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Pathogenic characteristics of candida albicans isolated from oral cavities denture wearers and cancer patients wearing oral prostheses

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Pathogenic characteristics of *Candida albicans* isolated from oral cavities of denture wearers and cancer patients wearing oral prostheses.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Candida albicans is a normal commensal carried by human in the oral cavity, digestive system and vagina. It can cause superficial to systemic opportunistic infection both in immunocompromised patients, hospitalized patients and otherwise healthy individuals. It has an ability to cause infection due to its virulence properties or pathogenic characteristics. These include the ability to adhere to host tissues and prostheses (eg. dentures), phenotypic switching, formation of germ tubes for tissue invasion and the production of a range of hydrolytic enzymes such as tissue damaging proteinases and phospholipases (Khan et al., 2010 and Samarananyake, 2002). During commensal and disease state, the number of *C. albicans* and their virulence in the oral cavity may fluctuate depending on the oral status, patient's general health, intake of antibiotics, the presence of prostheses and onset of candidiasis. The number and the virulence of this organism may act as a marker for the onset of disease process. This study investigated the virulence properties of *C. albicans* isolated from the oral cavities of healthy subjects and two vulnerable groups, denture wearers and cancer patients wearing oral prostheses.

1 Literature review

1.1 *Candida* species

Yeasts which belong to the genus *Candida* consists of imperfect unicellular dimorphic fungi which multiply by budding similar cells from their surface and form hyphae and/or pseudohyphae. They were earlier assigned to the family Deuteromycetes, indicating a lack of sexual reproduction. However, several pathogenic and non-pathogenic *Candida* species has been identified to have sexual reproduction (Webb et al., 1998).

Macroscopically, *Candida* spp. form cream-coloured colonies with a sour yeasty odour when grown under aerobic conditions on medium which has a pH range of 2.5-7.5 and a temperature range of 20-38°C. Growth is always detected in 48-72 hours, and on sub-culture may grow more rapidly. The ability of yeasts to grow at 37°C is an important characteristic to be considered in their identification from clinical specimens as most pathogenic species grow readily at 25°C and 37°C. Microscopically, all yeasts species are similar, Gram-positive but sometimes the shapes of the blastospores can vary from void to elongated or spherical. *Candida albicans* exhibits dimorphism with transition from ovoid budding blastospores (yeast cells) to parallel-sided hyphae (Webb et al., 1998).

The yeast exhibits a number of different morphological forms under different environmental conditions; i.e. budding yeast cell (blastorespore, blastoconidia) pseudohyphae, true hyphae and clamydospores (Figure 1.1). A hypha has been defined as a microscopic tube which contains multiple cell units divided by septa and which may arise from existing hyphae or from blastospores. They are known as germ tubes and grow continuously by apical extension. When

blastospores are produced in linear fashion without separating, a structure termed pseudohypha is formed. True septate hyphae are produced by some *Candida species*, such as *C. tropicalis*, under certain circumstances, but true hyphae are associated with *C. albicans*. The entire candidal cellular aggregate including hyphae, branches and lateral buds are referred to as a mycelium (Webb et al., 1998). The transition between these different morphological forms in response to diverse stimuli seems to be very important for fungal pathogenicity. Their morphology can change under a variety of environmental conditions, including response to physiological temperature of 37°C, a pH 7.0 or higher, CO_2 concentration of 5.5% or the presence of serum or carbon sources which stimulate the hyphal growth. The production of unicellular form is stimulated by low temperatures and acidic pH, absence of serum and high concentrations of glucose, (Karkowska-Kuleta et al., 2009).

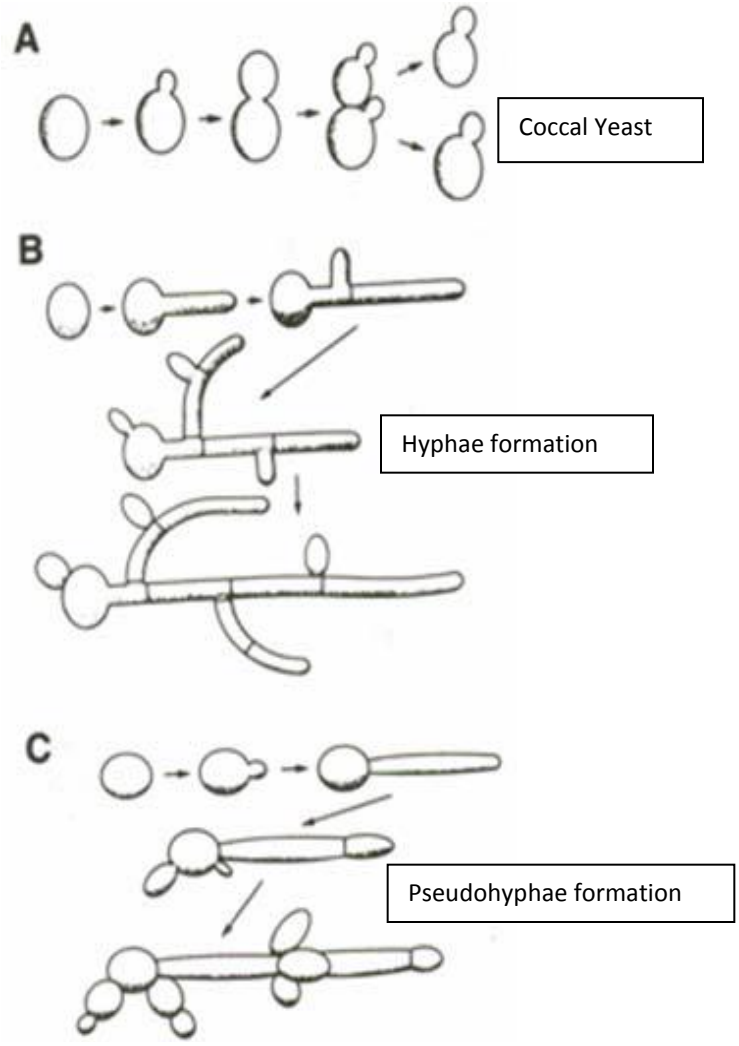

Figure 1.1 Growth forms of *Candida* species. [Source: Calderone (2002)]

1.1.1 *Candida albicans*

The fungus known as *C. albicans* was first isolated by Bennett in 1844 from the sputum of a tuberculosis patient (Collier et al., 1998). It is a human commensal that is also an opportunistic pathogen. Between 18% and 40% of individuals carry *C. albicans* in their oral cavity (Odds, 1988) and although predominantly associated with the dorsum of the tongue, it can be cultured from saliva, and from swabs of the cheek and tooth surfaces (Arendorf and Walker, 1980).

However, it survives poorly on dry surfaces (Odds, 1988) and can be viable for some time on moist objects. For example, it is isolated from the toothbrushes of most people who carry the yeast in their mouths, and survives in hand cream, cosmetics and on clothing (Cannon et al., 1995).

It is an oval, budding yeast-like fungus producing both blastospores and pseudomycellium in the tissue and exudates, and can be cultured at room temperature and at 37^0C. The most valuable test for rapid presumptive identification of *C. albicans* is the Germ Tube Test (GTT). Microscopic observation of the preparation will reveal that the short hyphal initials produced by *C. albicans* are not constricted at the junction of the blastoconidium and germ tube. Frequently, so many *C. albicans* blastoconidia, i.e. the unit of asexual reproduction produced by budding, also called blastospore, produce germ tubes that become entwined with each other, producing clumps of cells (Collier, 1998). Germ tube formation is the initial stage in the yeast-hyphal transition. The presence of hyphae seems to facilitate the candidal colonization on the mucosal surfaces and raises the *C. albicans* counts (Bilhan et al., 2008) and therefore, it is considered as one of the pathogenic characteristics.

C. albicans is the most prevalent yeast isolated from the human body as a commensal or as opportunistic pathogens (Cannon et al., 1995). However, many *Candida species* other than *C. albicans* also exist on the human host and cause opportunistic infections.

1.1.2 Pathogenic *Candida* species other than *C. albicans*

The genus *Candida* is comprised of about one hundred and fifty species including *C. albicans, C. tropicalis, C. parapsilosis, C. krusei; C. kefyr, C. glabrata, and C. guilliermondii* which are recognized as medically important pathogens causing diseases ranging from superficial mycoses to fatal infections (Cannon et al., 1995a). *Candida species* implicated in the development of oral candidiasis include *C. tropicalis* (2%), *C. glabrata* (15%), *C. parapsilosis* (15%), *C. guilliermondii* (6%) and *C. krusei* (1%) *(*Akpan and Morgan, 2002). *Candida dubliniensis* is often found in immunocompromised such as the elderly and HIV patients, and is found only in about 3.5% of healthy subjects.

Candida is the fourth most common cause of hospital-related bloodstream infections. Forty percent of patients who have *Candida* in their intravenous catheters have underlying fungemia while the case-fatality rate for catheter-related candidemia approaches 40% (Kuhn et al., 2004). The infections caused by *Candida spp*. result in increased length of hospital stay and medical costs that constitute an important public health problem (dos Santos, 2010).

According to Meurman et al., (2007), *C. glabrata* is considered a pathogen that causes infection only when detected with *C. albicans.* There have been several reports on oropharyngeal *Candida* (OPC) infections due to *C. glabrata* and it is now emerging as an important pathogen in both mucosa and bloodstream infections. It is commonly isolated from the oral cavities of HIV-infected individuals and it is the second-most common agent of candidemia in the United States

since the early 1990s. *Candida glabrata* associated with OPC infections in HIV- and cancer patients are more severe, more difficult to treat and are associated with systemic infections with a high mortality rate (Meurman et al., 2007).

Although *C. parapsilosis* is found as a commensal, it is the second most important pathogenic yeast of the *Candida* species (Kuhn et al., 2004, Ozkan et al., 2005). It often causes systemic infections especially related to foreign body insertions, neonates and hyperalimentation and particularly affects critically ill neonates and surgical intensive care unit (ICU) patients (Meurman et al., 2007). *Candida tropicalis* is found in 10-30% of non- *C. albicans Candida* (NCAC) infections and it is becoming an emerging pathogen globally. It is also detected in patients with neutropenia and bone marrow transplantation. The major contributory factors in the emergence of *C. tropicalis* include the increasing use of an antifungal regimine, the increasing number of immunocompromised patients, long-term use of catheters and complexity in treating underlying subclinical conditions coupled with antifungal drug intolerance (Meurman et al., 2007).

