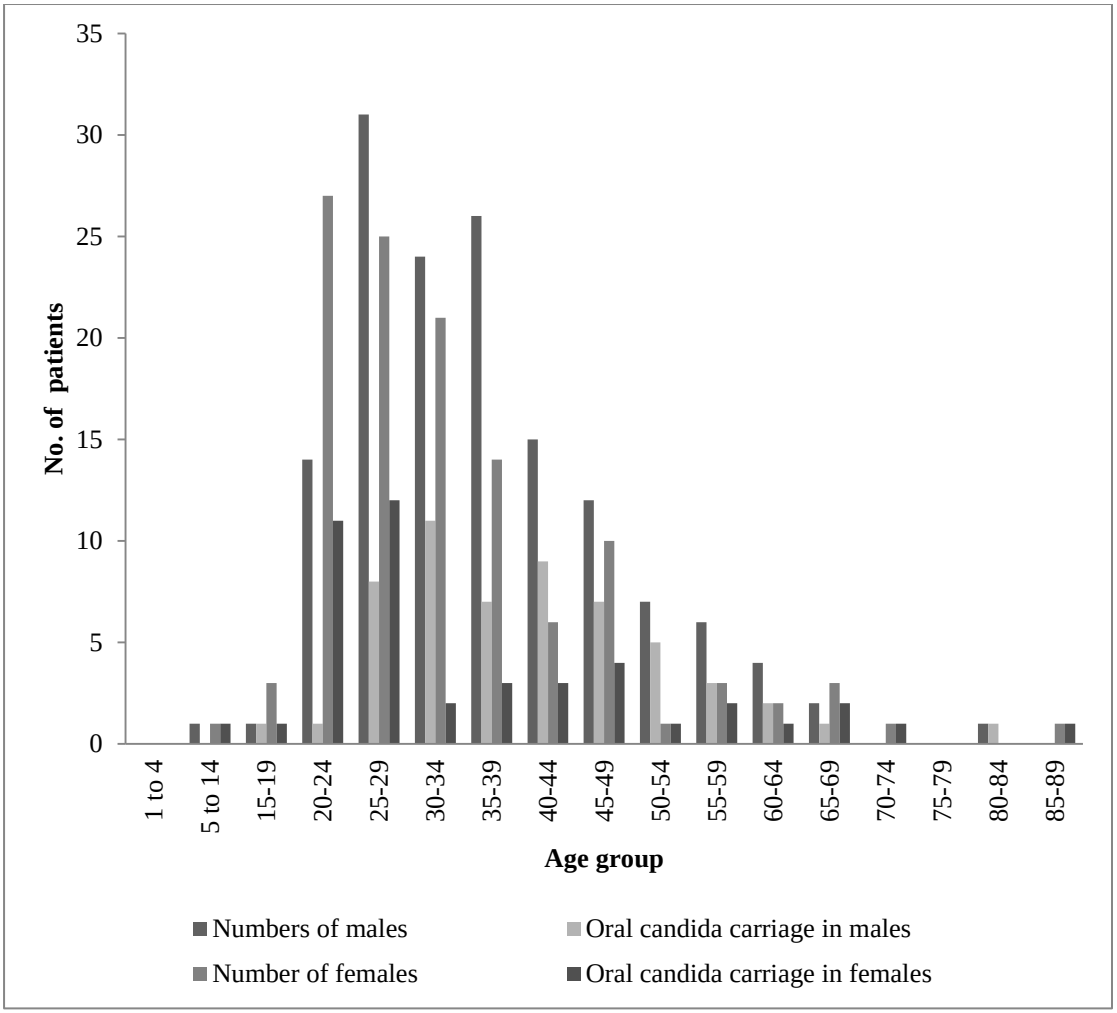


Fig. 4.9 Age and gender wise distribution of study subjects in HIV seronegative group and incidence of oral candida carriage

Note: Age ranges were represented in yrs.

4.1.1.5 MEAN ORAL CANDIDA CARRIAGE DENSITY

The colonies formed by culturing 0.1 ml of the oral rinse samples were calculated and converted into total colony forming units (CFU) /ml (Section 3.1.1.2 of Materials and Methods). Thus carriage density was calculated in the form of CFU/ml for culture positive samples.The values of CFU formed for every candida positive growth is shown in Table 4.1 and Table 4.2, earlier.HIV seropositive patients without OC yielded mean candida carriage density of 9,146 / ml which is far lesser than the mean candida carriage density in HIV seropositive individuals with OC 62,931 / ml**.** The difference in mean oral candida carriage density between these two groups was highly significant with p value < 0.001. It was also observed that, oral candida carriage density in HIV seronegative population was 2,696/ml, which was significantly lesser than the mean candida carriage obtained from Sub group 2, i.e., HIV seropositive individuals without OC ($p < 0.01$).

Oral candida carriage and its density in HIV seropositive subjects vary significantly in OC and asymptomatic carriage. It was also observed that significantly high rate of candida carriage was found in HIV seropositive subjects without OC (Sub group 2), as compared to healthy volunteers. These results are in agreement with other reports on candida carriage in HIV seropositive individuals (Ohmit et al. 2003; Sanchez –Vargas et al. 2005) However, Liu et al. (2006a), also showed that asymptomatic oral candida carriage found in HIV seropositive group was similar to that in healthy group.

4.1.1.6 *C. ALBICANS* VS NON ALBICANS CANDIDA (NAC)

a. PREVALENCE OF*C. ALBICANS*

C. albicans was the predominant candida species isolated from all the groups. A total of 313 candida isolates were obtained in the present study. Among them, 230 (73.48%) were *C. albicans* and 83 (26.51%) were NAC species. *C. albicans* / NAC ratio in study groups is shown in Fig. 4.10. In HIV seropositive subjects, sub group 1 showed highest isolation of *C. albicans* i.e., 95 (79.83%) out of 119 (total isolates). In sub group 2, isolation rate of *C. albicans* was 60.82% (59/97). The difference was

statistically significant with the p value < 0.01. Interestingly the HIV seronegative group showed isolation rate of *C. albicans* (79.38%) similar to that observed in sub group 1 (79.83%), whereas isolation of *C. albicans* in sub group 2 (60.82%) was significantly less compared with healthy HIV seronegative population; p value < 0.01.

In this study, sub group 1 (HIV seropositive individuals with OC); showed significantly higher rate of isolation of *C. albicans* as compared with sub group 2 which is comparable with other reports (Sanchez Vargas et al. 2005; Gupta et al. 2006;). In the present study, it is observed that the prevalence of NAC species were found more in HIV seropositive patients without OC, rather than the one with OC. However reports of increasing rate of isolation of NAC species were seen particularly in HIV seropositive patients with OC (Nadiger et al. 2008; Girish et al. 2009).

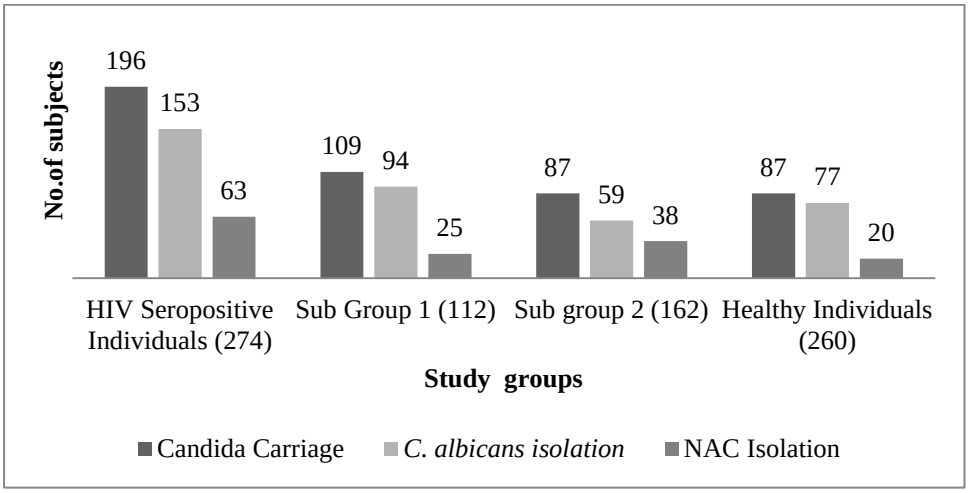


Fig. 4.10 Prevalence of *C. albicans* Vs NAC species (in percentage).

Note: In a few samples, mixed growth was observed and therefore total number of isolates when considered; exceeded the number of samples showing candida positive growth.

b. DISTRIBUTION OF NAC SPECIES

Various NAC *species* recovered in the present study are shown in Table 4.10. Distribution of NAC in the entire study population and in the individual groups is shown in Fig. 4.11**.** A total of 83 NAC species were isolated in the present study.

Table 4.10 Prevalence of NAC species in different study groups

	HIV Seropositive group		
NAC Species (n)	**Sub Group 1**	**Sub Group 2**	**HIV seronegative group**
C. tropicalis (27)	8	9	10
C. krusei (14)	3	10	1
C. guilliermondii (21)	7	9	5
C. zeylanoides (1)	1	0	0
C. parapsilosis (7)	0	6	1
C. glabrata (4)	1	1	2
C. kefyr (4)	3	1	0
C. lusitaniae (5)	2	2	1

There were a good number of NAC isolated in the present study from both HIV seropositive and HIV seronegative groups. *C. tropicalis* is the most predominant NAC isolated. A total of eight different NAC species were encountered in the study. *C. tropicalis* was the predominant isolate, with 27 out of 83 (32.53%) isolates among NAC species. It was followed by *C. guilliermondii*, 21/83 (25.30%) and *C. krusei,* 14/83 (16. 86%). *C. tropicalis* was the predominant NAC in both sub group 1 and HIV seronegative population with yielding 32% and 50% of the total NAC species respectively. However in sub group 2, *C. krusei* was the predominant NAC (26.31%; 10/38 of NAC isolates) species isolated. *C. tropicalis*, *C. glabrata*, *C. krusei* were the

most common NAC species found as oral colonizers in HIV seropositive individuals in the previous reports (Campisi et al. 2002; Vargas and Joly 2002; Gugnani et al. 2003; Ohmit et al. 2003; Sanchez – Vargas et al. 2005; Gupta et al. 2006; Liu et al. 2006a; Girish et al. 2009).

With regards to NAC isolation, the unique finding observed in this study is, isolation of *C. guilliermondii* as one of the predominant NAC species from both HIV seropositive and HIV seronegative subjects. This variation could be attributed to changes in geographical distribution of *Candida species*. It is important to note that *C. guilliermondii* shows high MIC for fluconazole, which are the common azoles used for treatment of OC (Menon et al. 2001). Predominant isolation of *C. guilliermondii* has been reported by Xu and Mitchell (2003) in their report on evaluation of healthy bjects. As a few other NAC speciesalso showed high degree of resistance to fluconazole, it is essential to speciate candida isolates from HIV seropositive individuals to facilitate the treatment of OC.

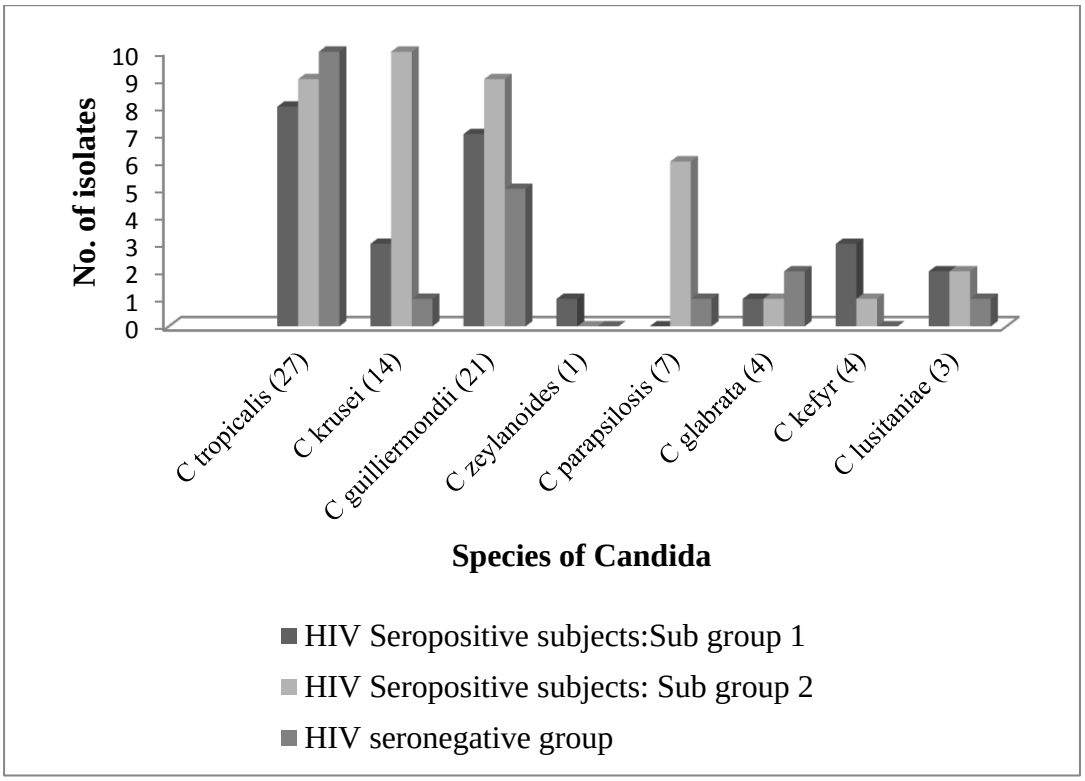


Fig. 4.11 Prevalence of each NAC species in the study population

Though *C. albicans* is the most common species to cause OC, rise in isolation of NAC both from OC and asymptomatic carriage is reported worldwide in recent years (Johnson et al. 1995; Samaranayake et al. 2001; Kevin et al. 2003; Lattif et al. 2004; Sanchez-Vargas et al. 2005) A few of these reports suggest that, increased prevalence of NAC species in HIV seropositive subjects may be due to previous exposure to fluconazole (Johnson et al 1995; Lattif et al. 2004; Nadiger et al. 2008).

4.1.2 FLUCONAZOLE SUSCEPTIBILITY PATTERN IN THE CANDIDA ISOLATES

Knowledge of general trend in fluconazole sensitivity pattern in candida isolates can give the necessary information on treatment strategies.

4.1.2.1 FLUCONAZOLE SENSITIVITY PATTERN IN CANDIDA ISOLATES FROM HIV SEROPOSITIVE SUBJECTS

A total of 153 *C. albicans* isolates from HIV seropositive group were evaluated for fluconazole sensitivity. Of these 94 isolates were from subgroup 1. Among these isolates, 88.29% (83/94) are sensitive to fluconazole (MIC < 8 µg/ml). Eight isolates (8.51%, 8/94) were resistant to fluconazole (MIC > 64 µg/ml). Dose dependant sensitivity was seen in 3.19% (3/94) (MIC between 16 to 32 µg/ml) of the isolates tested.

Table 4.11 Fluconazole sensitivity pattern in *C. albicans* Vs NAC

Species of Candida	Isolates from HIV Seropositive Individuals						Isolates from HIV Seronegative Group		
	Sub group 1			Sub group 2					
	Susceptible MIC< 8µg/ml **n (%)**	Susceptible (Dose dependent) MIC =16-32µg/ml **n (%)**	Resistant MIC>64µg/ml **n (%)**	Susceptible MIC< 8µg/ml **n (%)**	Susceptible (Dose dependent) MIC =16-32µg/ml **n (%)**	Resistant MIC>64µg/ml **n (%)**	Susceptible MIC< 8µg/ml **n (%)**	Susceptible (Dose dependent) MIC =16-32µg/ml **n (%)**	Resistant MIC>64µg/ml **n (%)**
C. albicans	83/94 (88.29%) ¶	3/94 (3.48%)	8/94 (8.5%)	52/59 (88.13%) §	3/59 (5.08%)	4/59 (6.77%)	73/77 (94.80%)	2/77 (2.59%)*	2/77 (2.59%)
NAC species	17/25 (68%) ¶¶	4/25 (16%)	4/25 (16%)	23/38 (60.52%)	6/38 (15.78%)	9/38 (23.68%)	12/20 (60%)	7/20 (35%) **	1/20 (5%)
Statistical correlation	¶, ¶¶, $\chi 2$ with Yates correction=0.96, DF=1, p> 0.05, not significant.			§, §§, $\chi 2$ with Yates correction =5.55, DF=1,p<0.05, significant.			*,**, $\chi 2$ with Yates correction = 0, DF=1,p=1, not significant.		

Recent studies have shown the increased resistance to fluconazole in *C. albicans* (Sanglardand Odds 2002; de. Bedout et al. 2003; Enwuru et al. 2008; Maninder et al. 2008; Nadgir et al. 2008). In the present study 88.29% of *C. albicans* isolates were sensitive to fluconazole and 8.5% were resistant. These findings are in accordance with that of de Bedout et al. (2003) and Enwuru et al. (2008). de Bedout et al. (2003) reported that 92.1% of *C. albicans* isolates were sensitive and 8% were resistant to fluconazole in their study. Similarly, Enwuru et al. (2008) reported that in their study, 86.7%of *C. albicans* isolates were sensitive to fluconazole and 10%were resistant. A study from North Karnataka showed 81.96% of *C. albicans* from OC cases were sensitive and 12.5% isolates were resistant to fluconazole (Nadiger et al. 2008). However this may be attributed to prolonged use of fluconazole which has lead to selection of drug resistant *C. albicans* or to expression of drug resistance in existing

sensitive strains of *C. albicans.* Johnson et al. (1995) showed that long term use of too low dose of fluconazole may be an important factor in the development of drug resistance in HIV seropositive patients with mucosal candidiasis; as such resistance was rare and transient in patients on intermittent short-term treatment.

Of the 59 *C albicans* isolates tested for fluconazole susceptibility, from subgroup 2, 88.13% (52/59) were sensitive to fluconazole. Fluconazole resistant isolates in this group were 6.77% (4/59). Dose dependent sensitivity was 5.08% (3/59). In sub group 2, i.e., HIV seropositive without OC, it was observed that 6.77% (4/59), *C. albicans* were resistant to fluconazole. Low level of fluconazole resistance (2.8%) was also reported from HIV/AIDS patients with only colonization in one of the previous reports (Xu et al. 2000). In another study Girish et al. (2009) reported that, 22.22% *C. albicans* isolated from HIV seropositive patients with asymptomatic candida carriage, were resistant to fluconazole.

Details of fluconazole sensitivity pattern of NAC isolates given in **Table 4.12**. NAC species appears to be consistently less sensitive to fluconazole than *C. albicans* among the sub group 1 and HIV seronegative subjects.

Table 4. 12 Fluconazole sensitivity patterns of NAC isolate among different study groups

	HIV Seropositive Group						**HIV Seronegative Group (20)**		
NAC Species of candida (n)	**Sub group 1 (25)**			**Sub group 2 (38)**					
	Susceptible MIC< 8µg/ml n (%)	**Susceptible (Dose dependent) MIC =16-32µg/ml n (%)**	**Resistant MIC>64µg/ml n (%)**	**Susceptible MIC< 8µg/ml n (%)**	**Susceptible (Dose dependent) MIC =16-32µg/ml n (%)**	**Resistant MIC>64µg/ml n (%)**	**Susceptible MIC< 8µg/ml n (%)**	**Susceptible (Dose dependent) MIC =16-32µg/ml n (%)**	**Resistant MIC>64µg/ml n (%)**
***C tropicalis* (27)**	6 (75)	1 (12.5)	1 (12.5)	7 (77.77)	1 (12.5)	1 (11.11)	9 (90)	1 (12.5)	-
***C krusei* (14)**	-	2 (66.66)	1 (33.33)	-	5 (50)	5 (50)	-	-	1 (100)
***C guilliermondii* (21)**	4 (57.14)	1 (14.2)	2 (28.57)	6 (66.66)	-	3 (33.33)	1 (20)	-	4 (80)

***C zeylanoides* (1)**	1 (100)	-	-	-	-	-	-	-	-
***C parapsilosis* (7)**	-	-	-	6 (100)	-	-	1 (100)	-	-
***C glabrata* (4)**	1 (100)	-	-	1 (100)	-	-	2(100)	-	-
***C kefyr* (4)**	3 (100)	-	-	1 (100)	-	-	-	-	-
***C lusitaniae* (5)**	2 (100)	-	-	2 (100)	-	-	1 (100)	-	-

Among 25 NAC isolates from subgroup 1, 68% (17/25) are sensitive to fluconazole. A 16% of the isolates (one isolate each of *C. tropicalis*, *C. krusei* and two isolates of *C. guilliermondii*) are resistant to fluconazole (4/25). Among 38 NAC isolates from sub group 2, 60.52% (23/38) were susceptible to fluconazole and 23.68% i.e., a total of 9 NAC (*C. tropicalis* - 1, *C. krusei*- 5, *C. guilliermondii* - 3) among the total 38 species are resistant to fluconazole.

Of the NAC isolates in sub group 1, 33.33% of *C. krusei* followed by *C. guilliermondii* (28.57%) and *C. tropicalis* (12.5%) were resistant to fluconazole. Mennon et al. (2001), showed in their study that *C. tropicalis* has higher MIC values for both fluconazole and itraconazole than *C. krusei* and *C. guilliermondii*. It is known that *C. krusei* shows intrinsic resistance to fluconazole (Kevin et al. 2003). In the present study also 50% of *C. krusei* showed resistance to fluconazole.

4.1.2.2 FLUCONAZOLE SENSITIVITY PATTERN OF CANDIDA ISOLATES FROM HIV SERONEGATIVE HEALTHY SUBJECTS

A total of 77 *C. albicans* were tested from HIV seronegative subjects. Out of these, 94.80% (73/77) *C. albicans* isolates were sensitive to fluconazole and 2.59% isolates were resistant (2/77) respectively which is presented in Table 4.11. Dose dependent sensitivity was seen in 2.59% isolates (2/77). Out of 20 NAC isolates 70% (14/20) were sensitive, 25% (5/20) were susceptible dose dependent and 5% (1/20) isolates were resistant to fluconazole as shown in Table 4.11 and 4.12.

Reports of recovery of resistant isolates from healthy persons who were never exposed to fluconazole exist (Goff et al. 1995; Xu et al. 2000). De-novo existence of fluconazole resistant strains in healthy persons was suggested by Xu et al. (2000). Therefore it can be suggested that, determination of MIC of fluconazole for *C. albicans* from individuals who never received fluconazole treatment is essential to treat the OC.

SUMMARY

There is increase in the oral candida carriage rate in the HIV seropositive individuals. HIV seropositive individuals with OC showed highest rate of candida carriage (97.32%). Rate of candida carriage in HIV seropositive individuals without OC (53.70%) was significantly more ($p<0.001$) compared to HIV seronegative healthy individuals (33.07%). In all the groups *C. albicans* was the most commonly isolated species (73.48%).

Here, one can also observe the predominance of *C. albicans* in infection process with increased resistance to antifungal agent, fluconazole. *C. albicans* isolates recovered from HIV seropositive group were more resistant compared to those recovered from healthy HIV seronegative group with showing 5.012% isolates being resistant to fluconazole (i.e., 3.48% in sub group 1, HIV seropositive subjects with OC and 6.77% in sub group 2, and HIV seropositive subjects without OC).

The isolation of 8 different NAC species shown in study groups with *C. tropicalis* (32.53%) was the predominant isolate followed by *C. guilliermondii*, 21/83 (25.30 %) and *C. krusei*, 14/83 (16. 86%). Isolation of *C. guilliermondii* in high number is one important outcome of this study, since this species develops resistance to fluconazole rapidly, the identification of the candida to species level and sensitivity testing is more vital.

Since it is also observed that there is an increase in oral candida carriage present in HIV seropositive patients, it was intended to screen the isolates from different study group for their adherence pattern onto Human Buccal Epithelial Cells (HBEC).

4.2 ANALYSIS OF ADHERENCE BEHAVIOR OF *C. ALBICANS* ISOLATES FROM HIV SEROPOSITIVE AND HIV SERONEGATIVE INDIVIDUALS ONTO HUMAN BUCCAL EPITHELIAL CELLS (HBEC)

The initial step in the establishment of colonization or disease occurs as the candida adheres onto the host cells. Several investigators have studied the adhesion pattern of *C. albicans* isolates from HIV seropositive patients by *in vitro* methods. There are contradictory reports found in the literatures on adherence pattern of isolates from HIV seropositive subjects (Imbert-Bernard et al. 1994; Sweet et al. 1995; Pereiro et al. 1997; Macura and Bort 2001). Therefore, in this part of experiments, oral *C. albicans* isolates from HIV seropositive individuals and healthy HIV seronegative individuals were evaluated for difference in their adherence behavior on HBEC.

4.2.1 RESULTS OF SCREENING STUDIES ON ADHESION OF *C. ALBICANS* ON HBEC

4.2.1.1 SCREENING STUDIES ON ADHESION OF *C. ALBICANS* ON HBEC COLLECTED FROM NORMAL HEALTHY HIV SERONEGATIVE INDIVIDUALS

A total of 19 oral *C. albicans* isolates from HIV seropositive group and 16 from HIV seronegative group were tested for the adherence to pooled normal HBECs. Gram stain preparations showing plain HBEC and HBEC adhered with *C. albicans* is given in Fig. 4.12a and 4.12b.

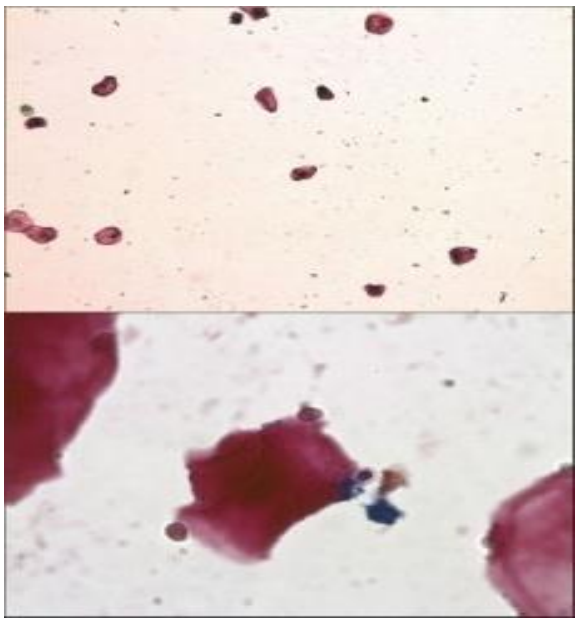


Fig. 4.12a. Gram stain preparation of HBEC collected from normal healthy individuals (×450x)

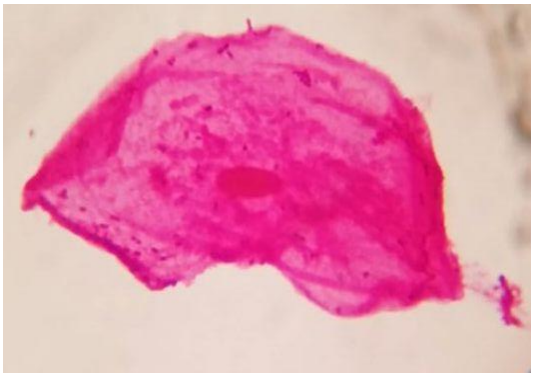


Fig. 4.12c. Gram stain preparation of HBEC unadhered with candida (×450x)

Evaluation of adherence of *C. albicans* isolates from HIV seropositive subjects and healthy individuals to normal, pooled; HBEC is presented in Table 4.13.

Table 4.13 Adhesion of *C. albicans* isolates from HIV seropositive and HIV seronegative group

		No. of. Isolates tested	**Percentage of HBEC showing adherence**	**No. of. candida cells /HBEC**
Isolates from HIV seropositive subjects	Total isolates	19	56.6	1.74
	Sub Group 1 (HIV seropositive subjects with OC)	6	55.5	1.8
	Sub Group 2 (HIV seropositive subjects without OC)	13	57.7	1.67
Isolates from HIV Seronegative subjects	Total isolates	6	29.1	1.33
Standard strain	*C. albicans* (MTCC 3017)	1	28.8	1.26

4.2.1.1.1 ADHERENCE PATTERN SHOWN BY ISOLATES FROM HIV SEROPOSITIVE GROUP

The isolates from HIV seropositive group showed more adherence to HBEC compared with the isolates of HIV seronegative group, with average percentage of HBEC adhered with candida being 56.61%. The average number of candida cells adhered per HBEC is 1.74. Adherence rate was slightly less for isolates from sub group: 1 (55.5%) compared to the isolates from sub group: 2, (57.7%), however, the difference was not significant.

4.2.1.1.2 ADHERENCE PATTERN SHOWN BY ISOLATES FROM HIV SERONEGATIVE GROUP

The isolates from HIV seronegative group showed average adherence rate of 29.1%. The average number of candida cells adhered per buccal epithelial cell is 1.33. Standard strain of *C. albicans* MTCC 3017 showed 28.77% of adherence on HBEC, with average of 1.26 candida cells adhering per HBEC.

4.2.1.1.3 STATISTICAL ANALYSIS OF THE RESULTS

The result from both the HIV seropositive and HIV seronegative groups is compared by using "t" test. *C. albicans* isolates from HIV seropositive individuals showed significant increase in the rate of adherence to the HBEC, compared to those isolates from HIV seronegative individuals with *p* value equal to 0.05, which is statistically significant.

4.2.1.2 ADHERENCE ASSAY OF CANDIDA ISOLATES ONTO POOLED HBEC COLLECTED FROM HIV SEROPOSITIVE INDIVIDUALS VS POOLED HBEC FROM NORMAL HEALTHY INDIVIDUALS

The adherence of candida isolates on HBEC isolated from HIV seropositive patients was more comparing to the results shown with adhesion on HBEC from normal healthy individuals. The average number of candida adhered per HBEC also increased with HBEC from HIV seropositive patients. However; the increase in adhesion was more with isolates from HIV seronegative group as shown in the table 4.14.

Table 4.14 Adhesion of *C. albicans* isolates from HIV seropositive and HIV seronegative group

		Results obtained with pooled HBEC from normal healthy individuals		**Results obtained with pooled HBEC from HIVseropositive individuals**	
Source for isolation	**Isolate tested**	**Percentage of HBEC adhered with candida**	**Average no. of candida adhered/ HBEC**	**Percentage of HBEC adhered with candida**	**Average no. of candida adhered/ HBEC**
HIV seropositive individuals	RL-112	80.55	2.68	83.73	5.52
	RL-24	72.56	2.03	84.61	5.72
HIV seronegative individuals	CN-192	41.69	1.158	75.84	3.83
	CN-102	55.75	1.95	72.08	3.35

C. albicans is an endogenous pathogen which is the most virulent of all pathogenic *Candida species* and is the most common cause of human candidiasis in immunocompromised hosts (Lima Neto 2009; Dos Santos Pinheiro et al. 2009).

Previous data showed higher rate of candida carriage and species variation in present in the HIV seropositive patients compared with HIV seronegative subjects. This variation may be due to several factors such as diet, oral hygiene, lack of access for HAART, long term treatment with fluconazole etc (Diz Dios et al. 2001; Menon et at. 2001; Gugnani et al. 2003; Nadagir et al. 2008; Girish et al. 2009). *C. albicans* was the most common isolate in both HIV seronegative and HIV seropositive subjects of this study. Increased association of *C. albicans* in the symptomatic stage may be resulting from the replacement of NAC by *C. albicans,* which may be attributed to its high adherence capacity to the HBEC in the presence of altered host immune response (Fidel 2006).

Recurrent oral infections with *C. albicans* are common in HIV patients and also *C. albicans* known to be more pathogenic than other species of candida. Therefore,*C. albicans* was selected to perform adherence study in the present research work. Lyon and Resende (2006) reported, that *C. albicans* adheres to host cells more than any other species of candida. This implicates the relationship between the adherence capabilities and ability of *C. albicans* to colonize mucosal surfaces. In this study, *C. albicans* isolated from the HIV seropositive individuals had shown strong adherence to normal buccal epithelial cells compared to the isolates from healthy individuals. Macura and Bort (2001) reported that the strongest adherence between fungi and epithelial cells isolated from a HIV seropositive subject. Similarly, in the present study also adherence was shown to be enhanced when epithelial cells isolated from HIV seropositive individuals were used. Pereiro et al. (1997) reported that the adherence of *C. albicans* isolated from patients in the initial stages of AIDS to oral mucous cells was less compared with the isolates from the subjects without HIV infection and adherence of *C. albicans* increased with the disease stage until it exceeded that of the normal subjects in proportion to the decrease in the CD4/CD8 ratio.

Interestingly, the percentage of adherence of *C. albicans* isolates from HIV seropositive subjects with OC (Sub group 1) (55.5%) was marginally less than the isolates from HIV seropositive individuals without OC (Sub group 2) (57.7%). Similar results from, Imbert-Bernard et al. (1994) showed that the adherence of most of the *C. albicans* isolates from HIV seropositive patients with clinical OC to HBEC was weak. Whereas Sweet et al. (1995) reported that, there is no significant difference in adherence detected between strains isolated from HIV seropositive or AIDS subjects, or between strains isolated from *C. albicans* carriers (low salivary *C. albicans* counts) or subjects with OC.

Adhesion is the corollary of interaction between the receptors on host cells and adhesin molecules on the microbe. A variety of factors govern adhesion. Change in adherence may be associated with the alteration of epithelial cell receptors in HIV seropositive subjects and change in the adhesins on candida strains (Mc Nutty et al. 2005). The change in the epithelial cell surface molecules of HIV seropositive individuals must have lead to increased adhesion in the present results also (Table 4.14). Thus studying the carriage rate of candida and their adherence throw light on the infection process. Findings of this study indicate that the increased adherence plays an important role in the pathogenesis of oral candida infections in the presence of predisposing condition like HIV infection.

4.2.2 ULTRA STRUCTURAL ANALYSIS OF ADHESION OF CANDIDA ON HBEC USING SCANNING ELECTRON MICROSCOPE (SEM)

The observations done in the present study indicated that candida cell substantially adheres on HBEC. Further studies were carried out to understand how they are adhered on HBEC and these studies were carried out by SEM analysis. Energy Dispersion X-ray Spectroscopy (EDX) was used to determine any change in the elemental composition before and after adhesion. The scanning electron microscopic observations were done with candida cells alone and when adhered to HBEC, and these results are presented in Fig. 4.13, 4.14. 4.15, 4.16.

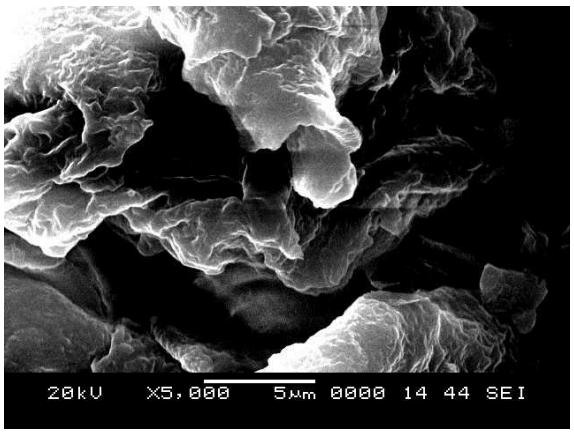


Fig. 4.13 SEM picture of HBEC

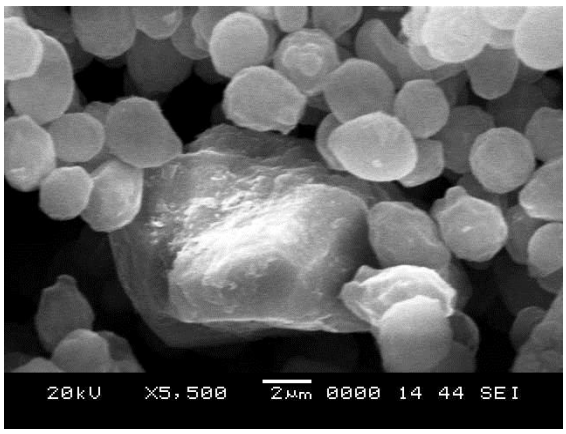


Fig. 4.14 HBEC with *C. albicans* (x 5,500)

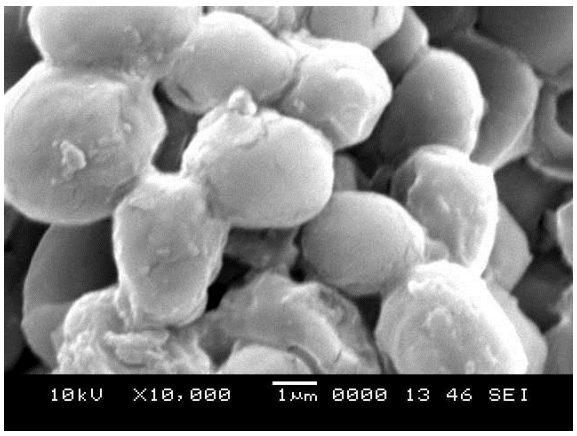


Fig. 4.15 SEM picture of *C. albicans* at 10,000x showing fibrillar budding structures

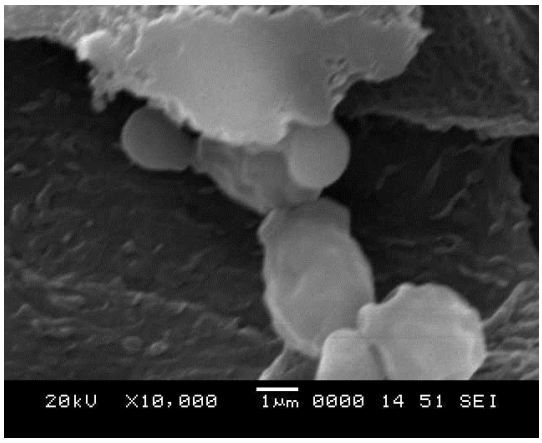


Fig. 4.16. *C. albicans* adhered on HBEC towards periphery of the HBEC, at 10,000x

Most of the candida cells presented with budding and presence of fibrillar structures was appreciable at 10,000x. In many instances the candida cells were seen adhered in towards the periphery of the HBEC**.** The findings of present study is in agreement with that of Henriques (2005a), who have observed that the candida cells adhered preferentially to the borders of outer surface of epithelial cells.

Ultra structural features on OC studied by Jayatilake et al. (2005) using Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) proved that though epithelial cells are not phagocytic in nature, during the process of candidiasis; yet they engulf candida through cytoplasmic process of encircling candida blastospores. Jayatilake et al. (2005) had used a tissue culture model based on Reconstituted Human Oral Epithelium (RHOE) and suggested that *C. albicans* invasion is a net result of interaction of physical, mechanical and enzymatic processes of the pathogen. The TEM pictures revealed that, hyphal elements penetrate the host cell membrane or traversing intracellular gap junctions of the epithelium and candida cells encircled by the epithelial cells and internalized in a manner similar to phagocytosis. They also observed cavitations on the host cell surface at the site of candida hyphal penetration and reported that this could be due to phospholipase activity.

The EDX was done to analyze types of elements present in all the samples and results are presented in Table. 4.15. a and b.

Table 4.15.a Details on the relative percentage of elements as measured with RL-24 and RL-112 in SEM: Experiment 1

At %	RL-24	HBEC	RL-24 with HBEC	RL-112	HBEC	RL-24 with HBEC
Total	100	100	100	100	100	100
Co K	0.03	-	0.01	-	0.01	0.03
Pb M	0.06	-	-	-		-
Cd L	0.00	-	-	-		-
Nb L	0.26	-	-	0.05		-
Zn K	-	0.06	0.05	0.00		0.06
Fe K	0.20	0.03	0.01	0.00	0.06	0.03
Ca K	0.04	0.02	0.04	0.00	0.02	0.02
K K	0.12	-	0.03	-	0.01	-
Cl K	0.03	0.00	-	0.00	-	0.00
S K	-	0.05	0.14	-	-	0.05
P K	0.29	0.02	-	-	-	0.02
Si K	-	0.03	-	-	0.00	0.03
Mg K	0.12	0.04	0.01	-	0.00	0.04
Na K	-	0.02	0.05	-	0.05	0.02
O K	21.04	14.01	14.97	8.89	33.66	14.01
N K	36.70	43.07	43.94	47.86	29.60	43.01
C K	41.14	46.27	40.25	43.80	36.59	42.67

Table 4.15.b Details on the relative percentage of elements as measured with RL-24 and RL-112 in SEM: Experiment 2

Mass %	RL-24	HBEC	RL-24 with	RL-112	HBEC	RL-112 with
C K	34.99	37.92	36.06	39.40	37.92	31.43
N K	36.40	44.57	45.34	49.58	44.50	29.64
O K	23.84	16.56	17.65	10.65	16.58	38.51
Na K	-	0.04	0.08	-	0.04	0.08
Mg K	0.21	0.08	0.02	-	0.08	0.01
Si K	-	0.06	-	-	0.06	0.00
P K	0.63	0.05	-	-	0.05	-
S K	-	0.11	0.34	-	0.11	-
Cl K	0.07	0.00	-	0.00	0.00	-
K K	0.32	-	0.08	-	-	0.02
Ca K	0.11	0.06	0.12	0.01	0.06	0.06
Fe K	0.80	0.13	0.04	0.02	0.13	0.22
Zn K	-	0.28	0.22	0.01	0.28	-
Nb L	1.71	-	-	0.34	-	-
Cd L	0.04	-	-	-	-	-
Pb M	0.88	-	-	-	-	-
Co k	-	0.12	-	-	0.12	-
Ni K	-	-	-	-	-	0.03
Total	100	100	100	100	100	100

Note (for both 4.15a and b): C; carbon, N;nitrogen, O;oxygen, Na;sodium, Mg; magnacium, Si;silicon, P;phosphorus, S;sulphur, Cl; chlorine, K;potassium, Ca; calcium, Fe; ferrous, Zn; zinc, Nb; Niobium, Cd; cadmium, Pb; lead, Co; cobalt, At %; relative percentage, and K; K orbit.

Candida glycan molecules in the cell wall play a role in candida- host interactions and other biological functions (Chaffin 1997). Glycoprotein are the proteins covalently attached to sugar units, either attached to the OH group of serine or threonine or through the amide NH_2 of asparginine (http://www.biology-online.org/dictionary/Glycoprotein). Glycan structures contain carbon, oxygen, hydrogen and nitrogen as the major structural elements. In the above table one can observe that the relative percentage of carbon is decreasing when candida adheres on to HBEC comparing to its percentage seen with plain candida cells, i.e., both RL-24 and RL-112. This may be due to the involvement of glycoprotein in adhesion of candida onto HBEC. Since the variation seen with relative percentage of other elements during different preparations was not persistent, no significant conclusion could be reached.

SUMMARY

It has been seen from the above results that, *C. albicans* isolates from HIV seropositive individuals showed significant increase in the rate of adherence to the normal HBEC, compared with those isolates from HIV seronegative groups with *p* value equal to 0.05, which is statistically significant. The adherence of candida isolates to HBEC collected from HIV seropositive individuals was also more, thus indicating that a few changes occurring even with the host cells under immunocompromised conditions. As a further procedure, the genetic similarity between the isolates was checked and compared with their adhesion behavior.

4.2.3 GENETIC ANALYSIS OF ORAL *C. ALBICANS* ISOLATES BY RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) AND COMPARISON OF THE GENETICALLY CONSTRUCTED GROUPS WITH THEIR ADHESION BEHAVIOR

The increase in both percentage of oral candida carriage and density of oral colonization was seen in HIV seropositive individuals. The oral candida isolates here showed enhanced adherence to HBEC, compared to the isolates from HIV seronegative individuals. With this background, the role of genetic changes occurring in isolates from HIV seropositive individuals was aimed to be determined in this part. The genetic similarity between isolates from HIV seropositive and HIV seronegative individuals was analyzed by a genotyping method called Randomly Amplified Polymorphic DNA (RAPD). The results obtained were utilized to construct the dendrogram and adhesion pattern of these isolates in relation to their genetic relatedness is compared and discussed.

RAPD analysis was performed with 48 oral *C. albicans* isolates. Among them 26 isolates were from HIV seropositive group and 22 isolates were from HIV seronegative group, details are given in Table 3.1 of Chapter "Materials and Methods". The primers used in the study are OPA-18 and OPE-18. The oligonucleotide sequences of these primers are given in Table 4.16. Photos of DNA profiles obtained for the two primers are given in fig, 4.17.