Candida famata is considered as an emerging pathogen associated with a range of conditions such as eye infections, intravenous catheter infections, fungemia and peritonitis in humans, particularly in immunocompromised patients. Although it has been isolated from foods, and human and animal tissues, it has been poorly studied (Pacheco et al., 2007). *Candida guilliermondii* has been documented to cause infection in patients undergoing surgical procedures, cause endocarditis in intravenous drug users and fungemia in immunocompromised patients. It has also been isolated in urinary tract infections. *Candida krusei* causes infection

mainly in the critically ill patients and is most often isolated from hematology patients with severe neutropenia (Meurman et al., 2007).

1.1.3 Colonization of *Candida albicans* in the oral cavity

Many healthy subjects carry *C. albicans* in their oral cavity. Colonization with *C. albicans* can lead to systemic infection when the host presents risk factors. Predisposing factors for colonization and candidiasis include immunosuppressive and cytotoxic therapies, treatment with broad spectrum antibiotics, AIDS, diabetes, drug abuse, use of catheters and indwelling devices. Depending on the underlying host defects, the microorganism may cause a wide variety of infections ranging from mucosal to life threatening disseminated candidiasis. Development of candidiasis depends on a delicate balance between the commensal or parasitic relationship (Mishra et al., 2007, Brawner and Cutler, 1989).

Once in the mouth, *C. albicans* is presented with a plethora of sites for adhesion to oral surfaces. These include the epithelial cells of buccal mucosae, the tongue, tooth surfaces, various oral prostheses such as dentures, and other oral micro-organisms that have already colonized these surfaces (Cannon and Chaffin, 2001). A warm, moist, generally nutrient rich environment is a situation that would enhance colonization. The growth in the oral cavity can be influenced by several factors such as temperature, redox potential/anaerobiosis, pH, nutrients (sugar, amino acids and peptides) and host genetics (Akpan and Morgan, 2002). Newborn usually become colonized with the mother's vaginal flora or other exogenous sources. Most people are colonized with a distinct strain of *Candida* and if the infection occurs, the infecting strain is the same as the colonizing strain (Meurman et al., 2007).

Salivary gland dysfunction can predispose to *Candida* infection such as oral candidiasis. Secretion of saliva causes a washing effect and removes organisms from the mucosa. Antimicrobial proteins in the saliva such as lactoferrin, sialoperoxidase, lysozyme, histidine-rich polypeptides, and specific anticandida antibodies, prevent the overgrowth of *Candida*. Therefore conditions or drugs that reduce salivary secretions such as Sjögren's syndrome, radiotherapy of the head and neck cancers, can lead to an increased risk of oral infections (Akpan and Morgan, 2002).

The prevalence of *Candida* in South African HIV-positive subjects was found to be significantly higher (75%) than in HIV-negative (68%) subjects (Hauman et al., 1993, Patel et al., 2006). In addition, HIV positive patients carry high quantities of *Candida* and variety of *Candida* spp. compared to the healthy individuals. Colonization rate in cancer patients is usually high and increases with radiation therapy. Head and neck radiotherapy and chemotherapy are associated with a significant increased risk for oral fungal infection. In patients receiving head and neck radiotherapy, the prevalence of clinical oral fungal infection was 7.5% pretreatment, 37.4% during treatment and 32.6% after the end of radiation therapy. Increased colonization during and after radiation therapy also leads to increased rates of clinical oral fungal infection (Bensadoun et al., 2011).

In a study by Schelenz et al., (2011), it was discovered that oral yeast colonization varied amongst different cancer groups and was highest in solid tumor patients (colonization rate

64.6%) compared with hematological malignancies patients (49.4%) or head and neck cancer patients (36.9%). Bensadoun et al., (2011) also mentioned that a wide variety of *Candida* species are carried by these cancer patients, for example, in patients receiving chemotherapy and/or radiation therapy to the head/neck, the oral cavity of 46.2% of patients were colonized by *C. albicans,* 16.6% *C. tropicalis,* 5.5% *C. glabrata* and 3% *C. krusei* (Bensadoun et al., 2011).

1.2 Oral candidiasis

The term "Candidiasis" is used to describe a primary or secondary infection caused by yeasts that belong to the genus *Candida* (Sini et al., 2007). Candidiasis is common among very young, the elderly, particularly in denture wearers, and it is avoidable with a good oral and denture hygiene program (Akpan and Morgan, 2002). In addition, it is common in immunocompromised patients such as those infected with HIV, cancer patients on chemo and radiotherapy and patients with diabetes mellitus. Approximately 50 percent of people with complete upper dentures will suffer from *Candida*-related Stomatitis (chronic erythematous candidiasis) whereas oropharyngeal candidiasis, more serious illness is highly prevalent in AIDS patients. Up to 60% of healthy individuals carry *Candida* in their oral cavities without clinical symptoms, but usually the yeast is present in low numbers in comparison to oral bacteria (Cannon and Chaffin, 2001).

1.2.1 Types of oral candidiasis

Oropharyngeal candidiasis has been classified into different categories depending on the type of lesions (Figure 1.2). It includes acute pseudomembraneous, acute and chronic atrophic, chronic hyperplastic, median rhomboid glossitis and angular cheilitis (Akpan and Morgan, 2002).

Pseudomembranous candidiasis (thrush) is characterized by white pseudomembranes consisting of desquamated host epithelial cells and fungal growth. The curd-like patches occur on the oral mucosa, palate and tongue. The membrane can easily be removed with a swab to expose an underlying affected tissue (Akpan and Morgan, 2002). In acute atrophic candidiasis, patient experience burning sensation, particularly on the tongue with a bright red mucosa (Akpan and Morgan, 2002).

In chronic conditions, hyperplastic candidiasis occurs as white lesions on the buccal mucosa or lateral part of the tongue which can progress to severe dysplasia and it is referred to as candidal leukoplakia (Akpan and Morgan, 2002).

Classification of Oral candidiasis

1. **Acute candidiasis**
   - Acute pseudomembranous candidiasis (thrush)
   - Acute atrophic or erythematous candidiasis
2. **Chronic candidiasis**
   - Chronic hyperplastic candidiasis or leukoplakia
   - Chronic atrophic denture related candidiasis
   - Median rhomboid glossitis
3. **Angular cheilitis or stomatitis**

Figure 1.2 Classification of oral candidiasis **[Source: Akpan and Morgan, 2002]**

Chronic atrophic candidiasis is characterized by localized chronic erythema of tissues underneath the dentures. Lesions usually occur on the palate and it can be diagnosed on the removal of dentures. Median rhomboid glossitis is a chronic symmetrical lesion on the center of the tongue anterior. In the 85% of cases *Candida* can be yielded from biopsies. It is often associated with steroid inhalation and smoking (Akpan and Morgan, 2002).

Angular cheilitis is an erythematous cracking at corners of the mouth and is usually associated with oral *Candida*, *Staphylococci* and *Streptococci*. A constant moist environment in this area predisposes to this lesion. Long term denture wearers usually suffer more with this condition (Akpan and Morgan, 2002).

1.2.2 *Candida* infections in denture wearers

Dentures are prosthetic replacement for individuals who have lost some of or all of their natural teeth. They assist to restore chewing functionality, return a normal appearance to the facial structure and improve the patient's ability to pronounce and enunciate during speech. For patients who are missing both maxillary (upper arch) and mandibular (lower arch) teeth, the use of complete dentures will give them a complete set of teeth. Dentures can also be constructed to replace only selected teeth on a particular arch. Fixed partial dentures are designed to replace missing teeth by cementing missing units with crowns cemented to appropriately prepared teeth (Zarb et al., 2013).

Wearing of dentures produces a suitable microenvironment for the growth of *Candida* with low oxygen and low pH (Akpan and Morgan, 2002). The development of oral candidiasis may be due to enhanced adherence of *Candida* species to acrylic, reduced saliva flow under the surfaces of the denture fittings, improperly fitted dentures, or poor oral hygiene (Gendreau and Loewy, 2011). In addition, growth of *Candida* and adherence to oral epithelial cells is enhanced by a high carbohydrate diet (Akpan and Morgan, 2002). Plaque containing oral bacteria and *Candida* on the inner surface of the denture harbors microorganisms causing inflammation of the mucosa (Samaranayake, 2002). *C. albicans* is considered the most important *Candida* species able to cause oral infections in denture wearers (Gasparoto et al., 2009).

In patients wearing dentures, *Candida* seem to be an important factor in the formation of denture-related stomatitis (DRS), which is a common inflammatory process that mainly involves the palatal mucosa when it is covered by complete dentures. DRS attack the mucosal area adjacent to dental prostheses and beneath the fitting surface of the denture, especially the maxillary dentures (Gasparoto et al., 2009). The pathological reactions of the denture bearing palatal mucosa may be known as denture-induced stomatitis, denture sore mouth, denture stomatitis, inflammatory papillary hyperplasia and chronic atrophic candidosis.

Denture-related stomatitis is still a dilemma in removable prosthodontics. Epidemiologic studies have shown that it is a relatively common fungal infection that affects approximately 50% to 65% of denture wearers (Darwazeh et al., 2001, Akpan and Morgan, 2002). It occurs mostly in women and the prevalence increases with age. Clinically, the inflammation is of varying degrees and classified using Newton's classification (Samaranayake, 2002).

There are different factors that cause denture stomatitis including traumatic occlusion, poor oral and denture hygiene, microbial factors and increased age of denture resulting in the accumulation of plaque on the denture, old age, dryness of mouth, systemic conditions, diabetes mellitus and immunodeficiency, nutritional deficiencies and medications (Naik and Pai, 2011,Gendreau and Loewy, 2011).

According to Akpan and Morgan, (2002), oral hygiene and topical antifungal agents are generally adequate for uncomplicated oral candidiasis. Oral hygiene involves daily cleaning of the oral cavity, tongue and dentures. Acrylic dentures have irregular and porous surfaces which facilitate the adhesion of *Candida* and brushing alone cannot remove them. Therefore, at night removal and disinfection of dentures is important. After disinfection, dentures should be allowed to air dry to kill adherent *Candida* on dentures. Regular oral and denture hygiene with regular oral examination can prevent most cases of oral candidiasis (Akpan and Morgan, 2002). In addition topical antifungal agents can be used.

1.2.3 Oral candidiasis in cancer patients

High percentages of cancer patients on cancer therapy carry *Candida* in their oral cavities and are at a great risk of developing oral candidiasis. In these patients with all types of cancers, the prevalence of oral colonization with *Candida* was 48% before treatment and during treatment increased to 72% (Lalla et al., 2010). This increased colonization affects the risk of oral infection development.

In patients undergoing head and neck radiotherapy, *Candida* colonization tends to increase throughout the course of treatment and remain increased if xerostomia occurs (Hancock et al., 2003). Prevalence of oral candidiasis is highest in patients with head and neck cancers in comparison to patients with solid tumors and haematological malignancy (Schelenz et al., 2011). Up to 73% of patients with head and neck cancers carry *Candida* in their oral cavities (Redding et al., 1999). Although *Candida albicans* is mostly carried by these patients, other *Candida species* such as *C. tropicalis, C. glabrata, C. krusei* and *C. kefyr* are also found (Paula et al., 1990).

1.2.4 Cancer patients with oral prostheses

Oral cavity is part of the upper aerodigestive tract that begins at the lips and ends at the anterior surface of the facial arch. Primary tumours of the oral cavity may arise from epithelium, minor salivary glands, tonsils, tongue or submucous tissues. Tumours of dental origin, bone tumours and tumours of neurovascular origin are common. The tongue, alveolus, gingivo-buccal sulcus, buccal mucosa are some of the common subsites of carcinoma. Oral cancer is traditionally defined as squamous cell carcinoma of the lip, oral cavity and oropharynx (Carnelio and Rodrigues, 2004). It is estimated that thousands of people die daily due to oropharyngeal malignancy. In addition, oral cancer is frequently associated with the development of multiple primary tumors. The primary tumors develop independently in the upper aerodigestive tract as a result of chronic exposure of the lining mucosal epithelium to carcinogens, and the theory is known as “field cancerization”. Oral cancer patients, who live five years after their initial

primary disease are diagnosed and treated, have up to a 35% chance of developing at least one new primary tumor during that time (Lingen et al., 2008, Parkin et al., 2001).

These patients are significantly challenged with both intra- and post-therapy oral complications resulting from radiation therapy. These complications can be divided into two groups on the basis of the usual time of their occurrence: 1) Acute complications occurring during therapy, which include oropharyngeal mucositis, sialadenitis and xerostomia, infections (primarily candidiasis), taste dysfunction. 2) Chronic complications which include mucosal fibrosis and atrophy, decreased saliva secretion and xerostomia, accelerated dental caries related to compromised saliva secretion, infections (primarily candidiasis), tissue necrosis, taste dysfunction, muscular and cutaneous fibrosis and dysphagia (National Cancer Institute, 2012). These adverse effects require additional palliative treatment to alleviate these troublesome symptoms (Weinberg et al., 2011). Therefore, cancer of the head and neck region can profoundly affect the quality of life of patients (Guttal et al., 2010).

Approximately 300 000 cases worldwide were diagnosed with oral cancers in 2003, making it the sixth most common malignancy in the world (Petersen PE, 2009). They are the most common cancers constituting almost fifty percent (50%) of all cancers diagnosed in males. The incidence is 3.8 to 11 per 100,000 population but the rates differ from region to region. Oral cancer predominantly affects men (Carnelio and Rodrigues, 2004, Petersen, 2009). A study by Fleming et al. (1982) showed that among South African Blacks, a high male: female ratio (7:1) is related to the differences in tobacco usage between the sexes. Oral cancer, like most other cancers, affects older individuals mostly over the age of forty (Carnelio and Rodrigues, 2004).

Malignant tumors of the maxilla and hard palate account for 1-5% of malignant neoplasms of the oral cavity. Majority of these lesions are squamous cell carcinomas. Most of these cancers are diagnosed late when they have affected the underlying bone. The procedures of choice for removal are alveolectomy, palatectomy, maxillectomy, which may require total or partial surgical reconstruction of the defect. This may be carried out using a wide range of microvascularized flaps which are supported by a single or multiple obturator prostheses (Tirelli et al., 2010). The maxillofacial prosthodontist, as a member of the surgical team, is able to aid in the recovery and rehabilitation of these patient by fabricating and placing a surgical obturator and prostheses (Figure 1.3). The prostheses support soft tissues after surgery and minimize scar contracture and disfigurement thereby having a positive effect on the patients' psychology and functions (Shambharkar et al., 2011).



Figure 1.3 Occclusal view of obturator Prosthesis. [Source: Ramaraju et al., 2010]

Rehabilitation via palatal obturators aims to restore the separation between the oral and nasal cavities which enable the patient to swallow, provide mastication, and mandibular support, support the soft facial tissues, re-establish speech and restore a smile (Tirelli et al., 2010). The majority of patients undergoing prosthetic treatment demonstrate the presence of yeast-like fungi on their oral mucosa. Patients treated for maxillary tumours reveal increased incidence of fungi. Post-surgical cavity inflammation due to *Candida* species commonly occurs in patients with removed maxillary tumour in whom the post-surgical wound is closed by means of an obturator (Więckiewicz et al., 2002).

The development of the pathological process is associated with the adherence of pathogenic fungi to tissues in contact with the prosthesis obturator. This effects the development of the fungi on the mucous membrane as well as the prosthesis. The cells of the fungus adhere permanently to the mucosal cells of the host because they have adhesins on their surface corresponding to the receptors that are present on the host's cells. The binding force depends on the number of receptors and the mutual affinity of the receptors and adhesins (Więckiewicz et al., 2002).

Hyposalivation as well as the oral prostheses increases the risk of colonization and infection. A number of constituents of saliva provide mucosal defense. Long-term dry mouth (sicca syndrome) can also contribute to persistent increased risk of OPC (Bensadoun et al., 2011). Salivary secretion is greatly suppressed by cancer therapy causing *Candida* cell overgrowth which gives rise to itching and burning pain of the oral mucosa (Umazume et al., 1995). Recent data on the frequency of OPC in patients with cancer is limited, and the prevalence of OPC is probably underestimated. It was reported as 5-60% in patients with solid tumors and 20-80% in

autologous bone marrow transplantation (Bensadoun et al., 2011). There is limited information available on the status of *Candida* carriage and virulence in patients with oral prostheses.

1.3 Pathogenicity of *C. albicans*

Pathogenesis is the ability of a microorganism to infect the host and produce disease resulting from the interaction of pathogen with host. Pathogenicity of the fungus (*Candida*) is complex, involving both yeast and host factors and depends on its ability to adapt to the tissue environment and to withstand the lytic activity of the host's defenses. Several factors including genes or gene products for example, enzyme molecules are known as virulence factors that are involved in this relationship and produce superficial to invasive infections in humans (Khan et al., 2010).

To produce disease in a patient, a fungus must actively invade tissues. Many human fungal pathogens are dimorphic, that is capable of reversible transitions between yeast and hyphal forms. The morphogenetic transition between these forms is often stimulated by growth in the host and correlated with host invasion (Khan et al., 2010, Molero et al., 1998). Determinants of pathogenicity are called virulence factors. Pathogenic microbes often possess a number of virulence factors and mechanisms. These factors determine whether the host lives or dies during host-microbe interactions (Khan et al., 2010).

The virulence factor can be assessed by comparing biological response in fungi with or without the factor. The most convincing evidence for a factor to be considered as a virulence determinant is the simultaneous loss of the factor and loss of virulence, and the regaining of virulence when the factor is restored. Virulence factors must help the pathogen to grow at elevated temperatures,

facilitated adherence, penetration and dissemination, or assist in resistance against innate immune defenses. The ability of a fungus to grow at 37°C and physiological pH is a virulence factor for fungi that invade deep tissue, and the transition to parasitic form is essential for the pathogenicity of dimorphic fungi (Khan et al., 2010).

The pH of the oral cavity also plays a role in the pathogenicity. According to Salerno et al., (2011), low levels of pH can favour the adhesion and the proliferation of *Candida* yeast. In fact, a pH of 3 is optimal not only for the adhesion of the yeasts, but also for the enzymatic activity of the proteinases that, together with the lipases, are the most important factors of virulence of *Candida* because of their cytotoxic and cytolytic effects. Moreover, high levels of carbohydrates present in the saliva can act as an additional nourishing source for the *Candida* yeasts. By metabolizing these sugars, they produce acid metabolic products and maintain a low environmental pH (Salerno et al., 2011).

Candida albicans possesses many virulence properties, such as the ability to adhere to host tissues and prostheses (eg. dentures), phenotypic switching, the ability to form germ tube for tissue invasion and the ability to produce a range of hydrolytic enzymes such as proteinases and phospholipases that hydrolyse peptide bonds and phospholipids respectively and cause tissue invasion and damage (Khan et al., 2010 and Samarananyake, 2002). The switching mechanisms of *Candida* may assist in pathogenesis by permitting the yeast to escape the action of antifungals, elude the immune system by altering surface antigens and maximize the attachment, colonization and invasion of a variety of body surfaces (Calderone and Fonzi, 2001). The virulence factors

required by *Candida* to cause infections may vary, shown in Figure 1.4, depending on the type of infection, the stage of infection, the site of infection and the nature of the host response (Naglik et al., 2003).

A number of factors contribute to the pathological state of transition from commensal yeast to pathogenic parasite in patients with malignancies. Cancer chemotherapy may lead to damage to the mucosal barrier that may result in epithelial atrophy and mucosal ulceration, which may be associated with increased adherence and invasion of *Candida* (Bensadoun et al., 2011).