Table 4.16 Details of primers used in RAPD analysis

Primer name	Oligonucleotide sequence	G+C content (%)
OPE-18	5'-GGACTGCAGA-3'	60
OPA-18	5'-AGCTGACCGT-3'	60

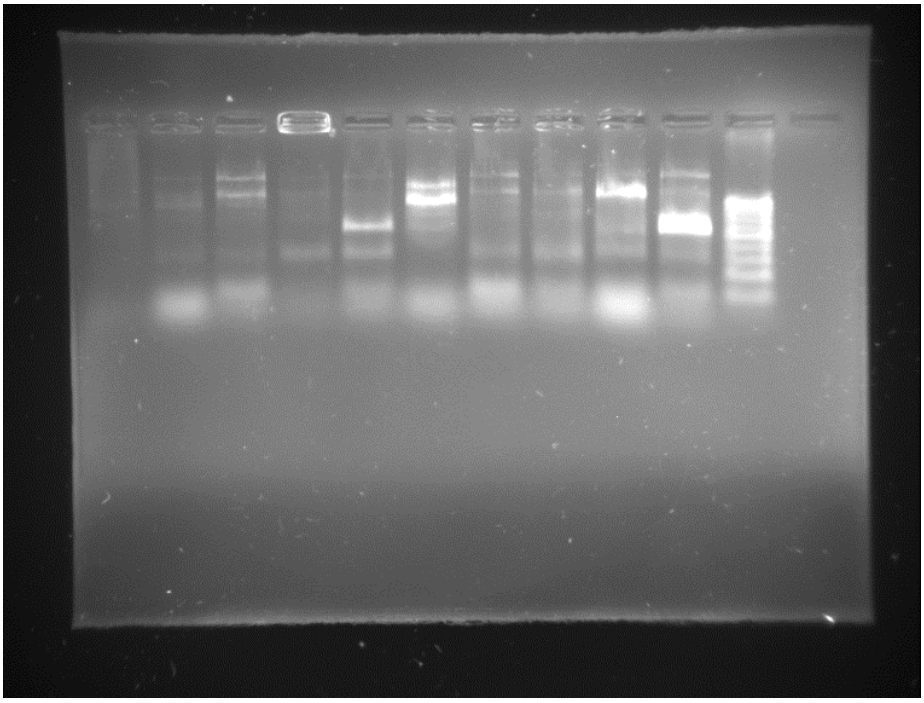


Fig 4.17 The RAPD profiles obtained for samples, From left CN-28, RL-09, RL-51, RL-11, RL-29, RL-08, RL-23, RL-112, RL-27, RL-14, Standard marker and CL-09 when tested with primer OPA-18.

Note: 12 Samples were run in each gel

4.2.3.1 RAPD ANALYSIS OF ISOLATES FROM HIV SEROPOSITIVE GROUP

Many of the samples did not amplify properly with OPE-18, but with OPA-18 good number of base pair bands was obtained. Table 4.17 gives the results obtained by samples from HIV seropositive group with both the primers.

Table 4.17 RAPD fragments derived from both the primers in isolates of HIV seropositive group

Sl.No	Isolates tested	Base pair bands obtained for each sample, with following primers	
		OPA-18	OPE-18
1	RL – 51	250, 570, 920, 1150,1350	1100
2	RL -11	250,1150,1350	560, 1100
3	RL – 29	250,570,1150,1350	560,1100,1200
4	RL – 8	570, 650, 720, 940, 1280	560, 1375, 1450
5	RL – 23	250, 720, 1150, 1350	1100, 1375
6	RL -112	250, 720,1150,1350	300,560,650
7	RL – 27	250, 570, 1100,1350,1400	1375
8	RL-14	250,570,1100,1350	650,
9	RL – 04	250, 570	650, 830, 1375
10	RL – 47	570, 650, 720	560, 650, 870
11	RL-17	570,650	560, 650
12	RL -13	250, 570, 650, 720, 1050, 1100	560, 870, 1100, 1200
13	RL -41	250, 570, 825	560,650
14	RL – 3	570, 650, 825	560
15	RL – 39	570	-
16	RL – 18	570, 650, 825,1050	650
17	RL – 09	570, 650, 825,1100	560

18	RL – 40	-	560
19	RL – 24	570,	300
20	RL – 20	-	300
21	RL - 29G	570, 650	-
22	RL- 02	650, 720, 825,	300,560
23	RL- 61B	650, 570	300
24	RL- 53	570	560

A. RESULTS WITH OPA-18

With OPA-18, the number of DNA fragments obtained was 250, 570, 650, 720, 825, 940, 1050, 1100, 1280 and 1350. Out of 26 isolates from HIV seropositive group, five of the isolates failed to give any DNA band with OPA-18. Out of the 22 isolates a few of them had similar type of reaction; e.g. as seen with RL-61B and RL-29G; RL-23 and RL-24.

A. **RESULTS WITH OPE-18**

With primer OPE-18, less base pair bands have been obtained. The DNA fragments obtained here were 300, 560, 650, 830, 870, 1100, and 1375. Among isolates from HIV seropositive group, 5 isolates (isolates RL-06, RL-26, RL-61B did not give bands with both the primers) did not give any DNA fragments with this primer. Many of the isolates resulted in production of single fragment. Three of the isolates failed to react and give DNA fragments with both the primers.

4.2.3.2 RAPD PROFILE ANALYSIS OF ISOLATES FROM HIV SERONEGATIVE GROUP

The isolates from HIV seronegative group also reacted less frequently with OPE-18. However the number of bands obtained for OPA-18 was lesser in these isolates compared to HIV seropositive group of isolates. The results are shown in Table 4.18.

Table 4.18 RAPD fragments derived from both primers in isolates of HIV seronegative group

Sl. no.	Isolates tested	Base pair bands obtained for each sample	
		OPA-18	**OPE-18**
1	CL-28	250, 720	300, 560, 940
2	CL-09	250, 920, 1150, 1350	1100
3	CN-172	250, 720	560, 830
4	CN-163	720, 1050	560, 830
5	CN-173	720, 1050	700, 830
6	CN-7/1	570, 720	750
7	CN -102	720, 1050	-
8	CN -31	720, 1050	560
9	CN -162	250, 570, 650, 725	560
10	CN -67	250, 570, 650, 720, 725	-
11	CN-28	250, 570, 720	-
12	CN-147	250, 650	-
13	CN-136	250, 570	-
14	CN -149	250, 570, 650	300,
15	CN-65	250, 570, 650	-
16	CN-111	570	-
17	CN-181	250,570,825	560, 830
18	CN-176	570	560, 940

19	CN-194	570, 650, 720, 1100	-
20	CN -192	570, 650	-
21	CN-188	570, 720	-

A. RESULTS WITH OPA -18

With primer OPA-18, one isolate did not give any band. A total of 21 isolates gave DNA polymorphism with OPA-18 and they exhibited 14 different sequences of DNA fragments either singly or in multiple. The DNA fragments obtained were, 250, 570, 650,720, 725, 1100, 1150 and 1350. DNA fragments 825, 940, 1050, 1280 which were obtained in isolates from HIV seropositive group were not present in isolates from HIV seronegative group. Similarly, DNA fragments 725, 1100, 1150 which were found in isolates from HIV seronegative group were not seen in isolates from HIV seropositive group. CN-163, CN-173, CN-102, CN-31 were shown similar results with giving two amplified DNA fragments, i.e., 720 and 1050. CN-149 and CN-65 gave 250, 750, 650 RAPD fragments. When observed manually many of the isolates look similar to each other by producing the same RAPD bands.

B. RESULTS WITH OPE - 18

With Primer OPE-18, the bands of DNA fragment obtained were 300, 560, 750, 830, 940 and 1100. Three bands 650, 870, 1375 which appeared in isolates from HIV seropositive groups were absent here. DNA fragments like 750, 940 were present only in HIV seronegative groups. Among 11 isolates which were positive for OPE-18 primer polymerization, gave 8 different sequences of DNA fragments either single or in multiple. A total of 13 isolates did not give any bands with OPE-18. Among the isolates which gave bands, 3 isolates namely, CN-172, CN-163, CN-181 gave similar results. Overall results with OPE-18 are presented with less number of fragments and many of the samples resulted in single fragments.

As a summary, when the RAPD profiles were assessed manually, it is found that two isolates from HIV seronegative group yielded similar polymorphic profiles with isolates in HIV seropositive group. When the isolates which may have given more than a single band, were considered two of them, i.e., CN-136 from HIV seronegative group and RL-04 from HIV seropositive group were given similar reaction (DNA base pair bands 250 and 570) when tested with OPA-18 primer. Among the two primers, many isolates failed to react with OPE-18. The polymerization did not occur for 19 (5 isolates from HIV seropositive group, 13 isolates from HIV seronegative group) isolates, when tested with OPE-18 primer. A few isolates, gave only single band with this primer. Therefore, only 38 isolates out of 48 were available to construct the dendrogram and to compare their adhesion behavior.

4.2.3.3 CONSTRUCTION OF THE DENDROGRAM

According to the results obtained in RAPD profiling, dendrogram was constructed using unweighted pair group control method. The strains were placed at different clusters according to their similarity co-efficient calculated and were subjected to dendrogram construction. The adhesion pattern obtained for each isolate in a cluster is compared further the clusters within and between the clusters.

It was found through the results obtained with 38 isolates that all these isolates were falling onto basically 4 groups, as shown in Fig. 4.18.

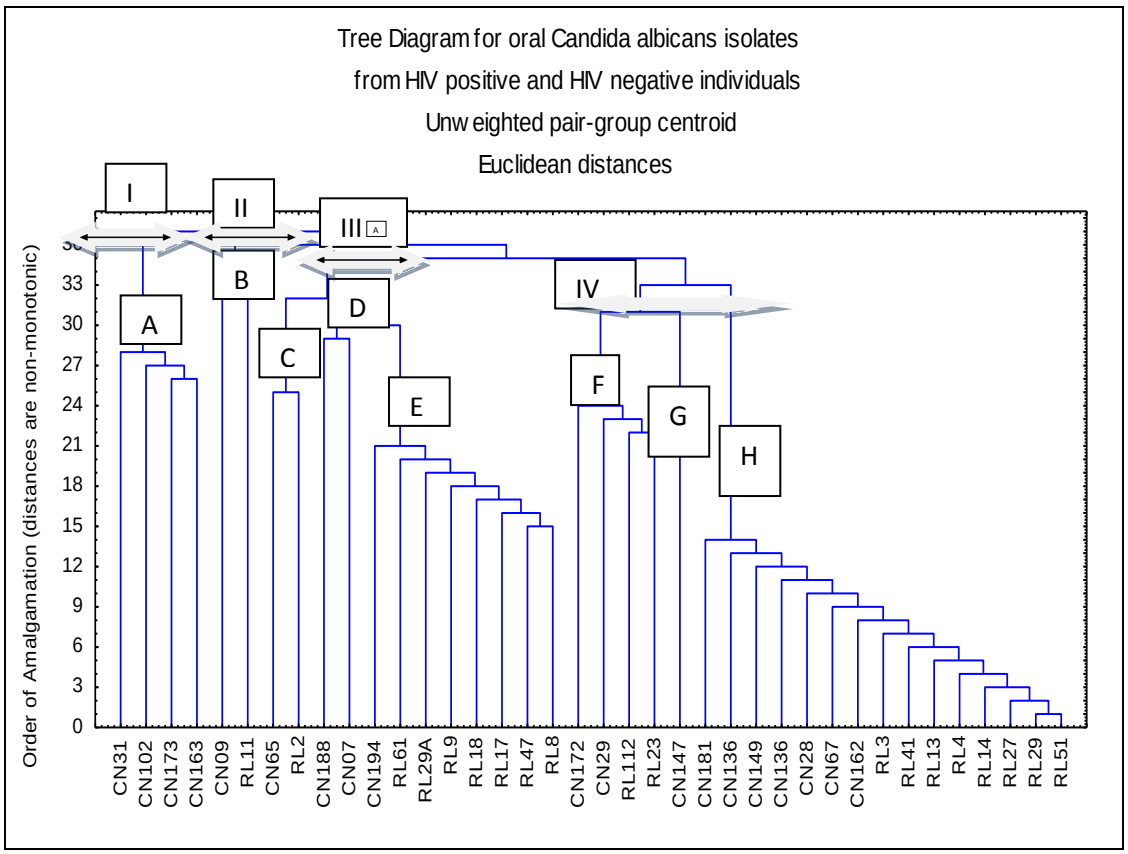


Fig. 4.18 Dendrogram based on the computed distances between 38 oral *C. albicans* isolates from both HIV seropositive and HIV seronegative individuals

According to the dendrogram formed, all the isolates tested were basically placed in 4 groups. Group I contains 4 isolates, which are from HIV seronegative group. Group II contains 2 isolates, one each from HIV seropositive group and HIV seronegative group are present here. Both Groups I and II contain only one isolate from HIV seropositive group. Group III contains 12 isolates, which were again divided into 3 subgroups. The third sub group here contains 7 isolates from HIV seropositive group. Group IV, is again a mixture of isolates from both the groups, among the 20 isolates found here 10 isolates from HIV seropositive group and 7 of them are placed in same sub groups. Remaining 10 of this group was the isolates from HIV seronegative group.

There are many different typing methods present for epidemiological typing of *C. albicans* namely, serotyping, biotyping, resistogram etc (Pizzo et al. 2005). One

can find the DNA microevolution occurring between the *C. albicans* isolates by genetic fingerprinting methods and correlate these results to explain the variations found in phenotypic expression (Bello et al. 2002). Variation in the gene expression can be analyzed in each strain, based on specific tandem repeats, sequences randomly distributed over the genome, which are highly polymorphic between the strains (Samaranayake et al. 2003 b). The genetic relatedness thus obtained by genetic fingerprinting can be utilized to find out the genetic distance between the strains of same species and isolates. While constructing the dendrogram, isolates will be placed as identical, related or non-related. The genetic finger printing technique can thus place *C. albicans* isolates into different groups, and place them in different clades according to their similarity co-efficient. A specific clade is formed by single ancestor and its descendants.

In the present part of the research work oral *C. albicans* isolates from HIV seropositive and HIV seronegative groups have been analyzed through RAPD analysis for their genetic relatedness and grouped according to their genetic profiles. Yet generally, RAPD has not been preferred by many investigators, due to its lack of reproducibility, though it is an easy and rapid method to perform and use for studying candida (Pujol et al. 1997; Bello et al. 2002). The primer used in the present study, i.e., OPA -18 shown reproducibility in the results of study by Gyanachandani et al. (1998), where set of 21 primers were tested for genotyping of *C. albicans*. The study with combining FTIR analysis and RAPD done by Sandt et al. (2002), suggested, also that these two procedures together can form strong epidemiological tool. The integrated approach can help in identifying the origin of an opportunistic infection. So using RAPD cannot be entirely disadvantageous for genetic finger printing.

Candida is a commensal of the oral cavity, and the number of colonizing candida increase with HIV infection. Colonization is the first event that is essential for infection and colonizing *Candida species* are frequently involved in the formation of OC (Patel et al. 2012). In the study by Hellstein et al. (1993), showed that major cluster of genetically similar *C. albicans* isolates contained 31% and 33% of strains from commensal and pathogenic group separately. Their result suggested common clonal origins for commensal and pathogenic strains in the same geographical locale, as in a dendrogram formation. In the present research work also one can see, in group

IV, sub group H contains isolates from both the groups in nearly the same number, i.e., 7 isolates from HIV seronegative and 8 isolates from HIV seropositive groups and they are placed together here. Vargas and Joly (2002), showed that out of 54 HIV seropositive patients monitored for candida carriage and genetic variation in candida isolates of their oral cavity, 54% (n = 6) patients who developed OC maintained genetically similar strains throughout the study period, with minor genetic variations. Remaining patients showed different patterns of variations throughout the study period such as, multiple strains, strain replacement and species replacement. When genetic profiling, is observed the most of the isolates from HIV seronegative group are closely related and were placed together with isolates from HIV seropositive group. Similar to this work, using the same primers in RAPD analysis intra specific polymorphism was previously demonstrated by Samaranayake et al.(2003b), among *C. albicans* isolates from HIV seropositive patients with and without buccal candidiasis. In HIV infection, changes in epithelial cell surface, reduction in rate of salivary flow, changes in oral microflora and changes in the immune status can be demonstrable. These factors may contribute to the selection of candida strains which are more virulent than the existing strains (Fidel 2006). *C. albicans* undergo spontaneous high-frequency switching under the influence of this surrounding environment. The switching thus caused will be reflected in their gene expression (Ernst 2000). The mutations occurring in candida can lead to micro variations due to various mechanisms, like mitotic cross over, recombination etc. (Poulter 1987).

C. albicans is diploid species, therefore can lead to generous heterozygosis (Poulter 1987). However, a few scientists reported predominant reproduces in a clonal mode according to previous references (Pujol et al. 1993; Anderson et al. 2001; Tavanti et al. 2004) Samaranayake et al. **(**2003b) reported that, the occurrence of genetic drift in *C. albicans* isolates was present in the oral cavity of HIV seropositive patients over a period of 12-months.

There are reports saying that the *C. albicans* strains colonizing the oral cavity are subjective to all the above said selective pressures which cause few genotypic and phenotypical changes and lead to emergence of strains with altered characteristics. The HIV seropositive patients tend to be colonized with single endogenous strain which can lead to repeated episodes of OC (Vargas and Joly 2002). In contrast to

these reports, a few studies show that in AIDS patients, original commensal can be replaced by entirely different genotype containing strains and this replacement occurs only once may be in the early stages of HIV infection or in some instances (Lupetti et al. 1995). In an infected area, there will be the presence of mixture of cells with nearly identical genome. The variation here expressed was known to be resulted from recombination and chromosomal ploidy shifts (Odds et al. 2006). In the present study also it has been shown that the strains from HIV seropositive individuals were placed in the same clusters where the isolates from healthy HIV seronegative individual oral cavity were present.

In the present study, only 20% (4 out of 20 isolates) of isolates from HIV seropositive patients gave similar genetic profiles with OPA-18 primer. With OPE-18, a total of 4 groups of isolates were found to have same genetic similarity. González et al. (2006) reported that profiles of RAPD analysis in *C. albicans* from buccal cavity of immunocompromised patients showed up to 85% similarity in their study.

4.2.3.4 DENDROGRAM BASED STUDIES ON RAPD PROFILES OF THE ISOLATES FROM ENTIRE STUDY GROUP AND THEIR COMPARISON WITH ADHESION BEHAVIOR

The RAPD profiles of *C. albicans* isolates in the study were compared with their adhesion pattern. Dendrogram formed among different groups and sub groups is shown in Table 4.19.

Table 4.19 Adhesion pattern seen among *C. albicans* in the groups and sub groups formed according to the dendrogram cluster formation

Groups	Sub Groups	No. of .isolates placed	No. of isolates from HIV seropositive group	No. of isolates from HIV seronegative group	** Percentage of epithelial cells adhered with candida	** No. of candida cells per HBEC
I	A	4	0	4	41.9875	1.255
II	B	2	1	1	37.13	1.3
III	C	2	1	1	46.53	1.45
	D	2	0	2	41.32	1.365
	E	8	7	1	61.158	1.46
IV	F	4	2	2	46.872	1.93
	G	1	0	1	38.46	1.79
	H	15	8	7	48.95	1.93
Total isolates tested		38	19	19		

Note: **Average percentage of adhesion was taken by adding the results obtained from all the members of the group

By referring to the Table 4.19, it is understood that the average percentage of HBEC adhered with candida didn't differ much between the Group I and II. When compared to adhesion pattern in group III, with 2 sub groups C and D, isolates from HIV seronegative group were in majority (2/3). Sub group E in the same group, mainly contains isolates from HIV seropositive group (7/8) showed high adhesion percentage compared to other sub groups. In group IV, subgroups F, G, H are included. Sub group H contains more number of isolates i.e., 15, with 7 from HIV seronegative group and 8 from HIV seropositive group. The adhesion percentage values for all the sub groups were 46.87%, 38.46% and 48.95% respectively. Here also the subgroup containing HIV seropositive isolates yielded highest adhesion i.e., 48.95% and also showed increase in average number of yeast cells adhered per HBEC. Though the adherence percentage for each strain was differing between HIV seropositive and HIV seronegative group and between the groups formed by RAPD profiling, yet large variation is not seen among the main groups or subgroups.

In the dendrogram construction, it is observed that isolates from HIV seropositive groups are commonly placed together. Same is true with isolates from HIV seronegative group. The isolates from both the groups are also seen in the same subgroup as in the case of sub group H in group IV. The sub group E of group III formed with more number of isolates from HIV seropositive group and showed highest percentage of adhesion as shown in Table 4.19.

When adherence behavior of each group of isolates is compared, there is no notable difference found among the groups of different isolates as formed in dendrogram. In accordance to these results, Xiaogang et al. (2003) compared *C. albicans* isolates from oral cavities of healthy volunteers, vagina of patients with vaginal candidiasis and from the environment for biofilm formation ability and genotypes. It has been found that, isolates were differing in their ability to form biofilm, cell surface hydrophobicity (CSH) according to their sources. The ability to form biofilm compared to genotypic variation revealed little correlation between biofilm formation and Multilocus genotypes as determined by PCR-RFLP at 16 polymorphic loci, regardless of source of strain. Strains with the same or similar Multilocus genotypes often showed different biofilm formation abilities. In contrast to

this, Zeng et al. (2008) performed genotyping of *C. albicans* isolates from Oral lichen planus, and accordingly obtained 4 types of genotypic profile. The isolates here showed statistically significant association with genotyping profiles and disease conditions. Adhesion and phospholipase activities of isolates also had significant correlation with genotyping profiles of the isolates.

SUMMARY

By comparison of RAPD genotypic profiles of the isolates in the present study it is found that, isolates from HIV seropositive group exhibit similarity to isolates from HIV seronegative group. As seen in isolates from HIV seropositive group, 3 of the isolates had given identical pattern of DNA polymerization. Genetic profiles of other isolates in this group are dissimilar, particular combination of band positions were confined to single isolates. In case of isolates from HIV seronegative group, 4 of the isolates had similar genetic profiles. However, most of the strains from HIV seronegative or HIV seropositive group were gathered into separate groups. By the present study, conclusions can be made that isolates from HIV seropositive individual genetically did not differ largely from HIV seronegative group isolates. Major cluster formed in dendrogram construction of this study had almost similar number of isolate from both the group. Though the adhesion pattern of individual strains in the group changes, dendrogram groups formed between isolates from HIV seropositive and HIV seronegative individuals have less genetic variability and they are placed together. Therefore, variation in adhesion and increased colonization, is not seen to be directly associated with genetic changes. The commensal candida isolates from HIV seronegative group were placed in the same cluster as with the isolates from HIV seropositive group, suggested that the isolates causing OC in HIV infection could be emerging from endogenous flora itself.

4.3 EVALUATION OF ADHESION OF CANDIDA AT VARIOUS EXPERIMENTAL CONDITIONS

Adhesion of *C. albicans* is reported to be varying depending on the experimental conditions provided (Senet 1998). In the present study, adhesion of *C. albicans* onto HBEC was evaluated under experimental conditions like, growth phases of candida, adhesion at different intervals of incubation period, different suspension media like PBS (pH 7.2), Artificial Salivary Medium (ASM) and different salt concentrations in PBS.

4.3.1 ADHESION AT DIFFERENT INTERVALS OF CONTACT TIME

Adhesion of candida at various time intervals of incubation was done and the results are shown in Table 4. 20 and Table 4.21. Two isolates, namely RL-112 and RL-24 were used in this study. Adhesion of candida onto HBEC attained constant values after 60 minutes of contact with the HBEC in both the isolates**.**

4.3.1.1 ADHESION PATTERN SEEN WITH ISOLATE RL-112

The data obtained are shown in Table 4.20. In isolate RL-112, 20.4% HBEC were adhered with *C. albicans* on only 20 minutes of incubation. The percentage of HBEC adhered with candida was increasing till 60 minutes of contact time by reaching a maximum of 45.6% adhesion. The adhesion on the HBEC was then shown to be running in nearly constant values, however at 180 minutes of incubation; again there was an increase in percentage of adhesion, and this increase continued up to 200 minutes of incubation also.

Table 4. 20 Adherence of isolate RL-112 onto normal HBEC at various time intervals

Sl. No	Time of Exposure (in minutes)	% of HBEC adhered with Candida (RL-112)	Average candida cells per HBEC
1	20	20.4	1.2
2	40	34.6	1.34
3	60	45.6	1.5
4	80	42.3	1.7
5	100	44.7	1.4
6	120	46	1.2
7	140	45.8	1.3
8	160	42.3	1.1
9	180	44.06	1
10	200	45.2	1.4

4.3.1.2 ADHESION PATTERN WITH ISOLATE RL-24

The data are displayed in Table 4.21. Here the percentage of HBEC adhered with candida was 23.2% at 20 minutes of contact time. The adhesion percentage here, increased till 80 minutes of incubation. The adhesion after 80 minutes was decreasing slightly, however the percentage was again increasing slightly towards 140, 160, 180, 200 minutes of incubation. In both the isolates the slight increase in adhesion though observed after 60 minutes, the values were running almost constantly.

Table 4. 21 Adherence of *C. albicans* isolates RL-24 to normal HBECs at various time intervals

Sl. No	Time of Exposure (in minutes)	Percentage of HBEC adhered with Candida (RL-24)	Average candida cells per HBEC
1	20	23.2	1.01
2	40	28.7	1.24
3	60	54.3	1.38
4	80	54.5	1.45
5	100	53	1.32
6	120	52	1.30
7	140	54.7	1.33
8	160	55	1.32
9	180	54.4	1.31
10	200	54.6	1.36

The average number of candida cells adhered per HBEC was changing, according to the contact time maintained. Both in RL-112 and RL-24, the average number of cells adhering per HBEC was less at 20 minutes, which was increased a little at 40 minutes and reached highest at 80 minutes of incubation. The incubation period for more than 80 minutes showed constant results in percentage of HBEC adhered with *C. albicans*. Both the isolates showed increase in average number of candida per HBEC at 200 minutes.

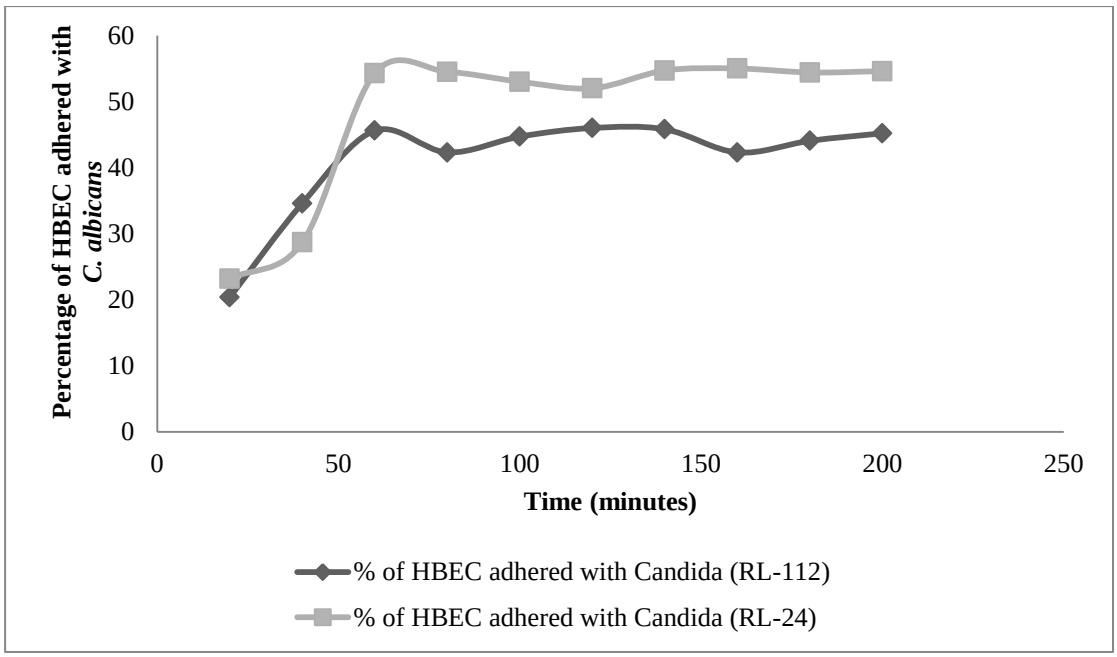


Fig. 4.19 Pattern of adhesion shown by both RL-112 and RL-24 isolates, when incubated with HBEC at various time intervals

According to the results with strains RL-112 and RL-24, the time of exposure was fixed as 60 minutes for later experiments. These results are in accordance with the study by Henriques (2005a), who showed that number of candida cells adhered to epithelial cells achieved a constant value after 60 min of contact with the surface of epithelial cells.

4.3.2 ADHESION ACTIVITY OF *C. ALBICANS* ALONG WITH DIFFERENT GROWTH PHASES

The growth curve of *C. albicans* was studied, to understand the different phases of growth. Effect of growth phases on adhesion activity of *C. albicans* cells was analyzed for the establishment of conditions required for further studies.

4.3.2.1 RESULTS WITH RL-24

The log phase for RL-24 began at the end of 10^{th} hr which was up to 19 hrs of incubation. Stationary phase was seen from 20^{th} to 41^{st} hrs of incubation. The variation shown at growth phases is explained in Fig. 4.20 for this isolate.

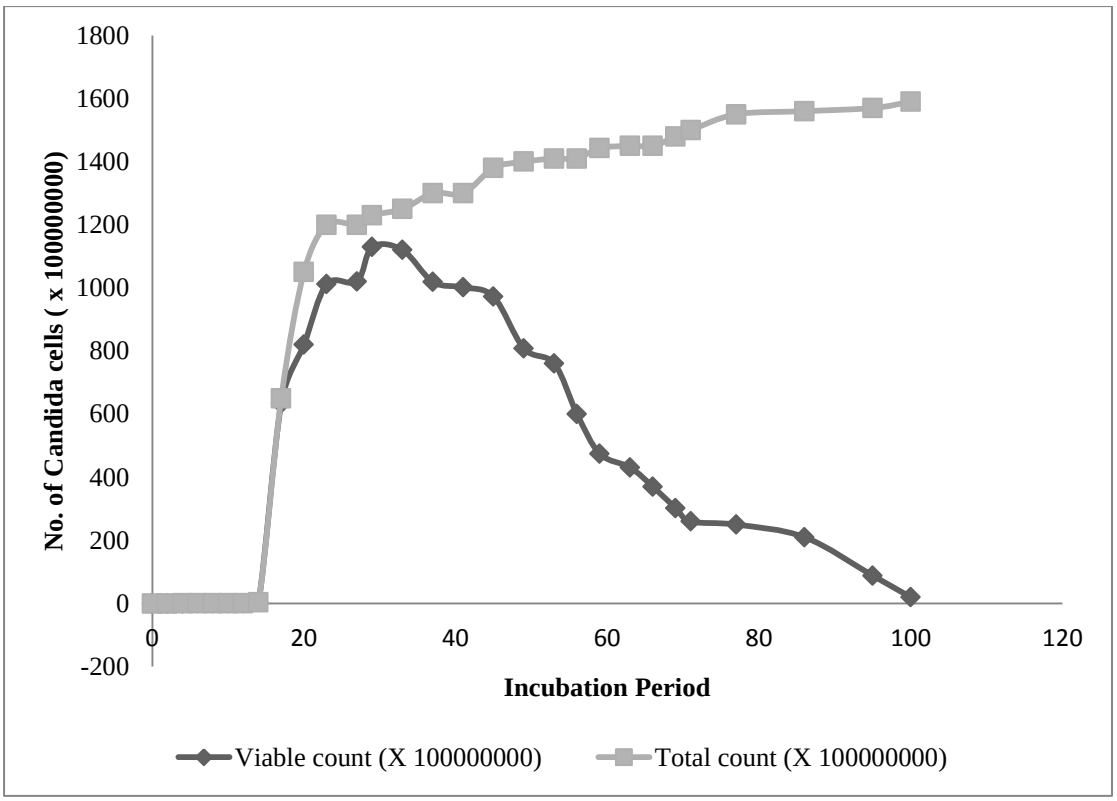


Fig. 4.20 Growth curve obtained for isolate RL-24

Higher candida adherence to HBEC was observed from 17th to 29th hrs (i.e., end of log phase and middle stationary phase) as shown in Table 4.22 and Fig. 4.21. Highest rate of HBEC adherence was noted at 23 hrs old candida cultures, i.e., in the stationary phase**.**

Table 4.22 Adhesion activities calculated in isolate RL-24, along the growth phases

Sampling Number	Incubation Period	% of HBEC adhered with candida cells	Average Candida cells/BEC
A1	0	20.78	1.13
A2	2	21.56	1
A3	4	21.36	1
A4	6	18.18	1
A5	8	23.33	1
A6	10	22.22	1.18
A7	12	25	1.3
A8	14	22.22	1.33
A9	17	45.45	1.4
A10	20	50	1.9
A11	23	62.5	1.9
A12	27	33.33	2
A13	29	32.72	1.83
A14	33	19.23	1.3
A15	37	21.11	1.43
A16	41	25	1.37

A17	45	21.42	1.4
A18	49	25.38	1.25
A19	53	27.39	1.2
A20	56	26.66	1
A21	59	24.7	1
A22	63	28.57	1
A23	66	20.52	1
A24	69	20.68	1
A25	71	23.04	1
A26	77	25.78	1
A27	86	26.66	1
A28	95	25.17	1
A29	100	24.79	1

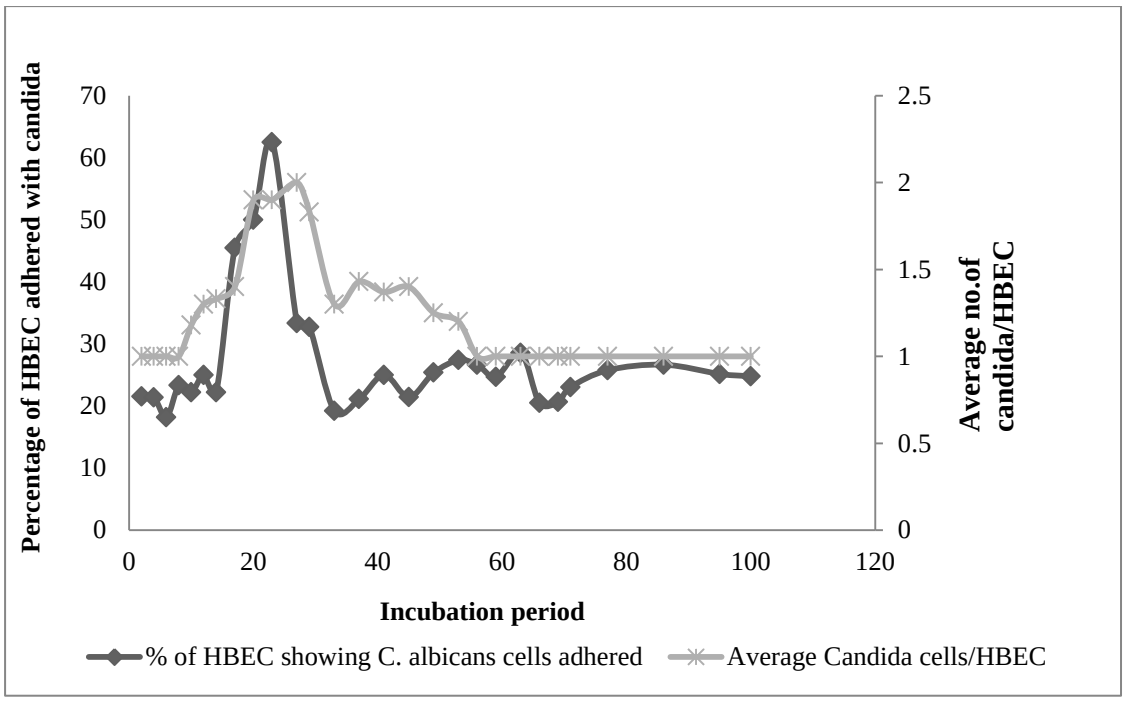


Fig. 4.21 Adhesion of candida isolates RL-24 along with its growth phase

4.3.2.2 RESULTS WITH RL-112

Isolate RL-112, showed log phase from the end of 8th hr which ended by 19th hrs of incubation. Stationary phase was seen from 20th hr to 49th hrs of incubation. Fig. 4.22 gives growth curve of strain RL-112.

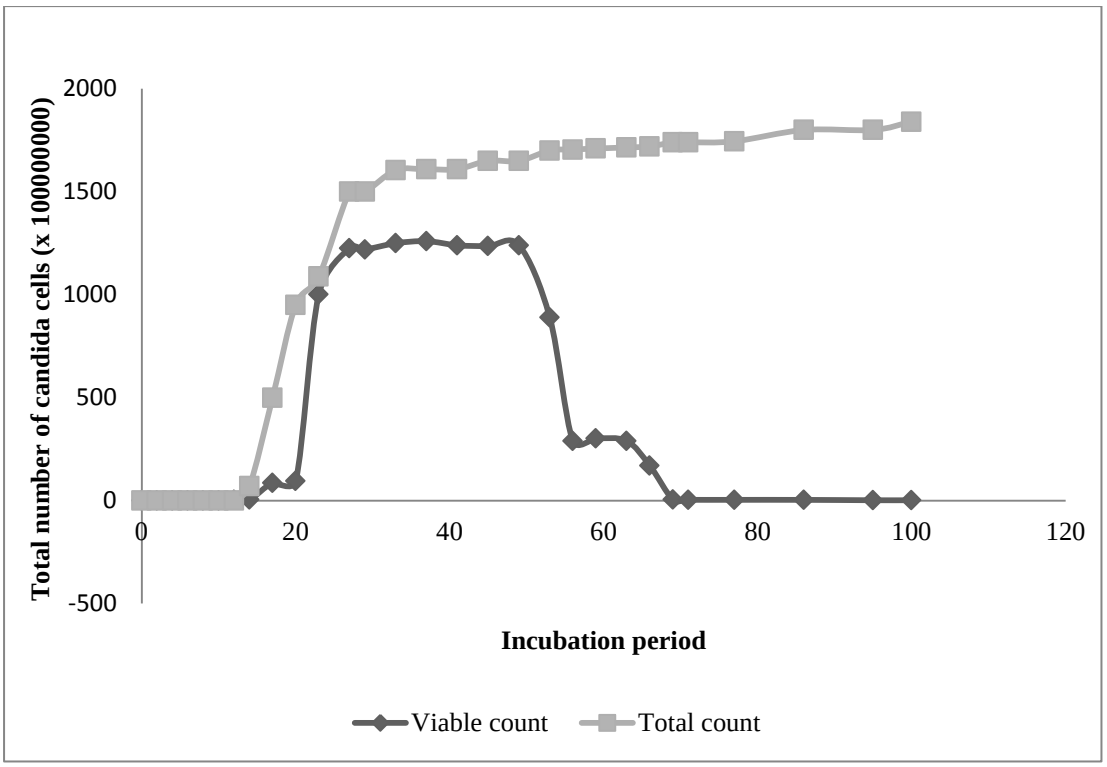


Fig. 4.22 Growth curve obtained for isolate RL-112

Higher rate of candida adherence to HBEC was observed from 14th to 33rd hrs (i.e., middle of log phase and middle stationary phase). Highest rate of candida adherence to HBEC was seen with 20 hrs (stationary phase) old candida cells as shown in Table 4.23 and Fig. 4.23. The cells at 41st to 45th hr were also showed high adherence, where cells were presented in stationary phase of their growth curve. After 49th hrs, this isolate entered into the phase of decline. The adhesion was less with cells taken from phase of decline.

Table 4.23 Adhesion activity as calculated for isolates RL-112 along with its growth phases

Number	Incubation Period	Percentage of HBEC adhered candida cells	Candida cells/HBEC
B1	0	17	1.2
B2	2	17.39	1.08
B3	4	21.73	1
B4	6	15.5	1
B5	8	13.33	1.02
B6	10	15.71	1.14
B7	12	21.05	1.16
B8	14	31.25	1.2
B9	17	36.73	1.22
B10	20	43.33	1.25
B11	23	37.5	1.3
B12	27	39	1.3
B13	29	37.58	1.33
B14	33	30.43	1.33
B15	37	29.04	1.6

B16	41	35	1.69
B17	45	35.29	1.87
B18	49	32.42	1.6
B19	53	30	1.25
B20	56	23.8	1.33
B21	59	16.66	1.4
B22	63	17	1.5
B23	66	20	1.3
B24	69	17.4	1.2
B25	71	18.88	1
B26	77	19.54	1.5
B27	86	13.33	1.23
B28	95	16.31	1.25
B29	100	9.52	1.3

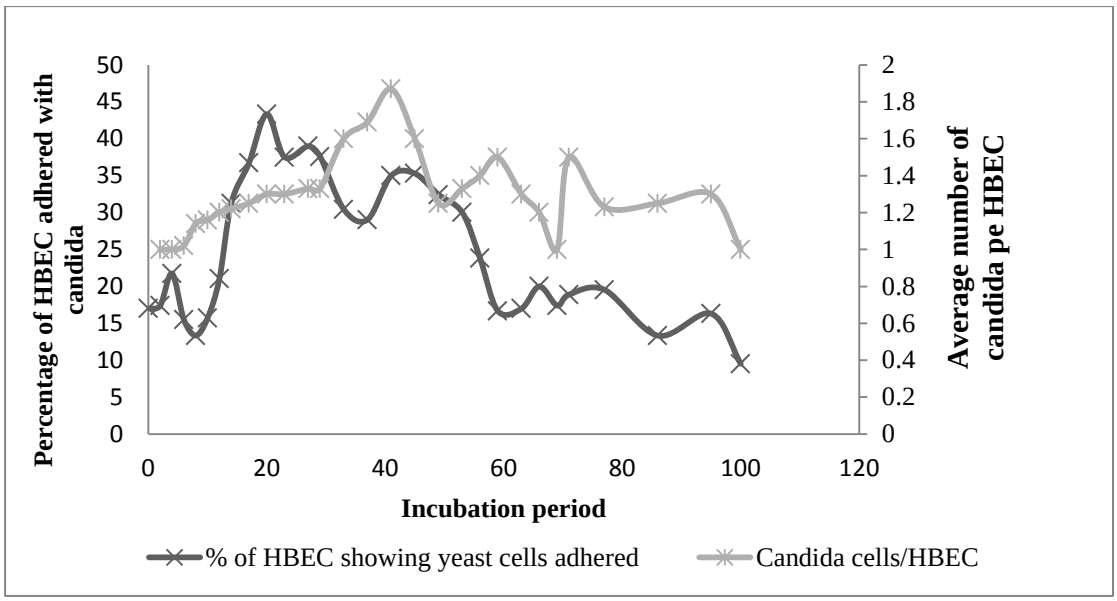


Fig. 4.23 Adhesion of RL-112 along with its growth phases

In the present study, it was observed that the adhesion of candida cells is high, at its stationary phase. Beggs (1985), study on adhesion to vaginal epithelial cells, proved that adhesion was greater with candida cells from stationary phase. Calderone and Braun (1991), in their review on adhesins of *C. albicans*, had reported the expression of mannoproteins in this fungus might depend on the growth phase and morphological forms. The effect of Cetyl Pyridinium Chloride (CPC), studied in connection with adhesion of candida on HBEC, showed preincubation of candida blastospores with CPC resulted in reduction of adhesion and reduction in Cell Surface Hydrophobicity (CSH) of the candida cells. The adhesion was significantly reduced with cells from stationary or exponential growth phases which were preincubated with CPC (Jones et al. 1995). These references thus emphasize the importance of understanding of the growth phases in candida adhesion related research. Another report showed adhesion is not dependent on growth phases (Dhadwar et al. 2003). Dhadwar et al. (2003) tested adherence of *Candida utilis* on cell supporting substrate coated with EAK 16 II, a self assembling oligopeptide. The candida showed significant decrease in the adherence on plastic surface after coating with the EAK 16 II. The authors reported that the changes observed in the cell adhesion and variation in morphology was not dependent on the growth phases of this fungus.

It has been reported that, expression of cell surface determinant seems to be a function of growth phase in *C. albicans* as a few mannose epitopes are expressed only in stationary phase of the growth curve (Brauner and Cutler 1984). To understand the adhesion of candida to host cells, Cameron and Douglas (1996) in their core research work had used candida cells from stationary phase to study the binding of candida to fucose containing lipids existing on the HBEC. Characterization of fucose binding adhesins of *C. albicans* has been studied with candida cells taken from stationary phase of growth i.e. at 24 hrs of incubation temperature was 37 ^{o}C at 150 rpm(Rotrosen et al. 1986). However the rationale behind selection of cells from stationary phase in the adhesion experiments has not been explained clearly.

Molecular work on growth phase of *C. albicans* suggest that many *C. albicans* genes encoding for mannoproteins and other cell wall proteins were abundantly expressed in stationary phase (Uppuluri and Chaffin 2007). Cell wall proteins, mainly mannoproteins are the major components for adhesion of candida onto host cells (Cutler 1991; Cotter and Kavanagh 2000). Michan and Peuyo (2009) have reported that transcription of putative genes responsible for thioredoxin and glutathione redox system component increased in copy number in late exponential and stationary phase respectively in the yeast but not under filamentous conditions. Both the systems mentioned here, are important in the pathogenesis of candida infection. This is indicative of importance of growth phase and morphological forms in the regulation virulence of pathogen.