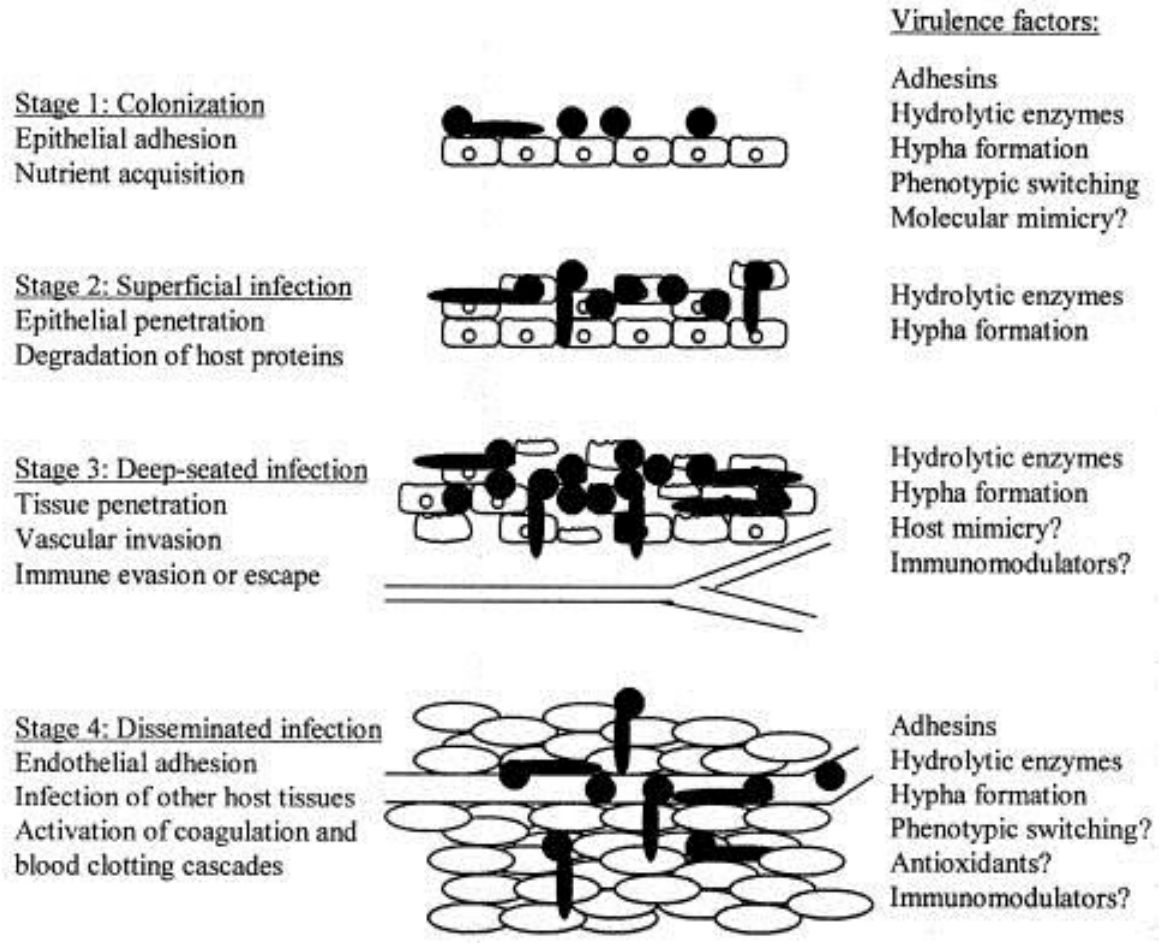

Figure 1.4 Schematic diagram illustrating virulence attributes to *C. albicans* pathogenicity. [Source: Naglik et al., 2003]

1.3.1 Germ tube formation

Germ tubes are the initial projections observed when *C. albicans* switches from yeast form to hyphal growth. Growth is polarized in *C. albicans* hypha, with continuous apical growth throughout the cell cycle and parallel cell walls at the septal junctions. The principal determinant in the development of disease is the ability of *C. albicans* to switch between yeast and hyphal forms rather than the individual morphologies (Nadeem et al., 2013). Germ tubes, that are short hyphal elements, are important in the penetration of adherent organism to the host epithelium. Researches have established that hyphal form is more virulent than yeast form. This phenomenon can be stimulated in the laboratory environment using blood serum.

The Germ Tube Test (GTT) is the most generally accepted and economical method used in the clinical laboratories for the identification of yeasts. Approximately 75% of the yeasts recovered from clinical specimens are *C. albicans* and the GTT usually provides a definitive identification of this organism within 3 hours. The GTT is specific for the identification of *C. albicans* with the exception of an occasional isolate of *C. tropicalis* that may rarely produce germ tubes whereas *C. dubliniensis* is also positive (Forbes et al., 1998). *Candida albicans* produce germ tube which is an initial stage of hyphae formation in the presence of host. In GTT, *C. albicans* is incubated in the presence of either horse serum or fetal calf serum which stimulates germ tube formation which is detected microscopically (Figure 1.6).

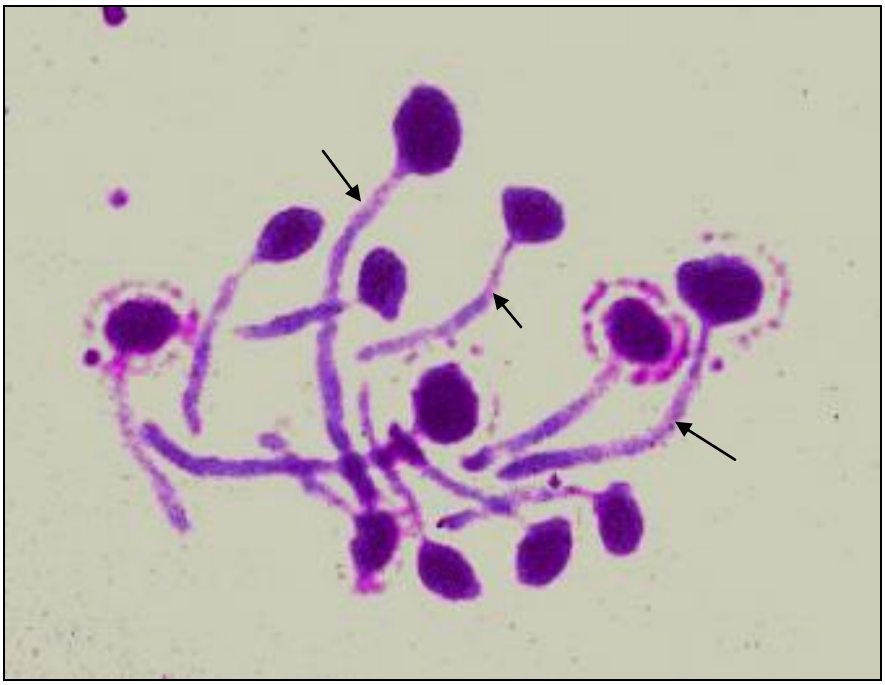

Figure 1.5 *Candida albicans* with germ tube after incubation at 37ºC.
Arrows shows germ tube formation. [Source: Photographed by the investigator]

1.3.2 Adherence

The ability of a *Candida* to invade different environments in the host organism is a result of great flexibility and adaptability of the fungi. This phenomenon is in part due to the presence of different adhesins connected with cell surface, which facilitate the first stage of infection called adherence (Karkowska-Kuleta et al., 2009). The ability of *Candida spp*. to adhere to an oral surface is crucial in colonization, persistence within the host and pathogenesis. Without attachment, the growth rate of *C. albicans* (about 1.5 hours per generation in a rich growth medium) is insufficient to maintain carriage in the mouth or gastro-intestinal tract. If yeast is unable to attach to a surface it will be removed by the mechanical washing action of saliva, swallowed, and destroyed by gastric acid. Therefore, yeast cells need to adhere to the surface of

mucous membranes. Also, the growth conditions in the oral cavity are not suitable unless the yeast cells adhere. Adherence allows yeast cells the chance to proliferate and colonize the host, with or without signs or symptoms of infection (Calderone and Fonzi, 2001). The balance between colonization and overt infection may be tipped toward infection in compromised individuals by changes in the expression of adherence ligands and receptors. *Candida* cells adhere to several host cell types, including epithelia (Figure 1.6), endothelial and phagocytic cells (Cannon et al., 1995).

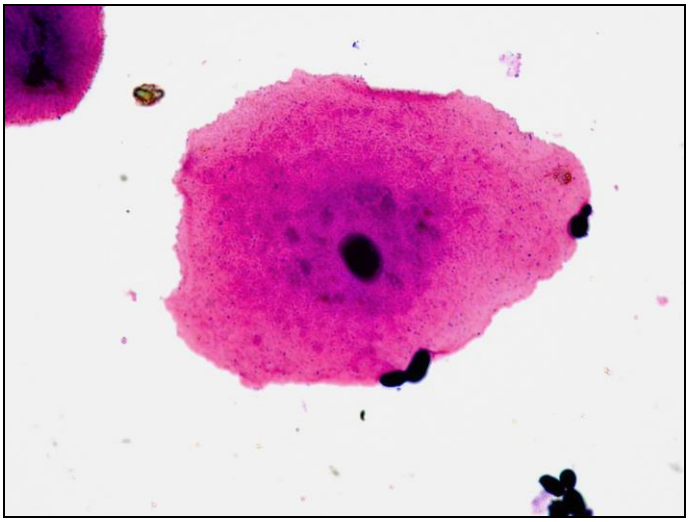

Figure 1.6 Light microscope image showing the adherence of yeast cells (black) to an epithelial cell (stained pink). [Source: Photographed by the investigator]

It is well-known that *C. albicans* adhesion to mucosa cells is enhanced by several factors such as germ tube production, phospholipase, protease, other extracellular enzymatic activities, carbohydrates, pH and temperature. Once this organism is established on the tissue surface, it produces germ tube or hyphae which will penetrate the host tissues for invasion. The ability of

hyphae formation can vary from strain to strain; therefore, virulence property can be determined by monitoring hyphae formation (Vidotto et al., 2003, Akpan and Morgan, 2002, Molero et al., 1998).

Strains of *C. albicans* with increased adherence ability are more pathogenic than strains that adhere less. There is a positive correlation between adherence and pathogenicity. For example *C. albicans* isolated from the oral cavities of HIV positive patients has increased adherence ability and therefore considered to have increased virulence (Sweet et al., 1995). Adherence occurs between moieties of the *C. albicans* cell wall and host surfaces (Cannon and Chaffin, 1999). *Candida albicans* presents adhesins on the cell wall surface, which are responsible for adhesion to epithelial and endothelial cells, serum proteins and extracellular matrix proteins (Chaffin et al., 1998, Karkowska-Kuleta et al., 2009).

Many cell-surface adhesins have been identified and characterized biochemically. These surface proteins may play a role in the adherence of *C. albicans* to extracellular matrix proteins and host cell surfaces. Genetic and molecular biology approaches have allowed for the investigation of adhesin function by isolating and expressing these genes in heterologous hosts (Cannon and Chaffin, 1999). One gene family of C. *albicans* called the agglutinin-like sequence (ALS) has been studied extensively since it encodes for adhesins that were observed to play a role in adherence. Members of ALS family are related to *Saccharomyces cerevisiae* agglutinin genes that enable cell-cell interactions during mating of haploid cells (Cannon and Chaffin, 1999,

Calderone and Fonzi, 2001). The gene family includes at least nine genes and has also been found in other *Candida species* such as *C. dubliniensis* and *C. tropicalis*. The proteins encoded by these genes have been found on the surface of *C. albicans* cells in the host environment and therefore these proteins play an important role in host-pathogen interactions (Hoyer et al., 2001).

The yeast/host cell interaction is also affected by external factors such as drug treatment. Antibiotic treatment can cause *C. albicans* overgrowth in the oral cavity by eliminating competing microorganisms and exposing additional sites suitable for colonization. Antifungals, however, can reduce the adherence of *C. albicans in vitro.* This effect may enhance drug efficacy. Pre-treatment of prostheses or mucosae may also help to prevent infection by reducing yeast adherence (Cannon et al., 1995).

Adherence of *C. albicans* to plastic medical devices (medical implants, prostheses and catheters) allows the organism to propagate and establish biofilms to release microorganisms from these biofilms which contributes to or initiates acute disseminated nosocomial infections. Several components of the microfibrillar surface layer of *C. albicans* are implicated in plastic binding and plastic adherence and appear to be mediated by cell surface hydrophobicity (CSH) through hydrophobic bonds between the plastic surface and the peptide moieties of the mannoproteins. These mannoproteins may play other significant roles in cell physiology rather than specifically bind to plastic surfaces. Salivary proteins, adsorbed to plastic material of dental or voice prostheses may also help colonization by fungal cells (Mishra et al., 2007).

1.3.3 Hydrolytic enzymes

Among the factors known to contribute to the pathogenicity of yeast, hydrolytic enzymes play a significant role, possibly being harmful to the host tissues when they are liberated by the fungi. These enzymes play a role in nutrition but also in tissue damage, dissemination within the human organism, iron acquisition and overcoming the host immune system, and therefore strongly contribute to fungal pathogenicity (Karkwoska-Kuleta et al., 2009). *Candida albicans* produces a range of hydrolytic enzymes that have various roles in the infection process. The two enzymes that have received the most attention are proteinases and phospholipases and are important virulence determinants (Haynes, 2001, Abaci, 2011).

Proteinases may act on the epithelial tissues and enable the fungal cells to adhere and hence penetrate (Kwon-Chung et al., 1985). These enzymes may also be involved in counteracting the host immune response because it is able to resist phagocytosis and intracellular killing (MacDonald and Odds, 1983). Secretion of proteinases by pathogens is mandatory in order to degrade the tissue barrier and obtain nutrition at the infection site. The extracellular proteolytic activity of *C. albicans* is due to aspartyl proteinase enzymes that constitute a family of enzymes SAPs, whose expression depends on both the yeast strain and the environment. Several observations suggest a major role for SAP proteins in the pathogenicity of candidiasis: a) SAPs are secreted by pathogenic *Candida* spp. *in vivo* during infection and there is a correlation between virulence and the level of proteinase production in both clinical isolates and laboratory strains of *C. albicans*, b) the enzymes are able to degrade a number of important defensive host proteins such as immunoglobulins and complement and c) genetically engineered strains with

disruptions in SAP genes were less virulent in an animal model of disseminated candidiasis. Therefore, proteinase production appears to enhance the ability of the fungal cells to colonize and penetrate host tissues and to evade the host immune system (Mishra et al., 2007).

Secreted aspartyl proteinases (SAPs) from *Candida* hydrolyze many proteins such as albumin, hemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary lactoferrin, interleukin 1β, cystatin A and Immunoglobulin. It has been reported that the production of SAPs also correlates with hyphal formation, adherence and phenotypic switching which highlights the complex role played by SAPs in the pathogenicity of *C. albicans* (Khan et al., 2010). These are extracellular enzymes and therefore can be detected in a laboratory environment. Growth of *C. albicans* on the culture media containing serum albumin, as a protein source, can show proteinase production around the colonies. This agar plate is opaque white due to the protein which can be stained with coomassie blue. Proteinase producing colonies would show colourless to light blue ring around the colonies.

Phospholipases activities are considered as putative virulence factors since they are associated with host cell penetration, adhesion to epithelial cells, invasion of epithelial cells, and interaction with host signal transduction pathways (Schaller et al., 1999, Mishra et al., 2007). These enzymes hydrolyze ester linkages of glycophospholipids and hence impart tissue invasiveness to *Candida* cells (Khan et al., 2010). Experiments involving the disruption of a gene that encodes for a phospholipase has shown a decrease in virulence and a reduced ability of *C. albicans* to penetrate host cells. This suggests that phospholipases play a role in causing damage to host cell

membranes. Furthermore *C. albicans* cells isolated from blood have higher extracellular phospholipase activity than commensal strain (Leidich et al., 1998, Khan et al., 2010). Phospholipases can be detected in a laboratory environment on agar plates containing Egg Yolk. Breakdown of phospholipids around the colonies creates opaque measurable zone.

Virulence of *Candida* depends on the production of these virulence factors. The production of these virulence determinants and the number *Candida* present in the host depends on the host immunity (Khan et al., 2010). During commensal state, the number of *Candida* and their expression of virulence factors in denture wearers and cancer patients with prostheses have not been studied.

1.4 **AIM**

The aim of this study was to compare the pathogenic characteristics of *C. albicans* isolated from the oral cavities of normal subjects, denture-wearers and cancer patients wearing oral prostheses.

1.5 OBJECTIVES

- To isolate and identify *Candida* species from the oral cavities of healthy subjects, denture-wearers and cancer patients wearing oral prostheses.
- To determine the quantity of *C. albicans* carried in the oral cavity of the three groups.
- To study pathogenic characteristics such as production of germ tube, hydrolytic enzymes, and the adherence ability of *C. albicans* isolated from the three groups.

CHAPTER 2

METHODS

2.1 Subject selection

A total number of 102 subjects were screened for *Candida* carriage during the period of 2011 to 2012. The HIV status of all the subjects was not known. The control group consisted of 48 healthy subjects who are staff members at Wits, Oral Microbiology laboratory and National Health Laboratories Services (NHLS) in Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). None of these subjects wore dentures or had systemic illness. The denture wearing group consisted of 35 patients with complete dentures having no signs and/or symptoms of denture related stomatitis. They were either attending clinics at Wits Oral and Dental Hospital or living in Golden Acres, Sandringham Retirement Home. The third group consisted of 19 patients with oral cancer and oral prostheses attending Wits Oral and Dental Hospital.

All the subjects agreed to participate in the study and signed consent forms. Ethical clearance was obtained from the Human Research Ethics Committee (Medical), University of Witwatersrand, Johannesburg (Appendix 6.2) and the clearance number is M110320.

2.2 Microbiological analysis

2.2.1 Sample collection and processing

Oral rinse samples were collected using the technique described by Samaranayake et al. (1986). Each subject was requested to rinse the mouth for 60 seconds with 10 milliliter of sterile distilled water and expectorate the rinse into a sterile universal bottle. Both denture wearers and cancer patients retained their prostheses during sample collection. Oral rinse samples were diluted to 1:10 and 1:100, and 100 µl of the diluted samples were spread on CHROMagar *Candida* agar (CHROMagar, Paris, France) and incubated aerobically at 37°C for 48 hours. The number of colonies on the CHROMagar plates was counted and the oral yeast carriage rate was expressed as colony forming units per milliliter (cfu/ml) of oral rinse. CHROMagar *Candida* allowed the identification of *Candida* species through the growth of yeast colonies and the colour of the colonies (Figure 2.1). Presumptive *Candida albicans* colonies appeared green whereas *C. tropicalis* blue and *C. glabrata* white. Single yeast colonies with different colours were subcultured on Sabouraud dextrose agar (SAB) until pure cultures were obtained and were further tested for the definite identification.

Note: List of media, reagents and the preparation procedures are given in Appendix 6.1.