4.3.3 ADHESION EXPERIMENTS IN ARTIFICIAL SALIVARY MEDIUM (ASM) VS PBS AS SUSPENDING MEDIUM

The results of adhesion activity in ASM Vs PBS are given in Table 4.24. Candida adhesion on HBEC was conducted in two different suspension media, PBS (pH 7.2) and ASM. Contents of ASM are given in chapter 3, "Materials and Methods" **Section 3.3.3 .1**. When adhesion was carried out in ASM, many of the isolates showed increased percentage HBEC being adhered with candida, than once in the PBS (pH 7.2) as suspension media.

With a few exceptions presents; 2 isolates from HIV seronegative group CN-176 and CN-181 had shown less adhesion in ASM comparing to PBS (pH 7.2) as

suspension medium. With isolates from HIV seropositive individuals; RL-112 also showed decreased adhesion in ASM. The results are also presented in Fig. 4.24.

Table 4.24 Adhesion pattern of *C. albicans* when suspended in Artificial Salivary Medium (ASM) (pH 6.8) and PBS (pH 7.2)

Sl. no	**Isolates tested**	**Adhesion of *C. albicans* at Artificial Saliva Medium (ASM)**		**Adherence of *C. albicans* strains on to HBEC while suspended in PBS (pH 7.2)**	
		Percentage of HBEC adhered with candida	Average number of candida cells per HBEC	Percentage of HBEC showing candida adhered on them	Average number of candida cells per HBEC
1	RL-24	91.58	3.3	72.1	2.19
2	RL-23	54.3	2.33	53.7	2.48
3	RL-26	84.3	2	80.9	2.91
4	RL-28	81	3.4	74	2.56
5	RL-27	87.8	3.55	63.8	2.1
6	RL-29	86.6	4.88	66.6	2.27
7	RL-112	40.6	1.9	63	2.25
8	CN-194	80.35	4.28	25.7	1.22

9	CN-176	44.6	1.7	73.2	2.46
10	CN-67	93.8	4.3	56.9	2.08
11	CN-102	86.9	3.75	50	1.4
12	CN-181	55	2.33	65.8	1.96
13	CN-188	90.69	4.2	46.5	2.04
14	CN-173	70.1	4.52	63.8	2.1
15	CN-31	67.8	3.73	66	2
16	CN-111	95.4	2.9	82.06	1.94
17	CN-192	93.2	4	50.5	1.89

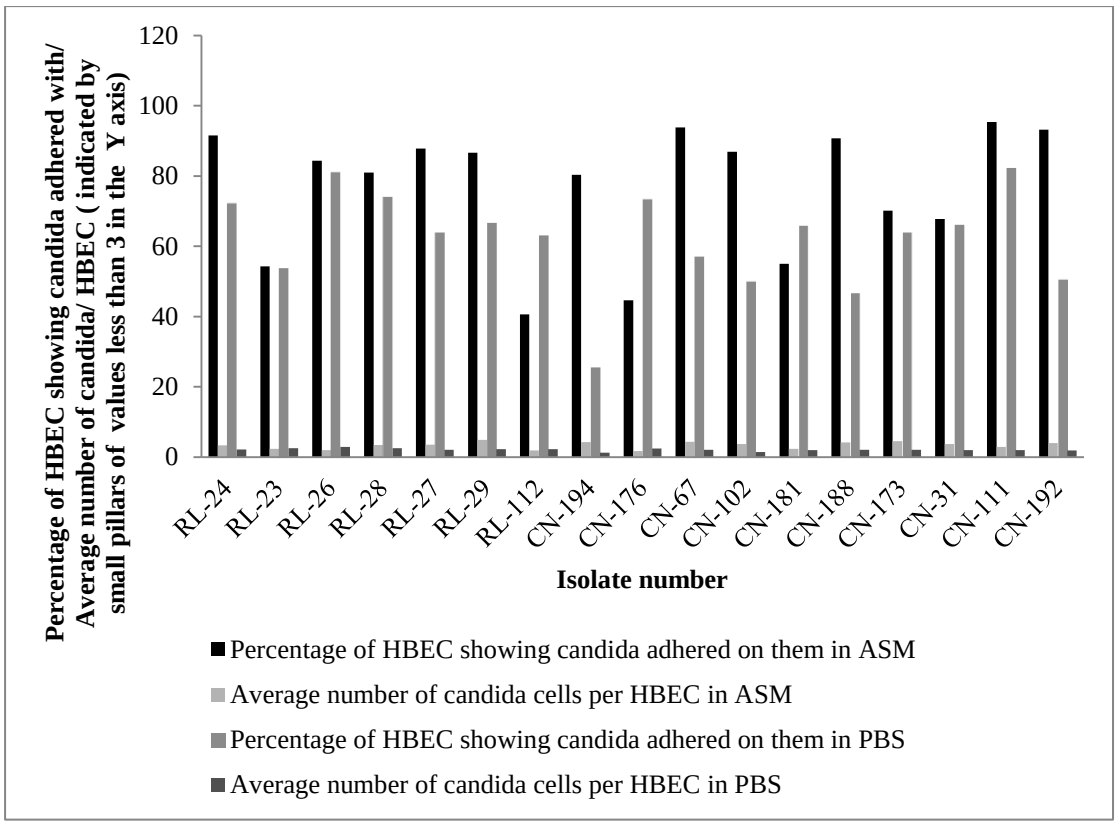


Fig. 4. 24 Adhesion pattern of *C. albicans* in ASM and PBS (pH 7.2) separately

According to the above study results; ASM showed enhancing effect on candidal adhesion. Human beings produce 1200 to 1500 ml saliva/day. Saliva has many immunoprotective actions. Xerostomia and hyposalivation are known to cause discomfort, pain while chewing and swallowing and also can predispose to tooth decay and other oral infections commonly OC (Napenas et al. 2009; Dodds et al. 2005). However, while studying the role of natural saliva on the adhesion of *C. albicans,* in the *in vitro* conditions results obtained are contrasting. Some researchers found that saliva helps in adhesion (Holmes et al. 2002). A few reports showed the results in opposite way (Umazume et al. 1995). Jin et al. (2004) tested human whole saliva and dietary sugars (glucose and galactose) for their effect on adhesion and biofilm formation in *C. albicans* using ATP bioluminescence, Tetrazolium (XTT) reduction assay and conventional Colony Forming Unit (CFU) evaluation. They reported that immobilized saliva coating had little effect on either candidal adhesion in initial incubation periods. However at longer incubation time both Human saliva and dietary sugars lead to higher pace of biofilm formation (incubation for 96 hrs).

Artificial saliva has been used in *in vivo* conditions for the patients who suffer with Xerostomia. Influence of artificial saliva in biofilm formation of *C. albicans* was tested in *in vivo* conditions by Silva et al. (2012). Two different artificial saliva preparations were used by the author's namely artificial saliva I which contained carboxymethyl cellulose and artificial saliva II which contained glucose oxidase, lactoferrin, lysozyme and lactoperoxidase. Silva et al. (2012) reported that artificial saliva I reduced the biofilm formation significantly compared with artificial saliva II and they concluded that carboxymethyl cellulose present in artificial saliva might be the reason for this. However the ASM preparation used in the present study did not contain carboxy methyl cellulose. In the present study, percentage of adhesion with the candida cells suspended in ASM was higher than the one suspended in PBS. The increased adhesion here may be due to the ion concentrations present in the ASM.

4.3.4 ADHESION REACTION AT VARIOUS IONIC CONCENTRATIONS OF NaCl IN PBS (pH 7.2)

Adhesion reaches towards the highest values when the ionic concentrations of PBS is at 0.318 M, and after this ionic concentrations, adhesion remains at higher side, but the differences run in constant values. However, average number of candida per HBEC was not varying in accordance to the ionic concentrations as observed here. The results obtained are given in Table 4.25 and Fig. 4.25.

Table 4.25 Adhesion reaction at various ionic concentration of NaCl in PBS as tested with the isolate RL-112

Sl. No.	Ionic concentration (in Moles)	Percentage of HBEC adhered with candida	Average number of Candida per HBEC
1	0.001	37.34	1.6
2	0.00316	33.47	1.6
3	0.01	39.7	1.7
4	0.0316	53.03	1.98
5	0.1	50	2
6	0.154	73.4	2
7	0.318	74	1.92
8	0.472	63.69	1.9
9	0.626	53.06	1.56
10	0.780	56.32	1.46
11	0.934	50.78	1.41
12	1.088	45.58	1.21

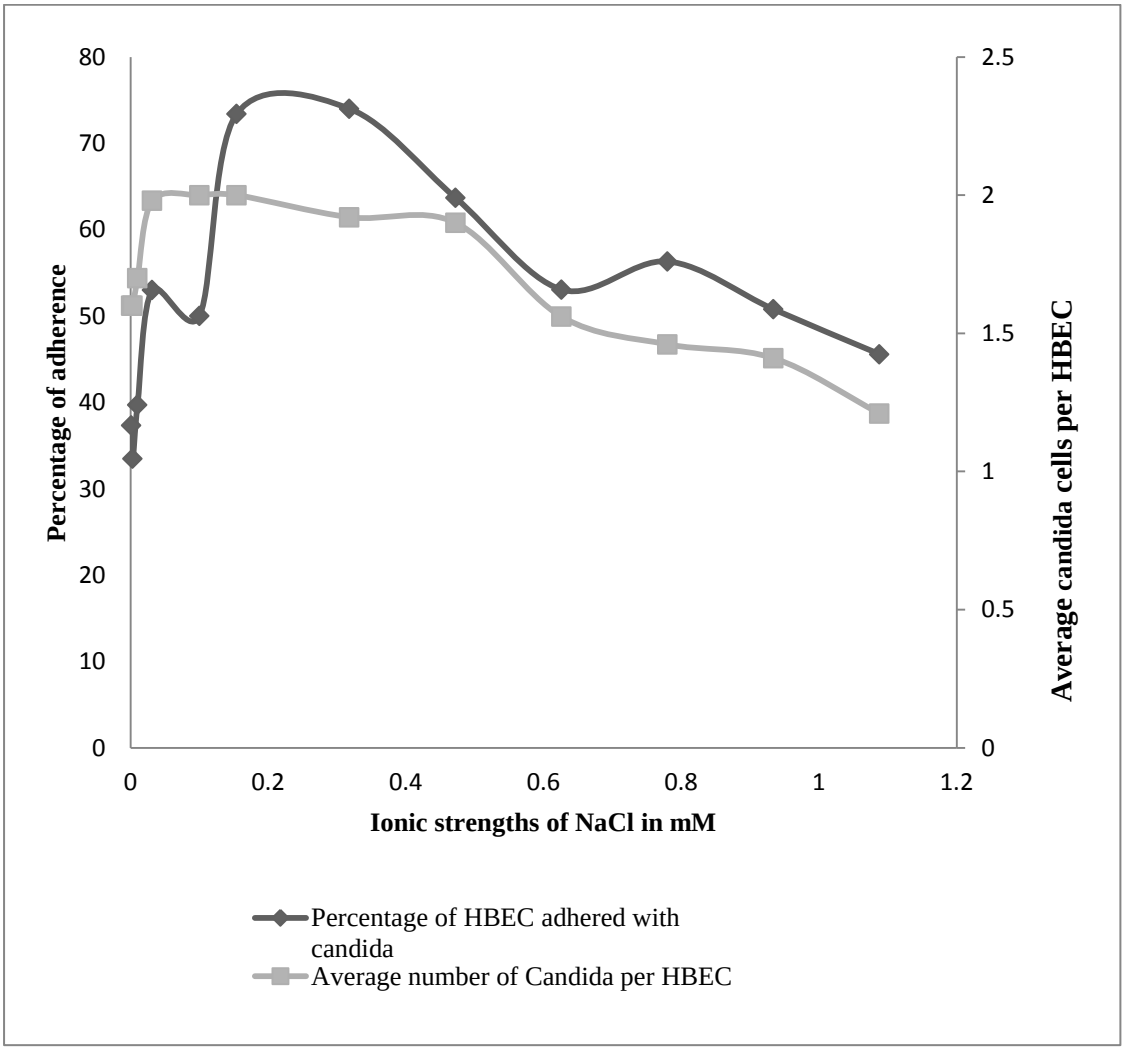


Fig. 4.25 Adhesion pattern of *C. albicans* (RL-112) at varying concentrations of NaCl in PBS

As shown in the results presented here the ionic concentrations are influenced on the adhesion of *C. albicans.* Cowell et al. (1998) also reported that adhesion of *Pseudomonas aeruginosa* on contact lenses was increasing when the salt concentration of media increases from 0.8% to 1.0% (w/v). However the adhesion of these bacteria in their study was not related to Cell Surface Hydrophobicity (CSH).

SUMMARY

With this part of the work, the best suitable condition for adhesion reaction to occur was analyzed and it is understood that the experimental conditions followed carry utmost importance in adhesion reaction. The cell surface components that are responsible for mediating adherence might have expressed more at stationary phase and that may be leading to increased adherence of these cells on HBEC. Similar observations have been reported in the literatures (Uppuluri and Chaffin 2007). The study is further proceeded to understand the mechanism involved in the adhesion process.

4.4 EVALUATION OF ADHESION MECHANISM INVOLVED IN ADHERENCE OF *C. ALBICANS* ONTO HBEC

Adhesion of candida onto HBEC involves both specific and non specific mechanisms as reported in literatures (Cotter and Kavanagh 2000). The specific mechanisms, involved are consisting of ligand-receptor interactions. Non-specific mechanisms involved in the adhesion are known to be electrostatic forces, aggregations and hydrophobic interactions (Henriques et al. 2007). Both types of mechanisms mentioned here were analyzed in the present research work for the role of these mechanisms on adhesion of the isolates of this study.

4.4.1 BIOCHEMICAL ASPECTS OF ADHESION MECHANISM

4.4.1.1 TESTING FOR LECTIN CARBOHYDRATE TYPE OF INTERACTIONS INVOLVED IN THE ADHESION

Lectin carbohydrate type of interactions involved in the adhesion was tested using various approaches. Candida cells and supernatants obtained from candida culture broth were tested for the presence of lectin activity. The adhesion reaction under the influence of carbohydrates and lectin Con A (Concanavalin A; a lectin isolated from *Canavalia ensiformis*) was checked by *in vitro* methods.

4.4.1.1.1 TESTING FOR PRESENCE OF LECTIN ACTIVITY IN CANDIDA CELLS

Candida cells were tested for lectin activity using intact cells and also the cells after sonication. Isolates obtained from HIV seropositive individuals, RL-112 and RL-24 were used for the lectin agglutination studies. Lectin agglutination studies were done here by using RBCs of A, B and O blood groups.

The washed cells of *C. albicans* gave positive results only with RBCs from O blood group. With RBCs from A and B blood groups, no hemagglutination reaction was seen. The cells were then subjected to sonication for 5 minutes with 30 sec on/off at 20 kHz, using sonicator VCX 130 by Sonic Vibra Cells. After sonication the suspension was centrifuged. Testing of pellets of cells and supernatant after sonication of the cells did not show any hemagglutination activity with any of the blood group RBCs. Therefore, it is understood that sonicated cell pellets and the supernatants obtained after sonication are negative for lectin activity. The results are shown in Table 4.26.

Table 4.26 Lectin activity exhibited by candida cells

Sl. No	**RL-112**		**RL-24**	
	30 hrs culture	**60 hrs of culture**	**30 hrs culture**	**60 hrs of culture**
Washed Candida cells	Positive (titre 32)	Positive (titre 32)	Positive (titre 32)	Positive (titre 32)
Sonicated pellets	Negative	Negative	Negative	Negative
Supernatant after sonication	Negative	Negative	Negative	Negative

4.4.1.1.2 TESTING FOR PRESENCE OF LECTIN ACTIVITY IN THE CULTURE SUPERNATANT OBTAINED FROM CANDIDA GROWTH MEDIUM

Testing of culture supernatant of strains RL-112 and RL-24 showed hemagglutination positive results with RBC's from O blood group. The hemagglutination failed to occur with RBC's from A, B and AB blood groups. The agglutination tested with 30 hr culture supernatant showed positive result with titer of 32, i.e. till 5th well of microtitre plate. The agglutination seen at 60 hrs gave positive result till 4th well i.e., 16 as the titre. The results are shown in Table 4.27.

Table 4.27 Lectin activity testing results of culture supernatant of candida growth medium after 30 and 60 hrs of the incubation separately

	Details on dilutions of supernatant solution tested for lectin activity								
Description for the sample tested	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**Blood groups of the RBCs**
RL-24 grown for 30 hrs	+	+	+	+	+	_	_	_	O
	-	_	_	_	_	_	_	_	A
	-	_	_	_	_	_	_	_	B
RL-24 grown for 60 hrs	+	+	+	+	_	_	_	_	O
	_	_	_	_	_	_	_	_	A
	_	_	_	_	_	_	_	_	B
RL-112, 30 hrs grown Culture	+	+	+	+	+	_	_	_	O
	-	_	_	_	_	_	_	_	A
	-	_		_	_	_	_	_	B
RL-112, 60 hrs grown Culture	+	+	+	+	_	_	_	_	O
	_	_	_	_	_	_	_	_	A
	_	_	_	_	_	_	_	_	B

Positive results of all the lectin activities occurred with 'O' blood group RBCs only. The carbohydrate moieties responsible for giving positive lectin activity reaction with'O' RBCs can be of fucose in nature (Yariv et al. 1967). Since the agglutination is negative with other blood group RBCs, it can be suggested that candida cells contain substances which have been acting as fucose recognizing lectins. The presences of fucose specific lectin like substances have previously been explained by Cameron and Douglas (1996). In support of this; in a study where HBEC were preincubated with lectins specific for fucosyl-containing glycosides showed decreased adhesion of *C. albicans* cells (Olsen 1990). The blocking adherence of adherence of candida onto HBEC in the presence of fucose is also noted in the results presented by Cameron and Douglas (1996). The minimal structural requirement for activity of fucose recognizing lectin found to be a Fuc-1 -2Gal ß determinant. Fuc-1 -2Gal ß determinant is found as H sugar sequence on all blood group substances of the ABO system and is known to be H antigen of the blood group, which can be found on HBEC. Cameron and Douglas (1996) reported that, the presence of H antigen on HBEC may have the implication on colonization of candida in the oral cavity. Presence of lectin-carbohydrate type of reaction is also reported by Brassart et al. (1991). They showed that the *C. albicans* strains are unable to adhere in the presence of a lectin which recognizes N-acetyl-D-glucosamine (NAGA). Precisely, all the above references along with results obtained in the present study observe that, candidal adhesion on HBEC involves lectin-carbohydrate type of interactions.

Any infectious disease starts with the adherence of infectious agent onto host cells. Adhesion behavior of the organism depends on the nature of ligands and adhesins. Both ligand and adhesins should have perfect complementary structures to fit each other. The precise expression of these molecules, at different underlying predisposing conditions can again decide the possibility for adhesion and disease progression (Senet 1998). Molecules present on the cell surface of *C. albicans* as well as a few proteins secreted by the fungus during their growth, studied and proved to be acting as adhesins. Mannan from *C. albicans* may provide a host recognition function for *C. albicans* (Calderone et al. 2000). In *C. albicans***,** mannoprotein, Glucan, chitin, cell wall proteins and lipids are known to be the possible adhesin molecules. Several

mannoproteins act as adhesions and these mannoproteins are known to give fibrillar or floccular appearance to outer most layers of the cells (Olsen 1990). The experiment conducted here has shown the evidence to the presence of some lectin like substances secreted to culture media during the growth of candida cells which can be more in early stages of growth. The secretion of these molecules decrease as the culture matures, as shown with results of 60 hrs of culture supernatant. The candida cells in intact form only yielded positive results on testing for lectin activity. Sonicated cell pellets and supernatant also gave negative results for lectin activity. Sturtevant and Calderone et al. (1997) reported that candidal adhesins act like lectins, which recognize carbohydrate residues on the host cells. Various mannoproteins like MP 37, MP 55, MP58, MP60, MP 66, MP 70, MP 130, and MP 165 were reviewed for their role in adhesion.

4.4.1.1.3 AGGLUTINATION REACTION OBTAINED FOR CON A WITH EIGHT*C. ALBICANS* ISOLATES

Four isolates were selected separately from HIV seropositive and HIV seronegative groups. All the 8 isolates gave strong positive results for agglutination reaction with Concanavalin A (Con A). The turbidity of the suspension of all the isolates was adjusted to OD 2 at 625 nm. The initial concentration of lectin used in this experiment was 1 µg/ml. Results are shown in Table 4.28.The agglutination seen up to 12 well, equal to 1: 4096 dilution of the initial concentration of lectin Con A. The lectin Con a binds specifically to mannose or glucose moieties (Sharon and Lis 1972). The strong agglutination with Con A indicated the presence of abundant quantity of mannose moiety on candida cell surface.

Table 4.28 Agglutination results with Con A

Subject group	Isolates tested	DILUTIONS OF CON A											
		2	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**4096**
HIV SEROPOSITIVE INDIVIDUALS	RL-112	+	+	+	+	+	+	+	+	+	+	+	+
	RL-24	+	+	+	+	+	+	+	+	+	+	+	+
	RL-29	+	+	+	+	+	+	+	+	+	+	+	+
	RL-23	+	+	+	+	+	+	+	+	+	+	+	+
HIV SERONEGATIVE INDIVIDUALS	CN-69	+	+	+	+	+	+	+	+	+	+	+	+
	CN-01	+	+	+	+	+	+	+	+	+	+	+	+
	CN-12	+	+	+	+	+	+	+	+	+	+	+	+
	CN-63	+	+	+	+	+	+	+	+	+	+	+	+

Olsen (1990) reported that preincubation of HBECs with Con A, results in inhibition of candidal adhesion onto HBEC. Tunicamycin which inhibits mannoprotein is also shown to inhibit adhesion of candida onto HBEC. Olsen (1990) also reported that methyl-alpha-D-mannoside inhibits adhesion of *C. albicans* on

HBEC and suggested that this substance behaves as analogous to the yeast adhesin or epithelial cell receptor. All the candida isolates used for Con A agglutination test in the present study showed strong agglutination reaction till the lowest dilutions of 1: 4096 of initial 1µg/ml Con A concentration and thus proving the presence of abundant mannan moiety on their surface. These reports prove the presence of mannose moieties on the candida cell surface. Colling et al. (2005) reported that yeast cells preincubated with various substances like various formulations of K-Y and heparin, divalent cations (Ca+ +, Mg+ +, Zn+ +, Cu + +) caused some surface modifications. These changes caused reduced adherence of yeast on to the microsphere and also diminished binding of Con A, thus prove the role of mannoprotein components in binding activity. Role of lectin-carbohydrate type of interactions can be further studied using a panel of carbohydrates and lectin Con A in adhesion reaction.

4.4.1.1.4 SCREENING FOR THE ROLE OF VARIOUS CARBOHYDRATES AND CON A ON ADHESION PATTERN OF *C. ALBICANS*

Results obtained by the work on effect of various carbohydrates, lectins on adhesion activity of *C. albicans* on HBEC are given in Table 4.29, 4.30 and Fig. 4.26 and 4.27.

Table 4.29 Adherence of *C. albicans*(RL-24) on HBEC, after pretreatment of the cells with various sugars and Con A

	Mannose	**Galactose**	**Dextrose**	**Sucrose**	**Fucose**	**Gal-Nac**	**Gluc-Nac**	**Con A**	**Fetuin**	**Mucin**	**Control slides**
adhered with candida	30.15	20.58	38	45.83	20.6	24.8	32.69	30.21	20.31	14.33	44.3
Average no. of candida per HBEC	1.42	1.83	1.21	1.45	1	1	1.35	2.3	1.6	1	1.64

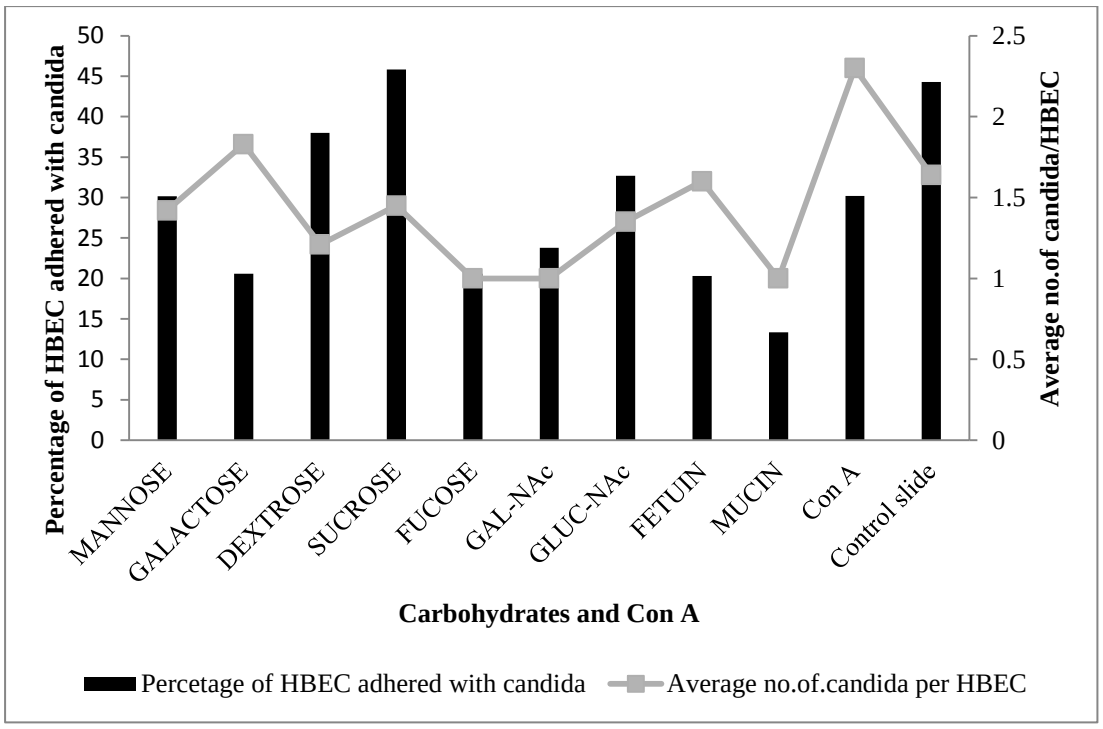


Fig. 4.26Adhesion reactions of candida cells after getting treated with sugars and lectins separately

Note: Reduction in adhesion was shown when the candida cells treated with fucose, galactose and mucin treated cells of RL-24

Table 4.30 Adherence of *C. albicans* (RL-112)on HBEC, after pretreatment with various carbohydrates and Con A

	Mannose	**Galactose**	**Dextrose**	**Sucrose**	**Fucose**	**Gal-Nac**	**Gluc-Nac**	**Con A**	**Fetuin**	**Mucin**	**Control slides**
Percentage of HBEC adhered with candida	28.78	22.3	36	44.34	18.6	24.6	31.34	29.66	19.54	15.27	47.2
Average no. of. Candida/ HBEC	1.31	1.74	1.39	1.78	1.3	1.02	1.56	1.8	1.5	1.24	1.38

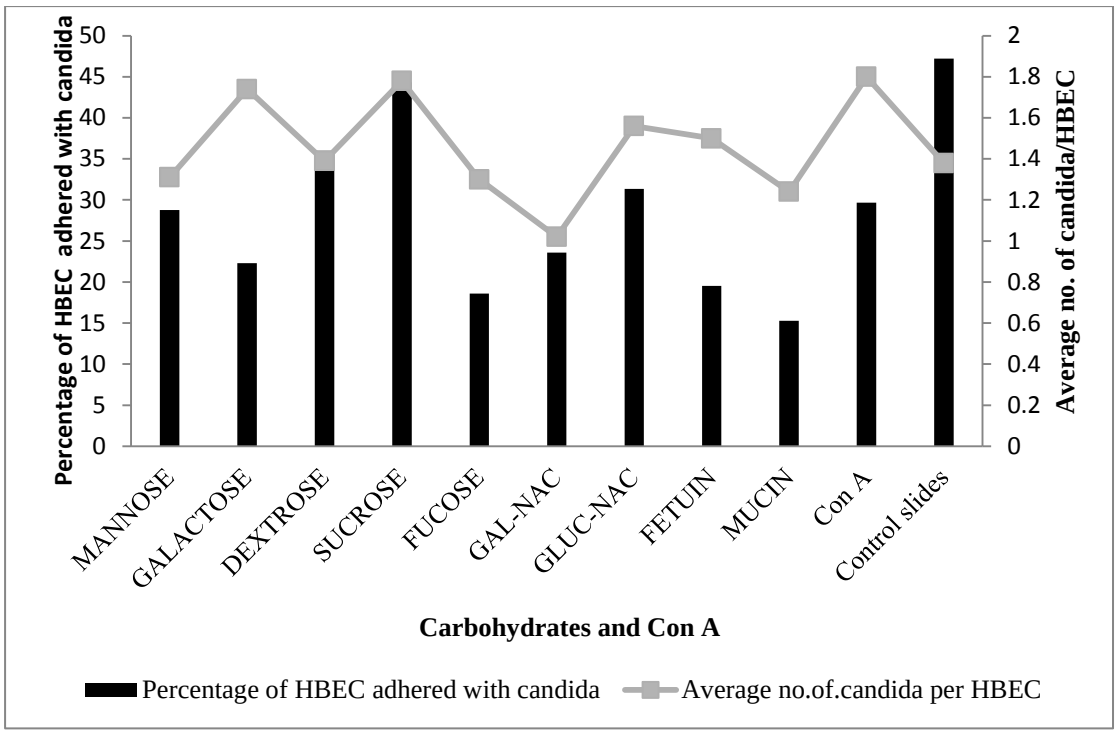


Fig. 4.27 Adhesion reactions of cells after it had been treated with sugars and lectins, separately. Maximum reduction in adhesion was seen when treated with mucin, fucose, galactose, Gal-Nac and fetuin treated cells of RL-112

The sugars like fucose, galactose and mucin treated candida cells showed the reduction in candida adhesion. Here with RL-112, reduction in inhibition of preincubated candida cells were in the following order, mucin >fucose> fetuin> galactose> Gal-NAc> mannose> Gluc-NAc> Dextrose. With RL-24, reduction in adhesion after preincubated candida cells was in the following order, mucin > fetuin> galactose > fucose > Gal-NAc> mannose> Gluc-NAc> Dextrose. With Sucrose preincubated candida cells showed slightly increased adhesion compared to the untreated cells.

Macura and Tondyra (1989) reported that sugars added to the test medium had varying effect on adhesion reactions. D-glucose, D-galactose and sucrose significantly (p less than 0.005) enhanced the adherence (p less than 0.025). D-xylose, D-ribose, D-fructose, maltose, lactose and raffinose had not shown any influence on adhesion process. However when fungal cells or epithelial cells were pretreated with glucose

and mannose, they did not show enhancement in adherence. Olsen (1990) reported that, L-Fucose, N-acetyl-D-glucosamine, or D-mannose, also show inhibition effects on candida adhesion onto HBEC. Similarly in the present study; reduction produced by mucin, gal-Nac and fetuin sugars was highest. In both the isolates of current study, sucrose did not exhibit any kind of inhibition effect to adhesion as compared to the results in untreated cells (control slides). Lectin typing of *C. albicans* using panel of peroxidase conjugated lectins namely Con A (binds to α-d-glucose/α-d-mannose), WGA (binds to N-acetyl-D-glucosamine), UEA I (binds to α-l-fucose residues) and PNA (binds to N-acetyl-D-galactosamine) in the concentration of 25 μg/ml showed strong positive reaction with *C. albicans* indicating the presence of the specific carbohydrate moieties recognized by these lectins on the cell wall suggested that these glycol conjugates may represent recognition molecules for interactions between the candida strain studied and the host cells (Lima-Neto et al. 2009).

Con A had reduced the percentage of adhesion of candida cells to HBEC. The candida cells incubated with Con A showed decrease in adhesion to 29.66% in RL-112 from 47.2% (untreated candida cells). In RL-24, the adhesion decreased to 30.2% in pretreated candida cells compared with the adhesion percentage of adhesion (44.3%) exhibited by untreated candida cells. Macura and Tondyra (1989), reported that Con A significantly inhibited the adherence of fungal cells to buccal epithelial cells both when it was added to the test medium (p less than 0.005) and when the fungal or epithelial cells were pretreated with Con A (p less than 0.001). With the comparison of above reports and the results obtained in the present study, it can be concluded that, a few carbohydrate moieties like mucin, fetuin, galactose etc. are present on the HBEC and play an important role in adhesion process through lectin carbohydrate type of interactions.

SUMMARY

The candida cells exhibit lectin activity in intact form. The culture supernatant also shows lectin activity. Lectin Con A, can strongly agglutinate candida cells and thus proving abundant mannose moiety on the candida cell surface. The incubation of candida cells with Con A and then subjected to adhesion showed also reduction in actual adhesion percentage, and thus proved the role of these mannose moieties in adhesion of candida on to HBEC. Treating with various carbohydrates and then performing adhesion reaction showed that, many of these carbohydrates especially mucin, fucose and galactose play important roles in adhesion. After the screening results for lectin activity in the cell and supernatant, further work was carried out to isolate and characterize cell wall proteins and secreted proteins of *C. albicans* in subsequent part of the study. Role of these proteins on adhesion of *C. albicans* onto HBEC were also evaluated.

4.4.1.2 STUDIES ON THE ROLE OF CELL WALL PROTEINS AND SECRETED PROTEINS OF *C. ALBICANS* ON THE ADHESION PROCESS

C. albicans contains a set of dynamic proteins which are distributed in its cell membrane and also a few of them secreted to the growth medium (Chaffin et al. 1998). Cell wall proteins act as major cell surface antigens that are recognized by mucosal receptors in infection process (Martinez et al.1998).

The structure and function of these proteins vary according to the surrounding environmental influence. Specific mannoproteins are synthesized during the hyphal morphogenesis (Marcilla et al. 1998). These factors may be determining the shift of commensal candida into a parasite of pathogenic characteristics and result in the formation of candidiasis (Senet 1998). Presence of OC and or other types of mucocutaneous candidiasis are commonly seen with AIDS patients who are having impaired immunity with T-cell deficiencies (Fidel 2006). In the present research work, *C. albicans* strains from two different patient groups were selected and analyzed for their protein profiling. The secreted proteins present in the growth medium are also studied for their role in adhesion.

Proteins undergo modification in their structure and function by post translational changes through several ways (Chaffin et al. 1998). Glycosylation is one such post-translational change occurring in glycoprotein structures. The present part of the study is aimed to detect the presence of glycosylation in the cell wall proteins among the commensal isolates and pathogenic strains, using lectin blotting method. Both cell wall proteins and secreted proteins were further studied for their role in adhesion. As mentioned previously, possibility of post translational modifications occurring in cell wall protein structures was studied using lectin blotting assays.

4.4.1.2.1 CELL WALL PROTEIN PROFILING OF THE ISOLATES AND LECTIN BLOTTING

4.4.1.2.1.1 ANALYSIS OF CELL WALL PROTEIN EXTRACTION METHODS AND SELECTION OF BEST METHOD WITH REQUIRED ADOPTING MODIFICATIONS

The method used for protein extraction plays a role in the outcome of the results and therefore the protein extraction method used was thoroughly standardized and during standardization a few important modifications to the existing method was carried out to get the best results.

For the extraction of candida cell wall proteins, initially two different methods were used, namely, a method using enzyme and detergents i.e., by Pitarch et al. (2002) and the other method by Feiz et al. (2006) where salts were used. Since the method by Feiz et al. (2006), gave comparatively poor band resolution as compared with the method of Pitarch et al. (2002), further protein extraction was tried with Pitarch et al. (2002) method. The results from both Pitarch et al. (2002) and Feiz et al. (2006) are shown in Fig. 4.28 and 4.29.

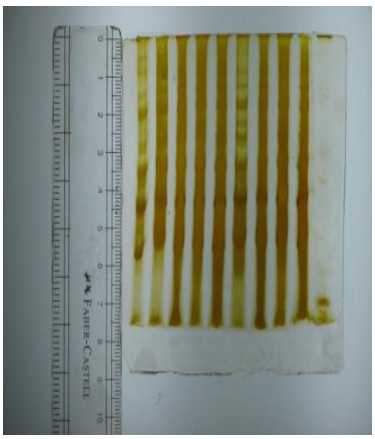


Fig. 4.28 Silver staining of SDS PAGE (10-17%) of cell wall proteins Feiz et al. (2006) method

Fig. 4.29 Silver staining SDS PAGE (10-17%) of . cell wall proteins by Pitarch et al. (2002) method

Though the extraction method explained by Pitarch et al. (2002), was chosen for further work, yet cell rupture step of this method was demanding a few modifications as it was seen that many cells remain intact even after performing the complete procedure of extraction. Therefore, a modification to the mechanical procedures of cell rupturing is suggested in Pitarch et al. (2002) method using a vortex mixer, by using two varieties of glass beads.

Two strains (RL-112 and CN-192) were used for evaluation of the method of Pitarch et al. (2002). Table 4.31 shows the effect of vortexing by two sizes of glass beads evaluated on two different strains of *C. albicans* viz. RL-112 and CN-192.

The parameters used to evaluate the quality of cell disruption were percentage of disrupted cells, protein concentration of the lysate, determination of viability of the cells and the number of clear bands given by SDS-PAGE. The samples were initially homogenized by vortexing with glass beads. The resulting lysate was evaluated by gram stain and semi-quantitative culture on SDA as given in Fig. 4.30 and 4.31 respectively and further subjected to extraction. The final yield was tested by protein estimation and SDS-PAGE electrophoresis. The SDS PAGE results of homogenization experiments are given in Fig. 4.32.

Table 4.31 Results obtained in Glass beads homogenization procedure followed by extraction with Method by Pitarch et al. (2002)

Isolate tested	**Time**	**0 min**		**3 min**		**6 min**		**9 min**		**12 min**		**15 min**		**18 min**	
	Bead size in mm	**0.5**	**3-5**	**0.5**	**3-5**	**0.5**	**3-5**	**0.5**	**3-5**	**0.5**	**3-5**	**0.5**	**3-5**	**0.5**	**3-5**
RL_ 112	Grams stain; % of cell disruption	0	0	26.1	9	65.1	40	78.2	60.7	74	66	89.4	70	92	76.9
CN-192		0	0	35.1	7.5	33.3	13.8	37.8	63.0	85.0	62.5	93.2	64.0	99.4	78.8
RL_ 112	Protein concentration µg/10 µl	5	8.5	7.0	8.5	8.0	10	9.0	12.5	12.5	13.0	13.5	13.5	16.5	14.5
CN-192		7.0	7.0	7.5	11.6	7.75	12.0	7.75	12.4	8.0	13.5	11.5	17.5	18.5	23.0
RL_ 112	Growth on SDA	+++	+++	++	+++	++	+++	++	+++	+	+	+	+	+	+
CN-192		+++	+	+++	+	+++	+	+	+	+	+	0	+	0	+
RL_ 112	SDSPAGE; Number of bands seen **Proteins MW in kDa**	15	4	18	7	23	8	27	10	28	19	16	20	21	26
CN-192		19	16	25	20	29	22	28	25	28	24	31	25	34	22

(+++) = > 500 colonies, (++) = > 200 and < 500 colonies, (+) = > 100 and < 200 colonies, < 100 is given as actual numbers

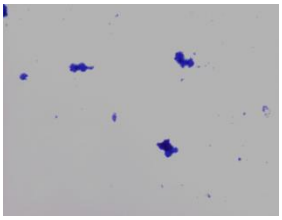
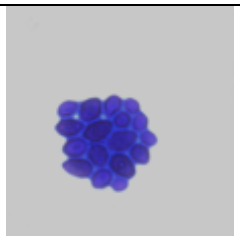
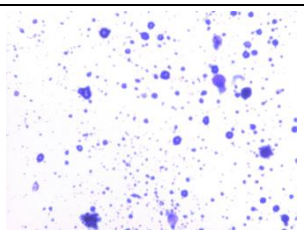
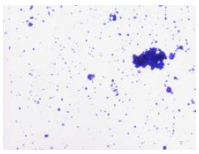
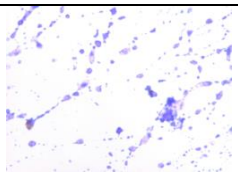
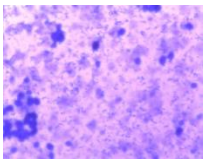
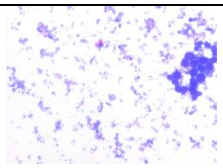
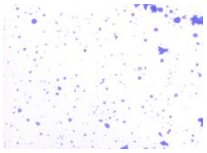
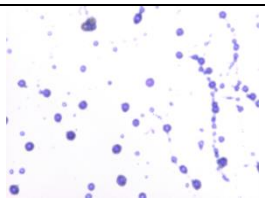
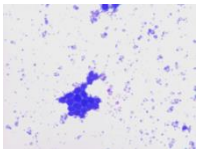
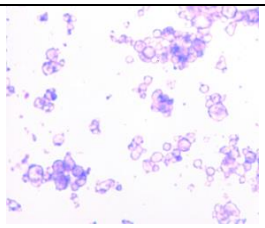
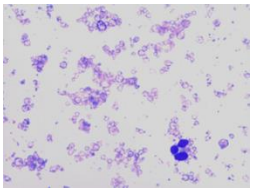
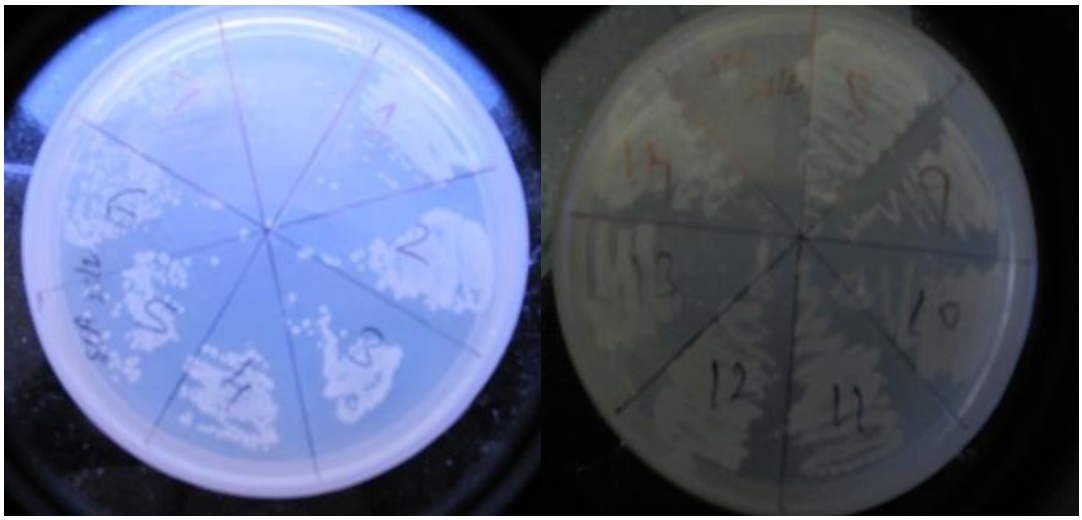
Sl. No	Glass bead beating done for time interval (minutes)	Small beads (1-3 mm)	Big beads (5 mm)
1	3		
2	6		
3	9		
4	12		
5	15		
6	18		

Fig. 4.30 Gram stain (1000x) morphology of candida cells subjected to various time intervals of Glass bead homogenization



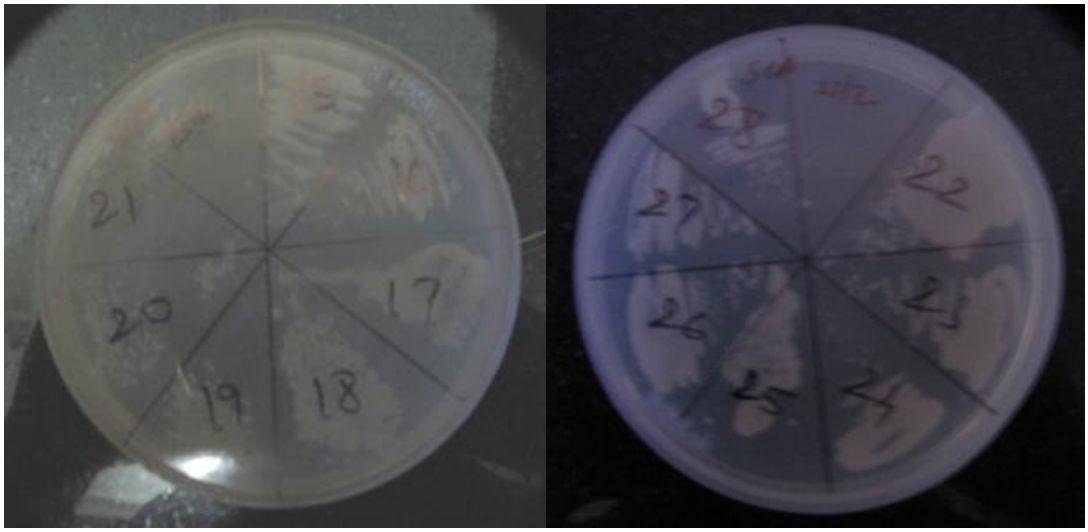


Fig. 4.31 Growth of candida on SDA, after subjected to various time intervals of glass bead homogenization

Note: The growth on SDA plate numbered 1-7 and 8-14 indicated culture grown after vortexing RL-112 using 0.5mm and 3 mm beads respectively at various time intervals were ranging from 0 to 18 minutes. The growth plates with numbers 15-21 and 22-28 indicated bead homogenization with glass beads for CN-192, by using 0.5 mm and 3-5 mm beads respectively at various time intervals ranging from 0 to 18 minutes.

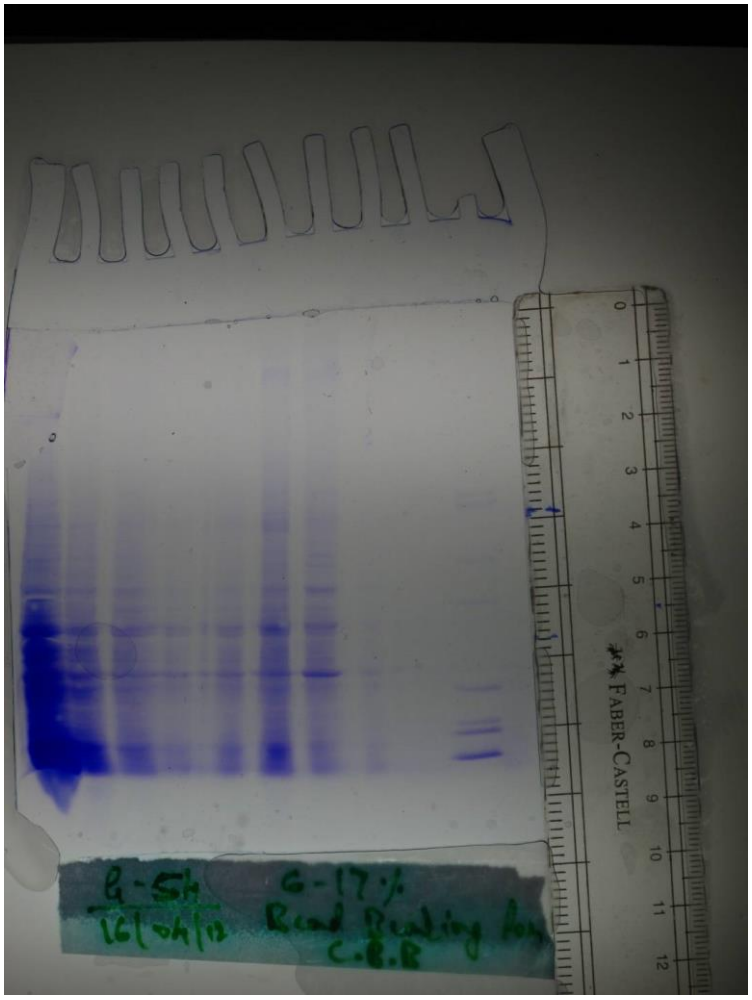


Fig. 4.32 SDS PAGE results of protein samples obtained after different time intervals of homogenization (CBB Staining)

Note: First left well till 7 wells samples were put from 0, 3, 6, 9, 12, 15, 18 minutes of bead homogenization using 0.5 mm beads. Right last well was put with standard protein marker.

The methods were intended to disrupt the cells maximally by increasing the vortexing time. The percentage of disrupted cells increased proportionately with vortexing time. At 15 and 18 minutes the disruption was around 90% using 0.5 mm beads. Vortexing for all time periods showed that the use of 0.5 mm beads were

always better than 3-5 mm beads in lysing the cells. The lysates were pelleted and the protein concentration was estimated. It is evident from Table 4.32., that the protein yield also increased with vortexing time. The protein yield was highest at 18 min of vortexing.

With 3-5 mm beads percentage of cells disrupted was 76.9% (RL-112) and 78.8% (CN-192) at 18 minutes of homogenization. At 18 minutes of homogenization with 0.5 mm beads, 99.4% of cells in CN-192 and 92.0% in RL-112 showed disruption and the band quality was better.

Better lysis and better recovery of cell wall proteins at 18 min of disruption is reflected by scanty or no growth from the lysate on SDA and more number of bands on SDS-PAGE. The strain CN -192 gave no growth after 18 min of vortexing indicating complete lysis. The same was reflected on SDS-PAGE as the number of bands was 34, the maximum recorded in the present study.

Candida cell wall proteins have been extracted using different methods by different researchers. The starting material and the method used for cell wall protein extraction, influence final outcome or quality of extracted proteins. Study of candida cell wall proteins was done using intact cells or cell wall material after cell disruption or by using proteins secreted into medium when protoplasts are synthesizing their cell walls (Farahnejad et al. 2004; Pitarch et al. 2002; Elorza et al.1983; Kapteyn et al.1995). Both the methods used in the present study were based on breakage of cells to isolate cell wall protein. In Method 1(Pitarch et al. 2002) and Method 2(Feiz et al. 2006), detergents and salts were used respectively. The initial experiments revealed the limitations of both the methods in candida cell wall protein extraction. It isobserved that Method 1(Pitarch et al. 2002), i.e., using detergents and chitinase yielded better quality of proteins, which were less contaminated with salts.

It was also found that protein extracts prepared according to the method of Pitarch et al. (2002), also had intact cells, indicating incomplete lysis of the cells in both the methods. These cells act as artifacts in SDS PAGE and the lysis of intact cells can release cytosolic proteins affecting the purity of the cell wall protein extract.

Therefore, complete breakage of all intact cells is crucial in the cell wall protein extraction.

From the results obtained from this study, it was found that, better lysis and better recovery of cell wall proteins were obtained at 18 min of disruption by 0.5mm beads. Same was reflected by scanty or no growth from the lysate obtained from above condition on SDA and more number of bands on SDS-PAGE.

Mechanical methods are commonly used to disrupt fungal cell walls, with the combination of other methods. Klimeckochab et al. (11) worked upon chemical, mechanical and osmotic shock in disrupting fungal cell wall and suggested that bead milling lead to good results in obtaining cell free extracts containing high concentration of soluble proteins. However, for particular species further adjustments are required. Okunghowa et al. (2007) suggested that the mechanical methods like sonication and French pressure cell press give good protein yield but glass beads provide a lesser quantity of protein and lead to the loss of protein activity.

Standardization of glass bead homogenization has to be done carefully by maintaining correct quantity of cells subjected to homogenization. Ratio of cells Vs glass beads, size of the glass beads and time for vortexing need to be carefully standardized. Kessler et al. (1959) had obtained clean cell wall by beating the cells with glass beads in a blender for 90 minutes aided by treatment with 0.25 M sucrose solution. In the original method of Pitarch et al. (2002), they had done the mechanical disruption of candida cells by bead beating, for 10 minutes with 30 S pulses in a bead beater. In the present research work it was done using simple vortex mixer, due to the unavailability of bead beater and sequentially increased homogenization time to test the efficacy of cell disruption.

An overnight NaOH extraction suggested by Pitarch et al. (2002), for the release of enriched fractions of proteins directly linked to 1-3 Glucan through their O-glycoside chains or by other alkali sensitive linkage. Since it was also shown by Kessler et al. (1959), that treatment with alkali can cause degradation of proteins linked with carbohydrates and lectin like proteins, this step has been omitted in the present extraction work.

In the method by Pitarch et al. (2002), along with detergents and mechanical disruption, the enzymatic treatment is also employed, using quantazyme and exochitinase separately. These enzymes can release glycolytic enzymes tightly trapped within Glucan-chitin complex (Pitarch et al. 2002). In contrast to this method, Klis et al. (2007), suggested the use of high concentration of detergents perturbs plasma membrane and they release cytosolic proteins. According to them, use of low concentrations of detergents, sodium phosphate buffer at pH 8 and low temperature at extraction avoid contamination of cell wall protein extraction with cytosolic proteins. Similarly, Feiz et al. (2006) who did not use enzymes and detergents for the extraction, suggested instead of NaCl, a low ionic strength buffer needs to be used during extraction which can prevent early release of cell wall proteins. They showed that 78% proteins released by Method of Pitarch et al. (2002), were intracellular proteins. However, Pitarch et al. (2002), demonstrated that, the proteins released by their method were from cell wall by showing failure to detect Sec 14p antigens, through immunoblotting procedure. Sec 14p is a marker of cytosolic contamination. Casonova et al. (1992) biotinylated cell wall proteins during their growth stages, and performed extraction of cell wall proteins using β-mercaptoethanol and β-glucanses, confirmed extracted proteins to be of cell wall origin by doing extravidin alkaline peroxidase reaction in western blotting.

SUMMARY

With the above findings it can be concluded that one can use the method by Pitarch et al. (2002), for extraction of candida cell wall proteins with proper homogenization by lysis of the cells using glass beads (0.5 mm) and simple vortex mixer. Modification adopted here made it easy to follow this method in laboratories which do not have bead beaters or blenders or sonicators.

4.4.1.2.1.2CELL WALL PROTEIN PROFILING AND LECTIN BLOTTING PATTERN OF THE ISOLATES FROM HIV SEROPOSITIVE AND HIV SERONEGATIVE GROUP

After setting up the protein extraction method and conditions required for performing SDS PAGE, the actual work on protein profiling was carried out. The proteins obtained here were further subjected for lectin blotting studies, to evaluate the carbohydrate moieties present in them.

I. PROTEIN PROFILING

Cell wall protein profiling was done with extracts of 34 *C. albicans* isolates obtained, from HIV seropositive patients and HIV seronegative healthy subjects and list of isolates tested for protein profiling are given in Table 3.4 of Chapter Materials and Methods. The protein profiling here revealed at least 36 proteins of molecular weights ranging between 8 kDa-151.4 kDa. SDS PAGE was conducted thrice for each candida isolate and reproducible results were obtained. In all the strains a few bands got more prominently stained , MWs of these bands are, 81.12, 74.13, 57.13, 46.17, 38.9, 38.02, 37.15, 31.62, 27.54, 26.92, 25.70, 22.39, 19.5, 18.62, 18.20, 14.8, 12.59, 12.8, 14.13 and 10 kDa. The pictures of SDS PAGE both stained by silver staining method and Coomassie Brilliant Blue are shown in Fig. 4.33, 4.34, 4.35, 4.36.Nodifference is found between protein profiles obtained from the isolates from HIV seropositive individuals and HIV seronegative group.Molecular weights obtained in all the 34 samples are given in Table 4.32.

Table 4.32 Molecular weights of protein bands obtained in all the 34 isolates tested and the protein profile obtained for individual isolate has been given (Gel percentage used were 10-14%, 10-15%, 10-17%, 12-17% and 5-20%)

MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	Isolates tested
75.86	83.18	97.72	125.9	128.8	**RL-18**
60.26	63.1	70.79	147.9	151.4	**CL-01**
75.86	83.18	97.72	125.9	128.8	**RL-04**
60.26	63.1	70.79	147.9	151.4	**CN-181**
60.26	63.1	70.79	147.9	151.4	**RL-03**
75.86	83.18	97.72	125.9	128.8	**CN-173**
60.26	63.1	70.79	147.9	151.4	**RL-06**
75.86	83.18	97.72	125.9	128.8	**CN-111**
60.26	63.1	70.79	147.9	151.4	**RL-09**
60.26	63.1	70.79	147.9	151.4	**CN-67**
75.86	83.18	97.72	125.9	128.8	**RL-17**
75.86	83.18	97.72	125.9	128.8	**CN-188**
60.26	63.1	70.79	147.9	151.4	**RL-13**
75.86	83.18	97.72	125.9	128.8	**CN-194**
64.57	75.86	77.62	87.1	97.72	**RL-112**
75.86	83.18	97.72	125.9	128.8	**CN-31**
75.86	87.1	91.2	97.72	104.7	**RL-24**
60.26	63.1	70.79	147.9	151.4	**CN-192**
33.88	41.69	43.65	56.23	91.2	**RL-23**
79.43	81.28	91.2	93.33	97.72	**CN-176**
75.86	83.18	97.72	125.9	128.8	**RL-28**
64.57	66.07	75.86	85.11	93.33	**CN-102**
81.28	85.11	87.1	93.33	107.2	**RL-29**
69.18	81.28	87.1	93.33	107.2	**RL-26**
81.28	85.11	87.1	93.33	107.2	**RL-27**
81.28	85.11	95.5	109.6	117.5	**RL-40**
81.28	85.11	95.5	109.6	117.5	**RL-41**
81.28	85.11	95.5	109.6	117.5	**RL-53**
81.28	85.11	95.5	109.6	117.5	**RL-47**
81.28	85.11	95.5	109.6	117.5	**CN-07**
81.28	85.11	95.5	109.6	117.5	**CN-65**
81.28	85.11	95.5	109.6	117.5	**CN-69**
81.28	85.11	95.5	109.6	117.5	**CN-62**
81.28	85.11	95.5	109.6	117.5	**CN-28**

MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa
48.98	50.12	53.7	56.23	60.26	66.07	67.61
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
48.98	50.12	53.7	56.23	60.26	66.07	67.61
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
48.98	50.12	53.7	56.23	60.26	66.07	67.61
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
48.98	50.12	53.7	56.23	60.26	66.07	67.61
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
48.98	50.12	53.7	56.23	60.26	66.07	67.61
48.98	50.12	53.7	56.23	60.26	66.07	67.61
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
48.98	50.12	53.7	56.23	60.26	66.07	67.61
32.36	39.81	41.69	43.65	47.86	56.23	60.26
48.98	50.12	53.7	56.23	60.26	66.07	67.61
35.48	41.69	43.65	52.48	54.95	60.26	69.18
33.11	35.48	**37.15**	43.65	45.71	47.86	57.54
		14.45	22.91	26.92	28.18	29.51
52.48	56.23	57.54	58.88	61.66	66.07	74.13
48.98	50.12	53.7	56.23	60.26	66.07	67.61
	18.2	31.62	38.02	56.28	57.54	58.88
57.54	58.88	64.57	66.07	74.13	75.86	79.43
39.81	43.65	53.7	57.54	58.88	61.66	64.57
58.88	61.66	64.57	66.07	74.13	75.86	79.43
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**
46.77	53.7	**57.54**	60.26	66.07	70.79	**74.13**

MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa
38.9	74	40.	41.69	42.66	44.67	45.71
22.91	55	24.	25.7	**27.54**	29.51	30.9
38.9	74	40.	41.69	42.66	44.67	45.71
22.91	55	24.	25.7	**27.54**	29.51	30.9
22.91	.55	24	25.7	**27.54**	29.51	30.9
38.9	74	40.	41.69	42.66	44.67	45.71
22.91	55	24.	25.7	**27.54**	29.51	30.9
38.9	74	40.	41.69	42.66	44.67	45.71
22.91	55	24.	25.7	**27.54**	29.51	30.9
22.91	55	24.	25.7	**27.54**	29.51	30.9
38.9	74	40.	41.69	42.66	44.67	45.71
38.9	74	40.	41.69	42.66	44.67	45.71
22.91	55	24.	25.7	**27.54**	29.51	30.9
38.9	74	40.	41.69	42.66	44.67	45.71
13.18	.6	16	18.62	22.91	28.18	29.51
38.9	74	40.	41.69	42.66	44.67	45.71
17.38	38	21.	25.12	26.92	30.9	33.88
22.91	55	24.	25.7	**27.54**	29.51	30.9
36.31	02	38.	41.69	43.65	44.67	46.77
38.9	74	40.	41.69	42.66	44.67	45.71
43.65	67	44.	47.86	52.48	53.7	56.23
				19.95	33.88	38.9
44.67	77	46.	50.1	52.48	53.7	56.23
26.92	36	32.	36.31	**38.02**	41.69	42.66
26.92	36	32.	36.31	**38.02**	41.69	42.66
26.92	36	32.	36.31	**38.02**	41.69	42.66
26.92	36	32.	36.31	**38.02**	41.69	42.66
26.92	.36	32	36.31	**38.02**	41.69	42.66
26.92	.36	32	36.31	**38.02**	41.69	42.66
26.92	.36	32	36.31	**38.02**	41.69	42.66
26.92	.36	32	36.31	**38.02**	41.69	42.66
26.92	36	32.	36.31	**38.02**	41.69	42.66

MW in kDa	**MW in kDa**	**MW in kDa**	**MW in kDa**	**MW in kDa**	**MW in kDa**	**MW in kDa**
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
						15.85
12.3	13.18	**14.13**	15.14	15.85	18.62	22.39
27.5	30.9	**31.62**	33.11	33.48	35.48	36.31
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12
13.8	15.85	16.98	**18.62**	**19.95**	23.44	25.12

MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa	MW in kDa
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
				9.33	**10**	11.52
13	13.8	16	**18.2**	20.4	**22.4**	**25.7**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**
			10.72	11.2	12.3	**12.59**

Fig. 4.33. SDS PAGE (12-17%) of samples from first lane standard protein molecular weight marker followed by cell wall protein samples from RL-13, RL-29, CL-01,RL-06, CN-67, RL-03, CN-181, RL-09, CN-192 (Silver stained)

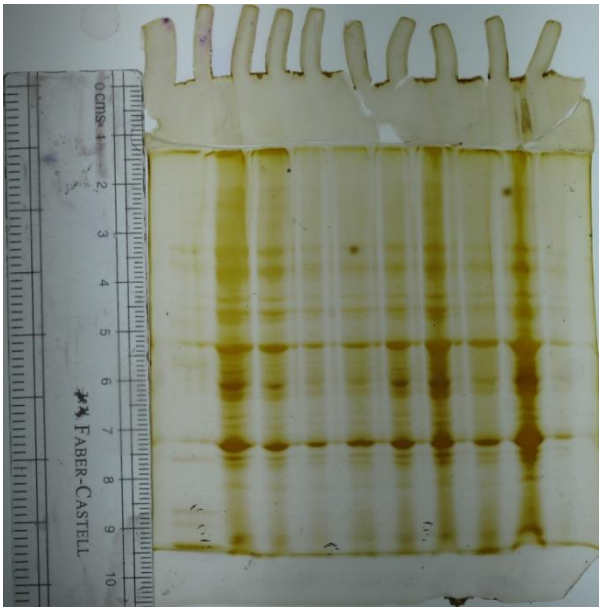


Fig. 4.34 SDS PAGE (12-17%) of samples; from left protein standard molecular marker followed by cell wall protein samples from CN-111, RL-17, CN-188, CN-194, RL-28, CN-173, RL-04, CN-31, RL-18. (Silver Stained)

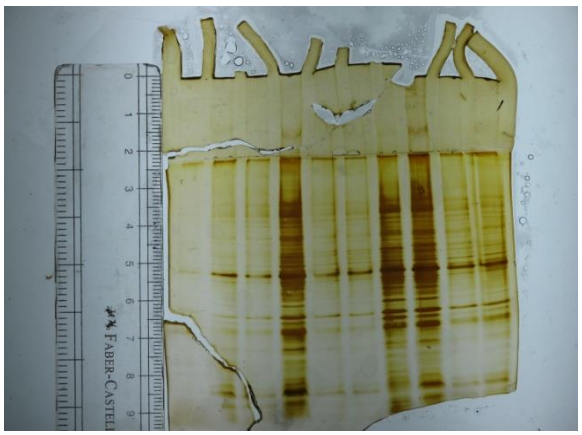


Fig. 4.35 SDS PAGE (10-14%) containing first left lane as standard protein marker followed by CN-192, CN-188, CN-181, CN-176, CN-102, RL-29, RL-28, RL-27, RL-28 (Silver staining)

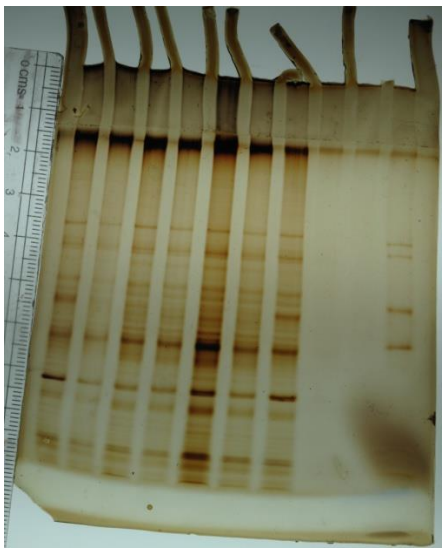


Fig. 4.36 SDS PAGE (10-15%) showing pictures of candida cell wall protein samples from the left serially RL-23, RL-23 (2nd sample), RL-24, CN-67, CN-111, CN-173, CN-181, CN-112, Sample buffer and last lane containing standard protein molecular weight marker (Silver staining)

III. ROLE OF CANDIDAL CELL WALL PROTEINS ON CANDIDA ADHESION

The HBEC, pretreated with cell wall proteins of candida was washed and subjected to adhesion. When compared for the reduction in adhesion, the cell wall protein treated HBEC showed 89.25% to 94.10% of reduction in adhesion when adhesion reaction was carried out with the same strain of candida whose cell wall proteins were used to treat the HBEC. When adhesion reaction carried out with different strains than the one used for pre treating the HBEC, percentage of reduction in adhesion shown was less. Results are given in Table 4.33 and Fig. 4.37.

Table 4.33 Role of cell wall proteins on adhesion of candida onto HBEC, as determined by *in vitro* adherence test

Sample	Percentage of HBEC adhered with candida	Average no. of cells per HBEC	Percentage of reduction in adherence
When CL-01 was exposed to the adhesion with plain HBEC	44.42	1.4	
When CL-01 exposed to HBEC which are pretreated with cell wall proteins of CL-01	1.62	1.2	94.10%
HBEC pretreated with cell wall proteins of CL-09 and adhesion was carried out with CL-01	11.68	1.43	73.82%
When CL-09 reacted with plain HBEC	30.07	1.41	
When CL-09 exposed to react with HBEC pretreated with cell wall proteins from CL-09	3.23	1.18	89.25%
HBEC pretreated with cell wall proteins of CL-01 and adhesion reaction was carried with CL-09	14.45	1.45	51.94%

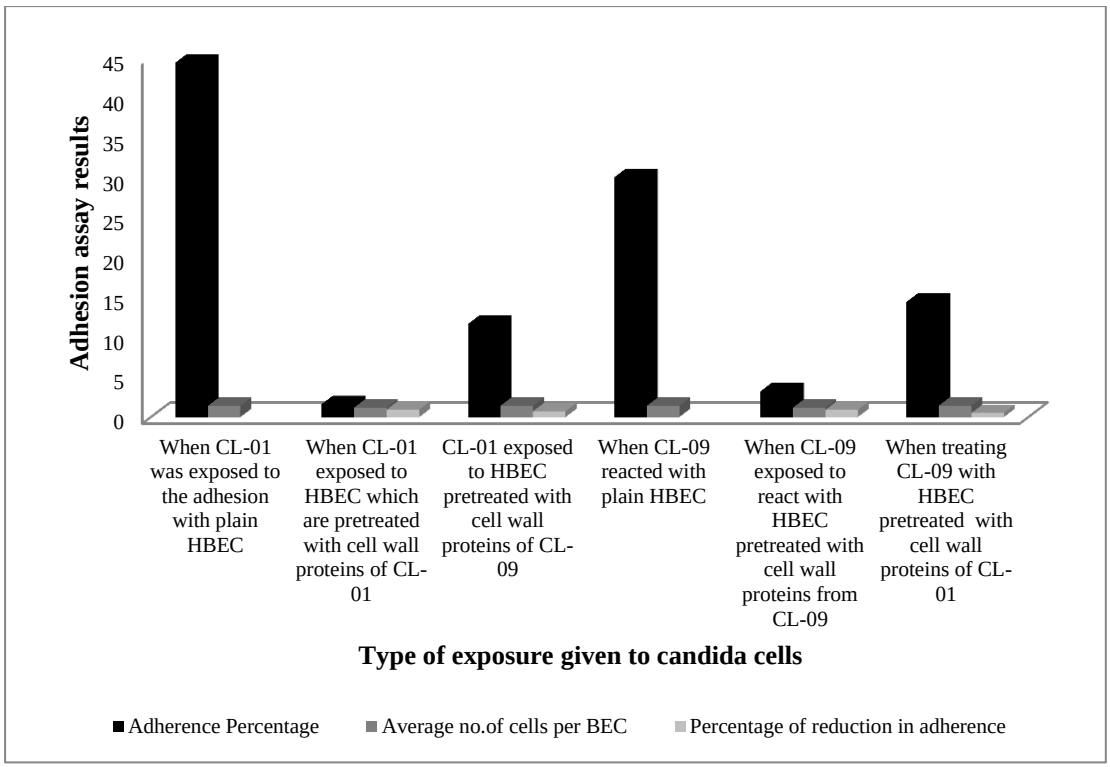


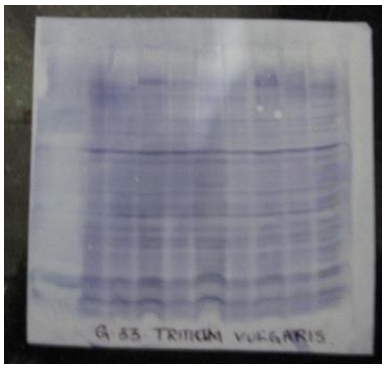
Fig. 4.37 Effect of candida cell wall protein on adhesion

There are a very few reports on the nature of cell wall proteins of *C. albicans* isolates from HIV seropositive subjects (Marcilla et al. 1998; Lopez-Ribot 1999; Shahid et al. 2006). In the work by Shahid et al. (2006), the membrane proteins of MWs of 73, 62, 60.5, 51, 42 kDa were obtained. In the heavily stained proteins one with MW 74.13 kDa obtained in the present study may be similar to the protein of MW 73, and this was observed by Shahid et al. (2006). Shahid et al. (2006), say that 42 kDa proteins expressed in their results can be the same as of 43 kDa protein obtained in the studies by Tavares et al. (1995). Antibodies produced against this protein can be immunoprotective in nature (Tavares et al. 1995). Cell wall protein profile of *C. albicans* isolates from HIV seropositive individuals in response to their fluconazole sensitivity pattern are reported previously, the study reports significant differences in cell wall protein components of fluconazole resistant and susceptible isolates of *C. albicans* (Shahid et al. 2006). In their work, fluconazole resistant strains formed separate cluster, when subjected to the protein profiling results of both fluconazole sensitive and resistant isolates, for cluster analysis. However in the present study, there is no change seen with protein profile of isolates from HIV

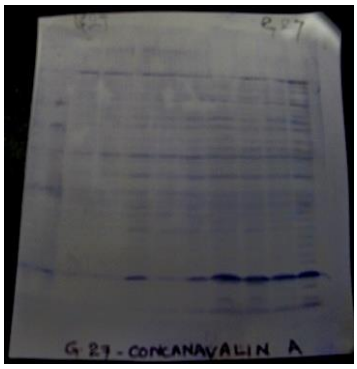
seroposiive and HIV seronegative groups. There is no difference found in the protein profile's results of fluconazole sensitive and resistant strains also.

IV. LECTIN BLOTTING

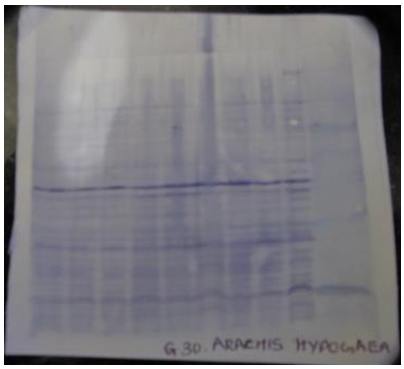
Four different lectins were used for this test. The results obtained in lectin blotting test is presented in Fig. 4.38 (i, ii, iii, iv). Results obtained are as follows.,



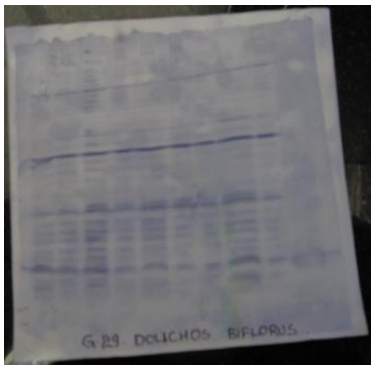
i.



ii.



iii.



iv.

Fig. 4.38 Lectin blotting Pattern of Candida cell wall proteins with
i) lectin from *Triticum vulgaris ii) Concanavalin A* (Con A) lectin

iii) lectin from *Arachis hypogaea* iv) lectin from *Dolichos biflorus*

A. RESULTS WITH LECTIN *CONCANAVALIN A* (CON A)

Lectin Con A can bind to α mannose and α glucose moiety and present on any glycolproteins (Sharon and Lis 1972). Number of bands detected from Con A in each specimen was more compared to other lectins used in this study. The MWs of bands detected ranged from MWs 154.9 to 12.87 kDa. No significant difference was seen in the bands detected between strains from HIV seropositive and HIV seronegative individuals by Con A.

B. RESULTS WITHLECTIN *DOLICHOS BIFLORUS*

Lectin from *Dolichos biflorus* binds to α-N-Acetyl galactosamine moiety exists in the protein structure (Sharon and Lis 1972). In the present study, this lectin detected 36 bands which was the highest. Bands detected by *Dolichos biflorus* in different experiments were ranging from MWs 131.8 kDa to 11.22 kDa. In addition to this, interestingly lectin from *Dolichos biflorus* recognized 5 extra proteins of high MWs 125.9, 120.02, 131.8, 112.2, 104.7, in *C. albicans* of HIV seropositive subjects. This result indicates that glycosylation of the cell wall proteins occurred in *C. albicans* of HIV seropositive individuals. This finding demands future work to be conducted in relation to find the role of glycosylation on virulence activity of the oral *C. albicans* isolates from HIV seropositive individuals.

B. RESULTS WITHWHEAT GERM AGGLUTININ (WGA); A LECTIN FROM *TRITICUM VULGARIS*

Lectin from *Triticum vulgaris* (Wheat Germ Agglutinin; WGA)binds to (gluc-NAC)$_2$ and Neuraminic N-Acetyl sugar moiety (Sharon and Lis 1972). In the present study this lectin has bound and detected 30 bands in each sample. Compared to isolates from HIV seronegative individuals, only one extra protein band at 109.6 kDa was detected by WGA in samples from HIV seropositive group. This result indicates that, *C. albicans* in HIV seropositive individuals are undergoing more glycosylation and this post translational modification might have some role in their virulence.

D. RESULTS WITHPEANUT AGGLUTININ (PNA);LECTIN *ARACHIS HYPOGAEA*

Lectin from *Arachis hypogaea* (Peanut agglutinin, PNA) recognizes β (1-3) gal NAC band of glycoprotein structure (Sharon and Lis 1972). In the present study, samples from HIV seropositive group showed one extra protein of MW 61.66 kDa, along with other common bands compared to HIV seronegative strains. This result again indicates possible glycosylation of this protein only in strains from HIV seropositive group.

Lectin blotting is the technique used in proteomics, to detect carbohydrate moiety on the biomolecules. It is an important and specific tool to identify post translational modifications such as glycosylation (Walker 2009). Glycosylation is a fundamental process in biopolymers found in the cells, where a carbohydrate will be added on to a protein or lipid and results in structural and functional changes in that molecule. Glycosylation of membrane proteins play a role in cell to cell adhesion via carbohydrate-binding proteins called lectins (Byrne et al.2007; Goldstein and Hayes 1978).

For the work of lectin blotting, the proteins of interest were separated by gel electrophoresis, blotted onto Nitrocellulose / PVDF membranes. The proteins were further analyzed using lectins as detecting reagents. Lectins are carbohydrate binding proteins and thus can analyze the glycan structures of glycoprotein. Biotinylated lectins and Avidin conjugated alkaline phosphate or Avidin conjugated with Horse Radish Peroxidase (HRP) was used. The complex formed subsequently revealed either by a chemiluminescent or a colorimetric reaction (Walker 2009). According to the present research work it is evident that, certain cell wall proteins in *C. albicans* isolates in HIV seropositive individuals undergo structural modification and that is found to be glycosylation and thus they differ from isolates from HIV seronegative individuals in their adhesion capacity.

Toriniwa and Komiya (2009) reported that the presence of glycosylation in Vero cells of vaccine preparations for Japanese encephalitis had resulted in higher

immunogenicity compared with conventional vaccine. The authors detected the presence of glycosylation in these vaccine cells using lectin blotting techniques. Adhesion activity of organism of present research interest, i.e., *C. albicans*, has also studied using lectin blotting. In *C. albicans* hydrophobic proteins undergo mild glycosylation. This change in glycosylation determines exposure of these protein regions at the cell surface and influences the adhesion of these candida cellsto HBEC (Fukazawa and Kagaya 1997). Thus understanding the protein glycosylation is a special requisite to pinpoint their role in normal and pathological states. The lectin blotting pattern of isolates from HIV seropositive group showed that, a few extra bands of proteins were detected while testing with lectins specific for N-acetyl glucosamine (gluc NAc) $_{2,}$ and β (1-3) gal NAc sugars, which were not detected with isolates from HIV seronegative individuals. These differences could be one of the reasons for increased adherence and colonization exhibited by candida isolates from HIV seropositive group. However, similar kind of work with lectin blotting of cell wall proteins from HIV seropositive and HIV seronegative group is not reported in literatures.

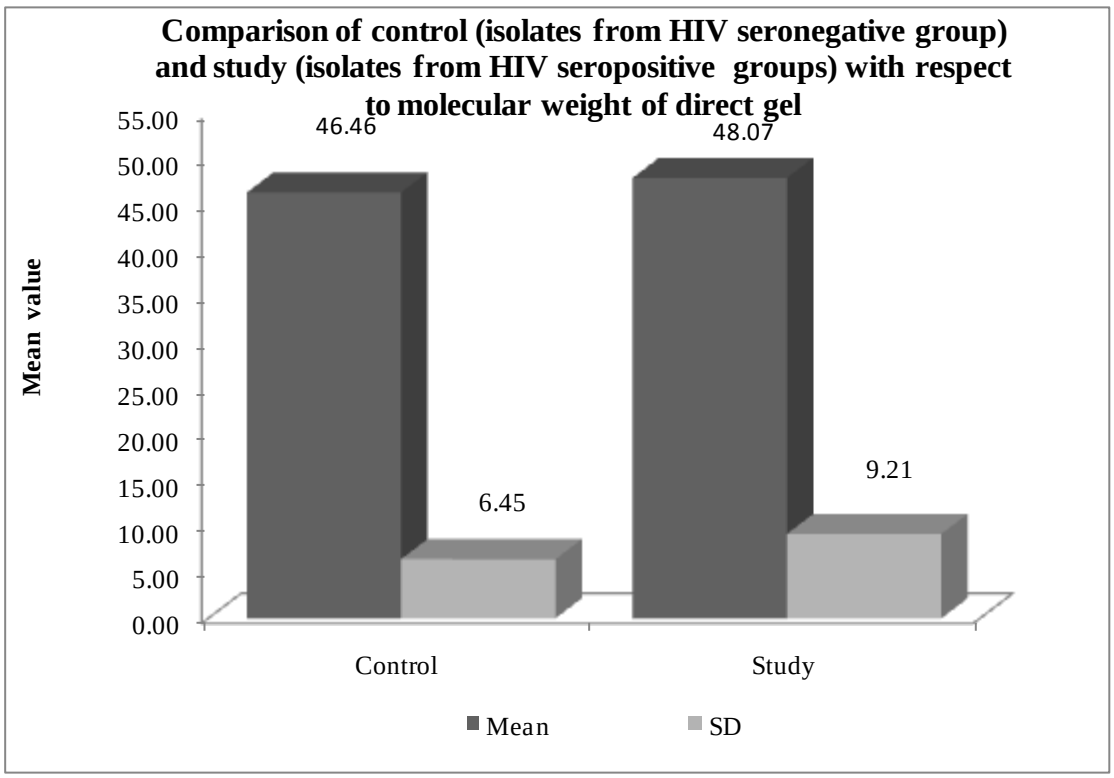
V. STATISTICAL TESTS USED TO ANALYZE THE RESULTS OBTAINED IN PROTEIN PROFILING AND LECTIN BLOTTING

Statistical Table 1 Mean and SD of molecular weight among groups

Groups	Mean	SD
Isolates from HIV seronegative group	41.96	9.88
Isolates from HIV seropositive group	44.98	11.83
Total	43.59	11.05

Statistical Table 2 Comparison of control and study groups with respect to molecular weight of direct gel by unpaired t test

Group	N	Mean	SD	t-value	p-value
Isolates from HIV Seronegative group	16	46.4558	6.4493	-0.5834	0.5637
Isolates from HIV Seropositive group	18	48.0664	9.2098		



Statistical Fig. 1

Note: In the figure, control indicates isolates from HIV seronegative group. Study indicates isolates from HIV seropositive group.

Statistically assays revealed that the protein profiling results obtained in isolates from HIV seronegative group did not differ significantly when compared to the protein profile obtained by isolates from HIV seropositive group.

Statistical Table 3. Mean and SD of molecular weight among groups and four different Lectins

Lectins	Mean	SD
WGA (Lectin from *Triticum vulgaris*)	38.96	8.57
Concanavalin A	47.45	14.14
PNA (Lectin from *Arachis hypogaea*)	42.66	10.46
Lectin from *Dolichos biflorus*	45.31	8.67
Total	43.59	11.05

Statistical Table 4. Mean and SD of molecular weight of Lectins among test groups and four control groups.

Interaction	Mean	SD
Control x WGA	38.65	9.48
Control x Con A	43.28	11.86
Control x PNA	41.52	9.18
Control x Lectin from *Dolichos biflorus*	44.38	8.68
Test x WGA	39.22	7.97
Test x Con A	50.96	15.23
Test x PNA	43.62	11.58
Test x Lectin from *Dolichos biflorus*	46.10	8.83
Total	43.59	11.05

Statistical Table 5Two way ANOVA with interaction between groups and Lectins on molecular weight

SV	Degrees of Freedom	Sum of Squares	Mean Sum Squares	F-value	P-value
Main effects					
Groups	1	317	316.9	2.7909	0.0972
Lectins	3	1314	438.0	3.8576	0.0110*
2-way interactions					
Groups x Lectins	3	263	87.7	0.7728	0.5112
Error	132	14987	113.5		
Total	139	16881			

*p<0.05

Statistical Table 6. Pair wise comparison of four Lectins by Least Square Difference post hoc procedure

Lectins	**WGA**	**Con A**	**PNA**	**Lectin from *Dolichos biflorus***
Mean	38.93566	47.11886	42.56726	45.23904
WGA	-			
Con A	P=0.0017*	-		
PNA	P=0.1563	P=0.0762	-	
Lectin from *Dolichos biflorus*	P=0.0146*	P=0.4618	P=0.2961	-

*p<0.05

A Least square post hoc procedure was applied to see the significant differences between two lectins; the results are presented in the above statistical table 6. It clearly shows that, WGA significantly differs with Con A and lectin from *Dolichos biflorus* at 5% level (p<0.05). It means that, the molecular weight is significantly higher in Con A as compared to WGA. But in other combinations the molecular weight is similar.

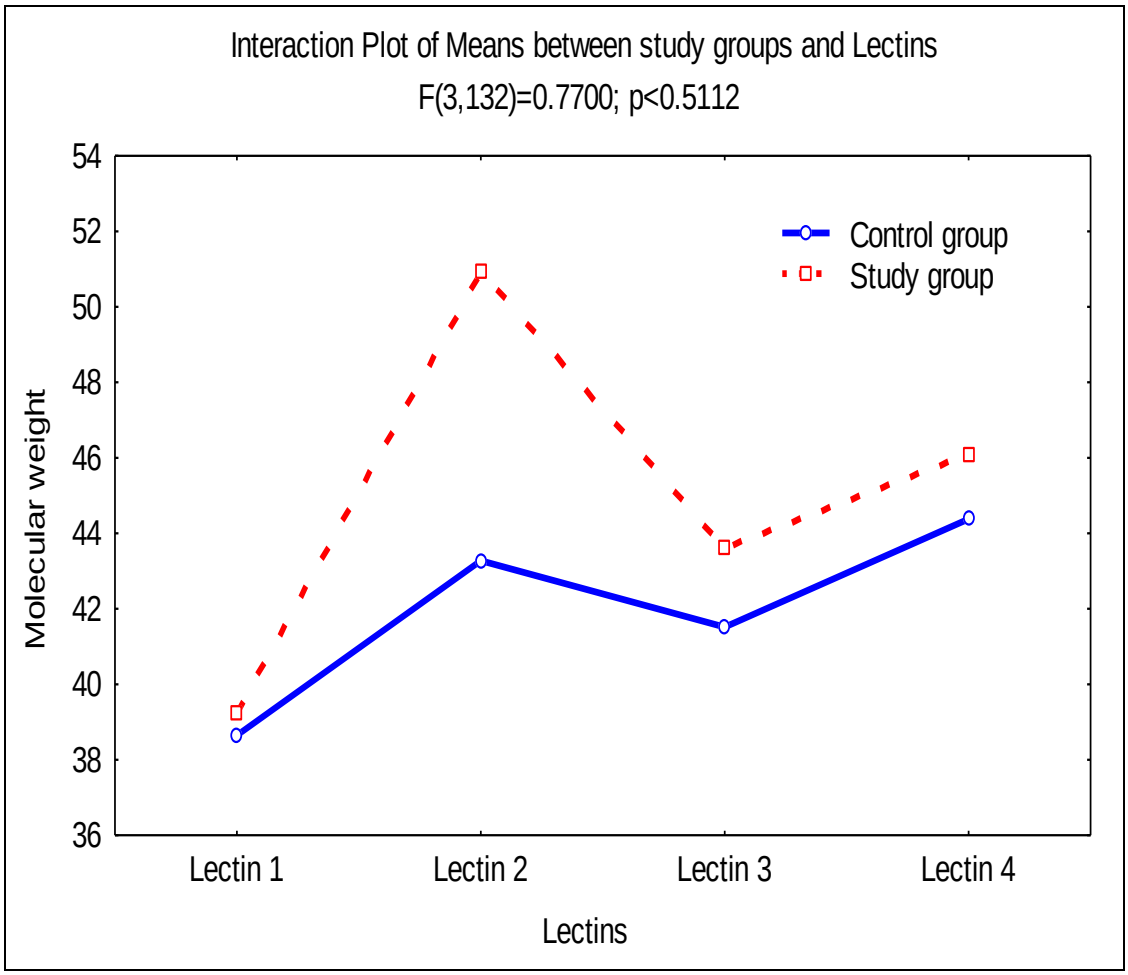
Statistical Table 7. Pair wise comparison of interactions between groups and Lectins by LSD post hoc procedure

Interaction	Control x WGA	Control x Con A	Control x PNA	Control x Lectin from *Dolichos biflorus*	Study x WGA	Study x Con A	Study x PNA	Study x lectin from *Dolichos biflorus*
Mean	38.6487	43.2752	41.5166	44.3802	39.2226	50.9625	43.6179	46.0979
Control x WGA	-							
Control x Con A	P=0.2216	-						
Control x PNA	P=0.4479	P=0.6414	-					
Control x lectin from *Dolichos biflorus*	P=0.1306	P=0.7697	P=0.4485	-				
Study x WGA	P=0.8741	P=0.2644	P=0.5269	P=0.1561	-			
Study x Con A	P=0.0009*	P=0.0353*	P=0.0100*	P=0.0709	P=0.0009*	-		
Study x PNA	P=0.1716	P=0.9246	P=0.5621	P=0.8333	P=0.2058	P=0.0355*	-	
Study x Lectin from *Dolichos biflorus*	P=0.0413*	P=0.4364	P=0.2073	P=0.6355	P=0.0488*	P=0.1617	P=0.4744	-

*p<0.05

The results of Statistical Table 7 shows that, WGAis significantly differs with Con A in the study group and lectin from *Dolichos biflorus* in isolates from HIV seropositive group at 5% level ($p<0.05$). It means that, the molecular weight is significantly higher in Con A in the study group and as compared to WGA in control. But in other combinations the molecular weight is similar.

The interaction effects of groups and lectins are also presented in the following figure.