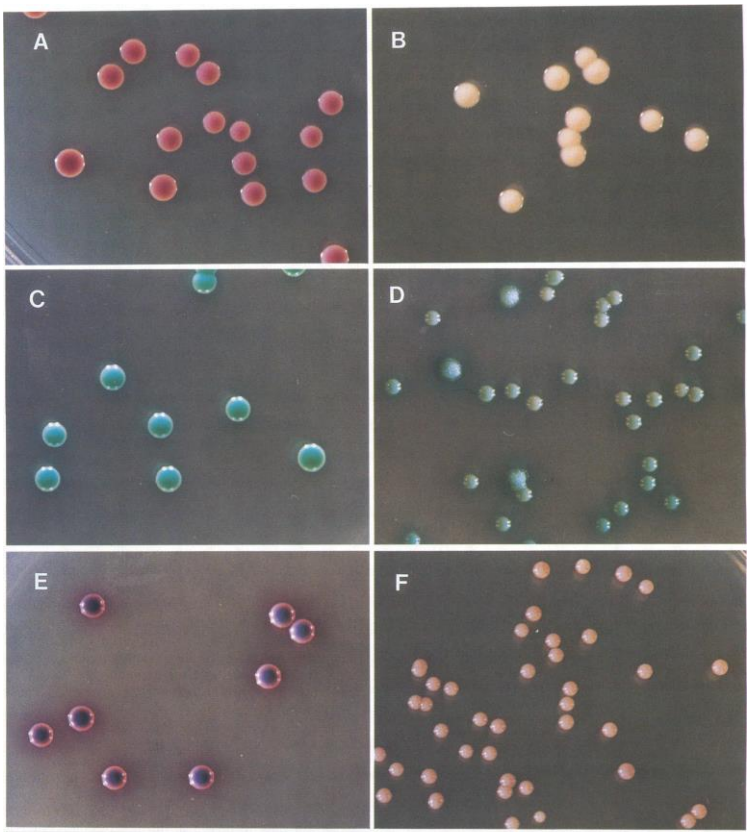

Figure 2.1 Colonies of different species of *Candida* on CHROMagar *Candida* medium. (A) Dark pink colonies of *C. glabrata* (B) Pale colonies of *C. parapsilosis* (C) Green colonies of *C. albicans* (D) Colonies of a *Geotrichumn sp*. (E) Colonies of *C. tropicalis (F) colonies of Trichosporon sp.* **[Source: Odds and Bernaerts, 1994]**

2.2.2 Identification

An API 20C AUX kit (Biomerieux, France) was used to confirm presumptive identification of all the *Candida* isolates. Pure cultures of each isolate was subcultured on SAB plates and suspended in 2ml sterile distilled H_2O equivalent to 0.5 McFarland turbidity standards. A 100 µl aliquot of this preparation was inoculated into the corresponding C medium tube that was provided with the kit. The inoculated C medium was transferred into the corresponding API 20C

strips. Each strip was incubated at 30°C for 48-72 hours. The strips were read for growth and cupules with turbidity significantly greater than of the negative control cupule were considered positive.



Figure 2.2 API 20C AUX identification system for *C. albicans*. [Source: photographed by the investigator]

Twenty strains of *C. albicans* were selected from normal subjects and denture wearers groups, and 14 from cancer patients with prostheses group. The virulence factors such as ability to adhere to the oral epithelial cells and form germ tube, and the production of proteinase and phospholipase of these strains were examined.

2.3 Germ tube formation

Germ tube formation was investigated using a technique described by Mackenzie (1962). A 0.5 ml of sterile horse serum was inoculated with a loopful of culture of *C. albicans*. Controls were set up using a known *C. albicans* (known to produce germ tube) as positive control and *C. parapsilosis* (do not produce germ tube) as negative control. The mixture was incubated for 2½ hours, aerobically at 37°C. A drop of suspension was placed on a clean microscopic slide using a capillary tube, covered with a coverslip and examined microscopically using the X10 and X40 objective lenses for the production of germ tubes. Cells that produced a germ tube at least twice the length of the cell were considered. Fifty yeast cells of each isolate were examined for germ tube formation and percentage was calculated.

2.4 Adherence assay

Adherence assays were performed using technique described by Ghannoum and Elteen (1986) with minor modification. The *C. albicans* cultures were inoculated into Sabouraud dextrose broth (Oxoid, Hampshire, England) and incubated at 37°C for 24 hours while shaking at 60rpm. Yeast cells were harvested by centrifugation (5000rpm, 15 minutes), washed three times with sterile distilled H_2O by repeated centrifugation, resuspended in 2 ml sterile distilled H_2O and adjusted to the concentration of 10^5 cells/ml. Buccal epithelial cells (BECs) were collected from the mouth of healthy (non-carrier of *Candida*) volunteer by gently rubbing the oral mucosal surface of the cheek with a sterile swab. The swab was then immersed in 4 ml sterile distilled H_2O. The cell suspension was centrifuged (5000rpm for 15 minutes), then the cell pellet was washed three times and resuspended into 2 ml sterile distilled water. Two milliliters of the cells and 2 ml of

yeast cell suspensions were mixed in sterile screw-capped bottle, incubated for 3 hours at 37°C while shaking at 60rpm. The mixture was filtered through a nylon mesh with 20 µm pores (Nucleopore GMBh, Germany) to remove non-adherent yeast cells. The BECs on the filter were washed twice with 5 ml sterile distilled H_2O and finally suspended in 2ml of sterile distilled H_2O. A drop of this suspension was mounted on a glass slide, air-dried, heat fixed and Gram stained. Adherence was determined microscopically by counting the number of adherent yeast cells per hundred BECs (Figure 2.3).

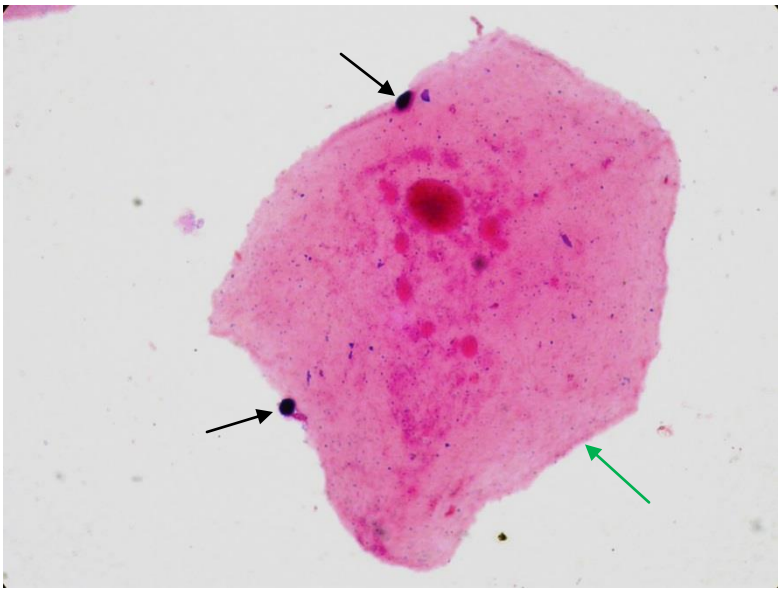

Figure 2.3 *C. albicans* cells (black arrows) adherent to the epithelial cell (green arrow) [Source: Photographed by the investigator]

2.5 Phospholipase Activity

Phospholipase production was detected using the egg yolk media (EYM) as described by Price et al. (1982). It contained 13g Sabouraud agar, 11.7g NaCl, 0.11g $CaCl_2$ and 20ml sterile egg

yolk emulsion. A suspension of fresh *C. albicans* culture containing 10^7 cfu/ml cells was prepared and 20µl was inoculated onto the four blank paper discs placed on the egg yolk agar plates, incubated aerobically for four days at 37°C. The diameters of colony (A) and the colony plus precipitation zone (B) were measured. The phospholipase activity of the isolates was considered positive when a precipitation zone was visible around the growth (Figure 2.4). The phospholipase activity (Pz) was expressed as Pz = A/B (in mm). The phospholipase activity of each isolate was recorded as the average of the four measurements. Pz of 1 indicated there was no phospholipase activity. Lower values indicated higher production of phospholipase.

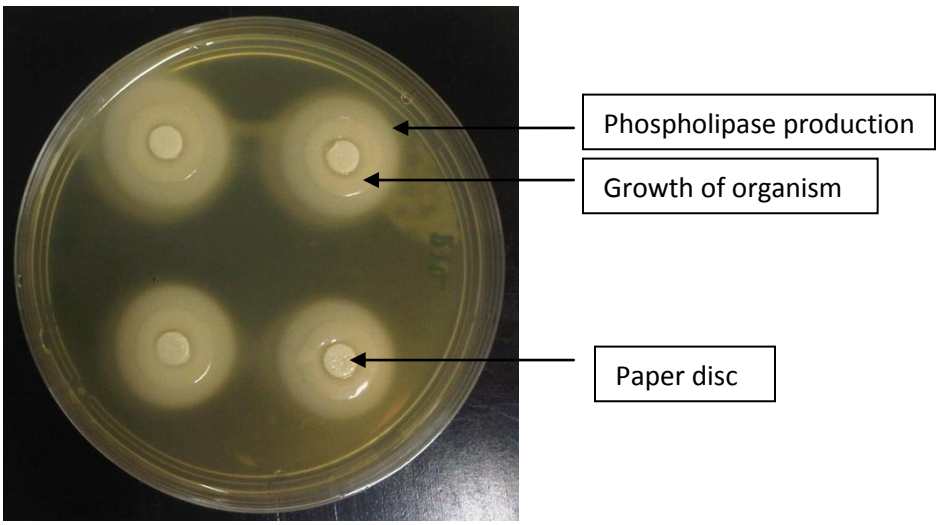

Figure 2.4 Phospholipase production by *C. albicans* on Egg Yolk media.

[Source: Photographed by the investigator]

2.6 Proteinase Activity

Proteinase production was detected using the modified method described by Aoki et al., (1990). A suspension of fresh *C. albicans* culture containing 10^7 cfu/ml cells was prepared and 20µl was inoculated onto the four sterile paper discs placed on the Yeast Carbon Base- Bovine Serum Albumin (YCB-BSA) medium plates. The medium contained 1.5% Columbia agar, 1.17% Yeast Carbon Base powder, 0.2% Bovine Serum Albumin (BSA) and 0.2% glucose. The plates were incubated aerobically at 37°C for fourteen days. The diameter of the colonies was measured and recorded. Plates were then stained with 0.5% Coomasie brilliant blue R250 (Pierce Biotechnology) for 20 min at room temperature and destained three times for 20 min at 37°C using a destaining solution (Appendix 6.1) and once with distilled water. The diameter of the colonies was measured before staining and the diameter of the clearing zones were measured after staining (Figure 2.5). Proteinase activity (Pr) was expressed as the ratio of diameter of colony to total diameter of colony plus clearing zone (in mm). The Pr value was taken as the average of the four measurements. When the Pr equaled 1, no proteinase activity was detected in the strain. Thus, a low Pr value indicated high enzyme production.