Statistical Fig. 1 Interaction plot of means between study groups and lectins

Note: Lectin 1: WGA Lectin 2: ConA Lectin 3: PNA,

Lectin 4: Lectin from *Dolichos biflorus*

Statistical Table 8. Karl Pearson's correlation coefficient among different Lectins in the control group

MW	WGA	Con A	PNA	Lectin from *Dolichos biflorus*
WGA	r=1.0000			
Con A	r=0.3703	r=1.0000		
PNA	r=0.1022	r=-0.3215	r=1.0000	
Lectin from *Dolichos biflorus*	r=0.7974*	r=0.0913	r=0.4924	r=1.0000

Statistical Table 9. Karl Pearson's correlation coefficient among different Lectins in the study group

MW	*WGA*	*Con A*	*PNA*	Lectin from *Dolichos biflorus*
WGA	r=1.0000			
Con A	r=0.8323*	r=1.0000		
PNA	r=0.0001	r=0.2860	r=1.0000	
Lectin from *Dolichos biflorus*	r=-0.1958	r=-0.1865	r=0.6364*	r=1.0000

*p<0.05

Statistical Table 10 Karl Pearson's correlation coefficient among different Lectins in total of control and study groups

MW	WGA	Con A	PNA	Lectin from *Dolichos biflorus*
WGA	r=1.0000			
Con A	r=0.6025*	r=1.0000		
PNA	r=0.0473	r=0.1041	r=1.0000	
Lectin from *Dolichos biflorus*	r=0.2947	r=-0.0470	r=0.5808*	r=1.0000

*p<0.05

Statistical Table 11 Multiple logistic regression analysis of control and study groups by different Lectins

Variables	Coefficient	Std. Err.	Z-value	p-value	95% CI	
Constant	3.6712	2.1161	-1.7300	0.0830	-7.8187	0.4763
WGA	0.6800	0.9953	-0.6800	0.4940	-2.6306	1.2707
Con A	1.4287	1.0158	1.4100	0.1600	-0.5622	3.4195
PNA	0.0376	0.8695	0.0400	0.9650	-1.6666	1.7419
Lectin from *Dolichos biflorus*	1.7622	0.8374	2.1000	0.0350*	0.1208	3.4035

*p<0.05

Log likelihood = -19.7491, LR chi^2 (4) = 8.7600, p= 0.0673

4.4.1.2.1.3 PROTEIN PROFILING AND LECTIN BLOTTING PATTERN OF SECRETED PROTEINS PRESENT IN THE CULTURE SUPERNATANT OF THE ISOLATES FROM HIV SEROPOSITIVE AND HIV SERONEGATIVE GROUP

I. PROTEIN PROFILING

The culture supernatants of four different isolates of *C. albicans* were studied for protein profiling and lectin blotting analysis. Only three proteins were obtained in each sample. SDS PAGE was done three times using different gradient gels (10-17% and 5-20%). The bands found were of MWs, 58.95, 50.50, 41.21 kDa respectively as shown in Fig. 4.39.

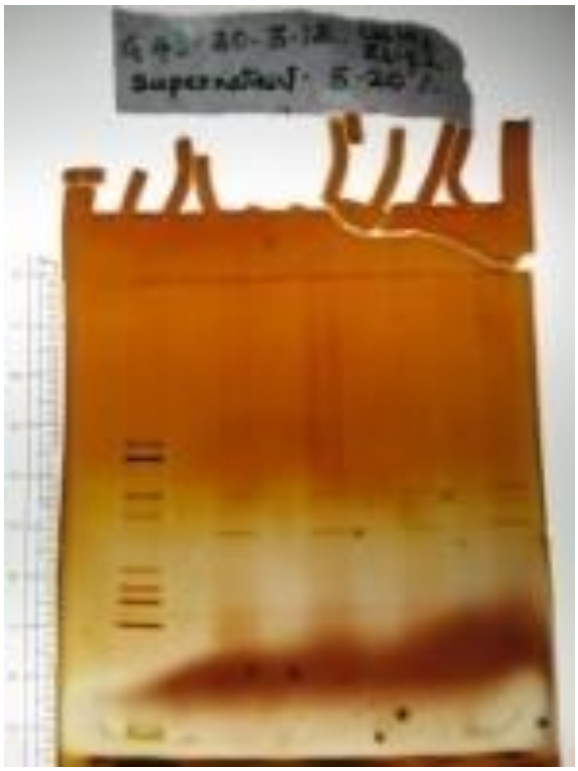


Fig. 4.39 Silver staining of SDS PAGE (5-20%) of proteins of *C. albicans* secreted to culture medium;

Three bands can be observed in the test sample. The protein molecular weight marker run in the first left lane.

Proteins secreted to culture medium in *C. albicans,* were previously studied to induce antibody production in rabbits. These antibodies were used for the detection of secretary proteins of candida in the clinical specimens (Juanm et al.1993). A fucose binding protein has also been isolated from culture supernatants of *C. albicans* showed role in adhesion of candida on to HBEC (Critchey and Douglas 1987). The work done in section 4.1.1 also showed that the supernatant contained some lectin activity. However, the hemagglutination shown here was restricted to only for 'O' blood group RBCs. For further analysis of carbohydrate moieties present on these proteins, lectin blotting was done.

II. LECTIN BLOTTING ANALYSIS DONE WITH SUPERNATANT PROTEINS

When lectin blotting of these proteins was performed, positive reaction was seen only with Con A. Other lectins used in the test are WGA, lectin from *Dolichos biflorus* andPNA. They did not show detection of any proteins, which is shown in Fig. 4.40. Since these proteins are detected by Con A alone, these results indicate the presence of only mannose and or glucose moieties in their structure. The proteins of culture supernatant did not possess β- gal (1-3) gal NAc, $(\text{gluc NAc})_2$ Neu NAc and α -gal NAc moieties as proved by their absence of reaction with other 3 lectins.

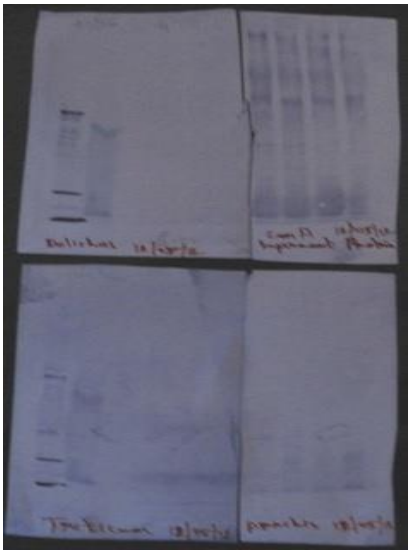


Fig. 4.40 Lectin blotting pattern of proteins of *C. albicans* secreted to culture medium

Note: Positive reaction was seen only with Con A.

Indirect testing for lectin activity of secreted proteins in the growth extracts of *C. albicans* showed agglutination only with "O" group RBCs, made to conclude that, these proteins contain fucose specific lectins like components secrets in spent medium. Cameron and Douglas (1996) reported that crude mannoproteins from culture supernatant of *C. albicans* contains fucose specific adhesins with MW more than 15.7 kDa containing approximately 52 amino acids. However, in the present study, the proteins of MW of 15.7 kDa were not found.

III. ROLE OF SECRETED PROTEINS ON CANDIDA ADHESION

The HBEC were pretreated with secreted proteins of candida, then washed and subjected to adhesion. When compared with the reduction in adhesion, the secreted protein treated HBEC showed reduction in adhesion of candida cells compared to the control HBEC which were plain or untreated. Results are shown in Table 4.34. Here, the average number of candida cells adhering per HBEC was also reduced when tested with pretreated HBECs.

Table 4.34 Studies on effect of Protein of *C. albicans* strains secreted to culture supernatants on adhesion pattern of candida to HBEC

Isolates tested	Adherence of candida cells to plain HBEC		Adherence of candida cells to HBEC preincubated with supernatant protein		Percentage of reduction in HBEC adhered with candida
	Percentage of HBEC adhered By candida	No. of candida cells per HBEC	Percentage of HBEC adhered by candida	No. of candida cells per HBEC	
RL-112	76.2	1.96	57.6	1.91	24.40
RL-24	65.7	1.52	44.6	1.47	32.11
CN-192	62.0	1.96	40.4	1.67	34.83
CN-111	64.3	1.96	55.6	1.65	13.53

Here it is observed that the secreted proteins of *C. albicans* play a role in adhesion of candida onto HBEC. However reduction is not as strong as shown in cell wall protein experiments. The experiment here was conducted with the isolates from HIV seropositive individuals, RL-112 and RL-24 and with the isolates from HIV seronegative individuals, i.e., CN-192 and CN-111. RL-112, has been isolated from HIV seropositive patient with OC, had shown high percentage of HBEC adhered with candida. However the reduction pattern in adhesion after the HBEC treated with supernatant proteins was similar among, in all the isolates. Secreted aspartyl proteinases, phospholipase B and lipases are the extracellular hydrolytic enzymes of *C. albicans*. Secreted aspartyl proteinases (SAP) are known to be involved in adhesion to HBEC in *in vitro* experimental conditions. Higher SAP producing candida caused more colonization in liver, spleen and kidney of mice (Fallon et al. 1997).

Pepstatin, a proteinase inhibitor treatment caused reduction in *C. albicans* invasion of the mucosa in different experiments proved the role of SAP in adhesion (Ollert et al. 1993). Increased SAP production was seen with *C. albicans* strains isolated from HIV seropositive patients than isolates from HIV seronegative healthy carriers and also with HIV seronegative individuals with OC lesions (De Bernadis et al. 1992). Isolates from patients in advanced stages of HIV infection have more production of SAP than patients with early stages of infection (De Bernardis et al. 1992; Ollert et al. 1993). This leads to the assumption that isolates with more virulence might be selected in HIV infection. It was shown that elevations in SAP production occur in candida due to the influence of HIV antigens like GP 120, GP 160, GP 41 (Gruber et al. 1998; Naglik et al. 2003). The secreted proteins isolated in the present study had shown to be involved in adhesion; however their biochemical characterization and further elucidation of their role in antigenicity can be performed as future perspective.

4.4.1.2.1.4 INCUBATION OF EPITHELIAL CELL MEMBRANE PROTEIN (EP) WITH CANDIDA CELL WALL PROTEINS (CP)

4.4.1.2.1.4.1 PROTEIN PROFILING

The candida cell wall protein (CP), epithelial cell membrane proteins (EP) and proteins after adhesion (CEP) were subjected for gel electrophoresis (10-17% and 12-20%). The EP and CP were mixed in the quantity of 250 µl each and kept for incubation at 37 ^{o}C at 150 rpm for 2 hrs. The solution was centrifuged and washings were collected in another micro centrifuge tube and named as WP assuming that a few proteins might be present in washing which were supposed to be unadhered proteins. Finally; the gel electrophoresis was run using the following samples, CP, EP, CEP and WP along with standard molecular weight marker in each set of gels. The results are given in Table 4.35a and 4.35b. In these experiments it is found that on incubation of CP with EP, a few proteins disappear and those are listed in the respective tables. These proteins may be washed away or may not be participating in adherence. In the experiments shown in Table 4.35a, only a few epithelial proteins did not exist in CEP, however all CP were retained after adhesion

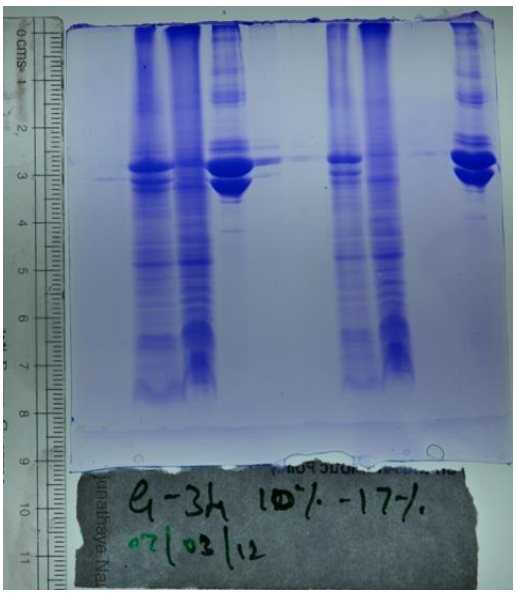


Fig. 4.41a SDS PAGE (10-17%) gel with experiments involved with cell wall proteins of candida and HBEC, (CBB Staining)

Note: From left; first lane contained standard protein marker, lane2nd represents CEP, 3rd lane is containing CP, 4th well is EP, 5th and 6th with washings, 7th with CEP, 8th lane is representing CP, 9th lane is with sample buffer and 10th well is representing with again EP (CBB staining used in standardization only).

Fig. 4.41b SDS PAGE (12-20%) gel with experiments involved with cell wall proteins of candida and HBEC (Silver staining)

Note: From left; first well contains standard protein marker, 2nd lane represents only sample buffer, third well represents cell wall protein of RL-24, 4th well represents Cell membrane protein of HBEC, wells 5,6 and 7 represent WP (supernatant), 8, 9 and 10th well are containing CEP (candida and epithelial cell wall proteins combined).

Table 4.35aProtein profiles obtained with EP, CP and CEP, when tested at 10-17% of the gel (G-34 B)

EP MW in kDa	**CP** MW in kDa	**CEP** MW in kDa	**WP (Proteins obtained in wash)** MW in kDa	**MW of the heavily stained bands** MW in kDa	**Proteins present only in CEP** MW in kDa	**Proteins of EP not observed in CEP** MW in kDa	**Proteins of CP not observed in CEP** MW in kDa
120.2	120.2	120.2	53.7	56.23	NIL	112.2	NIL
112.2	79.43	79.43		53.7		109.6	
109.6	77.62	77.62		51.29		97.72	
97.72	67.61	67.61		47.86		100	
100	66.07	66.07		46.77		91.2	
91.2	64.57	64.57		45.71		89.13	
89.13	61.66	61.66		43.65		83.18	
83.18	58.88	58.88		27.54		81.28	
81.28	57.54	57.54		18.2		56.23	
79.43	53.7	53.7		17.38		51.29	
77.62	50.12	50.12		16.2		46.27	
61.66	45.7	45.7		12.88		45.71	
58.88	42.66	42.66		12.59		43.65	
56.23	39.81	39.81		12.3			
53.7	34.67	34.67					
51.29	33.11	33.11					
47.86	32.36	32.36					
46.27	30.2	30.2					
45.71	29.51	29.51					
43.65	27.54	27.54					
42.66	23.99	23.99					
34.67	22.91	22.91					

	20.42	20.42					
	19.5	19.5					
	18.2	18.2					
	17.38	17.38					
	16.2	16.2					
	13.8	13.8					
	12.88	12.88					
	12.59	12.59					
	12.3	12.3					
		53.7					
		47.86					
		45.71					

Table 4.35bProtein profiles obtained with EP, CP and CEP, when tested at 10-17% of the gel (G 34)

EP MW in kDa	**CP** MW in kDa	**CEP** MW in kDa	**WP (Proteins obtained in wash)** MW in kDa	**MW of the heavily stained bands** MW in kDa	**Proteins present only in CEP** MW in kDa	**Proteins of EP not observed in CEP** MW in kDa	**Proteins of CP not observed in CEP** MW in kDa
123	85.11	81.28	57.54	67.61	81.28	123	85.11
120.2	79.43	77.62	53.7	66.07	77.62	120.2	79.43
114.8	72.4	61.66	51.29	64.57	43.65	114.8	72.4
112.2	66.07	58.88	46.77	61.66	27.54	112.2	66.07
100	61.66	56.23		58.88	20.42	100	50.12
97.72	58.88	53.7		17.38	17.38	97.72	46.7
89.13	56.23	51.29		16.6	16.6	89.13	42.66
85.11	53.7	47.86		16.22	16.22	85.11	36.31
83.18	51.29	45.71			12.3	83.18	32.36

81.28	50.12	43.65			12.02	79.43	30.2
79.43	46.7	40.74			11.48	72.4	29.51
72.4	45.71	39.8			11.22	69.18	28.18
69.18	42.66	38.9				66.07	25.12
67.61	39.81	30.9				64.57	
66.07	38.9	30.2				51.12	
64.57	36.31	27.54				46.77	
61.66	32.36	23.99				41.61	
58.88	30.2	22.91				42.66	
56.23	29.51	20.42				40.74	
53.7	28.18	17.38				33.11	
51.29	25.12	16.6					
51.12	23.44	16.22					
47.86	22.91	12.3					
46.77		12.02					
45.71		11.48					
41.61		11.22					
42.66							
40.74							
33.11							

In the experiments shown in Table 4.35b., a few of the proteins from both epithelial cells and *C. albicans* did not retain after adhesion. Proteins which are participating in adhesion may be retained and shown with lane CEP.

4.4.1.2.1.4.2 PROTEIN PROFILING AND LECTIN BLOTTING DONE ON THESE SAMPLES

I. RESULTS WITH CON A

Con A detects α- Man and α- gluc moiety on the glycoprotein (Sharon and Lis 1972). Both CP and EP solution gave all their bands in combination except proteins of MW 47.86 kDa of EP, 39 kDa of both CP and EP. The protein bands of MWs 22, 21.38, 25.12 appeared extra in washings. But the same were not seen with EP or CP. This indicated that all the mannoproteins and glucose containing moieties are either retained or not, get altered during adhesion. The bands retained in both, after adhesion are 101.2, 51.29, 47.86, 45.2, 40.2 and 26.4.

II. RESULTS WITH LECTIN FROM *DOLICHOS BIFLORUS*

Lectin from *Dolichos biflorus* detects α-gal NAc (Sharon and Lis 1972). Here after adhesion high molecular weight proteins of epithelial cell membranes, i.e., 120.2, 112.2, 108.2, 104.7, 100, 96.1 kDa disappeared on combining with candida cell wall proteins. Other proteins like 59.3, 56.28, 52.2, 48.98, 44.65, 20, 11.2, and 9.5 kDa were also disappeared. However, all candida proteins were retained. Here also proteins of 48.98 and 44.65 kDa were found in the washings. These results indicated that these proteins did not participate in adhesion.

III. RESULTS WITH WGA

WGA detects gluc $(NAc)_2$ and NeuNAc (Sharon and Lis 1972). The proteins of heavy MW from epithelial cell membrane, named 123, 117.5, 114.8, 109.6 kDa were not retained on combination of epithelial cell proteins with candida cell wall proteins. Other proteins from EP, 81.28, 70.79, 61.66, 54.95, 37.51, 31.62 and 26.3 kDa disappeared after combination.

IV. RESULTS WITH PNA

The candida protein bands ranging from MW 61.6 to 14.8 kDa were seen under the gradient gel of 10-17%. A few bands like 42.66, 25.12, 16.22, 15.49, 14.79 and 14.8 kDa were heavily stained. A total of 20 bands obtained. With PNA, epithelial proteins showed 29 bands, which were identified and 57.54, 56.28, 20.89, 20.42, 16.6and 14.3 kDa were the bands heavily stained among them. Once *C. albicans* cell wall proteins were made to combine with EP and when gel was run and lectin blotting done with PNA, showed absence of all heavy molecular weight protein bands, i.e., from 120.2, 112.2, 104.7, 95.5, 75.86 and 79.43 kDa of EP, when observed in CEP. However, washing contained only 4 proteins of MW 54.74, 48.98, 44.65, and 26.92 kDa and among them 48.98 kDa is the EP protein disappeared on adhesion. Remaining 3 proteins found in the washings were from CP. But these 3 proteins were also seen in the lane of CEP. Therefore final conclusion was made that, as PNA binds to β – (1-3) gal NAc, the higher MW proteins of EP with this specific carbohydrate moiety do not participate in adhesion along with one low MW protein i.e., of MW 48.98 kDa.

SUMMARY

The results of biochemical experiments revealed that the adhesion of candida onto HBEC is carried out strongly through lectin–carbohydrate interaction mechanisms. It is proved that the extraction procedure can be modified to give good results and can be achieved with the use of vortex mixer. The HIV seropositive isolates do not differ in their mere protein profiling pattern comparing to the isolates from HIV seronegative group. However, on lectin blotting, it was revealed that the candida isolates from HIV seropositive individuals showed the occurrence of glycosylation in a few of their cell wall proteins. The cell wall proteins are the most important factors in carrying on the adhesion. The role of cell wall protein on adhesion of *C. albicans* on HBEC is strain specific. The secreted proteins of *C. albicans* are also involved in adhesion, but in lesser proportion as shown in the adhesion inhibition experiments.

4.4.2 ANALYSIS OF ADHERENCE BEHAVIOR OF *C. ALBICANS* ISOLATES IN RELATION TO THE PHYSICOCHEMICAL FACTORS OF THE CELLS

Surface properties of *C. albicans* and HBEC were analyzed for their effect on adhesion. Cell Surface Hydrophobicity (CSH) was determined by contact angle method. Zeta potential values for the cell were obtained by calculating cell surface Electrophoretic mobility using Zeta Pals instrument (Brookhaven Instruments Corporation). The information obtained in this part of the work was compared with adhesion reaction at those particular experimental conditions to understand the influence of cell surface factors on the mechanism of adhesion.

4.4.2.1 CELL SURFACE HYDROPHOBICITY (CSH) DETERMINATION

Contact angle was measured for each strain grown in YNB and then suspended in DW and the one preincubated in artificial saliva and then subjected to analysis is presented in the Table 4.36, 4.37 and 4.38.

In the present study of 17 strains, a few isolates with more contact angles showed higher adhesion. Between *C. albicans* isolates from HIV seropositive and HIV seronegative groups, no significant variation is seen.

4.4.2.1.1 RESULTS OF CONTACT ANGLES WITH PBS (pH 7.2) SUSPENDED CELLS

It can be seen that, for polar components such as water, the contact angle was ranged from 12.94^0 to 35.32^0. In formamide contact angle was ranging from 14.28^0 to $38.29^{0.}$ With diiodomethane (apolar component) the contact angle values obtained were 37.56^0 to 69.32^0.

Table 4.36 Adherence patterns of *C. albicans* strains on HBEC, and contact angle formation results when suspended in PBS (pH 7.2)

	Adherence of *C. albicans* strains on to HBEC while suspended in PBS (pH 7.2)		**Contact angle values in polar solution**		**Contact angle values in apolar solution**
Sample number	Percentage of HBEC showing candida adhered on them	Average number of candida cells per HBEC	Water	Formamide	Di-iodomethane
RL-24	72.10	2.19	20.21^0	25.28^0	47.94^0
RL-23	53.70	2.48	21.21^0	14.28^0	52.97^0
RL-26	80.90	2.91	27.57^0	28.61^0	45.00^0
RL-28	74.00	2.56	30.69^0	38.39^0	44.26^0
RL-27	63.80	2.1	35.32^0	28.91^0	39.25^0
RL-29	66.60	2.27	20.55^0	20.63^0	42.22^0
RL-112	63	2.25	30.05^0	33.84^0	38.93^0
CN-194	25.70	1.22	17.53^0	18.18^0	55.31^0
CN-176	73.20	2.46	21.61^0	22.42^0	40.41^0
CN-67	56.90	2.08	12.94^0	15.17^0	38.21^0
CN-102	50.00	1.4	23.69^0	21.28^0	37.56^0
CN-181	65.80	1.96	30.51^0	30.96^0	50.71^0
CN-188	46.50	2.04	28.18^0	33.45^0	45.84^0
CN-173	63.80	2.1	23.89^0	22.33^0	69.32^0
CN-31	66.00	2	31.39^0	32.99^0	51.15^0
CN-111	82.06	1.94	29.95^0	26.22^0	47.92^0
CN-192	50.50	1.89	32.42^0	31.39^0	52.29^0

The water contact angle for isolates from HIV seropositive group varies from 20.21^0 to 35.32^0. For formamide, contact angle range was 14.28^0 to 38.39^0 and for Di-iodomethane 38.93^0 to 52.97^0. The water contact angle for isolates from HIV seronegative group varies from 12.94^0 to 32.34^0. For formamide, the contact angles were ranging from 15.17^0 to 33.45^0 and for Di-iodomethane 37.56^0 to 69.32^0. There is no significant difference found in contact angles of HIV seropositive and HIV seronegative isolates.

4.4.2.1.2 RESULTS OF CONTACT ANGLES WITH CELLS SUSPENDED IN ARTIFICIAL SALIVARY MEDIUM (ASM) (pH 6.8)

With ASM, water contact angle was 10.14 till 27.41 and formamide 8.49 till 27.24. Candida strains had reduced contact angles when they were grown and suspended in ASM as presented in Table 4.36. Di-iodomethane also showed reduction in contact angle values when its values were compared with candida strains suspended in PBS the contact angles shown here were 28.97^0 to 44.03^0. Similarly, here also there is no significant difference observed between HIV seropositive and HIV seronegative group of isolates, in their contact angle values. The contact angles obtained for HIV seropositive individuals in water were varying from 16.5^0 to 25.84^0. Formamide is 10.82^0 to 22.86^0. Di-iodomethane is 31.61^0 to 41.8^0. In case of HIV seronegative subjects, the contact angles in water obtained are from 10.41^0 to 27.41^0, in formamide 8.63^0 to 19.98^0 and in Di-iodomethane it is 28.97^0 to 42.06^0.

Table 4.37 Adherence patterns of *C. albicans* strains on HBEC, and contact angle formation results when suspended in ASM (pH 6.8)

Sl no	Isolates tested	Adhesion of *C. albicans* at Artificial Saliva medium (ASM)		Contact angles in polar solution		Contact angle values in apolar solution
		Percentage of HBEC showing candida adhered on them	Average number of candida cells per HBEC	Water	Formamide	Di-iodomethane
1	RL-24	91.58	3.3	19.00^0	17.97^0	37.22^0
2	RL-23	54.30	2.33	25.15^0	13.20^0	41.8^0
3	RL-26	84.30	2	25.84^0	20.22^0	34.66^0
4	RL-28	81.00	3.4	17.24^0	25.71^0	36.10^0
5	RL-27	87.80	3.55	22.86^0	15.75^0	39.99^0
6	RL-29	86.60	4.88	17.86^0	27.24^0	31.61^0
7	RL-112	40.60	1.9	16.5^0	10.82^0	38.19^0
8	CN-194	80.35	4.28	18.28^0	15.95^0	34.00^0
9	CN-176	44.60	1.7	16.93^0	19.98^0	31.90^0
10	CN-67	93.8	4.3	10.14^0	15.22^0	33.99^0
11	CN-102	86.90	3.75	27.41^0	16.84^0	33.37^0
12	CN-181	55.00	2.33	17.35^0	16.02^0	42.06^0
13	CN-188	90.69	4.2	16.41^0	8.49^0	30.82^0
14	CN-173	70.10	4.52	14.13^0	17.18^0	28.97^0
15	CN-31	67.80	3.73	27.00^0	8.63^0	37.41^0
16	CN-111	95.40	2.9	23.50^0	16.39^0	31.61^0
17	CN-192	93.20	4	14.88^0	19.33^0	44.03^0

Table 4.38 Difference in contact angle measured for normally grown cells and the one grown in ASM

	Contact angles values when cells suspended in Distilled water			Contact angles values when cells were pre-incubated in artificial saliva and then suspended Distilled water		
	Contact angle values in polar solution		Contact angle values in apolar solution	Contact angle values in polar solution		Contact angle values in apolar solution
Isolates tested	Water	Formamide	Di-iodomethane	Water	Formamide	Di-iodomethane
RL-24	20.21^0	25.28^0	47.94^0	19.00^0	17.97^0	37.22^0
RL-23	21.21^0	14.28^0	52.97^0	25.15^0	13.20^0	41.8^0
RL-26	27.57^0	28.61^0	45.00^0	25.84^0	20.22^0	34.66^0
RL-28	30.69^0	38.39^0	44.26^0	17.24^0	25.71^0	36.10^0
RL-27	35.32^0	28.91^0	39.25^0	22.86^0	15.75^0	39.99^0
RL-29	20.55^0	20.63^0	42.22^0	17.86^0	27.24^0	31.61^0
RL-112	30.05^0	33.84^0	38.93^0	16.5^0	10.82^0	38.19^0
CN-194	17.53^0	18.18^0	55.31^0	18.28^0	15.95^0	34.00^0
CN-176	21.61^0	22.42^0	40.41^0	16.93^0	19.98^0	31.90^0
CN-67	12.94^0	15.17^0	38.21^0	10.14^0	15.22^0	33.99^0
CN-102	23.69^0	21.28^0	37.56^0	27.41^0	16.84^0	33.37^0
CN-181	30.51^0	30.96^0	50.71^0	17.35^0	16.02^0	42.06^0
CN-	28.18^0	33.45^0	45.84^0	16.41^0	8.49^0	30.82^0

188						
CN-173	23.89^0	22.33^0	69.32^0	14.13^0	17.18^0	28.97^0
CN-31	31.39^0	32.99^0	51.15^0	27.00^0	8.63^0	37.41^0
CN-111	29.95^0	26.22^0	47.92^0	23.50^0	16.39^0	31.61^0
CN-192	32.42^0	31.39^0	52.29^0	14.88^0	19.33^0	44.03^0

The contact angle compared in Table 4.38 shows that the values decrease with cells suspended in ASM, which indicates decrease in hydrophobicity. However, if the adhesion is dependent on Hydrophobicity, one would expect decrease in adhesion here, but cells suspended in ASM showed increased adhesion, thus proving that there is some other factor, may be ionic concentrations present in the ASM causing enhancement in adhesion.

4.4.2.1.3 ADHESION IN ASM

All the strains except RL-112, CN-176 and CN-181 showed more adherence when adhesion experiment medium was artificial saliva. Results are given in Table 4.37. Since the experiments in ASM done in previous section**; 4.3.3**, the Fig .4.24 can be referred, which is given in page number.

Contact angle measured by Henrique (2005a) , showed the *C. albicans* strains had high contact angle in apolar solution, both in the case of saline and artificial saliva suspending medium. In the artificial saliva both the *C. albicans* strains tested show similar contact angles for water and formamide. In the artificial saliva the cells gave comparatively decreased contact angle than in the saline solution. The enhanced adhesion pattern of cells in ASM did not correlate with their decreased CSH. Surface tension and hydrophobicity were not found to be related and have role in the ability of candida to adhere to epithelium in the study by Henrique, (2005). There are many factors in the natural saliva which may not be reproducible in the artificial saliva. However it can be due to high ionic compounds present in ASM as one of the reasons

to promote increased adhesion. There are reports saying about the absence of required amount of saliva to be one of the causes for the OC. This is more evident with cancer patients under radiotherapy, who experience decreased saliva secretion (Xerostomia) due to which dry mouth conditions exist and the patients show OC most often (Umazume et al. 2000). However there is another report where saliva showed to promote the adhesion of *C. albicans* to epithelial cells (Holmes et al. 2002). In present study also ASM showed increased effect on adhesion and limitation of ASM is the absence of immunological components of the host. The *in vivo* status with natural saliva and immunological components together may bring inhibition in adhesion as explained in the protective action of saliva.

The results with CSH and adhesion to HBEC are in accordance with the reports from Henrique et al. (2002) and also with Blanco et al. (2010). Blanco et al. (2010) showed in their experiment that, CSH forms an important factor when considering adherence of *C. albicans* onto plastic surfaces, and the most hydrophobic strains were most adherent to plastic and produced biofilms highly. They observed that there was no significant correlation occurred with CSH and adherence of candida cells onto HBEC.

CSH was measured by binding of styrene microspheres with yeast cells. Yeast cells were preincubated with various substances like various formulations of K-Y and heparin. Divalent cations (Ca+ +, Mg+ +, Zn+ +, Cu + +) caused some surface modifications. These changes caused reduced adherence of yeast on to the microsphere and diminished binding of Concanavalin A. Here one can note that some of the binding activities are due to mannose containing surface components and also it states that the formulations used as above have implications to develop as therapeutic agents and that might block yeast binding to tissues (Colling et al. 2005). These references explain the importance of surface factors in adhesion of candida on inanimate objects than host cells. Therefore, conclusion from the current study can be laid as; CSH may not be playing any role in the adhesion of candida onto HBEC.

4.4.2.2 THE SURFACE CHARGES AND ADHERENCE BEHAVIOR OF CANDIDA

4.4.2.2.1 THE ELECTROPHORETIC MOBILITY (EPM) OF CANDIDA CELLS AT DIFFERENT IONIC CONCENTRATION SOLUTIONS

The effect of different ionic concentrations of KCl, starting from 0 mM till 100 mM both on zeta potential and adhesion was observed. The details of Electrophoretic mobility and zeta potential for both Rl-112 and RL-24 are given in the Table 4.39 and Table 4.40 respectively.

4.4.2.2.1.1 ELECTROPHORETIC MOBILITY AND ZETA POTENTIAL OF ISOLATE RL-24

The EPM and Zeta potential results are presented in Table 4.39. The Electrophoretic values were varied from -2.93 till -0.56 in case of negative charge ranges. Zeta potential of -640.36 to -123.43 was seen. The variation in the EPM was not proportional to the ionic concentration as such. Only in a few ranges like ionic strengths varying from 10 mM till 25 mM, it was going towards more negative charge. From 35 till 55 mM again the EPM was increasing towards positive charges. The values at ionic strenghts 10, 15, 95 and 100 mM were positive in surface charges. The candida cells here had more negative EPM, once at 0 and 5 mM concentrations of KCl. There after constantly at 35 mM and 40 mM the more negative EPM was seen. However, from 60 mM KCl concentrations till 100 mM EPM was almost giving more negative values with exceptions of values at 75 and 80 mM concentrations of KCl. Similarly zeta potential also has more negative values from 65 mM onwards till 95 mM concentrations of KCl.

Table 4.39 Zeta potential and EPM of *C. albicans* (RL-24) at various ionic concentrations

Sl. No	Concentrations of KCl in mM	Sample No.	EPM	Zeta potential (in mV)
1	0	A0	-0.63	-137.35
2	5	A5	-2.86	153.85
3	10	A10	0.05	-625.78
4	15	A15	0.26	11.7
5	20	A20	-0.90	57.21
6	25	A25	-2.26	-197.04
7	30	A30	-0.67	-493.3
8	35	A35	-1.29	-145.9
9	40	A40	-1.46	-282.05
10	45	A45	-0.61	-319.27
11	50	A50	-0.61	-132.35
12	55	A55	-0.56	-132.35
13	60	A60	-2.42	-123.43
14	65	A65	-1.89	-528.31
15	70	A70	-2.02	-412.28
16	75	A75	-1.19	-441.12
17	80	A80	-1.04	-259.77
18	85	A85	-1.72	-226.27
19	90	A90	-2.93	-376.63
20	95	A95	0.99	-640.36
21	100	A100	1.23	216.33

4.4.2.2.1.2 ELECTROPHORETIC MOBILITY AND ZETA POTENTIAL OF ISOLATE RL-112

The results are presented in Table 4.40. The values-0.14 to -1.95 was observed as EPM of the negative charge. Candida cells in some ionic concentrations, like in 55, 70, 80, 85, 90 and 100 mM gave both EPM and zeta potential in positive charge. Negative Zeta potential in the range of-350.55 till -30.86 was seen. The positive range of zeta potential of 27.16 till 165.48 was observed.

Table 4.40 Zeta potential and EPM of *C. albicans* (RL-112) at various ionic concentrations

Sl. No	KCl. Concentrations in mM	Sample No.	EPM	Zeta potential (in mV)
1	0	B0	-1.43	-313.05
2	5	B5	-0.14	-30.86
3	10	B10	-0.26	-56.2
4	15	B15	-1.25	-273.41
5	20	B20	-1.07	-233.47
6	25	B25	-1.95	-4.26
7	30	B30	-1.25	-272.39
8	35	B35	-0.7	-153.35
9	40	B40	-0.02	-4.76
10	45	B45	-0.41	-89.07
11	50	B50	-1.6	-350.55
12	55	B55	0.76	165.48

13	60	B60	-0.99	-216.31
14	65	B65	-1.09	-237.92
15	70	B70	0.12	27.16
16	75	B75	-0.54	-118.27
17	80	B80	0.61	132.27
18	85	B85	0.59	128.47
19	90	B90	0.33	72.73
20	95	B95	-1.39	-303.58
21	100	B100	0.63	138.36

4.4.2.2.2 ADHERENCE BEHAVIOR OF CANDIDA ISOLATES AT DIFFERENT IONIC CONCENTRATIONS OF KCl IN PBS

The results obtained in adhesion experiments conducted at different ionic concentrations of KCl in PBS are explained in Fig. 4.42 and 4.43.

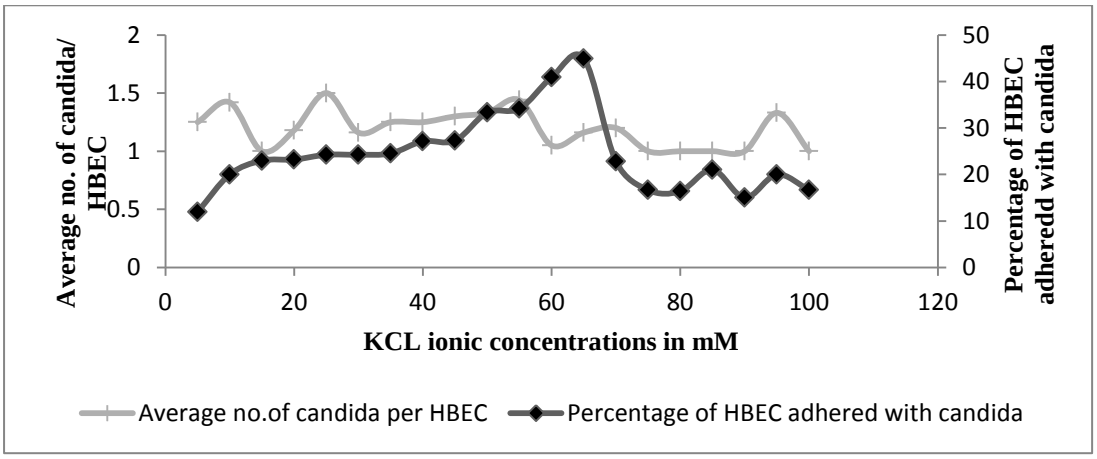


Fig. 4.42 Adhesion behavior of RL-24 at various KCl solutions of various ionic strength

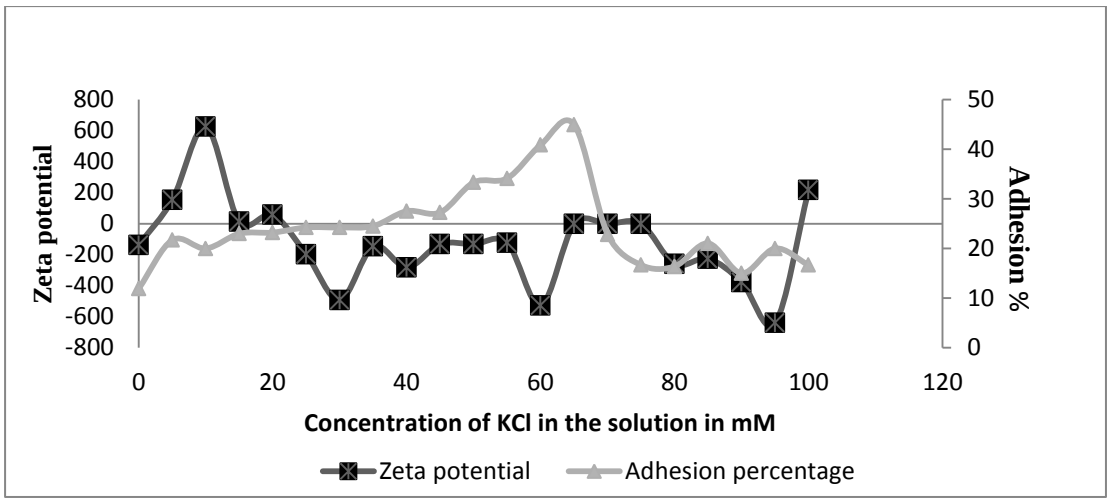


Fig. 4.43 Comparison for zeta potential and adhesion pattern for RL-24 isolate under varying concentrations of KCl in PBS

RL-24, (Fig. 4.43) showed high zeta potential at the ionic concentrations and of 0 to 20 mM in KCl. Thereafter the values for zeta potential of the cells were reduced as shown in the graph. On the other hand the adhesion was more at KCl with 50 mM concentration, where zeta potential was 0.76. Again at KCl solutions with 60 and 65 mM concentrations, adhesion was increasing and zeta potential was reducing. When adhesion was slowly decreasing and zeta potential values were increasing i.e. with ionic concentrations of more than 75 mM of KCl. Therefore, here at certain ionic concentrations like from 25 mM till 75 mM, and also at 100 mM of KCl, adhesion of candida seems to be inversely proportional to the changes occurring in zeta potential.

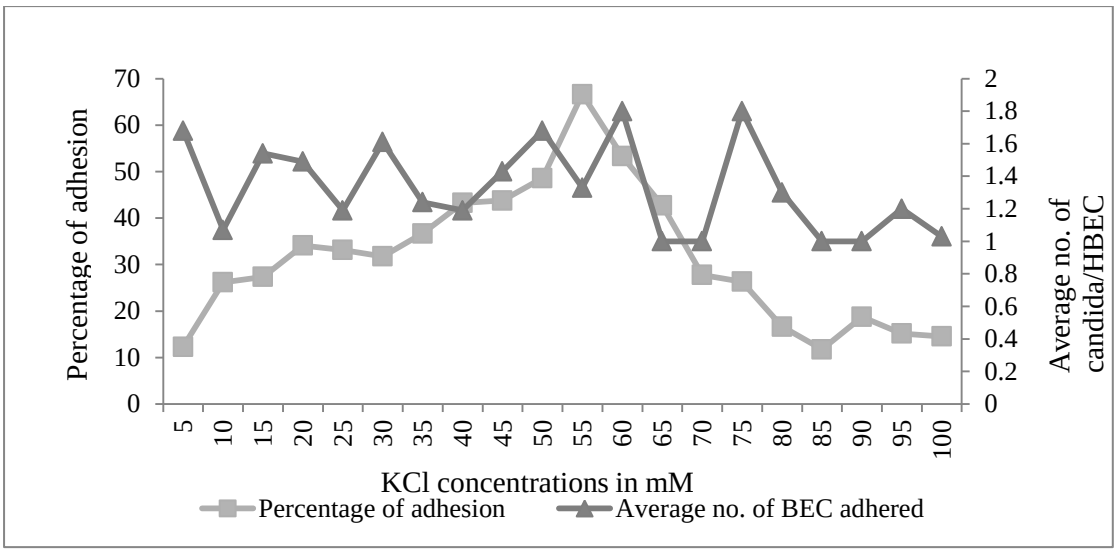


Fig. 4.44 Adhesion behavior of RL-112 in varying molar concentrations of KCl solutions

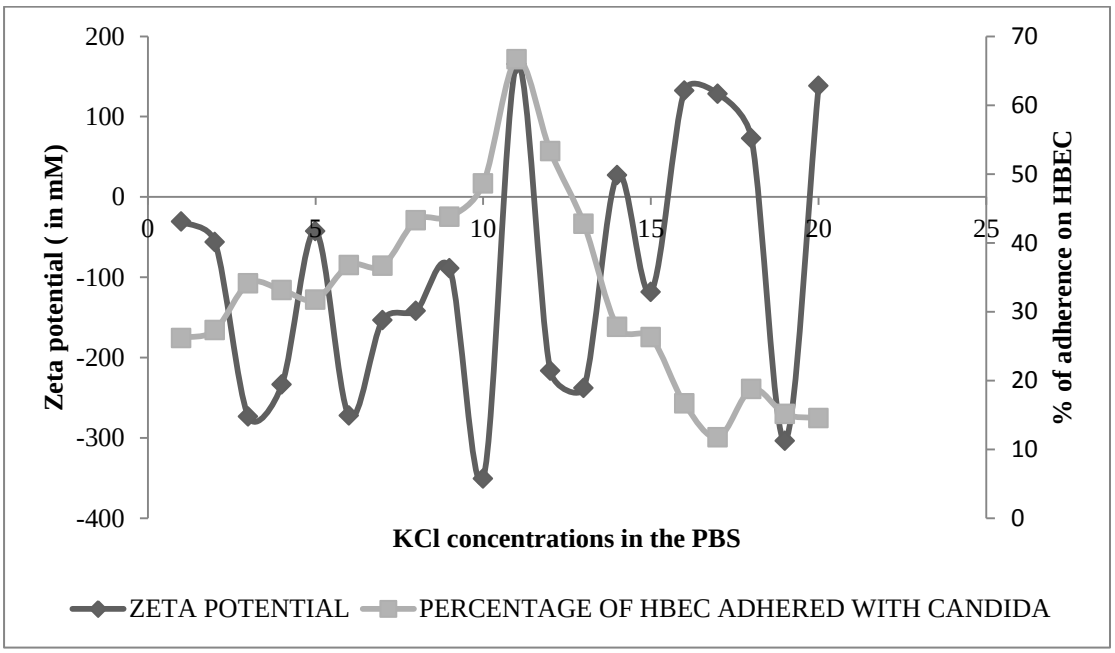


Fig. 4.45 Comparison for zeta potential and adhesion pattern for RL-112 isolate under varying concentrations of KCl in PBS

In RL-112, the zeta potential was reducing at 25 mM KCl in PBS, and was increasing at 40 mM KCl. The adhesion was running independent of zeta potential changes at a few KCl concentrations like in 40 mM (Fig. 4.45). At 60 mM of KCl, the increased zeta potential values were observed and which were correlated with enhanced adhesion seen at the same concentration. At KCl concentration 70 to 90 mM adhesion was decreased and zeta potential was increased. However in both the isolates the correlation between zeta potential and adhesion along the different KCl ion concentration of the PBS was not regularly followed. It was observed that at high ionic concentration the zeta potential turns to be positive. At the smaller range of ionic concentrations like 25 mM till 50 mM, both the isolates of candida had given negative zeta potential values. In many of the ionic concentrations of KCl, adhesion of candida onto HBEC was shown to be inversely proportional to zeta potential values. Thus indicating the electrostatic forces like zeta potential plays a role on candidal adhesion at certain ionic concentrations only.

The zeta potential measurements give information on electrostatic interactions between particles subjected to binding. The binding or immobilization of the particles on to each other not only depends on the chemical components, but also on their surface charges and surface charge potential (Schultz et al. 2008). This is clearly demonstrated even for biological molecules, for e.g., during enzyme immobilization.

Schultz et al. (2008) studied the role of zeta potential in the immobilization of enzyme CALA (*Candida Antarctica* A-type lipase) as a model protein on to non-porous magnetic micro particles with epoxy (M-PVA E02), carboxy (M-PVA C12) and amine (M-PVA N12) terminations. They observed that the CALA, being negatively charged protein, showed maximal binding with M-PVA N12 beads, since these carrier particles had slightly positive zeta potential. As with the magnetic particles the negative zetapotential was increasing, electrostatic repulsion also increased and binding becomes less efficient, therefore lower binding efficiency of CALA with M-PVA E02 beads followed by M-PVA C12 beads was observed.

Role of surface charges and zeta potential were studied with bacteria and yeast in connection with their adhesion onto inert surfaces by various researchers. Uyen et al. (1989), conducted adhesion studies on a few oral bacteria on polymethylmethacrylate (PMMA) a denture based material under varying ionic concentrations of buffer. They reported that, the zeta potential of bacterial strains as well as PMMA reduced as the ionic strength of buffer solution was increased. This change elevated number of adhering *S. mitis, S. sanguis* and *S. mutans* on PMMA. Adhesion of *C. albicans* to this acrylic surface of dentures was preceded by the adhesion of all the above mentioned indigenous bacteria. Thus it is shown here that, increased ionic concentration resulted in decreased zeta potential which in turn facilitates increased adhesion.

Shepard et al. (2010) studied on EPM in *Pseudomonas aeruginosa* cells at different NaCl concentrations. The bacterial cells were most hydrophobic in contact with 0.1 mol/L NaCl solutions as measured by contact angle method. Interestingly, adsorbed cell layers in 0.3 mol/L NaCl had a lower IR absorption intensity, and were more hydrophilic, indicating that other factors influence microbial adhesion on the cell-surface approach distance and hydrophobicity. Electrophoretic mobility values varied with NaCl concentration with suggests that the compression of charged polysaccharides increases polysaccharide which in turn charge density and reduce the flow of liquid through polysaccharide layer and which affect effective potential at the interface, CSH and cell surface approach distance.

Surface charge plays a role in bacterial biofilm formation also. Van Merode et al. (2007) reported that homogenous *Enterococcus faecalis* strains form biofilms more prominently in the presence of other microbial species with a considerably less negative zeta potential.

Environmental conditions in which growth of the microbes occurs play a role in CSH and zeta potentials of the cell, both in bacteria and candida in separate incidences. Jones et al. (2001) showed, the growth of the *C. albicans* in an atmosphere containing 5% v/v CO_2 significantly increased their cell surface

hydrophobicity and reduced the zeta potential. These changes resulted in increased adherenceof cells to both PVC and silicone. Pre-treatment with pooled human saliva, significantly decreased their cell surface hydrophobicity and increased their adherence to either biomaterial in comparison to yeast cells that had been pretreated with PBS ($p<0.05$). But the microbial zeta potential had no consistent effect. Treatment of silicone and PVC with saliva significantly reduced both the advancing and receding contact angles and micro rigidity which in turn decreased adherence of saliva-treated microorganisms to these biomaterials. Therefore it is demonstrated here that the zeta potential is not essential in adhesion process. Rad et al. (2004), after studying zeta potential and adherence in gram positive bacteria like *Corynebacterium* species, *Staphylococcus* and *Streptococcus* , gram negative bacteria like *E.coli* and *Ps. aeruginosa* and two isolates of *C. albicans* reported that there is no correlation found between the CSH and zeta potential of the organisms.

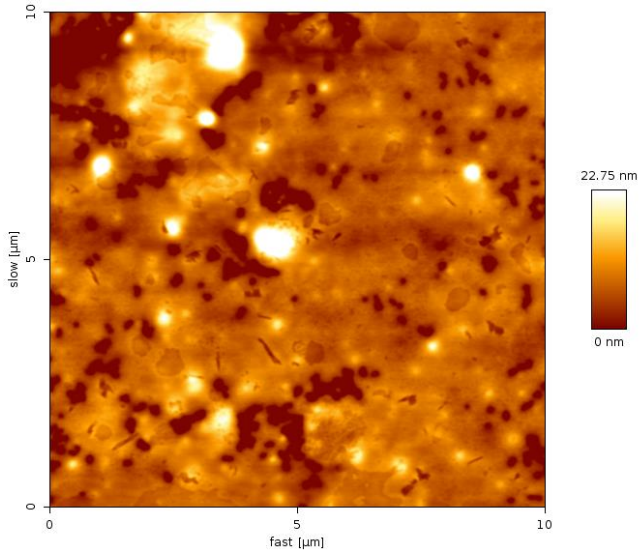
SUMMARY

By referring to the results presented, it can be inferred that the role of CSH, revealed that the changes occurring with CSH is independent of the percentage of adherence. Therefore, conclusion from the current study can be laid as; CSH may not be playing any role in the adhesion of candida onto HBEC.

When considering the surface charges and zeta potential of the cell, at the lower range of ionic concentrations like 25 mM till 50 mM, both candida isolates had shown negative low zeta potential values. In many of the ionic concentrations of KCl, adhesion of candida onto HBEC was shown to be inversely proportional to zeta potential values. Thus showing that electrostatic forces like zeta potential plays a role on candidal adhesion at certain ionic concentrations only.

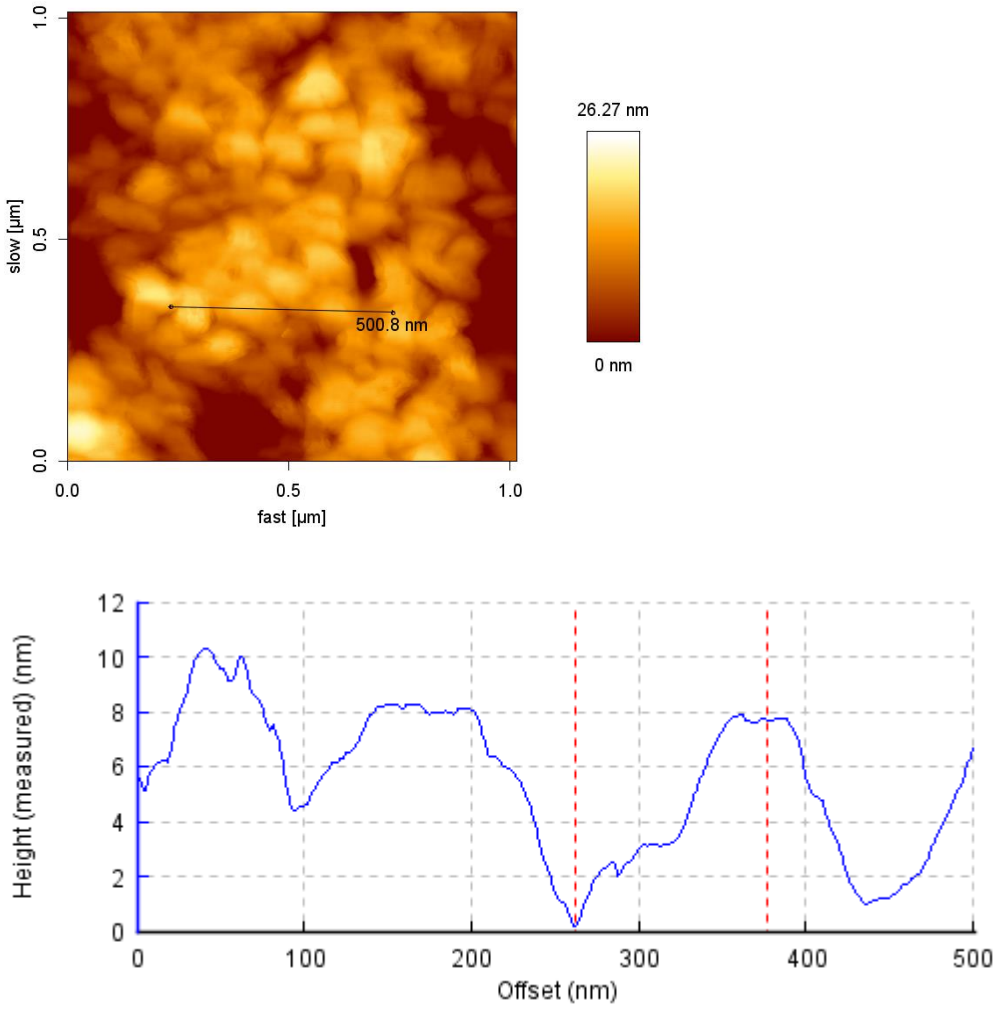
4.4.2.3 ATOMIC FORCE MICROSCOPY (AFM) STUDIES ON SURFACE TOPOGRAPHY OF *C. ALBICANS* AND HBEC

The candida cells and HBEC screened through AFM and results are presented in Fig.4.46 and 4.47. The informations on surface roughness of both the cells were obtained.



Zoom into 2 micron region (Height Image)
Average roughness : 2.443 nm
RMS roughness : 3.526 nm.

Fig 4.46 AFM picture of *C. albicans*



Cross section : 8.2 nm
Average roughness : 2.85 nm
RMS roughness : 2.95 nm.

Fig. 4.47 AFM picture of HBEC

4.5 STUDIES OF CANDIDAL CELL SURFACE FUNCTIONAL GROUPS AND THEIR INVOLVEMENTS IN ADHESION

Fourier Transform Infrared (FTIR) Spectroscopy was first developed for analytical chemistry purposes in 1905 by William Coblenz, who was the first to study spectral data bases of molecules and constructed tables of wave lengths at which various materials absorbed IR light. At present, FTIR had been utilized for complex biological samples to allow their characterization based on molecular information (Naumann et al.1991).

FTIR technique has been utilized for understanding the mechanism of adhesion of *C. albicans* for conducting different experiments. In the present study, changes occurring in functional groups on candida cell surface with relation to adhesion were aimed to be understood by performing FTIR approaches. A total of 20 isolates were tested for FTIR analysis. Ten isolates each from HIV seropositive and HIV seronegative groups were tested separately.

The use of FTIR for understanding the mechanisms of candidal adhesion was carried out in the present study using various approaches; i.e., FTIR analysis of *C. albicans* throughout their growth phases; FTIR analysis of strains suspended in PBS and artificial saliva and comparison with adhesion behavior; FTIR analysis of HBECs adhered with candida; FTIR analysis of cell wall proteins of *C. albicans* and cell membrane proteins of HBEC before and after adhesion.

4.5.1 FTIR ANALYSIS OF *C. ALBICANS* FOR COMPARISON OF THE SPECTRAL PATTERN WITH THE ADHESION BEHAVIOR AT DIFFERENT GROWTH PHASES

The FTIR spectra of isolates were done to compare changes in spectral pattern during their growth phases. The FTIR spectral results obtained here were compared to adhesion of organism at different growth phases. Due to the spectral variation with regards to carbohydrate, amide and lipid contents of the organism along with its growth phase, there could be changes in their adhesion pattern. Therefore here it was

aimed to use FTIR to know whether biochemical groups would influence adherence or not.

4.5.1.1 FTIR SPECTRA OF VARIOUS CANDIDA CELLS AT DIFFERENT GROWTH PHASES

Spectra of different *C. albicans* strains were in the similar range when compared to the spectral pattern obtained by the other researchers (Isabelle et al. 1998; Elsayed et al. 2010). The Table 4.41 and 4.42, gives the information on the peaks obtained from different growth phases for each isolate tested. Fig. 4.48, 4.49, 4.50, 4.51 shows the spectral pattern of a few isolates numbered CN-31, CN-67, RL-23 and RL-26 throughout their growth phases. The reference figure for candida FTIR spectra also is given at Fig. 4.52 and Fig 4.53, which has been done by the researchers Isabelle et al. (1998) and Elsayed et al. (2010), respectively.

Table 4.41 FTIR spectra (wave number) of isolates from HIV seropositive group along their growth phases

		(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})
RL-28	DECLINE	3278.8			2924.3
	STATIONARY	3274.9			2924.9
	LOG	3250.8			2924.2
	LAG	3271.4			2925.8
RL-27	DECLINE	3261		3596.7	2922.7
	STATIONARY	3279	3629	3546.5	2927.9
	LOG	3278.9			2925.6
	LAG	3279.7			2924.6
RL-112	DECLINE	3274.8	2923.4	2049.6	1635.6
	STATIONARY	3276.3	2923.9		1636.1
	LOG	3299.4	2926.6		1635.7
	LAG	3277.9	2924.1		1635.6
RL-26	DECLINE	3279.5	2924.6		1638.2
	STATIONARY	3274.4	2925.1		1629.9
	LOG	3286.7	2926		1631.9
	LAG	3363.4			1633.1
RL-40	DECLINE	3278.8	2923.8	2857	1739.3
	STATIONARY	3279.2	2924.3		
	LOG	3346.3	2926.6		
	LAG	3274.9	2925.1		
RL-24	DECLINE	3277.2	2923.2		
	STATIONARY	3276.1	2924		
	LOG	3263.8	2925.6		
	LAG	3275	2926.3		
RL-23	DECLINE	3271.9	2926.1		
	STATIONARY	3267.4	2924.6		
	LOG	3265.5	2926.8		
	LAG	3353.1	2927.4		
RL-18	DECLINE	3275.9	2924.4	2860.2	1739.3
	STATIONARY	3276.2	2925.1		
	LOG	3273.3	2923.7		
	LAG	3274.4	2925.2		
RL-09	DECLINE	3277.8	2923.5	1637.7	1541.8
	STATIONARY	3278.8	2924.1	1638.8	1541.7
	LOG	3273.9	2926.3	1635.6	1541.5
	LAG	3276.3	2924.4	1634.3	1538.9
RL-29	DECLINE	3272.6	2924.5	1633.3	1538.6
	STATIONARY	3269.6	2919.3	1631.1	1532
	LOG	3271.2	2925	1631.1	1538.6
	LAG	3280.3	2926.1	1632.3	1540.9

(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})
1635.6	1540.9	1400.4	1240	1035.5		
1632.3	1540	1399.3	1234.8	1038.3		
1632.6	1538.4			1043.2		
1628.2	1538.2	1398.1	1230.9	1050		
1637.5	1537.6	1392.3	1236.7	1043.2	685.1	521.4
1654.9	1577.8	1458.1	1229.7	1052.2		
1631.8				1033.1		
1631.5	1540.8			1057.4		
	1542.2	1454.4	1401.9	1239.1	1026.2	
	1539.5		1401.2	1238.5	1030.3	
	1547.5	1457.5	1403.7	1226.4	1066.1	989.6
	1538.4	1451.2	1397.9		1044	
	1541.2		1400.1			
	1538.4		1401	1242.3	1031.2	
	1538.4	1445.2	1381.5	1234.4	1059.3	
					1067.6	
1637.7	1540.8		1451.2	1402.7	1242.2	
1638.8	1542.7			1403.2	1241.5	
1638	1558.8			1403.7	1229.6	1071.2
1635.6	1540		1454.4	1400.2	1236.6	1042.7
1636.6						
1632.9	1539.3					
1632.9	1537.9					
1631.2	1542.4			1401.9	1234.7	
1634.8	1539.6		1401.4	1238.4	1038.8	
1630	1535.3		1400.8	1235.4	1045	
1631.6	1530.2		1396.2	1226.4	1050.9	
1630.1	1543.5		1401.5	1234.5	1055.5	
1637.7	1540.8	1451.2	1402.7	1242.2	1029.6	
1638.3	1541.8		1401.8	1239.7	1028.1	
1632.1	1543.9				1043.4	526.9
1635.4	1539.1		1400.1	1234.9	1043.7	
	1403		1245.4	1030	2569	
	1402.1		1240.2	1027.9		
1454.3	1401		1233.4	1050.7	527	
	1401.1		1235.3	1044.2		
		1398.7		1034.9		
1452.2		1389	1239	1033.6		
		1398.4	1234.6	1043.4		
		1399.9	1234.2	1047.5		

(cm^{-1})	**(cm^{-1})**	**(cm^{-1})**
855.9	526.6	
1029.6		
1028.9	681.8	520.3
1000.6		533
1033.8		
1027.6		
1040		
1052.2		

Table 4.42 FTIR spectra seen in isolates of HIV seronegative group along their growth phases

		(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})
CN-176	decline	3270.8	2921.7		
	Stationary	3262.9	2922.2		
	Log	3363.7			
	Lag	3369			
CN-192	decline	3273.8		2924.6	
	Stationary	3271.3		2925.9	
	Log	3273.2		2925.7	
	Lag	3343.5		2927.2	
CN-102	decline	3277.4	2924.2		
	Stationary	3267.2	2924.7		
	Log	3273.2	2924.8		
	Lag	3652.8	3596.5	3424.8	3256.3
CN-67	decline	3273	2924		
	Stationary	3268.9	2924.2		
	Log	3284.9	2925.2	2359.9	
	Lag	3269.3	2924.8	2361.1	
CN-188	Decline	3283.6		2923.5	
	Stationary	3270		2926.9	
	Log	3266		2925.5	2354
	Lag	3599	3275	2918.7	2350.5
CN-111	Decline	3282.1		2925	
	Stationary	3274		2927.5	
	Log	3267.1		2925.3	
	Lag	3364			
CN-31	decline	3273.3	2923.1		
	Stationary	3258.1	2921.7		
	Log	3273.7	2924		
	Lag	3357.3	2926.7		
CN-181	decline	3274	2923.9		
	Stationary	3272.9	2927.1		
	Log	3275.1	2927.3		
	Lag	3047.2	2891	2579.6	2358.3
CN-194	decline	3275.6		2924.1	
	Stationary	3555.3	3272.2	2926.7	
	Log	3273.4		2924.5	
	Lag	3284.2			
CN-173	decline	3276.3		2923.2	
	Stationary	3271.3		2924.3	
	Log	3275.7		2923.9	
	Lag	3344.1		2928.7	

	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})	(cm^{-1})
		1635.3	1540.4		1398.9	
		1632	1537.1		1398.2	1236.5
		1631.8	1539.1	1455.4	1402.5	1237.1
		1633.5				
1636.7		1540.7	1400.4			1039.2
1631.7		1538.2	1399.4		1233.3	1039.6
1629.3		1537	1398.3		1230.3	1045.2
1630.7		1542.2	1402.3		1234.7	1050.7
		1635.4	1540		1399.8	
		1628.5	1537.1		1400	1237.3
		1631.8	1537.9		1397.1	1234.6
2927.3		1630.2	1532.2		1392.9	1234
		1635.1	1539.9		1400.8	
		1624.7	1539.5		1401	1238.4
		1632.3	1538.3			
		1628.3	1539		1395.8	1231
1637		1541.6	1401.9			1037.9
1625.5			1401			1034.1
1633.1		1538.9				1042.2
1632.8		1540.5		1397.9		1035.4
1634.8		1540.5				1029.8
1627.5		1536.4	1401.7			1029.4
1628.3		1540.4	1454.4	1399.7	1236.1	1041.9
1632.2			1403.8			1068
		1636	1540.3		1401.2	
		1631.2	1540.8		1401.5	
		1630.5	1536.2		1396.7	1235.5
		1631.1	1542.7		1401.5	1235.3
		1635.4	1541.2			
		1629.9	1538.6			
		1632.1	1538		1398	1227.8
2024.2	1981		1512.9			
1634.9		1538				1036
1635		1534.5				1032.5
1632.7		1539.5	1454.8	1398.6	1231.7	1057.4
1633.2		1542.1	1402.4		1235.2	1072.6
1634.3		1537.5			1038.1	
1631.7		1533.8	1369.9	1233.4	1039.2	
1632.1		1531.4	1397.9	1236.9	1043.9	520.5
1631.2		1543.8	1402.7	1235.6	1052.9	

(cm^{-1})
1035.8
1026.6
1067.1
1070.1
1032.5
1036.4
1044.5
1048.5
1033.5
1041.8
1046.2
1045.8
503.6
1036.7
1031
1046.1
1049.1
1038.1
1048.2
1051.1

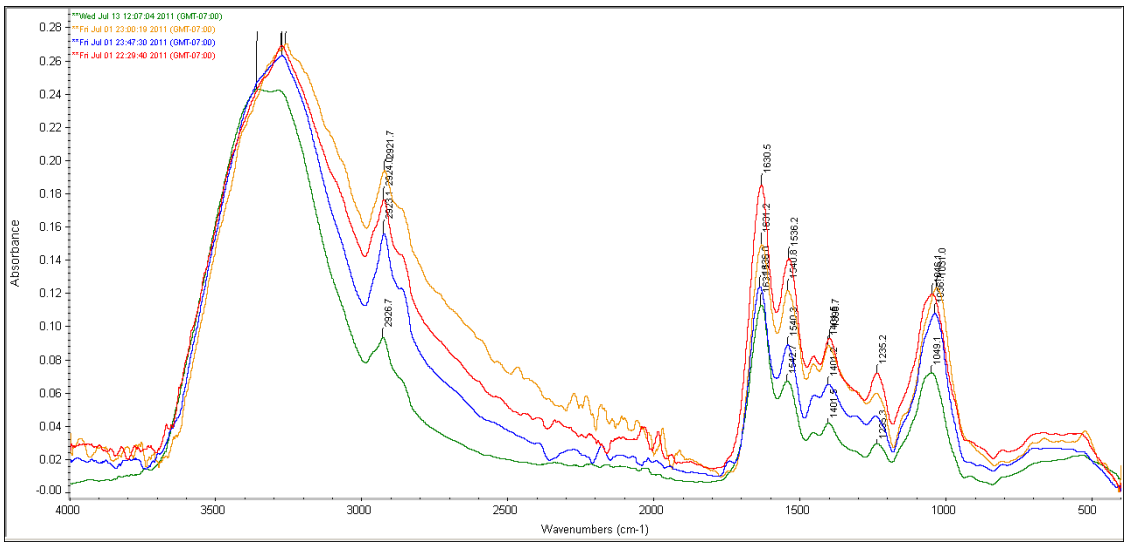


Fig. 4.48 the FTIR spectra of sample CN-31 here color green indicates for lag, red for log, Orange for stationary phase and blue for decline phase of peaks

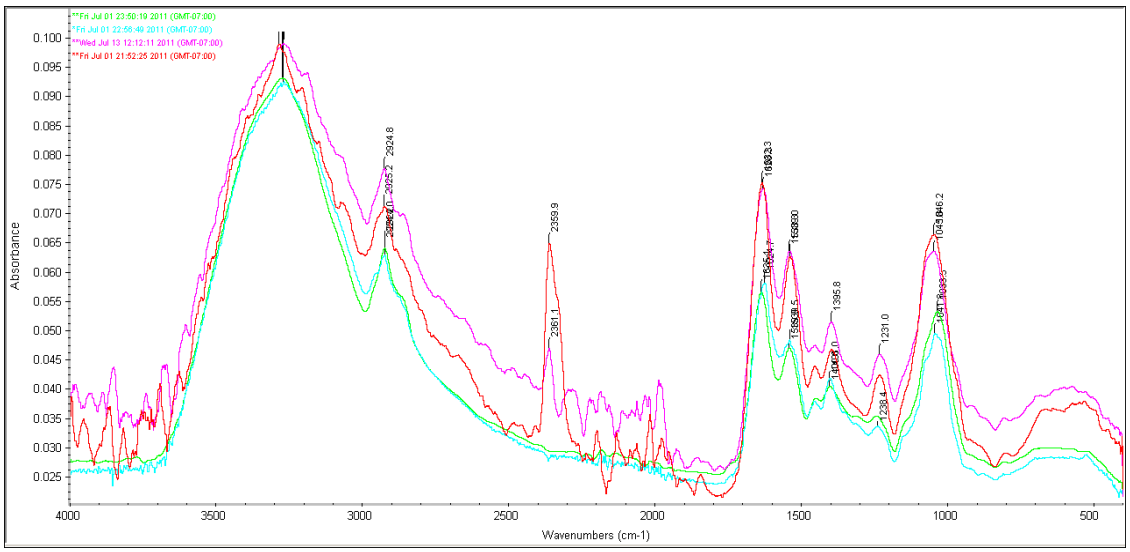


Fig. 4.49 FTIR spectra of CN-67, The curves of color pink for lag, red for log, green for stationary phase and blue for phase decline are represented in the figure.

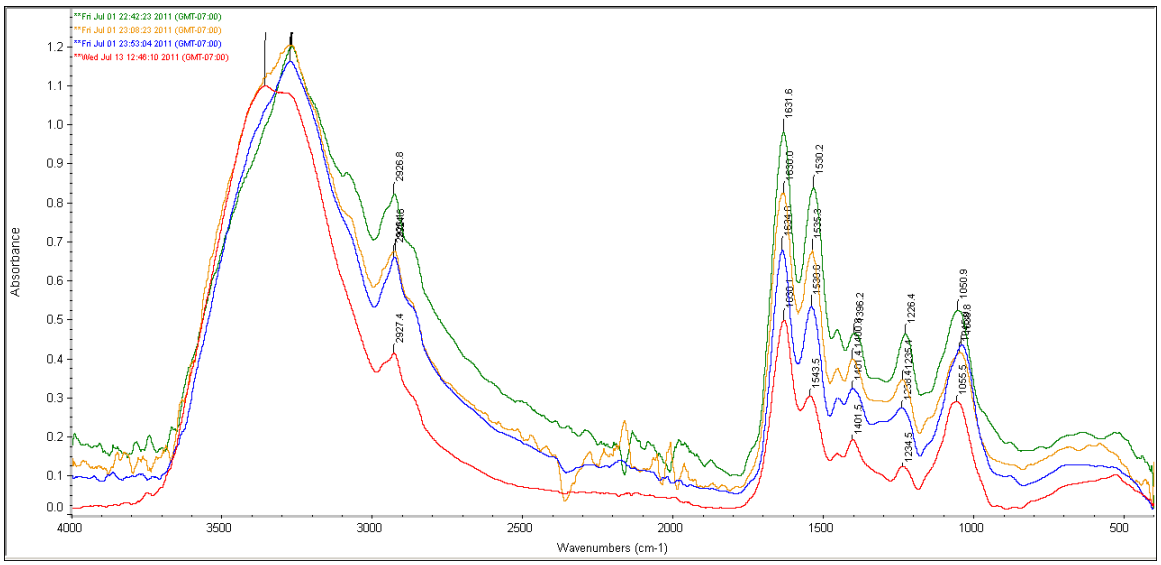


Fig. 4.50 FTIR spectra of RL-23. The colors, red for lag, green for log; orange for stationary blue for phase of decline are represented.

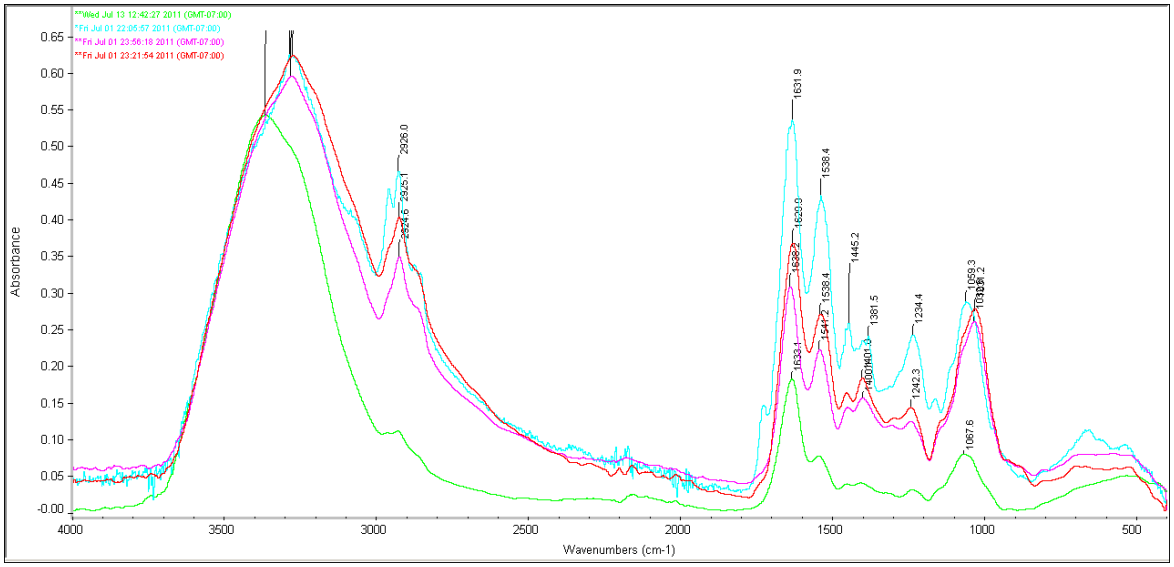


Fig. 4.51 FTIR spectra of RL-26. Pink for lag, green for log, red for stationary phase and pink for Phase of decline are given.

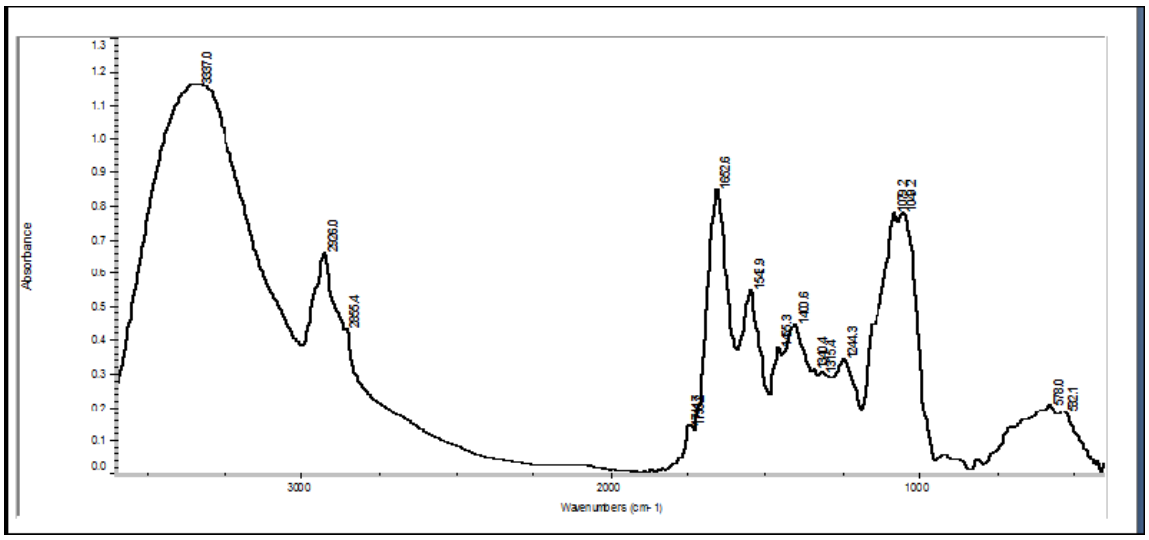


Fig. 4.52 FTIR spectra of *C. albicans* from Isabelle et al. (1998).

Note: A FTIR spectrum in Fig 4.53 represents IR spectra of two candida obtained from two different patients (grey and black lines) and shows only polysaccharide absorption region (900-1200 cm). The most commonly obtained peaks of wavelengths of absorption seen in the range of 998-1106 /cm were considered for identification of candida as shown by other researchers (Isabelle et al. 1998).

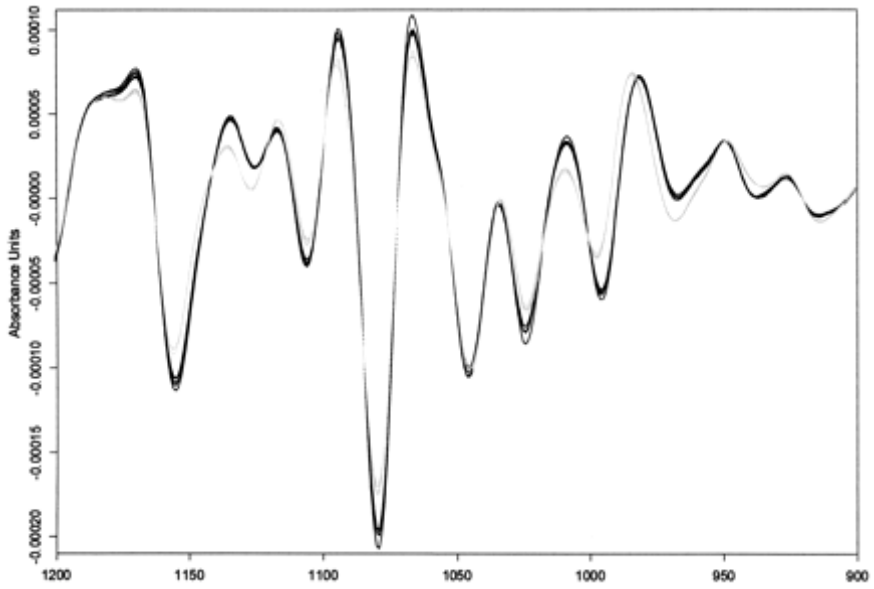


Fig. 4.53 FTIR spectra of *C. albicans* from Elsayed et al. (2010).

All the minor or observable differences of absorption peaks corresponding to the functional group represented were given in detail in Table 4.41 and 4.42. One could not find any major difference in the vibrational spectra obtained in the isolates from HIV seropositive or isolates from the HIV seronegative individuals. Spectral peaks of all 20 isolates gave similar kind of absorption peaks, with minor variation at the wavelengths. Over all peaks of absorption corresponding to mannan, glycogen, beta (1-3) glucans, beta (1-6) glucans, Amide I, Amide II, Asymmetric CH_3 modes, and deformation, CH_2 asymmetric deformation , P=O asymmetric stretching, phosphoryl diester bands of nucleic acids, cellular protein, cell membrane and cell wall components were seen.

Uniformly, in all the isolates absorption peaks for asymmetric CH_3 modes showed very slight variation with increase or decrease in only one unit. Amide I peak had decreased value at stationary phase in a few samples; in a few of them it remained constant throughout all the growth phases. Peaks for Amide II band decreasing towards death phase in a few samples and this also varied between the samples as shown in the Table 4.41 and 4.42. Absorption of mannan and glucans peaks decreased towards death phase.

4.5.1.1.1 FTIR PATTERN IN ISOLATES FROM HIV SEROPOSITIVE GROUP

In all the HIV seropositive group isolates, stationary phases contain more bands. 1600 - 1200/cm series corresponding to amide I and II groups were shown with maximum number of absorption peaks at stationary phase. A peak at 3200/cm (absorption of lipids) range decreases towards log and stationary phase and becomes constant at death phase and rarely shown to be increased in death phase. Peaks of 2900/cm series showed one or 2 units variation and these peaks also indicate lipid contents. At 1050/cm (glycogen peaks) series decreases towards stationary and death phases. 1390/cm series also decreases towards stationary and death phases, which are corresponding to CH_2 asymmetric deformation.

4.5.1.1.2 FTIR PATTERN IN ISOLATES FROM HIV SERONEGATIVE GROUP

In candida isolates from HIV seronegative group, stationary phase cells showed more number of bands than any other phase of cells, which was same as in HIV seropositive group samples also. The bands were more towards 1600-1200. Bands towards 1632/cm (Amide I) increase in stationary phases and decreases in death phase. The range, 1540/cm decreases to 1534/cm towards death phase or sometimes remains unchanged. This is corresponding to Amide II bands.

In this study FTIR analysis was used due to the advantages of FTIR over the other methods. To know the chemical composition of a microbial surface, X-ray photo electron spectroscopy (XPS) and FTIR can be used. However, there are many advantages in using FTIR over other methods, for e.g.: sample preparation for XPS needs centrifugation, freezing, Lyophilization and these steps can cause variation in the measurement. Organisms growth medium culture conditions may also lead to further variations and make this technique suitable mainly for metals than microbes (Van der Mei et al. 2000). Since sample preparation procedure in FTIR is easy and it will not alter the cell surface composition, its applicability to biological surface is more relevant.Studies of FTIR are more acceptable because it doesn't need reagent, and less laborious procedures involved in doing FTIR. FTIR analyses can yield reliable reports under proper standardization (Van der Mei et al. 2000). Change in culture parameters (e.g., culture age, medium composition, medium pH, temperature, humidity, and storage mode) can give difference in spectral reflections, as shown by Essendoubi et al. (2005). Therefore in the present research work, some experiments were carried out only for the standardization of culture conditions and sample preparations, and the best conditions were maintained throughout.

Spectra of different *C. albicans* strains of this study were in the similar range compared to the spectral pattern observed with *C. albicans* spectra from available references (Essendoubi et al. 2005; Elsayed et al. 2010). The most commonly obtained peaks of wavelengths of absorption obtained in this study were in the range of 1106-998/cm, which is observed commonly in all the microbial samples examined

previously. In isolates from HIV seropositive group and the strains from i.e., HIV seronegative healthy individuals, no difference in the vibrational spectra was obtained. However, this work is first of its kind, where FTIR has been used to know the difference between candida isolates obtained from HIV seropositive patients and healthy individuals.

References with microbiological studies using FTIR, explain that, in region I (Figs. 4.50 and 4.51) major spectral variations in amide I and amide II possibly due to C=O stretching vibrations (~1650 /cm) and N–H deformation or bending (~1540 /cm) of amides associated with proteins can be seen (Elsayed et al. 2010).

Region II, commonly called the fingerprint region (1300–900/cm), contains vibrational features of cellular proteins (including enzymes), nucleic acids, and cell membrane and cell wall components (Goodacre et al.1996).C–O–C stretching vibrations of polysaccharides associated with the bacterial cell wall and cell membrane peptidoglycan layer and lipopolysaccharide outer leaflet is assigned to the region 1200–900 /cm (Kansiz et al.1999; Choo-Smith et al.2001; Filip and Hermann et al. 2001; Lin et al.2004).Nivens et al. (1993) reported that different bands obtained at 1200-900/cm are mainly due to different carbohydrate content and glycoform distributions.

Vibrations in the range of ~1455 and ~1398 /cm occur from CH_3 asymmetric and CH_2 symmetric deformation of proteins respectively. Vibrations due to P=O asymmetric stretch of the phosphodiester backbone of nucleic acids occur at ~1242 /cm and in P=O symmetric stretch of the nucleic acid ribose or deoxyribose moieties occur at ~1080 /cm according to Kaniz et al. (1999), and Filip and Hermann (2001)

The range from 1657.9-1649.4 represents two distinct helical structures in the protein assigned to alpha –helix structures of the protein. The range 1682-1673/cm assigned to turns and 1691 to beta sheets and turns, 1619 to side chains and aggregates of proteins(Nivens et al. 1993).

Kates (1986) reported that 1360 to 1480 /cm and 900-1200/cm Mixed region assigned to polysaccharide region. The lipid ester region is starting from 1720 to 1760

/cm. So this region containing absorption from the C=O bond of fatty acid esters and highly variable reflecting the dependence of fungal lipid content on culture conditions. However this region is also known to be influenced by light diffraction phenomenon. This investigator also says that, the frequencies observed at 1450 and 1400 /cm is probably related to the structure of the side chains (CH_2 and Carboxyl) of the proteins. Also 1748 /cm absorption peak was assigned to C=O stretching in esters of glycerides (Kates 1986).

Toubas et al. (2007) had taken spectra from 500-400 /cm, 750-3100 /cm for *C. albicans* strain classifications. Peaks in the range of 2800-3000/cm had important contribution from absorption of lipids.

4.5.1.2 RESULTS OF ADHESION ASSAY

Adhesion assay of candida cells throughout their growth phases shown in Table 4.43. All 20 organisms showed increased adherence on HBEC when tested from log and stationary phase cells.

Table 4.43 Adhesion pattern of the isolates throughout their growth phases

			Percentage of HBEC adhered with candida/ average number of candida per HBEC			
Sl. No	Isolates	Lab No.	Lag Phase	Log Phase	Stationary Phase	Phase of decline
1	Isolates from HIV seropositive group	RL-24	18.9/1. 625	56.1/ 2	54.05/ 1.9	52.22/ 1.66
2		RL-23	9.81/ 1.35	33.3/ 1.84	60.37/ 2.78	46.5/ 1.3
3		RL-26	10.5/ 1.3	63.3/ 2.3	69.8/ 1.4	58.8/ 1.83
4		RL-28	10.9 / 1.47	51.84/ 2.87	57.66/ 2.95	60/ 2.8
5		RL-27	33.3/ 1.5	70.4/ 2.0	83.9/ 2.88	73.7/ 1.8
6		RL-29	38.49/ , 2.4	52/ 2.17	67.57/ 2.35	60.6/ 2.08
7		RL-09	23.34/ 1.2	54.56/ 1.6	64.93/ 1.5	60.90/ 1.4
8		RL-18	22.45/ 1.4	70.64/ 1.7	80.67/ 1.25	78.67/ 1.1
9		RL-40	26.89/ 1.3	67.98/ 1.8	75.98/ 1.67	63.32/ 1.5
10		RL-112	15.89/ 1.2	58.32/ 1.3	64.34/ 1.3	53.96/ 1.2
11	Isolates from HIV seronegative healthy individuals	CN-194	9.52/ 3.2	18.28/ 1.18	57.92/ 1.39	47.63/ 3.
12		CN-176	11.88/ 1.24	24.6/ 1.1	48.88/ 2.39	38.2/ 1.45
13		CN-67	10.33/ 1.75	53.4/ 1.9	62.46/ 2.71	51.15/ 2.04
14		CN-102	22.42/ 1.99	48.58/ 1.65	61.6/ 4.8	50.0/ 1.7
15		CN-181	9.66/ 1.3	35.94/ 2.24	53.98/ 3.22	45/ 1.6
16		CN-188	33.18/ 1.92	58.88/ 2.73	64.2/ 2.4	58.9/ 3.0
17		CN-173	9.66/ 2.3	25/ 1.41	47.45/ 1.8	44.79/ 1.9
18		CN-31	19.03/ 1.7	32.15/ 1.85	48.4/ 1.7	58.6/ 1.85
19		CN-111	11.48/ 2.19	61.43/ 1.88	82.69/ 2.3	62.5/ 2.2
20		CN-192	35.25/ 2.41	56.3/ 1.8	74.07/ 2.015	73.9/ 3.0

4.5.1.3 COMPARISON OF FTIR RESULTS AND ADHESION PATTERN OBTAINED THROUGHOUT THE GROWTH PHASE

Each band absorbs a specific wave-length within the FTIR spectra, revealing a specific fingerprint for each molecule. Here final spectra are generated by the sum of the absorption of all atom-atom bonds (Roscini et al. 2010). Knowing the changes in biochemical finger printing of candida cell throughout their growth phases and comparing this with adhesion pattern of the organism in similar conditions, will give insight about the type of functional group which influences the adhesion process.

C. albicans cells showed more adhesion onto HBEC when taken from stationary phase. This could be due to more secretion of the adhesion molecules on cell surface of candida at this particular phase of growth. In the present research work, more number of peaks has observed at stationary phase compared to other phases of growth.