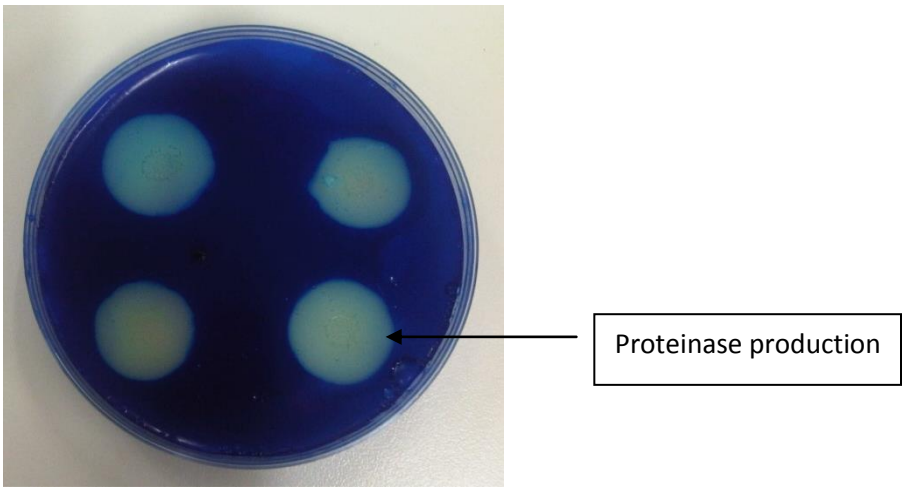

Figure 2.5 Proteinase production by *C. albicans.*

[Source: Photographed by the investigator]

2.7 Statistical analysis of data

Scheffe test for pairwise comparison and chi square test was used to compare the *Candida* counts in these study groups.

The results of the virulence factors i.e. germ tube formation, adherence, proteinase and phospholipase production by the strains of *C. albicans* isolated from the 3 groups were compared using a Kruskal-Wallis test for pairwise comparison and a chi square test.

Note: Statistical analysis of results is given in Appendix 6.3.

CHAPTER 3

RESULTS

3.1 Isolation and identification of *Candida* species

3.1.1 Normal healthy subjects

Forty nine subjects were screened for the *Candida* carriage, shown in Table 3.1. Their ages ranged from 21-70 years with the mean of 34 years and consisted of 9 males and 40 females. Twenty one subjects were colonized with *C. albicans* and the mean *Candida* count in the oral cavity rinse was 326 cfu/ml.

Table 3.1 *Candida* carriage in Normal healthy subjects

Subject no.	Age/gender	Count cfu/ml	Identification
N1	56/M	**0**	-
N2	24/F	20	*C. albicans*
N3	35/F	30	*C. albicans*
N4	30/M	**0**	-
N5	36/F	**0**	-
N6	25/F	**0**	-
N7	38/F	**0**	-
N8	51/M	945	*C. albicans*
N9	25/F	20	*C. albicans*
N10	30/M	**0**	-
N11	24/M	80	*C. albicans*
N12	28/M	**0**	-
N13	30/M	10	*C. albicans*
N14	28/F	**0**	-
N15	24/M	1058	*C. albicans*
N16	27/F	60	*C. albicans*
N17	28/F	**0**	-
N18	29/F	**0**	-
N19	29/F	**0**	-
N20	35/M	**0**	-
N21	26/F	**0**	-

Table 3.1 *Candida* carriage in Normal healthy subjects (continues)

Subject no.	Age/gender	Count cfu/ml	Identification
N22	26/F	2146	*C. albicans*
N23	25/F	**0**	-
N24	25/F	**0**	-
N25	21/F	550	*C. albicans*
N26	23/F	**0**	-
N27	23/F	**0**	-
N28	28/F	**0**	-
N29	25/F	**0**	-
N30	39/F	240	*C. albicans*
N31	38/F	70	*C. albicans*
N32	41/F	**0**	-
N33	34/F	80	*C. albicans*
N34	25/F	**0**	-
N35	35/F	10	*C. albicans*
N36	29/F	**0**	-
N37	29/F	**0**	-
N38	23/F	**0**	-
N39	30/F	**0**	-
N40	55/F	150	*C. albicans*
N41	46/F	20	*C. albicans*
N42	40/F	**0**	-
N43	58/F	60	*C. albicans*
N44	58/F	1108	*C. albicans*
N45	40/F	100	*C. albicans*
N46	35/F	**0**	-
N47	25/F	**0**	-
N48	60/F	40	*C. albicans*
N49	70/F	50	*C. albicans*
	Mean age 34 M9+ F40	**Mean count 326 cfu/ml/ coloniser**	**21 - *C. albicans***

3.1.2 Denture wearers

Thirty five denture wearers were screened for the *Candida* carriage, shown in Table 3.2. The mean age of this study group was 75 years with 17 males and 18 females. Twenty individuals were colonized with 20 *C. albicans* and the mean of *Candida* count was 9878 cfu/ml.

Table 3.2 *Candida* carriage in Denture wearers

Subject no.	Age/gender	Count cfu/ml	Identification
D1	70/F	350	***C. albicans***
D2	60/F	2106	*C. glabrata*
		49300	*C. dubliniensis*
D3	88/M	410	***C. albicans***
		240	*C. glabrata*
D4	86/F	76300	***C. albicans***
		3520	*C. famata*
D5	72/M	8000	***C. albicans***
		1254	*C. glabrata*
D6	75/M	**0**	-
D7	80/M	475	***C. albicans***
D8	68/M	**0**	-
D9	72/M	660	***C. albicans***
D10	65/M	**0**	-
D11	82/M	3700	*C. glabrata*
		10	***C. albicans***
D12	65/M	**0**	-
D13	69/F	**0**	-
D14	73/M	**0**	-
D15	74/F	100	*C. parapsilosis*
D16	86/F	2200	***C. albicans***
D17	80/F	395	*C. guilliermondii*
		265	*C. tropicalis*
D18	67/F	75200	*C. glabrata*
D19	78/F	**0**	-
D20	75/M	**0**	-
D21	69/F	**0**	-
D22	F	642	*C. glabrata*
D23	M	380	*C. glabrata*

Table 3.2 *Candida* carriage in Denture wearers (continues)

Subject no.	Age/gender	Count cfu/ml	Identification
D24	71/M	520	***C. albicans***
D25	70/M	480	***C. albicans***
D26	M	48950	***C. albicans***
		1460	*Cryptococcus neoformans*
D27	M	8000	***C. albicans***
		7400	*C. glabrata*
		2050	*C. famata*
D28	F	63400	***C. albicans***
		4650	*C. tropicalis*
D29	F	35000	***C. albicans***
		20	*Cryptococcus neoformans*
D30	M	8042	***C. albicans***
		640	*Trichosporon mucoides*
		420	*Kodamaea ohmeri*
D31	81/F	2690	***C. albicans***
		33700	*C. glabrata*
D32	65/F	15110	***C. albicans***
		21650	*C. glabrata*
D33	90/F	50	***C. albicans***
		264	*C. glabrata*
D34	86/F	3840	***C. albicans***
		1500	*C. glabrata*
D35	88/F	4475	***C. albicans***
		550	*Cryptococcus neoformans*
		350	*C. glabrata*
	Mean age 75 Age unknown 7 M17/F18	**Mean count 9878 cfu/ml/ coloniser**	**20- *C. albicans***

3.1.3 Cancer patients with prostheses

Nineteen oral cancer patients with prostheses were screened for the *Candida* carriage, shown in Table 3.3. The mean age of this study group was 70 years with 6 males and 13 females. Fourteen

patients were colonized with *C. albicans* and the mean *Candida* count in the oral cavity rinse was 13977 cfu/ml.

Table 3.3 *Candida* carriage in Cancer patients with oral prostheses

Subject no.	Age/gender	Count cfu/ml	ID
		73000	***C. albicans***
		6243	*C. glabrata*
C1	83/M	96000	*S. cerevisiae*
		1250	*C. krusei*
C2	F	864	*C. tropicalis*
C3	76/M	110	***C. albicans***
C4	57/F	115	***C. albicans***
C5	51/F	**0**	-
		7600	***C. albicans***
C6	63/F	48300	*C. glabrata*
		7075	***C. albicans***
		125	*C. tropicalis*
C7	M	70	*C. glabrata*
		59425	***C. albicans***
C8	73/F	8855	*C. glabrata*
		21005	***C. albicans***
C9	84/M	12550	*C. glabrata*
		2864	***C. albicans***
C10	88/M	1248	*C. glabrata*
C11	67/M	3600	***C. albicans***
		2400	***C. albicans***
C12	75/F	100	*C. glabrata*
C13	64/F	**0**	-
C14	66/F	10	***C. albicans***
C15	77/F	345	***C. albicans***
C16	72/F	45200	***C. albicans***
C17	68/F	1990	***C. albicans***
	55/F	3500	*C. glabrata*
C18		1510	*C. krusei*
C19	71/F	**0**	-
	Mean age 70 Age unknown 1 M6+ F13	**Mean count 13977 cfu/ml/ coloniser**	**14 - *C. albicans***

3.1.4 *Candida* carriage

The carriage of *Candida species* in the normal subjects, denture wearers and cancer patients with prostheses was 42.86%, 74.29% and 84.21% respectively (Table 3.4). All though the groups were not matched for the risk factors, the carriage of *Candida species* between the normal subjects and denture wearers and cancer patients with prostheses were significantly different ($p<0.01$).

Table 3.4 Carriage of *Candida species* in the three groups

Study Group	**No. of subjects/ patients screened**	**No. of subjects/ patients with *Candida species***	**Prevalence (%) of *Candida species***
Normal subjects	49	21	42.86*, **
Denture wearers	35	26	74.29*
Cancer patients with prostheses	19	16	84.21**

***: p<0.01, **: p<0.01**

3.1.5 Multiple *Candida* colonization

Several *Candida* species were isolated from the denture wearers and cancer patients with prostheses (Table 3.5). Normal subjects yielded only *C. albicans* strains. In contrast, 28.57% of denture wearers carried only one strain of yeast and 45.72% carried multiple yeast strains. In

cancer patients with prostheses, 36.84% of the patients carried single yeast strain and 47.37% carried multiple strains of yeasts.