Similar work done with *Escherichia coli* and *Listeria innocua* on FTIR spectral features for detection and discrimination of different bacterial strains and between growth phases, state that spectral variations (1800-1300 /cm) will be mainly seen with log, the stationary and the death phases which makes these stages obviously distinguishable from each other and from the lag phase because of unique amide I and amide II bands (proteins) in the range of ~1650 and ~1540 /cm (Hamazah et al. 2008). In the isolates studied here, amide range was very prominently seen with log and stationary phases. Previously mentioned investigators found relatively minor variations in the range of ~1455 and ~1398 /cm from CH_3 and CH_2 asymmetric and symmetric deformation of proteins. Similar results are obtained in present study also. To compare or hypothesize why HIV seropositive group strains are more adherent to HBEC, specific range of peaks was carefully observed but it did not reveal many changes between the two groups.

The following conclusions made by comparing the adhesion and FTIR spectral pattern. In stationary phase adhesion is shown to be increasing. Similarly in FTIR analysis also, more bands are observed towards log and stationary phases in most of the samples, irrespective of the groups to which they belong. The bands from 1600 to 1200 are increasing towards the stationary phase and this range is corresponding to amide I and amide II groups and represents protein structures. Therefore it can be said here that increased adhesion is the direct effect of increased protein production in the cell, during specific phase i.e., end of log phase and stationary phases.

4.5.2 FTIR ANALYSIS AND ADHESION BEHAVIOR OF STRAINS IN PBS AND ARTIFICIAL SALIVA

As per previous experiments, the isolates showed increased adherence when growth was carried out in ASM. FTIR analysis was done taking the isolates which are grown in ASM, and changes in their spectral pattern was evaluated in comparison to the isolates grown in standard condition mentioned in the study. Results from FTIR analysis are mentioned in Table 4.44.

Table 4.44FTIR spectra for samples treated with PBS Vs Artificial Saliva

	Lab No	Artificial Saliva (pH 6.8)				PBS (pH 7.2)			
		3000-4000 **Wave number (cm^{-1})**	2000-3000 **Wave number (cm^{-1})**	1200-2000 **Wave number (cm^{-1})**	500-1200 **Wave number (cm^{-1})**	3000-4000 **Wave number (cm^{-1})**	2000-3000 **Wave number (cm^{-1})**	1200-2000 **Wave number (cm^{-1})**	500-1200 **Wave number (cm^{-1})**
Isolates from HIV Seropositive individuals	RL-24	3824	2921.4	1639	1029	3276.1	2924	1632.9	1027.6
		3293.2	2359.8	1537.9	561.2			1539.3	
			2210.5	1399.5					
	RL-23	3275.6	2922.4	1639	1027.5	3267.4	2924.6	1630	1045
			2358	1540.2	561.4			1535.3	
								1400.8	
								1235.4	
	RL-26	3823.6	2925	1737.5	1051.9	3274.4	2925.1	1629.9	1031
		3264.6	2543.3	1644.4	845.9			1538.4	
			2357.9	1366.9	521.4			1401	
			2207.1					1242.2	
	RL-28	3306.2	2932.7	1641.3	1028.9	3274.9	2924.9	1632.3	1038.3
			2355.8	1452	563.1			1540	
								1399.3	
								1234.3	
	RL-27	3648.1	2927.5	1975	1028.3	3279.7	2924.6	1631.5	1057.4
		3248	2357.6	1641.1	561.5			1540.8	

			2019.9	1542.6					
				1404.2					
	RL-29	3307	2927.3	1641.3	1026.3	3269.6	2919.3	1631.1	1033.6
			2354.2	1403.3	561			1532	
			2015.2					1452.2	
								1389.1	
								1239	
	RL-112	3681.9	2355	1976.1	1029.2	3279.2	2924.3	1638.8	1028.9
		3323.5	2024.4	1738.9	563			1542.7	681.8
				1641.8				1403.2	520.3
				1539.8				1241.5	
				1369.6					
				1219.2					
Isolates from HIV Seronegative individuals	CN-194	3712.8	2358.1	1988.5	1027.7	3555.3	2926.7	1635	1032.5
		3637.9	2186.2	1642.2	562.4	3272.2		1534.5	
		3276		1451.6					
	CN-176	3863.8	2925.2	1639.7	1026.4	3262	2922.2	1632	1026.6
		3799.7	2544.8	1397.8	561.3			1537.1	
		3575.5	2473.2					1398.2	
		3281.4	2359.7					1236.5	
			2182.7						
			2260						
	CN-67	3864.1	2924.9	1646.3	1027	3268.9	2924.2	1624.7	1041.8
		3800	2472.8	1543	561.8			1539.5	
		3739.3	2358.9	1393.6				1401	
		3582.3	2267					1238.4	
		3251.6	2184.1						
	CN-102	3840	2916.9	1972.5	1027.9	3267.2	2924.7	1628.5	1036.4
		3730.2	2358.5	1642.1	562.4			1537.1	
		3657.7	2157.8					1400	
		3201.4						1237.3	
	CN-181	3967.9	2923.2	1976.7	1027.9	3272.9	2927.1	1629.9	1048.2
		3838	2479.3	1741.4	562.4			1538.6	

		3732.9	2358.6	1642.6					
		3568.8	2167.4	1514.2					
		3363.3							
	CN-188	3843.9	2917	1979.5	1053.8	3270	2926	1625.5	1034.1
		3739.7	2537.7	1647.9	849			1401	
		3669.4	2467.2		516.5				
		3571.3	2359.5						
		3319.3	2171.2						
	CN-173	3993.7	2861.6	1637.2	1030.8	3271.3	2924.3	1631.7	1039.2
		3749.5	2354.1		564.3			1533.8	
		3242.5	2237.1					1396.9	
			2163.2					1233.4	
	CN-31	3990.1	2969.1	1985	1028.2	3258.1	2921.7	1631.2	1031
		3902.4	2888.8	1920.2	681.5			1540.8	
		3813.9	2747.5	1786.1	567.2			1401.5	
		3616.6	2516.7	1717.1					
		3527.6	2345	1653.1					
		3473.5	2133.3	1369.1					
		3356.9		1228					
		3293.7							
		3242.8							
		3090							
		3016.8							
	CN-111	3279.4	2919.5	1979.8	1029.6	3274	2927.5	1627.6	1029.4
			2357.4	1640.1	563.4			1536.4	503.6
				1540.9				1401.7	
				1402.9					
	CN-192	3578.4	2943.7	1982.4	1028.7	3271.3	2956.9	1631.7	1039.6
		3322.8		1739.1	559.9			1538.2	
		3207.5		1640.8				1399.4	
		3019		1536				1233.3	
				1367.5					
				1218.6					

In general, isolates from the both groups showed extra bands at the range of 3800-3500/cm, corresponding to fatty acid esters. Along with the prominent amide bands the samples showed bands in the range of 1972-1989/cm which were assigned for fatty acids, with the isolates grown in ASM. This range of spectra were absent in the cells grown in normal conditions. In ASM grown cells extra bands were seen corresponding to symmetric CH_3, Asymmetric CH_3, symmetric CH_2 and asymmetric CH deformation seen in the range of 2500-2100/cm.

With the isolates from HIV seropositive individuals, the peaks for amide I and amide II were increasing with the cells grown in ASM. However out of 8 samples, 6 gave decreased peaks for glycogen in ASM grown set. In one of the remaining isolate the peak is similar for both the conditions. Only in one isolate the glycogen peak increased from 1034.1 to 1053.8. ASM grown cells bands towards symmetric CH_2 modes show increased wave number in both HIV seropositive group samples by 2 or 3 points. But in HIV seronegative group isolates, this peak decreased in 6 of the isolates (CN-31, CN-102, CN-181, CN-173, CN-111, CN-194), and increased in CN-188. The decrease in wavelength was from 4 to 9 points as observed in these isolates. In case of one sample values for symmetric CH_2 modes were similar in both the cases (CN-176). With FTIR peaks for isolates from HIV seropositive individuals, Amide I and Amide II region peaks were increasing as well as peaks for CH_3 asymmetric deformation increased with cells grown in ASM. Peaks for P=O symmetric stretching was variable. These peaks can represent cellular proteins, nucleic acids, cell membrane and cell wall components.

Candida adhesion on HBEC was conducted in two different suspension media, PBS (pH 7.2) and ASM. Contents of ASM are given in section 3.3.3.1of Chapter 3; Materials and Methods. Adhesion carried out in ASM showed increased percentage of HBEC adhered with candida cells on HBEC, than in PBS (pH 7.2). The results of adhesion activity in ASM Vs PBS were given in Fig. 4.24 and Table 4.24 of section 4.3.3 earlier.

Conclusion can be derived from these observations that here also peaks at amide I and amide II regions are increasing. Peaks for asymmetric deformation increase in ASM grown cells compared to the normal cells. All these changes are

directly proportional to the increased adhesion in ASM grown cells. This again emphasizes role of amide groups in adhesion reaction.

4.5.3 FTIR ANALYSIS OF CANDIDA CELLS ADHERED ONTO HBEC, BEFORE AND AFTER ADHESION

Adhesion involves fitting up of the different molecules presented from both the cells, i.e., candida and HBEC. The emergence or deletion of any functional group here can give the information on molecules involved in the adhesion. The same is been concentrated to detect from this part of work.

The FTIR Spectra obtained in this set of experiments are given in Table 4.45. The spectra obtained in each case are given in Fig. 4.54 and 4.55.

Table 4.45 FTIR analysis of plain *C. albicans* and plain HBEC comparing to the HBEC when treated with for adhesion in PBS (pH 7.2)

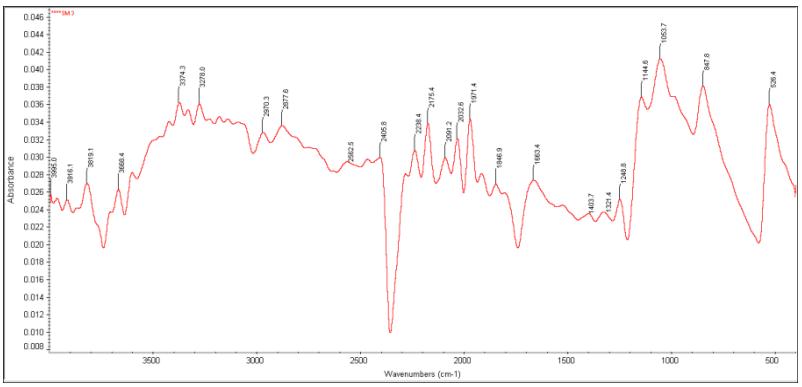
	Experiment 1, where candida cells used were RL-28 for adhesion process			**Experiment 2, where candida cells used were RL-112 for adhesion process**		
Sl. No	Candida cells (RL-28) **Wave number (cm^{-1})**	Epithelial cells **Wave number (cm^{-1})**	Epithelial cells 1adhered with candida **Wave number (cm^{-1})**	Candida cells (RL-112) **Wave number (cm^{-1})**	Epithelial cells **Wave number (cm^{-1})**	Epithelial cells adhered with candida **Wave number (cm^{-1})**
1		3995				
2		3916.1		3958.7		
3		3819.1		3818.6		3830.1
4		3278		3710.6		3727.8
5		3668.4			3642.3	
6	3274.9		3257.3	3266	3260.6	
7	2924.9	2970.3		2919	2922.7	2918.9
8		2877.6				
9		2562.5				

10		2405.6	2408.2	2358.4	2358.8	2357.6
11		2238.4				
13			2157.3			2225.2
14		2175.4		2180		
15		2091.2		2065.1		2098.9
16		2032.6				
17		1971.4		1962.2		2004
18			1790			
19		1846.9				
20	1632.3	1663.4	1649.3	1642.6	1640.7	1639.9
21	1540			1534.9	1534.2	1529.3
22						
23	1399.3	1403.7		1400.3	1399.4	1400.2
24		1321.4				
25	1234.8	1248.8		1251.5		1253.7
26		1144.6				
27	1038.8	1053.7	1062	1045.1	1040.5	1039.6
28			977.1			
29			842			
30					666.5	
31			509.8	586.1		569.1

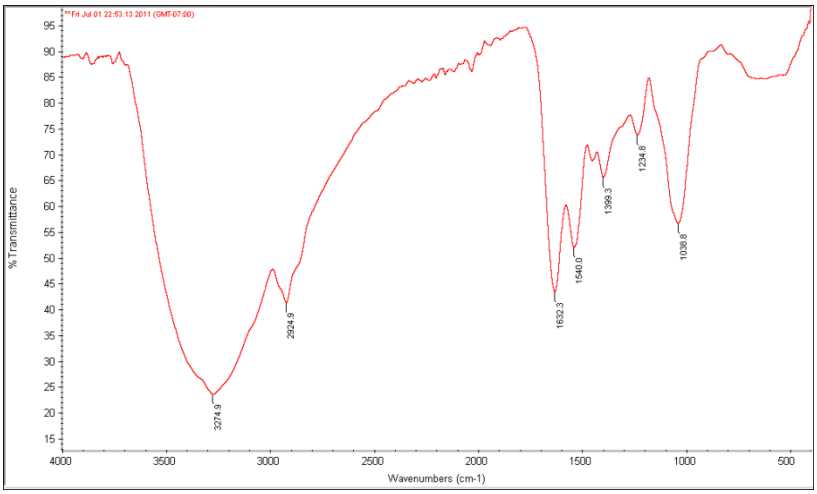
4.5.3.1 RESULTS OF EXPERIMENTS DONE WITH RL-28

The experiments were carried out by taking the spectra of candida cells alone, HBEC alone and the HBEC adhered with candida cells. The peaks of absorption were compared with each other. The bands of individual cells disappeared on adhesion, or retained, or stretched more or less was noticed carefully. Higher bands like 3966-3668.4/cm found in epithelial cells were disappeared after getting adhered by candida. 2924/cm (Lipids) stretch and 2238.4/cm disappeared from HBEC. 3274/cm peak had decreased to 3257.3/cm when epithelial cells were adhered by candida. A few of the bands retained with slight changes in their excitation peaks, they are, 1632.3/cm to 1663.3/cm range, corresponding to amide I bands, 1399-1248 corresponding to cellular proteins, nucleic acids, cell membrane and cell wall components. Surprisingly, in this set of experiment 1790/cm is one of the new peaks of absorption

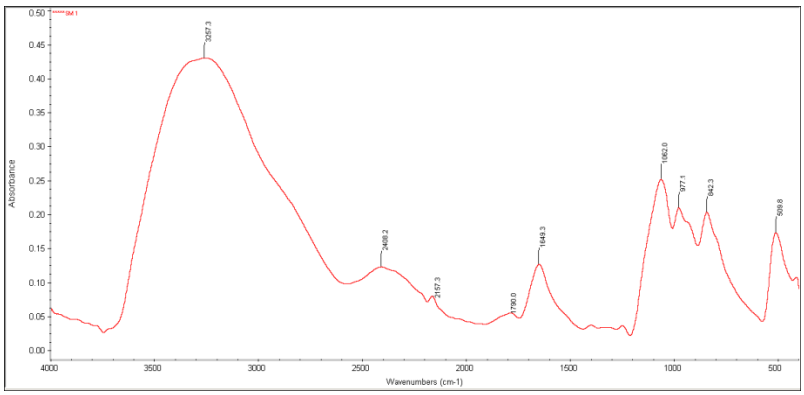
appeared after adhesion of candida on HBEC. This peak corresponds to the functional group representing CH_3 asymmetric deformation.



i. Epithelial cells



ii. RL-28 Candida cells



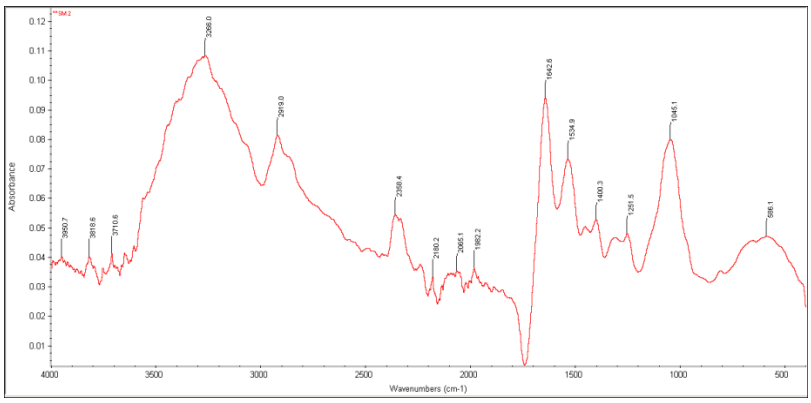
iii. HBEC adhered with candida RL-28

Fig 4.54 FTIR spectra of RL-28 with HBEC before and after adhesion

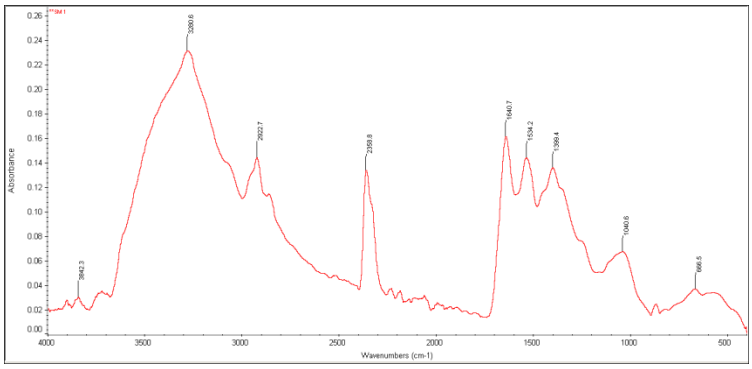
4.5.3.2 RESULTS OF EXPERIMENTS DONE WITH RL-112

Another set of experiment with the same conditions done with isolate RL-112 of showed the following results. A few bands of candida cells retained after adhesion on HBEC were, 3818.6, 3830.1, 3710.6 as 3727.8/cm corresponding to lipids or may be due to retention of moisture. The peak 1962.2/cm as 2004/cm (Lipid group), 1534/cm as 1529.3/cm (Amide II functional group), 1251.5/cm as 1253.7/cm (P=O Asymmetric stretching in phosphodiester bands of nucleic acids group) were retained after adhesion.

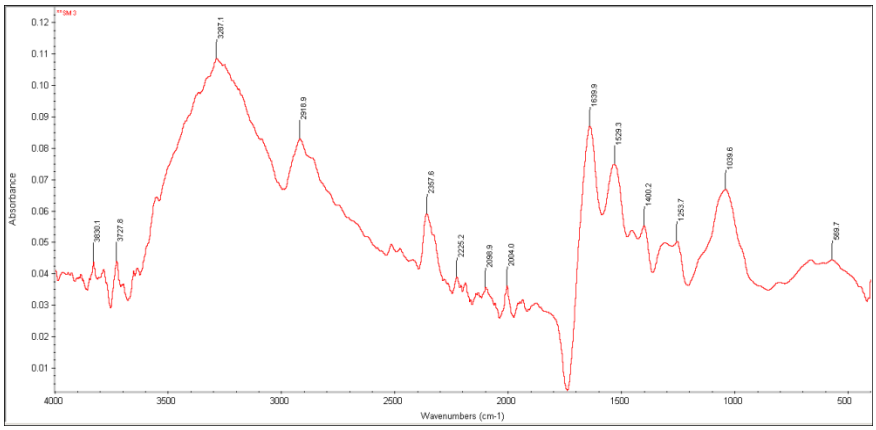
A few peaks present both in candida and epithelial cells retained after adhesion as shown in the representing Fig. 4.55. The spectra were mainly corresponding to Amide I and II groups and they demonstrate involvement of these groups in adhesion process. 1399/cm of epithelial cells and 1400.3/cm of candida cells presented as 1400.2/cm on adhesion, which is assigned to CH_2 asymmetric deformation. A peak 1251/cm stretching corresponding to P=O asymmetric stretching of phosphodiester bands of nucleic acids was retained after adhesion. Bands for mannan seen in candida or these may be peaks of C-O-C stretching or cellular proteins occurring both in epithelial cells get retained after adhesion.



iii. RL-112 Candida cells



ii. Epithelial cells



iii. HBEC Adhered with RL-112 candida cells

Fig 4.55 FTIR spectral pattern of RL-112 before and after adhesion

4.5.3.3 CONCLUSIONS

By evaluating the results from both the sets of experiments on candidal adhesion, following conclusions can be laid: Amide I peak of *C. albicans* increased after adhesion, compared to the peaks in unadhered candida cells. Amide II bands decreased after adhesion by comparing to candida and HBEC wave lengths. Peak in the range of 1063 to 1027 which represents glycogen groups and C-O-O stretching of polysaccharides were not retained after adhesion. Asymmetric CH_2 modes obtained after adhesion were similar to the peaks obtained with plain candida cells. A new band i.e., 1790/cm corresponding to CH_3 asymmetric deformation was seen after candida adhered onto epithelial cells.

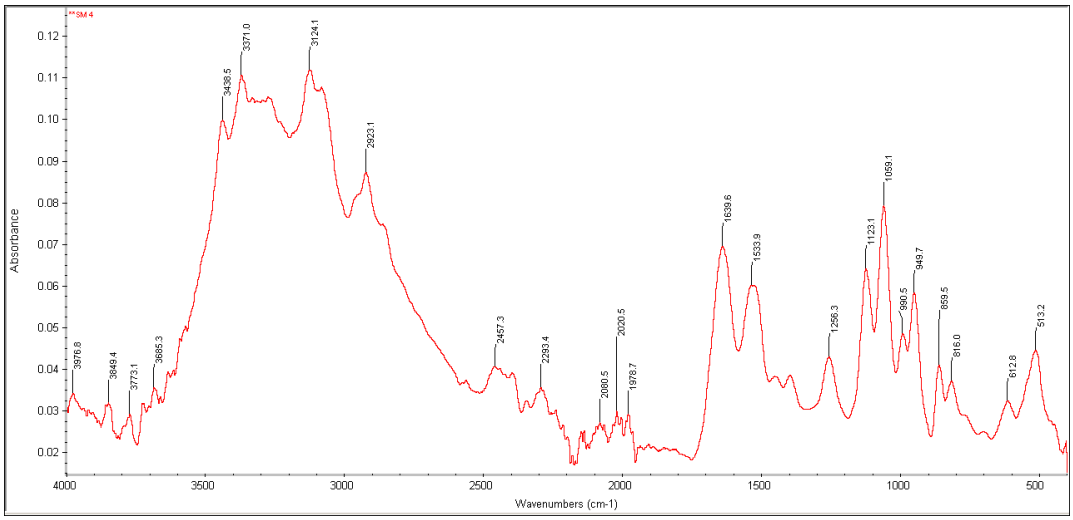
4.5.4 FTIR ANALYSIS OF CELL WALL PROTEINS OF *C. ALBICANS* AND CELL MEMBRANE PROTEINS OF HBEC

To have more precise picture of adhesion, the IR spectra of plain candida cell wall proteins, epithelial cell membrane proteins were compared. Here also emergence or deletion of any IR peak was aimed to be determined, after incubating both the proteins for certain time interval. Results obtained in this set of experiments are shown in Table 4.46 and Fig. 4.56.

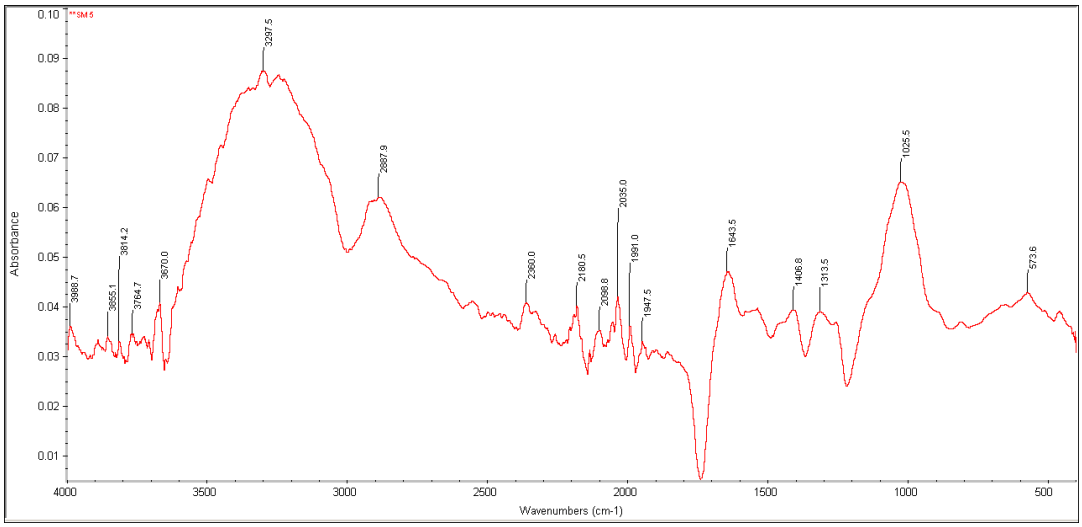
Table 4.46 FTIR analysis of cell wall proteins of *C. albicans* and HBEC separately and when made to react with each other for adhesion in PBS pH 7.2

Sl. No	Epithelial Protein Wave number (cm^{-1})	Candida Protein Wave number (cm^{-1})	Candida Cell Wall Proteins and Epithelial Cell Proteins Combined Wave number (cm^{-1})
1	3976.8	3968.7	3952.4
2	3849.4	3855.1	
3		**3814.2**	**3818.9**
4	3773.1	3764.7	3736.3
5	**3685**	**3670**	**3699.2**
6	3438.5		

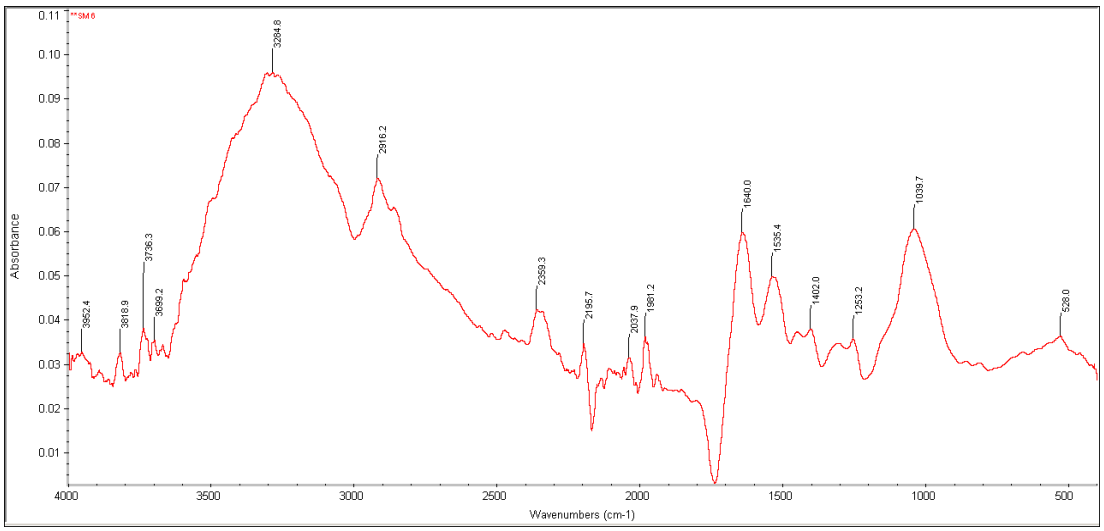
7	3371		
8		3297.5	3284.8
9	3124.1		
10	2923.4	2887.9	2916.2
11	2457.3		
12		**2360**	**2359.3**
13	2293.4		
14			2195.7
15	**2080.5**	**2080.5**	2180.5
16		**2035**	**2037.9**
17	2020.5		
18	1978.7		1981
19		1991	
20		1947.5	
21	**1639.6**		**1640**
22		1643.5	
23	**1533.9**		**1535.4**
24		**1406.8**	**1402**
25		1313.5	
26	**1256.3**		**1253.2**
27	1123.1		
28	1059.1	1025.5	1039.7
29	990.5		
30	949.7		
31	859.5		
32	816		
33	612.8		
34	513.2	573.6	528



i.Epithelial cell protein



ii. Candida cell protein



iii. Candida cell wall protein and epithelial cell membrane protein combined

Fig. 4.56 FTIR spectral pictures of experiments with candidal cell wall protein and epithelial cell membrane proteins

Here it can be observed that, a few of the peaks of absorption ranges retained similar peaks after adhesions, which are highlighted in bold font in the Table 4.46. Other than these peaks, a few peaks were seen only in candida cell wall protein, epithelial cell wall protein and on combination of epithelial protein with candida cell wall proteins. A few bands seen only in candida proteins were retained on adhesion, but were slightly changed in their peak of absorption, e.g.: 2180.5/cm as 2195.7/cm, 2035/cm as 2037.9/cm, 1406.8/cm as 1402/cm, 1025.5/cm as 1039.7/cm, 573.6/cm as 528/cm and were corresponding to fungal lipid contents, CH_3 asymmetric deformation, P=O Asymmetric stretching in phosphodiester bands of nucleic acids or mannans, β glucans and glycogens respectively.

Among epithelial cell proteins, 3485.5/cm for lipids, 3124.5/cm for lipids functional group, 2020.5/cm for lipids, 1256/cm for polysaccharide contents i.e., C-O-C stretching were retained after adhesion process. The peak of 1947.5/cm corresponding to lipid group disappeared on adhesion. In brief, there was some increase or decrease in the pre-existing bands of both epithelial cells and candida cell wall proteins after adhesion. Absolutely no emergence of entirely new peak has occurred. The occurrence of new peak was observed only when intact candida cells

adhered on to HBEC as explained previously. Broadly speaking, lipids, polysaccharides and amide groups are retained after adhesion and may be participating in the adhesion.

Use of FTIR to understand mechanisms of adhesion was seen with various investigators on different organisms (Nivens et al. 1993; Deepika and Charalampopoulos 2010). Adhesion of *Catenaria*, soil fungus zoospores to germanium crystal showed adhesive proteins of zoospores contained amide I and II bands, absorption of these proteins measured as increase in amide II band. The researchers say here that adhesion onto germanium crystal surface in the ATR flow cell shows adherence was involved with amide I and II bands of the proteins. A FTIR was used to study adhesion of *Lactobacilli* on gastrointestinal mucosa and influence of surface characteristics of the bacteria involved in the adhesion. (Deepika and Charalampopoulos **2010)**

In current study the amide I (1700-1600/cm) and amide II (1580-1510/cm) bands were seen commonly in the adhesion, when tested with intact cells or with extracted proteins. The amide I band originates from C=C stretching vibration of the peptide group, which depends on hydrogen bonding and coupling along the protein chain. Amide II band is due to C-C and C=N stretching vibrations in N-H bending. Amide I band is most sensitive to secondary structures of protein. In many references the protein secondary structures was predicted by decomposition of the amide I band into its components through curve fitting procedures (Natallelo et al. 2005).

The candida cells and HBEC alone had both amide I and II regions, which were retained after adhesion of these and showed increased peaks. This suggested involvement of peptide groups in adhesion. In artificial saliva grown cells, peaks for various amide groups are increasing comparing to normally grown cells, suggesting the role of these amide groups in enhancing adhesion.

SUMMARY

The FTIR spectral analysis pattern obtained by candida isolates from HIV seropositive and HIV seronegative individuals did not differ significantly. The peaks for bands corresponding to amide I and amide II groups were increasing at stationary phase. Parallel to this, adhesion of the candida cells on HBEC was also shown to be more at stationary phase. The candida cells grown and suspended in artificial saliva showed increased peaks for amide groups compared to the normally grown cells, suggesting the role of these amide groups in enhanced adhesion by the cells grown in ASM. When the HBEC adhered with candida was tested and compared with plain candida and plain HBEC, emergence of new peak corresponding to functional group representing CH_3 asymmetric deformation was observed. Finally, FTIR analysis proves that the role of amide group containing biomolecules is involved in adhesion.

4.6 STUDIES ON SUBSTANCES WHICH BLOCK THE ADHERENCE OF ORGANISM TO RESPECTIVE LIGANDS IN MOST EFFECTIVE MANNER

The biochemical nature of the candida cells, surface features like Cell Surface Hydrophobicity (CSH) and zeta potential were extensively studied and role of these factors in adhesion is elucidated in previous parts of the present research work. As the continued part, screening a few substances for their antiadhesion activity has been done. In this regard plant extracts, surfactants and sub inhibitory concentration of fluconazole were used for antiadhesion approach.

4.6.1 EFFECT OF VARIOUS PLANTS EXTRACTS ON ADHESION PATTERN OF *CANDIDA ALBICANS* ONTO HBEC

Five different plants were used for adhesion inhibition study, the details about the materials used from each plant is given in Table 3.6 of Materials and Methods Chapter.Entire study results from all the different experimental conditions for RL-24 and RL-112 are shown in Fig. 4.57 and 4.58 respectively. Reduction in the adhesion was analyzed by comparing to the control slides and the percentage of reduction

calculated. Results of gram staining of the plant extract treated candida cells did not show any morphological variations.

Briefly, as explained in the Materials and Methods, four different conditions were employed to carry out the experiment. They are explained as follows,

Condition A: Candida cells treated with plant extracts were exposed to plain (untreated) HBEC.

Condition B: Untreated candida cells were exposed to pretreated HBEC.

Condition C: Both candida and HBEC were treated with the extract and adhesion was carried out in plant extract, instead of PBS (pH 7.2).

Condition D: Untreated candida cells were exposed to untreated HBEC. This condition was taken as control.

Both RL-24 and RL-112 strains showed overall reduction in adhesion after pretreatment with plant extracts and when the reaction took place in PBS, pH 7.2. Details of conditions followed for each extract and abbreviations used for the ease of explaining the results are given in **Table 4.47**. Results of adhesion reaction and adhesion pattern with candida pretreated with plant extracts and subjected for adhesion is given in the **Table 4.48 and 4.49**.

Table 4.47 Details of conditions followed for each extract and abbreviations used

Sl. No	Abbreviation used	Description for the Plant extract type
1	SC 10%, aq	Singapore Cherries, 5 gm extracted using 50 ml Distilled water
2	SC 20% aq	Singapore Cherries, 10 gm extracted using 50 ml Distilled water
3	SC 10%, eth	Singapore Cherries, 5 gm extracted using 50 ml Ethanol
4	SC 20% eth	Singapore Cherries, 10 gm extracted using 50 ml Ethanol
5	AL 10%, aq	Aloe vera, 5 gm extracted using 50 ml Distilled water
6	AL 20% aq	Aloe vera, 10 gm extracted using 50 ml Distilled water
7	AL 10%, eth	Aloe vera, 5 gm extracted using 50 ml Ethanol
8	AL 20% eth	Aloe vera, 10 gm extracted using 50 ml Ethanol
9	NM 10%, aq	Neem, 5 gm extracted using 50 ml Distilled water
10	NM 20% aq	Neem, 10 gm extracted using 50 ml Distilled water
11	NM 10%, eth	Neem, 5 gm extracted using 50 ml Ethanol
12	NM 20% eth	Neem, 10 gm extracted using 50 ml Ethanol
13	LG 10%, aq	Lemon grass, 5 gm extracted using 50 ml Distilled water
14	LG 20% aq	Lemon Grass, 10 gm extracted using 50 ml Distilled water
1	LG 10%, eth	Lemon Grass, 5 gm extracted using 50 ml Ethanol
16	LG 20% eth	Lemon Grass, 10 gm extracted using 50 ml Ethanol

17	T0	Tea tree oil
18	CN	Untreated candida cells were exposed to untreated HBEC (Condition D)

RESULTS WITH ISOLATE RL-24

Table 4.48 and Fig. 4.57 explain the results obtained with RL-24.

Table 4.48 Effect of plant extracts on RL-24 under different experimental conditions

	Extraction condition	**Neem**		**Aloe vera**		**Lemon grass**		**Singapore cherries**		**Tea Tree Oil (TTO)**	
		***A**	*** B**	***A**	*** B**	***A**	*** B**	***A**	*** B**	***A**	*** B**
Condition A	10% (Aqueous)	13.475	70.93	23.52	49.27	11.89	74.35	24.16	47.89	8.57	81.51
	20% (Aqueous)	4.24	90.85	24.78	46.55	10.9	76.49	23.27	49.81		
	10% (Ethanol)	20	56.86	24.15	47.91	12.68	72.65	25.38	45.25		
	20% (Ethanol)	3	93.56	26.99	41.78	11.6	74.98	25.6	44.78		
Condition B	10% (Aqueous)	28.9	37.66	36.9	20.41	30.8	33.56	42.8	7.68	38.9	16.09
	20% (Aqueous)	29.3	36.8	38.3	17.39	34.9	24.72	37.3	19.54		
	10% (Ethanol)	39	15.88	37.3	19.54	39.98	13.76	33.3	28.17		
	20% (Ethanol)	26.4	43.05	39.4	15.01	37	20.19	37.2	19.76		
Condition C	10% (Aqueous)	26.9	41.98	25.9	44.13	17.8	61.6	34.8	24.74	24.9	46.25
	20% (Aqueous)	18.3	60.53	27.8	40.03	26	43.92	29.2	37.01		
	10% (Ethanol)	26	43.92	18.3	38.96	22	52.55	23.3	49.74		
	20% (Ethanol)	22	38.74	26	40.03	28	39.6	22.2	52.11		

Note:*A indicates the actual percentage of HBEC adhered with candida cells,*** B** indicates the percentage of reduction in adhesion in particular condition, compared to the results of control experiments (Control D slides, where perecentage of HBEC adhered with candida was 46.36%).

Condition A

From 41.78% to a maximum of 93.56% reduction in candida adherence was observed. A drastic inhibition of candida adhesion with neem 20% aqueous and ethanol extracts was observed. Neem (20%, ethanol extraction) gave best result with 93.56% reduction in candida adhesion as compared to the control. Neem aqueous (20%) extraction also had good results with 90.85% reduction in adhesion. Similarly, Lemon grass extracts (aqueous and ethanol extracts) treated candida cells also showed reduction in adhesion to HBEC, ranging 72.65% to 76.49%. Tea tree oil had given second best effect with 81.51% reduction in adhesion. Aloe vera, both aqueous and ethanol extracts, Singapore cherries (aqueous and ethanol extracts) have less inhibition effect on adhesion of candida, in the range of 41.78% to 49.27% as compared to condition D (control slide).

Condition B

In this condition untreated candida cells are exposed to the pretreated HBEC. Reduction in adhesion was not high in this condition. Highest reduction was seen with ethanol extraction of 20% Neem (43.05%). Neem, 10% aqueous and 20% aqueous extracts reduced the adhesion to 37.66% and 36.80% respectively. All other extracts showed nearly similar pattern of reduction in adhesion. Least reduction was seen with Singapore cherry, 10% aqueous, i.e., 7.68%.

Condition C

In this condition, both candida and HBEC were treated with the extract and adhesion was carried out in plant extract as suspending medium, instead of PBS (pH 7.2). Under this experimental condition highest of 61.60% of reduction was seen**.** Singapore cherries 20% ethanol extracts gave good reduction (52.11%) as compared to its aqueous counterpart (37.01%). Lemon grass 10% aqueous extraction, gave best inhibition with 61.60% reduction in adhesion followed by neem 20% aqueous extraction which gave 60.53% reduction. Apart from this, ethanol extracts of both lemon grass (10%) ethanol extraction and SC (20%) in both aqueous and ethanol

extracts, showed 52.55% and 52.11% of reduction respectively. All other plant extracts gave similar results, least reduction seen with Singapore cherry (10%) aqueous extract, i.e., 24.94% reduction.

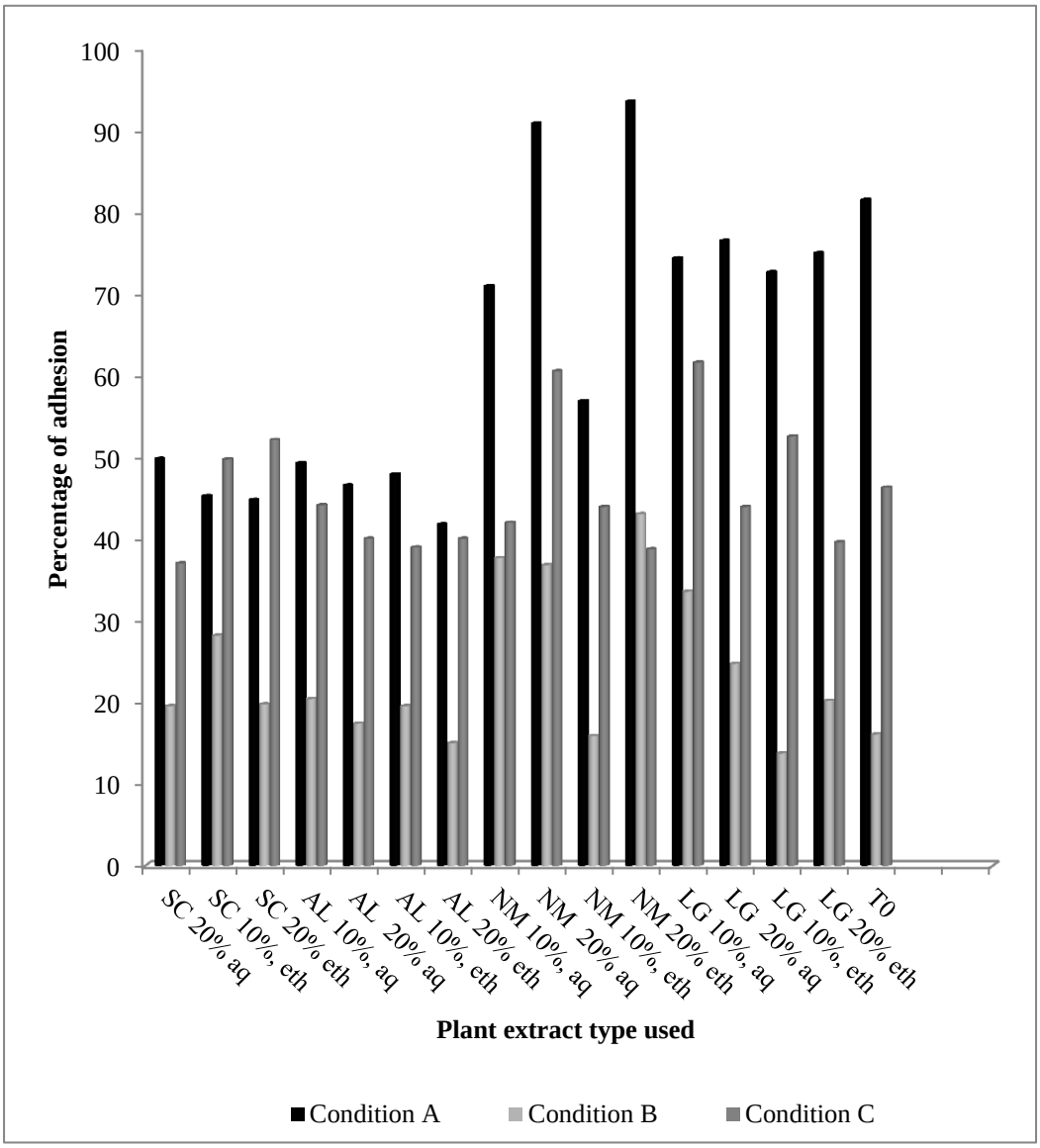


Fig. 4.57 Reduction in adhesion in RL-24 under different experimental conditions as compared to Control condition (under control condition; percentage of HBEC adhered with candida was 46.36%)

RESULTS OBTAINED WITH ISOLATE RL-112

The results obtained for RL-112 is given in table 4.49 and Fig. 4.56.

Table 4.49 Effect of plant extracts on RL-112 under different experiments

	Extraction condition	Neem		Aloe vera		Lemon grass		Singapore cherries		Tea Tree oil	
		***A**	*** B**	***A**	***B**	***A**	*** B**	***A**	***B**	***A**	***B**
Condition A	10% Aqueous	26.4	42.11	21.93	51.91	14.7	67.76	21.78	52.24	14	69.39
	20% Aqueous	12.5	72.59	17.88	60.79	11.56	74.65	23.47	48.53		
	10% Ethanol	19.73	56.73	24.7	45.83	18.97	58.4	23.79	47.83		
	20% Ethanol	7.35	83.88	24.3	46.71	17.03	62.65	20.98	53.99		
Condition B	10% Aqueous	27.5	39.69	50.4	-10.53	38.6	15.35	50	-9.65	52.2	-14.46
	20% Aqueous	50	-9.65	51.2	-12.28	43.5	4.61	53.7	-17.76		
	10% Ethanol	48.76	-6.93	48.6	-6.58	48.9	-7.24	41.66	8.64		
	20% Ethanol	37.76	17.19	47.7	-4.61	50	-9.65	41.86	8.2		
Condition C	10% Aqueous	12.1	73.62	21.7	52.68	15.07	67.14	22.5	50.94	31	32.4
	20% Aqueous	8.8	80.81	20.7	54.86	16.09	64.91	27.6	39.82		
	10% Ethanol	12.1	73.62	24.4	46.79	24.39	46.82	27.2	40.69		
	20% Ethanol	10	78.14	23.7	48.32	24.04	47.58	24.01	47.65		

Note:*A indicates the actual percentage of HBEC adhered with candida cells,***B** indicates the percentage of reduction in adhesion in particular condition, compared to the results of control experiments (Control D where 45.6% HBEC were adhered with candida).

Condition A

Ethanol, extracts of Neem (20%) showed reduction of 83.88% followed by 74.65% of reduction by lemon grass (20%) (Aqueous). Similarly Neem (20%) aqueous extract showed 72.59%, Tea tree oil, showed 69.39%, Aloe vera (20%) aqueous extracts resulted in 60.79% of reduction in adhesion. Remaining plant extracts showed similar results.

Condition B

Under this experimental condition, candida cells showed increased adhesion unlike that seen in Condition A. Singapore cherries aqueous extracts , Aloe vera both aqueous (10 and 20%) (-10.53% and -12.28%) and ethanol extract (10 and 20%) (-6.58% and 4.61%), lemon grass ethanol extract (10 and 20%) (-7.24% and -9.65%) and tea leaf oil (-14.46%) extracts showed increase in adhesion. Even with aqueous extracts of neem 20% and 10% ethanol extracts increase in (-9.65% and -6.93%) candidal adhesion to the pretreated HBEC were seen as compared to the control condition. Aqueous extracts of Lemon grass and ethanol extracts of Singapore cherries showed slight reduction in percentage of adhesion with pretreated HBEC. However, with neem 10% in aqueous extraction, the HBEC showed 39.69% of reduction in adhesion as compared to the control slide.

Condition C

Under this condition the adhesion behavior of RL-112 varied slightly as compared to RL-24. Here both 10 and 20% of the aqueous and ethanolic extracts of neem had reduced the adhesion to 73.62%, 80.81%, 73.62% and 78.19% respectively. Lemon grass aqueous (10%) extracts gave good reduction compared to other extracts (67.14%). When both cells were pretreated with plant extracts and later subjected to adhesion, best reduction was seen in the following order; Neem 20%,aqueous 80.81% > neem 20%, ethanol, 78.19% > neem 10% ethanol and 10% aqueous, 73.62%> lemon grass 10% , aqueous 67.14% > LG 20% aqueous, 64.91%. Aloe vera,

Singapore cherries, lemon grass ethanol extracts and tea tree oil did not differ much in their inhibition pattern. In many of the situations, reduction in adhesion was more with 20% of the extracts irrespective of aqueous or ethanol extraction or types of plant materials used.

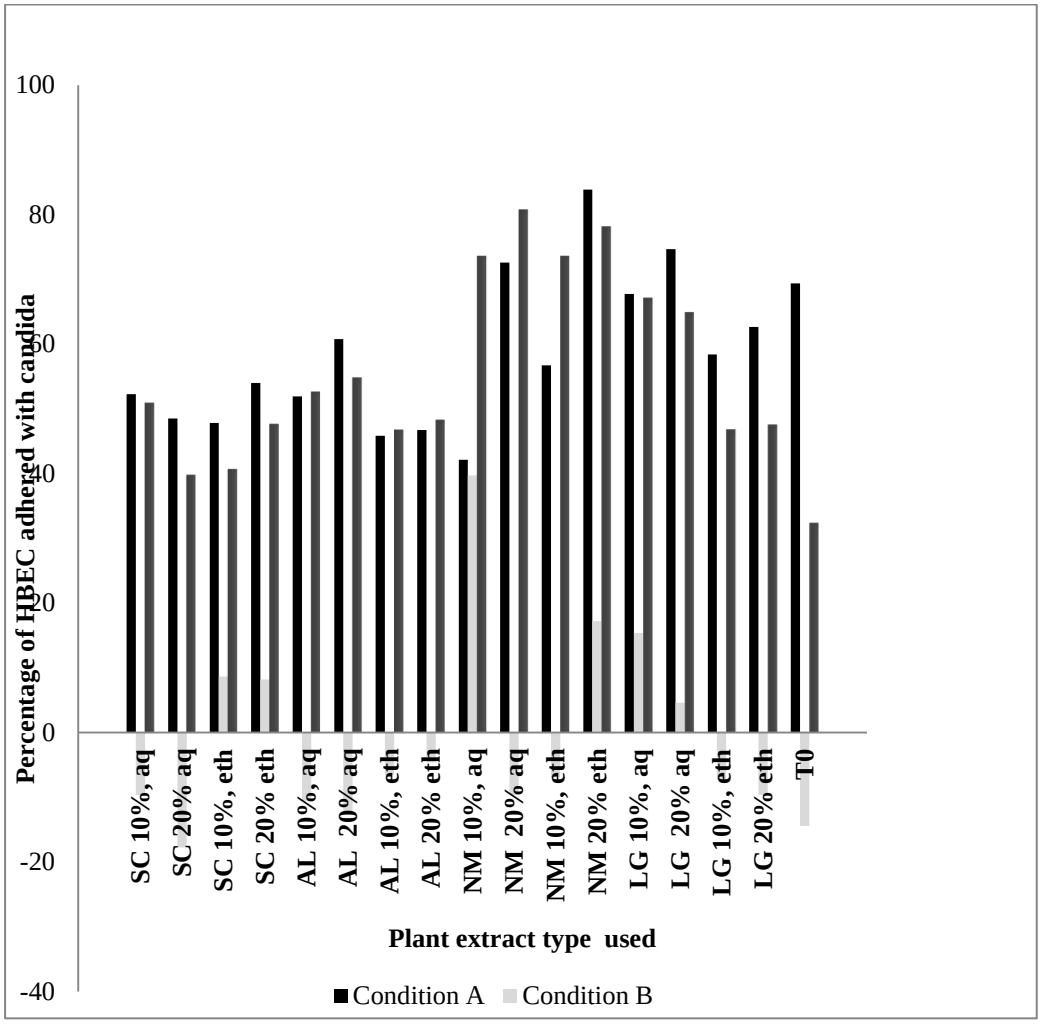


Fig. 4.58 Reduction in adhesion with candida isolate RL-112, when compared to the control condition, in which 45.6% HBEC were adhered with candida

The emerging infectious diseases and alarming increase in drug resistance can prompt studies on alternative type of prevention, for example the antiadherence treatment. In the current study two *C. albicans* isolates namely RL-112 and RL-24 isolated from OC lesions presented by HIV seropositive individuals were used. OC is

one of the most prevalent oral lesions seen among HIV seropositive individuals. Fluconazole is the drug of choice, for treatment of OC (Scully 1994). Recent reports from other countries and India have showed increased prevalence of fluconazole resistant strains of *C. albicans* from HIV seropositive individuals with OC (Mennon et al. 2001; Gugnani et al. 2003; Liu et al. 2006a; Nadagir et al. 2008). In this context, to evolve an antiadherence therapeutic approach, screening of plant extracts for their antiadherence activity is attempted in the present study.

Receptor analogues, adhesin analogues and surface modifying agents are employed to inhibit the adhesion of microbes onto host cells. Number of organisms developing resistance to antiadhesive substances seems to be less compared to antimicrobial agents. However, limitation of this kind of therapy is the requirement of multiple antiadhesion agents to counter each type of adhesin of infecting pathogen (Ofek et al. 2003b). Therefore, though antiadhesion therapy as an important approach exploited in recent years one can understand that yet there are both advantages and limitations present in this type of therapy.

Plant extracts and surfactants were evaluated for their anticandidal/ antiadhesive properties both on HBEC and inert surfaces by various researchers (Ghannoum 1990; Polaquiniet al. 2006; Taweechaisupapong et al. 2005; Mondello et al. 2006; Lyon et al. 11; Liu et al. 2006b). Patel et al. (2009) showed *Dodonaea viscose var.angustifolia* (Sand Olive plant) crude extract can inhibit adhesion of *C. albicans* isolated from HIV seropositive and HIV seronegative individuals, on oral epithelial cells. Mouth rinse with date extract had also proven to decrease adhesion of 3 species of candida on HBEC (Abu-Elteen 2000). In the present study Aloe vera gel, lemon grass, neem leaf, ripened fruits of Singapore cherries and tea tree oil were chosen for their antiadhesive action. All these plant materials except tea tree oil were available in the locality of the laboratory where work has been carried out.

Due precaution was taken while collecting and selecting plant materials with regards to their origin, identification and human usage. In other studies, various solvents such as acetone, dichloromethane, methane, hexane, water etc., were used for extraction. In the present study, distilled water and ethanol were used as extraction solvents, similar to the studies conducted by Polaquini (2006) and Thaweboon and

Thaweboon (2011). A few researchers have used oil forms of plant materials (Taweechaisupapong et al. 2012). Due to the unavailability of tea tree in the locality around the laboratory, tea tree oil was used in readymade form and was purchased from Falcon, Bangalore, India.

In the present study it is found that among the five plant extracts evaluated, neem and lemon grass were found to be more effective in reducing the adhesion followed by tea tree oil. Whereas, Aloe vera and Singapore cherry extracts were not very effective. However, in one set of the experiments (Condition A in RL-112); Aloe vera was also found to be effective in inhibiting the adhesion. Unlike the persistent activity of neem extract, results were not persistent with other plant extracts screened.

In general, neem is known to have antimicrobial activity against a broad range of organisms (Kareru et al. 2010). In the present study the neem extract showed maximum antiadhesive activity. Similar observation was made by Polaquini et al. (2006) though composite resins were used as adhesion matrix.

In the present study lemon grass was found to be the second best plant material in inhibiting the adhesion. Taweechaisupapong et al. (2012) reported that lemon grass oil (1.7 mg/ml) exhibited 80% candidicidal activity towards the cells in preformed biofilm.

Another promising candidate for inhibition of candidal adhesion observed in the present study was tea tree (*Malaleuca alternifolia*) oil. Tea tree oil (TTO) proved to have anti-candidal effect. TTO exhibits *in vivo* and *in vitro* anti-candidal activity against vaginal candidiasis produced both by azole resistant and susceptible strains of *C. albicans* (Mondello et al. 2006).TTO and terpinen 4-ol administrated 3 hrs and 24 hrs after candida infection in mice with fluconazole sensitive and fluconazole resistant *C. albicans* strains separately, reduced symptoms of Oral Candidiasis (OC) and viable candida cell number in their oral cavity (Ninomiya et al. 2012). Terpinen rich TTO at sub inhibitory concentration (0.016-0.25%) are shown to inhibit biofilm formation and adhesion of *C. albicans* on polystyrene, HBEC and HeLa cells (Sudjana et al. 2012). The present test results with TTO in condition A, where only candida is treated with TTO, the outcome is promising.

Aloe vera also showed good result in inhibiting the adhesion in the condition A, where only candida cells were pretreated with plant extracts and in condition C, where both the type of cells were pretreated with the plant extracts. In case of Aloe vera, leaf without gel and only the gel were used separately to study the antimicrobial activity against both bacteria and fungi (Agarry et al. 2005). In the present study, gel of Aloe vera was used. Gel is proved to have better antimicrobial effect on *Staphylococcus aureus* and *Pseudomonas aeruginosa* and fungus *Trichophyton metagrophyte* than leaf extracts of Aloe vera. However, gel did not show any inhibitory effect on *C. albicans* (Kareru et al. 2010). Therefore it can be suggested here, using other preparations of Aloe vera might give different and/or improved results towards adhesion inhibition, which can be further focused.

Singapore cherry plant *(Muntingia calabura)* is consisted to be antiseptic, antispasmodic, hypotensive, cardio – protective and anti-carcinogenic by the researchers (Kaneda 1991; Nivedita et al. 2009). However, reports on its antimicrobial or antiadherent nature of this plant are sparse. The ripened fruits of Singapore cherry used in the present study showed reducing effect on adhesion. However compared to other extracts the effect produced was significantly less.

All the plant materials used here can be recommended for *in vivo* use also and therefore the effect of these extracts in *in vivo* conditions can also be evaluated. The specific effect of these extracts on adhesins and cell surface properties like CSH and ultra structural modification has to be elucidated as shown in other references (Sangetha et al. 2009; Taweechaisupapong et al. 2012).

Interestingly, the observations made on the pretreated HBEC with plain plant extracts in adhesion reaction did not give promising result; this could be due to the effect of these plant extracts mainly on candidal cells rather than on HBEC.

4.6.2 EFFECT OF SURFACTANTS ON ADHESION PATTERN OF *CANDIDA ALBICANS* ONTO HBEC

Results of adhesion with surfactants are given in Table 4.50 and Fig. 4.57. SDS had reduced the adhesion of candida cells to HBEC, more strongly than the other surfactant agents. This may be due to the action of SDS on the cell wall proteins which are responsible for adhesion. With other surfactants the adhesion of the cells remained almost nearer to or higher compared to isolates from HIV seronegative group in both the strains (RL-24 and RL-112).

Table 4.50 Effect of surfactants on adhesion of *Candida albicans* on HBEC

	RL-24		RL-112	
	Percentage of HBEC adhered with candida cells	Candida cells/HBEC	Percentage of HBEC adhered with candida cells	Candida cells/HBEC
SDS	28.85	1.54	28.3	1.575
Tween 20	52.65	2.09	53.15	1.82
CPC	55.65	1.865	39.2	1.28
EDTA	41.05	1.605	35.9	1.335
Control Slides	44.3	1.64	47.2	1.38

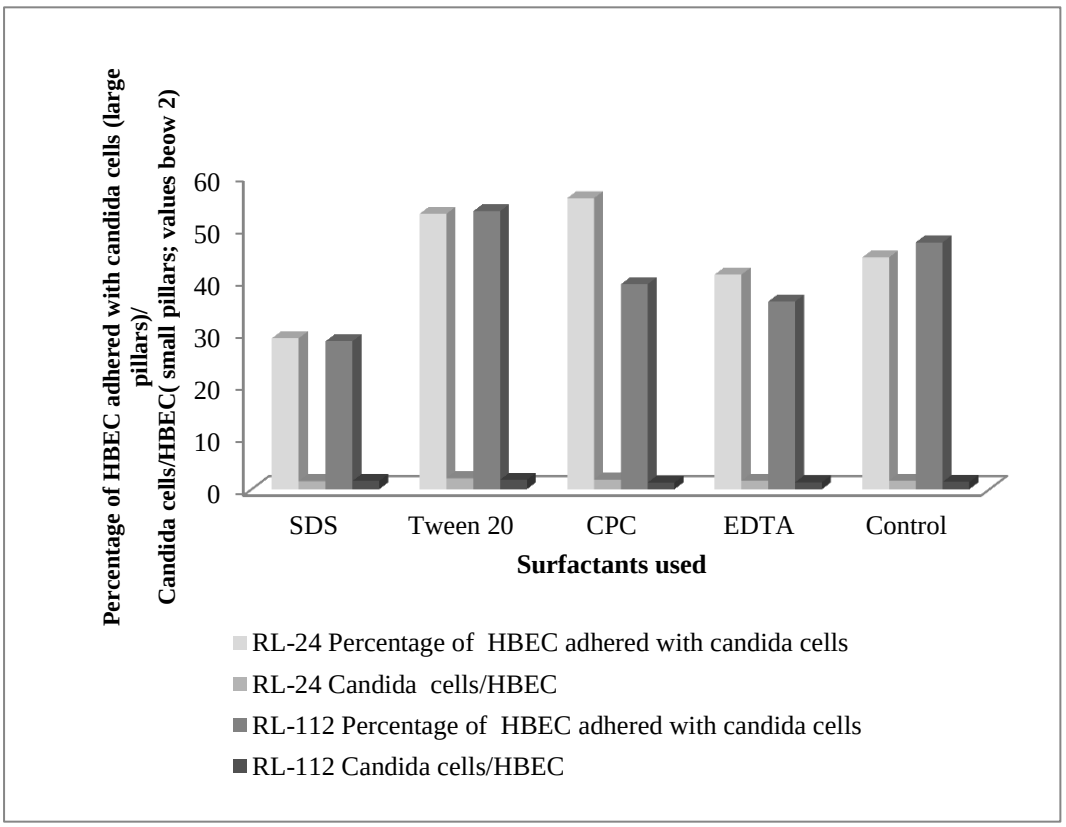


Fig. 4.59 Results of adhesion with surfactants (Control indicates the adhesion reaction without untreated candida and HBEC cells)

Surfactants usually decrease the hydrophobicity of candida cells and reduce the adhesion to HBEC, as demonstrated by using nanoparticles coated with surfactants by McCarron et al. (2007). Different surfactants like SDS (Sodium Dodecyl Sulphate, anionic), CTAC (Cetyl tri-Methyl Ammonium Chloride, cationic), EDTA and Tween 20, were tested in the present study for their effect on adhesion of *C. albicans* onto HBEC.

CTAC, SDS, N-hexadecyl-N-N'-dimethyl-3-ammonio-1-propane-sulfonate (HPS) and octyl phenoxy polyethoxyethanol (Triton X-100) were tested for their effect on viability, adhesion ability and exoenzyme production by *Candida species* by Lyon et al. (2011) and reported that, all surfactants demonstrated to be capable of inhibiting the adhesion of Candida species to buccal epithelial cells (HBEC) and decreasing the proteinase production. Similarly, Pizzo et al. (2010) also reported that *in vitro* exposure of HBEC to mouth rinse products, containing Chlorhexidine 0.2%

(CHX), Cetyl Pyridinium Chloride (CPC) 0.05% reduced the candida adhesion in significant manner. Tobgi et al. (1987) reported that Chlorhexidine mouth rinses were effective in decreasing candidal adhesion even at 0.002% concentration. Faria et al. (2011) reported that, the Chlorhexidine digluconate (0.12%) containing mouth washes were effective in preventing the adhesion of microbes along with C. Goldberg et al. (1990) reported that CPC enhanced adhesion of *C. albicans* along with a few bacteria onto hexadecane. They explained that these enhanced action was due to, CPC diminishing cell surface charges of the treated cells and increasing the cell surface hydrophobicity. In the present screening work with surfactants, SDS is found to be efficient. Similarly, Dusane et al. (2008) reported a nonionic surfactant, lauryl glucose found to be effective in preventing biofilm formation and adherence of *C. albicans* and *C. lipolytica* and other bacterial strains, on polystyrene and glass surfaces.

4.6.3 EFFECT OF SUB INHIBITORY CONCENTRATION OF FLUCONAZOLE ON ADHESION

Fluconazole had reducing effect on adhesion at its concentration of 0.125µg/ml. The effect of fluconazole on adhesion is represented in Table 4.51.

Table 4.51 Effect of sub inhibitory concentration of fluconazole on adhesion of *C. albicans*

	Candida cells exposed to fluconazole and allowed for adherence	**Untreated candida cells tested for adhesion with HBEC**
Percentage of HBEC showing candida cells adhered on them	33.67	47.2
Average number of candida per HBEC	1.46	1.38

Henriques et al. (2005b) reported that sub inhibitory concentration of antibiotics and antifungal agent exhibit strain dependent action on biofilm formation of *Staphylococcus epidermidis*, *C. albicans* and *C. dubliniensis*. Short exposure to sub-lethal concentrations of antifungal agents, nystatin (6xMIC), amphotericin B (8xMIC), 5-fluorocytosine (8xMIC), ketoconazole (4xMIC) and fluconazole (4xMIC), for 1 hr in case of *C. albicans*, and later removed antifungal agents and incubated for germ tube formation. Exposure to nystatin and amphotericin B almost completely inhibited germ tube formation of all the isolates (mean percentage reduction of 97.68% and 97.52%, respectively; $P<0.0001$), while ketoconazole suppressed this activity to a lesser degree (30.84%; $P=0.0174$). However, 5-fluorocytosine- and fluconazole-mediated germ tube suppression was minimal (12.63% and 15.93%, respectively; $P=0.3255$ and $P=0.3791$) (Ellepola and Samaranayake 1998). Thus results obtained with sub inhibitory concentrations of fluconazole in the present study also in accordance with above described findings.

However, biofilm of *C. albicans* challenged by sub inihibitory MICs of fluconazole tend to secrete higher quantities of Secreted Aspartyl Proteinases (SAP) (Mores et al. 2011). Miyake et al. (1990), also reports that sub-inhibitory concentrations of antifungal drugs decrease electrorepulsive forces between *C. albicans* strains and acrylic surfaces thus enhances the adherence of candida to acrylic surfaces.

According to the objectives laid, the current study has successfully revealed several aspects of adhesion of *Candida albicans* onto HBEC (Human Buccal Epithelial Cells).

C. albicans was successfully isolated from the oral cavity of HIV seropositive individuals. The prevalence of Oral Candidiasis (OC) was studied in HIV seropositive individuals of the geographical region of the study. Species of candida involved in both oral colonization and infection were extensively studied, in both male and female HIV seropositive subjects from different age group. The entire data obtained was compared with age and sex matched control group comprising of healthy, HIV seronegative subjects. The most important fact noted down is, 40.8% of HIV seropositive patients of this geographical area had OC. The *C. albicans* was the most prevalent species found in both colonization and infection. However, emergence of Non Albicans Candida (NAC) was also found, with *C.guilliermondii* being one of the most prevalent NAC, which is reported rarely. This species is known to acquire resistance to fluconazole very rapidly. Fluconazole resistant *C. albicans* of HIV seropositive group were found to be 8.51%. Thus, these observations gave base line data for the clinician to consider speciation and fluconazole sensitivity testing as important evidence with regard to treating HIV seropositive patients with OC.

C. albicans isolated from HIV seropositive individuals when tested for adherence on HBEC, found to be significantly more adhesive compared to the isolates from healthy individuals. The genotyping by RAPD method was done to know the correlation between adherence property and genetic similarity between the two groupsof isolates. It was found that the isolates from both the groups sometimes are placed in the same dendrogram clusters. They have similar base pair fragments amplified, in many of the isolates. The clusters contained mixtures of organisms which differ in their adherence behavior broadly. The ultra structural study on adhesion using Scanning Electron Microscopy (SEM), revealed candida cells contain fibrillar structures on their surface and these cells preferably bind to periphery of the HBEC.

CHAPTER 4. SUMMARY AND CONCLUSIONS

The experimental conditions required for adhesion through the present studies revealed the following aspects. The growth curve of candida studied showed the cells from late log phase and stationary phase are more adherent to HBEC. In *in vitro* conditions, the optimum incubation period required for the adhesion to occur is found be 60 minutes. The artificial saliva medium used as suspension medium for the adhesion reaction showed more HBEC get adhered with candida, than with the reaction done in PBS (pH 7.2).

The mechanisms of adhesion are known to be containing both specific and nonspecific factors, as reported in literatures. The biochemical analysis of candida cells was done to understand the adhesion process occurring in the fungus. The different sugars, lectin *Concanavalin A* (Con A) were used to understand the role of lectin-carbohydrate interactions in adhesion process. The sugars like fucose, mucin and fetuin etc. showed their role in adhesion process. The candida cells were reacting strongly with the Con A, and thus showing the presence of mannose moiety in abundance and involvement of mannose in adhesion. The cell wall protein profiling and lectin blotting of isolates from HIV seropositive individuals revealed the presence of few additional bands detected with different lectins. This indicated the presence of glycosylation of the cell wall proteins of *C. albicans* isolates from HIV seropositive individuals, which was not found in the isolates from healthy individuals. This was a unique finding in the present study which can add up to the present knowledge and open up new direction in candida research. Cell wall protein and supernatant proteins have role in adhesion of candida onto HBEC. However, the cell wall proteins were very strongly involved in adhesion than the excreted or secreted proteins.

Use of FTIR (Fourier Transform Infrared Spectroscopy) to understand candidal adhesion was again a rare approach according to the extensive literature survey done. The FTIR was used to know the changes in functional groups along the growth phase of candida isolates from HIV seropositive and HIV seronegative groups. Functional groups expressed did not differ between the isolates of both the groups. However, amide groups were found to be playing certain role in adhesion. When

HBEC adhered with candida was studied, the emergence of a new band representing CH_3 asymmetric binding was found.

The physicochemical factors of candida cells, i.e.., Cell Surface Hydrophobicity (CSH) and zeta potential studies carried did not show any specific role in adhesion of candida on host cells. Therefore, it is proved, that the biochemical molecules or specific mechanisms of interactions play specific role when candida adheres to other biological matter.

As the final objective, the inhibiting molecules like different surfactants, plant extracts and fluconazole in sub MIC (Minimum Inhibitory Concentration) were screened for their effect on adhesion. The surfactant Sodium dodecyl Sulphate (SDS), plant extract from neem, lemon grass and fluconazole (sub MIC) were found to be most successful agents in inhibiting adhesion of candida onto HBEC.

The entire course of work, explained the status of OC and colonization and candida carriage in HIV seropositive patients of certain geographical area, adhesion behavior of *C. albicans* isolates, mechanisms of adhesion and also specific substances which can inhibit the adhesion. The findings of the present research work indicate that, a lectin- carbohydrate interaction is the strongest mechanism involved in the adhesion. The glycosylation occurring in certain proteins of *C. albicans* from HIV seropositive individuals may be the reason for their enhanced adherence on to HBEC.

APPENDIX I

PREPARATIONS OF CULTURE MEDIUM AND PROCEDURES FOLLOWED IN SPECIATION OF CANDIDA

1.1. SABOURAUD DEXTROSE AGAR (SDA)

Components	g/L
Peptone	10
Dextrose	20
Agar	40

All the components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.6. Sterilization of the medium was done by autoclaving at 15 lb/in^2 pressure for 10 minutes.

1.2. GERM TUBE TEST

A small portion of isolated colony to be tested was suspended in test tube containing 0.5 ml of pooled human serum. The test tube was incubated at 35 ^{o}C for two hours. A drop of yeast serum suspension was placed on microscopic slide overlaid with cover slip. The preparation was examined microscopically for the presence of germ tubes. The appendage that is half the width and 3-4 times the length of the yeast cell from which it arises is called germ tube. There is no constriction at the origin of the germ tube from the cell.

1.3. CORN MEAL AGAR

1.3.1. PREPARATION OF THE MEDIUM

Components	g/L
Cornmeal	40
Agar	20
Tween 80 (1%)	10 ml

Cornmeal was dissolved in 500 ml of water and heated at 60 ^{o}C for 1 hr and filtered through filter paper. pH was adjusted to 6.6-6.8, agar was added and dissolved. The volume was made up to 1000 ml by adding remaining amount of water. Sterilization was done by autoclaving at 15 lb/in^2 pressure for 15 minutes.

1.3.2. INOCULATION OF CORNMEAL AGAR

A small amount of colony of candida was picked with sterile straight wire and with holding the inoculating wire at about 45- degree angle inoculation has to be done on to the centre of 1 cm x 1 cm corn meal agar block placed on sterilized glass slide. The agar block was then covered with a sterile cover slip and placed in sterile petri dish having moistened filter paper. The petri dish was incubated at 25 ^{o}C (Koneman 2006). The culture was observed after 72 hours of incubation. Isolates were identified on the basis of microscopic morphological features of the growth on corn meal agar (Larone 2002).

1.4.SUGAR ASSIMILATION TEST

1.4.1. PREPARATION OF THE MEDIUM

A. BASAL MEDIUM

Components	Quantity
Bromocresol purple (1.6%)	0.2 ml
0.1 N NaOH	1 ml
Noble agar	2 g
Deionized water	90 ml

Heated to dissolve the contents.

B. STOCK CARBOHYDRATE SOLUTION

Components	Quantity
Yeast Nitrogen Base	0.67 g
Appropriate carbohydrate	1 g
Deionized water	10 ml

The contents were dissolved properly. Medium was prepared by adding stock carbohydrate solution to the melted agar base and the preparation was mixed thoroughly. The pH was adjusted to 7.0. The final preparation was dispensed into screw-cap tubes and allowed to solidify in slant position. Prepared tubes were stored in the refrigerator.

1.4.2. INOCULATION OF THE MEDIUM

A loopful of pure culture was suspended in 9 ml sterile distilled water. Each slant was inoculated with 0.1 ml of yeast suspension along with controls. The tubes were incubated at 25 °C. The tubes were examined on 7^{th} and 14^{th} day for abundant growth and acid production indicated by change in color. Sugar assimilation was considered positive when tubes showed growth and the indicator changed from purple to yellow. Tubes showing no growth and no color change were considered negative.

1.4. YEAST NITROGEN BROTH (YNB) STOCK SOLUTION

Components	Quantity
Yeast Nitrogen	6.7 g
Distilled water	100 ml

The 20 ml of stock YNB solution was taken and to this 500 mM galactose and 2 g of dextrose was added. The volume was made up to 100 ml by adding sterile distilled water. The final preparation was filter sterilized by the membrane filter (0.1µm Sartorius).

1.5. YEAST LONG TERM STORAGE MEDIUM (YEAST EXTRACTS PEPTONE DEXTROSE MEDIUM- YEPD)

Components	g/L
Yeast extract	10
Peptone	20

The components are dissolved in 1 litre of distilled water and pH was adjusted to 5.8. The entire medium is sterilized by autoclaving at 15 psi for 20 minutes at 121 ^{o}C. A 50 ml of filter sterilized dextrose solution added to this medium.

1.4.1. PROCEDURE FOR INOCULATION

A. A 1 ml of YEPD medium was dispensed to storage vial of capacity 2 ml. Following this a 225 µl of 80% glycerol is added under sterile precautions, the final percentage of glycerol should be 15.
B. The candida colonies were inoculated to this medium, vortex mixed for 30 s for thorough mixing and immediately transferred to deep fridge.

APPENDIX II

CELL WALL PROTEIN EXTRACTION IN *CANDIDA ALBICANS*

The reagents were prepared in thoroughly cleaned glasswares and were rinsed in distilled water and dried. Analytical grade chemicals and deionized water is used for the preparation. All buffers were stored at 4 ^{o}C for several weeks.

1.1. LYSIS BUFFER

Components	g/L
Tris HCl	1.2112
PMSF	0.0871
pH adjusted	7.5.

1.2. EXTRACTION BUFFER

Components	g/L
Tris HCl	6.0570
SDS	20
DTT	1.5424
EDTA	37.2
pH adjusted	7.5.

1.3. PROTEIN ESTIMATION BY FOLIN LOWRY METHOD

1.3.1. PREPARATION OF REAGENTS REQUIRED

a. REAGENT A

Reagent A was prepared by dissolving NaOH 1.25 g in 250 ml Dist water. Sodium carbonate (6.25 g) was added to this and dissolved to make the volume up to 250 ml.

b. REAGENT B

Reagent B was prepared by adding 0.125 g of CuSO4 penta hydrate to 10 ml in DW.

c. REAGENT C

Reagent C was prepared by adding 0.25 g Sodium Potassium tartrate was added to 10 ml DW.

d. REAGENT D

Reagent D was prepared by adding solutions C: B: A in the ratio of 1:1:100

e. REAGENT E

Reagent E was prepared by mixing Folin's Cio-calteu's reagent D/W in the ratio 1:2. Reagent E was prepared just before adding to the tubes for estimation, as this cannot be stored.

f. PROTEIN STANDARD

Bovine Serum Agar (BSA) in 10 mg was dissolved in 100 ml Distilled water. The preparation was stored at 4 ^{o}C.

1.3.2. PROCEDURE FOLLOWED FOR THE TEST

The standard calibration curve was obtained by following the procedure as given in the table below along with determining the protein concentration expressed in the test sample in 1µg /ml.

Tube	**Protein Standard Added (in ml)**	**D/W (in ml)**	**Reagent D (in ml)**		**Reagent E (in ml)**	
1	0.0	1	2	**Kept for 10 minutes**	0.25	**Kept for 20 minutes and absorbance was read at 750 nm**
2	0.1	0.9	2		0.25	
3	0.2	0.8	2		0.25	
4	0.4	0.6	2		0.25	
5	0.6	0.4	2		0.25	
6	0.8	0.2	2		0.25	
7	1.0	0	2		0.25	
Test	Sample 0.1	0.9	2		0.25	

Calibration curve for Lowry's method for the determination of protein concentration was drawn. The linear curve was obtained. The absorbance obtained for the test sample was plotted and protein concentration was determined.

APPENDIX III

SDS POLYACRALAMIDE GEL ELECTROPHORESIS

1.1. PREPARATIONS OF STOCK SOLUTIONS FOR SDS PAGE

30% ACRYLAMIDE WITH 0.8% BIS-ACRYLAMIDE

Sl. No.	Chemicals	Quantity added
1	Acrylamide	30 g
2	Bis-acrylamide	0.8 g
3	Distilled Water	100 ml

1.2. SODIUM DODECYL POLYACRYLAMIDE GEL ECTROPHORESIS (SDS PAGE)

The gel was prepared by mixing the components as given in the table below. The gradient gel was obtained by pouring the percentage solutions to different tubes and allowing them to flow in a specified flow rate; between the plates of the electrophoresis unit.

Contents	5-15%		4.5% Gel
	A	B	6 ml
Tris HCl pH 8.8	1.74 ml	1.72 ml	-
4XTris HCl pH 6.8	-	-	1.25 ml

Stock Acrylamide solution	1.154 ml	3.45 ml	0.75ml
10% SDS *Added only when it was not present in your Tris buffer preparation*	35 µl	35 µl	50 µl
Water	4.05 ml	1.13 ml	3 ml
10% Ammonium Persulphate	35 µl	35 µl	50 µl
TEMED	7 µl	7 µl	5 µl
Total	6.96 ml	6.30 ml	6 ml

Preparation of various percentages of gradient gels was done by using following formula.

	Monomer %	**30% Acrylamide Stock (ml)**	**Tris HCl 8.8 Ph(ml)**	**Water (DI) (ml)**	**Sucrose (g)**
Upper solution	5	3.3	5	11.6	-
	10	6.7	5	8.3	-
Lower solution	10	6.7	5	6.7	3
	12	8	5	5.3	3
	15	10	5	3.3	3
	17	11.366	5	2	3

	20	13.3	5	0	3
APS and TEMED TEMED	Added 30 µl and 7µl respectively when final solution volume is 6.5 ml in each case.				

1.3. SAMPLE BUFFER (2x)

The sample buffer was prepared by following formula.

Components	Quantity added
Tris (0.5 M, pH 6.8)	2.5 ml (4x stacking gel buffer)
SDS (10%)	4 ml
Glycerol (100%)	2.0 ml
Beta-mercaptoethanol	0.8 ml
	or
1M DDT	0.5 ml
Stock solution of Bromophenol blue (of 0.1% Concentration)	300 µl

The sample buffer was made up to 10 ml by adding distilled water and pH was adjusted to exactly 6.8. Sample buffer was stored only for 10 days in the refrigerator and 1 month in deep freezer; not more that.

1.4. SAMPLE PREPARATION FOR LOADING

Protein sample was mixed with sample buffer, according to the concentration of protein. SDS concentration in the sample buffer was adjusted so that it was not

exceeding 200mg/30µl of sample. To the final volume beta-mercaptoethanol was added up to 10%. Vortexed thoroughly, before keeping for heating. The sample prepared was boiled for 3 minutes/ 65 ^{o}C for 15 minutes. The samples were kept in 4 ^{o}C till the use.

Before loading the sample both upper and lower tanks of the electrophoresis unit was sufficiently filled, any leak is there in the lower tank was checked and it was sealed with molten agar.

The sample was vortex mixed again thoroughly before loading into the gel. To each sample well maximum volume fixed to load was 50µl. Empty wells were filled with sample buffers. After loading sample to the well, immediately electrophoresis was started. The constant voltage of 150 V was used during the electrophoresis.

1.5. GEL STAINING SOLUTIONS AND PROCEDURES FOLLOWED

1.5.1. COMASSIE STAINING

1.5.1.1. COMMASSIE BRILLIANT BLUE (CBB) R250 (0.1%) PREPARATION

A 100 mg of CBB weighed in a watch glass and transferred in to a 100 ml beaker. CBB was first dissolved with around 1 ml of methanol, once dissolved completely, methanol was added up to 50 ml. Following this 10 ml and 40 ml of glacial acetic acid and water were added respectively. This solution was filtered into a 100 ml reagent bottle and stored at room temperature.

1.5.1.2. PREPARATION OF FIXATIVE

Any one of the following was used as fixative.

A.	B.	C.
10%(V/V) Acetic acid 65% (V/V) DW 25% Isoproponal	12.5% Trichloroacetic acid in DW	50 ml Methanol 10 ml glacial acetic acid 40 ml DW

1.5.1.3. PREPARATION OF DESTAINER

Two types of preparations were done

A. DESTAINER (STRONG)

Component	**Quantity**
Methanol	50 ml
Glacial acetic acid	10 ml
DW	40 ml

B. DESTAINER 5%

Component	**Quantity**
Methanol	5 ml
Glacial acetic acid	7 ml
DW	88 ml

1.5.2. SILVER STAINING OF THE GELS

Step	Procedure	Holding time Maintained	Remarks
1	The gel was treated with solution containing 60 ml 50% Acetone stock, 1.5 ml TCA stock and 25µl Formalin	5 min	Performed on shaker
2	Distilled water Rinse was given	3x5 s	
3	Distilled water Rinse was given	5 min	Performed on shaker
4	Distilled water Rinse was given	3x5 s	
5	Gel was pretreated with 60 ml 50% Acetone stock	5 min	Performed on shaker
6	Gel was treated with 100 µl sodium thiosulphate stock with 60 µl 37% Formaldehyde in 60 ml DW. Fresh Sodium thiosulphate stock was prepared every time	1 min	Performed on shaker
7	Distilled water rinse was given	3x5 s	
8	Gel was impregnated with 0.8 ml Silver Nitrate stock with 600 µl 37% formaldehyde in 60 ml distilled water	8 min	Performed on shaker and chilled solutions were used.
9	Distilled water Rinse was given	2x5 s	
10	1.2 g Sodium carbonate + 25 µl 37% formaldehyde + 25 µl Sodium thiosulphate	0-20 s	Immediately bands will start

	in 60 ml distilled water		developing.
11	Over coloration was stopped by using with 1% glacial acetic acid	Immediately	
12	Gel was rinsed in DW		
13	Gel was preserved in 1-1.5% glycerol		

APPENDIX IV

ELECTROTRANSFER OF PROTEINS TO PVDF MEMBRANE

1.1. WESTERN BLOT TANK BUFFER

Chemicals	Quantity (2050 ml was prepared as per instruments require)
Tris HCl	6.2115 g
Glyceine	29.52 g
Methanol	410 ml
Water	Added 1640 ml water.

1.2. STAINING OF PVDF MEMBRANE

1.2.1. PONCEAU STAIN STOCK

Component	Quantity
Ponceau Stain (powder)	2 g
TCA	30 g
Sufosalicylic acid	30 g
DW	100 ml

Working solution was prepared by adding 10 ml of the Stock Ponceau S to the 90 ml of DW.

1.2.2. PONCEAU STAINING PROCEDURE FOLLOWED

The membrane was immersed in Ponceau stain (working solution) for about 5-10 minutes in dark with shaking. Once the membrane is completely dark red, stain was discarded. Destaining of the membrane was done with the DW, until the washed water remains colorless and desired contrast between the bands and background obtained.

APPENDIX V

LECTIN BLOTTING

1.1. PREPARATION OF ALKALINE PHOSPHATSE BUFFER

Sodium Chloride 100 mM, magnesium chloride 5 mM, Tris HCl 100 mM were added to 1000 ml of DW and pH was adjusted to 9.5. This solution was prepared just before colorimetric reaction.

1.2. PROCEDURE FOLLOWED FOR LECTIN BLOTTING

A. The PVDF membrane with the transferred protein was washed in distilled water –3x5 min
B. The membrane was treated with blocking solution for 1 hr at room temperature.
C. PVDF membrane was incubated with biotinylated Lectin for 2 hr
D. PVDF membrane was immersed in 200 ml PBS –Tween 20 for 10 min and washed with the same solution. The procedure was repeated for 6 times.
E. PVDF membrane was treated with blocking solution with Extravidin-Alkaline Phosphatase (1:2000 diluted) for 1 hr.
F. Wash in PBS containing 5%Tween 20 for 6 times with prior incubation of the membrane in the solution for 10 min every time.
G. Finally incubation of the blotted membrane was done with156µl BCIP stock solution and 312 µl NBT stock, solution in 50 ml of alkaline phosphatase buffer for 10-20 min.
H. Blotted proteins developed purple hue. The membranes were preserved in a clean box for further evaluation as the color remains permanent.

CHAPTER 6

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PUBLICATIONS AND PRESENTATIONS DONE FROM THE PRESENT THESIS WORK

PUBLICATIONS

- Pavithra , Gopala Mugeraya , G. Srinikethan, B.M.Swamy (2012). **" Studies on Adhesion Activity of *Candida albicans* to Human Buccal Epithelial Cells in Relation to Its Growth Phases."***J Biosci Tech,* Vol 3 (1), 2012, 449-454.

- Pavithra , R. D. Kulkarni , B.M.Swamy , Gopala Mugeraya , Srinikethan G , Sharanbasu KA . (2013). **"A method for efficient extraction of cell wall proteins from *Candida albicans."*** *Indian J Clin Res R Review*. 5(16), 01-07.

CONFERENCE PROCEEDINGS

- Presented a poster titled **"Studies on Adhesion Activity of *Candida albicans* to Human Buccal Epithelial Cells in Relation to Its Growth Phases."** at National Conference on "Recent Advances in Mycological Research" of Mycological Society Of India (MSI) on 8th& 9th, February, 2011, University of Madras, Chennai, Tamil Nadu**.**
    - **Authors: Pavithra,** Gopal Mugeraya, G.Srinikethan, B.M.Swamy.

- Presented a poster titled: **"Analysis of protein extraction methods for cell wall protein study of *Candida albicans"***at National Seminar on " Biomolecules & Biocatalysts" at St. Aloysius College, Mangalore on 8th & 9th March 2012.
    - **Authors: Pavithra,** B.M.Swamy, Gopal Mugeraya, G. Srinikethan , R.D.Kulkarni and Sharanabasu.

- Presented a poster titled "Effect of neem extracts on adhesion of *Candida albicans* onto Human Buccal Epithelial cells - an *in vitro* study" at CME on

"Current Challenges in Clinical Microbiology." at Department of Microbiology, Kasturba Medical College, Manipal University, Manipal on 9th March 2013.

- **Authors: Pavithra,** Shubhada, C., B.M.Swamy, G. Srinikethan, Gopal Mugeraya, R.D. Kulkarni , Pradeep K and Pravinchandra K. R**.**

1.

BRIEF BIODATA OF THE RESEARCH SCHOLAR

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5. **Educational Qualification** : Degrees obtained (Begin with Bachelor's Degree)

Degree	Institution	Field(s)	Year
B. Sc	St. Aloysius College Mangalore	General microbiology	1999
M. Sc	KMC, Mangalore, Karnataka	Medical Microbiology	2002

6. **Publications**

1. Research articles published (National): 11
2. Research articles published (International): 2

7. Research/Training Experience.

- A project work on food adulteration --- St. Aloysius College Mangalore, Karnataka- Performed Lab. Experiments to find out food adulteration.
- Cold Sterilization Of Gutta Percha Points An invitro Comparative study between 5 Different agents at different concentrations — Gutta percha points which were used in root canal procedures were tested in the our laboratory for successive and fast disinfection with various commonly available disinfectants.

- Effect of garlic extract on *Enterococcus, C. albicans* flora of Dental Plaque: Allocin, the extract from garlic was tested for its effect on various oral cavity organisms using standard MIC testing methods.
- Study of type of bacteria and their viability within human supra-gingival dental calculus : using anaerobic and aerobic culture methods.
- "Comparative evaluation of oral colonization of Candida in HIV seropositive individuals with special reference to fluconazole sensitivity" **RGUHS Bangalore funded** Project.
- Comparison of adherence properties of oral *C. albicans* isolates from HIV infected patients and Healthy HIV non-reactive individuals on Human buccal epithelial cells of Healthy individuals- an *in vitro* study.
- Study of microbiology of fungal sinusitis: On process.
- Evaluation of neonatal candidemia in our hospital with testing for antifungal susceptibility testing: Going on.

Workshops and Trainings attended

Sl. No	Duration	Institution	Particulars of work done
1	Jan 11-23rd 2010.	Manipal Life Science Centre, Manipal. Karnataka	IISc AND National Academy of Science sponsored workshop on "Biotechnology and modern molecular biology" Got hands-on training in molecular biology tools.
2	October 22nd 2009	JNMC, Belgaum Karnataka	CME "Biomedical Communication" –Scientific writing and presentation.
3	Janaury:2009	NITK, Surathkal Karnataka	TEQIP Sponsored Workshop on Emerging Trends in Environmental Biotechnology (TW-ETB-2009): Training in Instrumental analysis, mathematic modulation in research.
4	July 08-Aug 08,	NITK Surathkal Karnataka	AICTE Sponsored Short Term Training Program In Industrial Biotechnology- Got hand-on training in Instrumental analysis used in research
5	Aug 2005	SDMCMS & Hospital-Dharwad	Teacher Training Programme – training on Teaching in medical subjects