Table 3.5 Multiple *Candida* colonization of the three study groups

Study Group	No. of patients with no *Candida* spp. (%)	No. of patients with single *Candida* spp. (%)	No. of patients with multiple *Candida* spp. (%)
Normal subjects **N=49**	28 (57.14%)	21 (42.85%)	-
Denture wearers **N=35**	9 (25.71%)	10 (28.57%)	16 (45.72%)
Cancer patients **N=19**	3 (15.79%)	7 (36.84%)	9 (47.37%)

Table 3.6 shows a higher occurrence of mixed/multiple *Candida* species characterized in denture wearers and cancer patients. Greater variety of yeasts was isolated from the denture wearers than the cancer patients.

Table 3.6 Various fungi including *Candida species* isolated from the three study groups

Isolated *Candida species*	Normal subjects (21 Strains)	Denture wearers (49 Strains)	Cancer patients (29 Strains)
C. albicans	21 (100%)	23 (46.9%)	15 (78.9%)
C. glabrata	-	13 (26.5%)	8 (27.6%)
C. tropicalis	-	3 (6.1%)	2 (6.9%)
C. parapsilosis	-	1 (2.0%)	-
C. guilliermondii	-	1 (2.0%)	-
C. famata	-	2 (4.1%)	-
C. dubliniensis	-	1 (2.0%)	-
C. krusei	-	-	2 (6.9%)
Cryptococcus neoformans	-	3 (6.1%)	-
Kodamaea	-	1 (2.0%)	-
S. cerevisiae	-	-	1 (3.5%)
Trichosporon spp.	-	1 (2.0%)	-

3.1.6 *Candida* count in the study groups

The mean±SD *Candida* counts in the normal subjects, denture wearers and cancer patients were 326.05±549.4, 18616.5±25967 and 59084.63±176407.7 cfu/ml respectively, shown in Table 3.7. The *Candida* counts were significantly higher in the two study groups than the normal subjects ($p<0.01$). Therefore, the *Candida* counts were categorized as shown in Table 3.8. The results showed that 63% of cancer patients with prostheses carried higher numbers of *Candida* in their oral cavity (>1000 cfu/ml) than the denture wearers (42.86%).

Table 3.7 *Candida* count (Mean ± SD) in the colonized study groups

(overall of $p<0.01$)

Study group	*Candida* count (cfu/ml) Mean ± SD
Normal subjects N=49	326.05 ± 549.4
Denture wearers N=35	18616.5 ± 25967
Cancer patients N=19	59084.63 ± 176407.7

Table 3.8 *Candida* count (cfu/ml) in the study groups

Study Group	No. of patients with range of *Candida* counts (cfu/ml)			
	0 (%)	1-100 (%)	101-1000 (%)	>1000 (%)
Normal subjects N=49	28 (57.14)	14 (28.57)	7 (14.29)	0
Denture wearers N=35	9 (25.71)	1 (2.86)	10 (28.57)	15 (42.86)
Cancer patients N=19	3 (15.79)	1 (5.26)	3 (15.79)	12 (63.16)

3.2 Pathogenic characteristics of *C. albicans* isolated from the study groups

The results of the germ tube formation, adherence to epithelial cells, proteinase production and phospholipase productions by the strains of *C. albicans* isolated from the study groups are shown in Table 3.9 to Table 3.12. The results of the pathogenic characteristics of the strains isolated from the study groups are summarized in Table 3.13.

3.2.1 Germ tube formation

All the strains isolated from study groups produced germ tube (Table 3.9, Table 13 and Figure 3.5). However, the percentage of *C. albicans* cells with germ tube varied between the study groups. They were 41%, 43% and 57% in the strains isolated from normal subjects, denture wearers and cancer patients with prostheses respectively. The overall difference in the germ tube formation between the study groups was weakly significant (p=0.03) as shown in Table 3.13.

Table 3.9 Germ tube formation (GTF) by *C. albicans* isolated from Normal subjects, Denture wearers and Cancer patients with prostheses.

Normal subjects		**Denture wearers**		**Cancer patients with prostheses**	
Subject no	**GTF (% yeast cells)**	**Subject no**	**GTF (% yeast cells)**	**Subject no**	**GTF (% yeast cells)**
N2	18	D1	42	C1	50
N3	12	D3	62	C3	62
N8	24	D4	26	C4	76
N9	30	D5	46	C6	68
N11	66	D7	48	C7	52
N13	68	D9	54	C8	62
N15	78	D11	60	C9	56
N16	40	D16	46	C10	64
N22	12	D24	38	C11	78
N25	40	D25	44	C12	62
N30	20	D26	20	C14	28
N31	42	D27	44	C15	80
N33	40	D28	48	C16	32
N35	34	D29	24	C17	28
N40	52	D30	24	**Mean±SD**	**57.17±17.48**
N41	12	D31	32		
N43	62	D32	76		
N44	58	D33	62		
N45	36	D34	24		
N48	80	D35	44		
Mean±SD	**41.2±21.85**	**Mean±SD**	**43.2±15.11**		

The results of germ tube formation were further analyzed using Scheffe pair wise comparison.

Figure 3.1 showed the germ tube formation results which are significantly high (p=0.05) in strains isolated from cancer patients (57.17) compared to the normal subjects (41.2). However, the significance was marginal and could be due to the wide spread of the results of normal subjects. There was no significant difference between the strains isolated from denture wearers (43.2) and normal subjects (41.2). In addition, there was no significant difference between the results of denture wearers and cancer patients with prostheses.

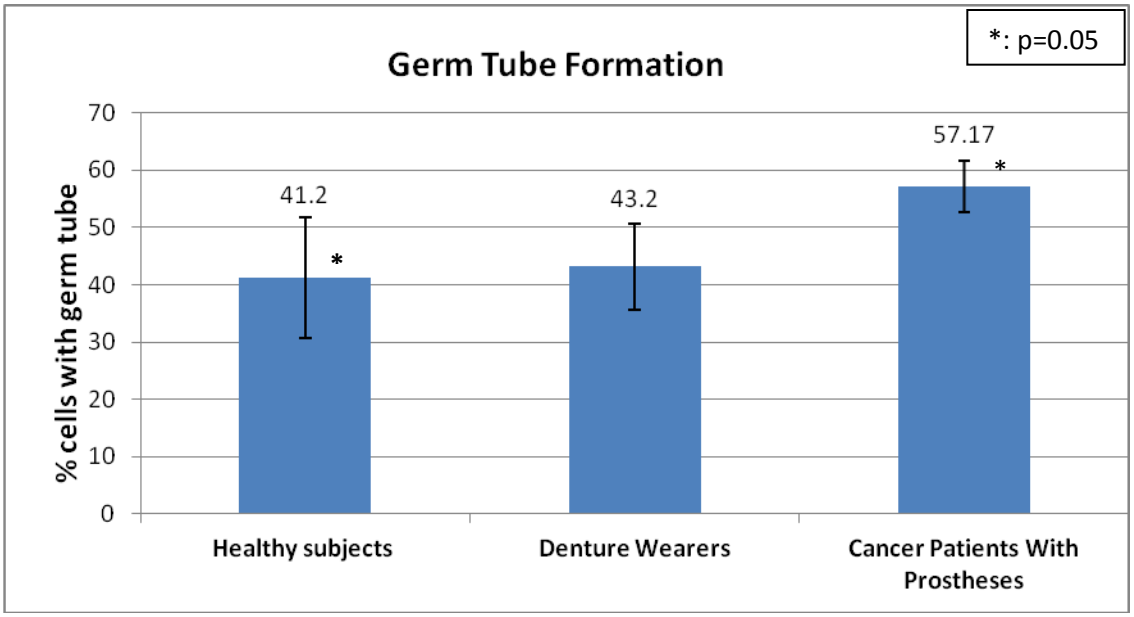

Figure 3.1 Germ tube formation by *C. albicans* isolated from normal subjects, denture wearers and cancer patients with oral prostheses

3.2.2 Adherence assay

All the strains isolated from the study groups adhered to oral epithelial cells (Table 3.10, Table 3.13 and Figure 3.5). The mean number of yeast cells isolated from normal subjects, denture

wearers and cancer patients with prostheses that were adherent to the epithelial cells were 343.4, 694.8 and 818.7 respectively. The overall difference in the results was statistically significant ($p<0.01$).

Table 3.10 Adherence of *C. albicans* isolated from the study groups to oral epithelial cells.

Normal subjects		**Denture wearers**		**Cancer patients with prostheses**	
Subject no	**Adherence (yeast/100 BECs)**	**Subject no**	**Adherence (yeast/100 BECs)**	**Subject no**	**Adherence (yeast/100 BECs)**
N2	940	D1	360	C1	652
N3	156	D3	520	C3	912
N8	208	D4	692	C4	1102
N9	584	D5	232	C6	894
N11	428	D7	152	C7	508
N13	488	D9	468	C8	878
N15	520	D11	376	C9	1230
N16	320	D16	600	C10	968
N22	148	D24	1312	C11	880
N25	80	D25	272	C12	640
N30	268	D26	696	C14	568
N31	103	D27	1120	C15	1278
N33	64	D28	742	C16	706
N35	224	D29	600	C17	246
N40	116	D30	464	**Mean±SD**	**818.71±286.1**
N41	64	D31	1750		
N43	1103	D32	980		
N44	980	D33	744		
N45	192	D34	956		
N48	364	D35	860		
Mean±SD	**343.4±267.6**	**Mean±SD**	**694.8±391.44**		

The results of adherence assay were analyzed using Scheffe pair wise comparison.

Adherence of strains isolated from denture wearers was significantly higher than strains isolated from the normal individuals (p=0.01) as shown in Figure 3.2. Similarly, adherence of strains isolated from cancer patients with prostheses was significantly higher than strains isolated from the normal individuals (p<0.01). However, there was no significant difference between the results of denture wearers and cancer patients. This may be due to the greater standard deviations in the results.

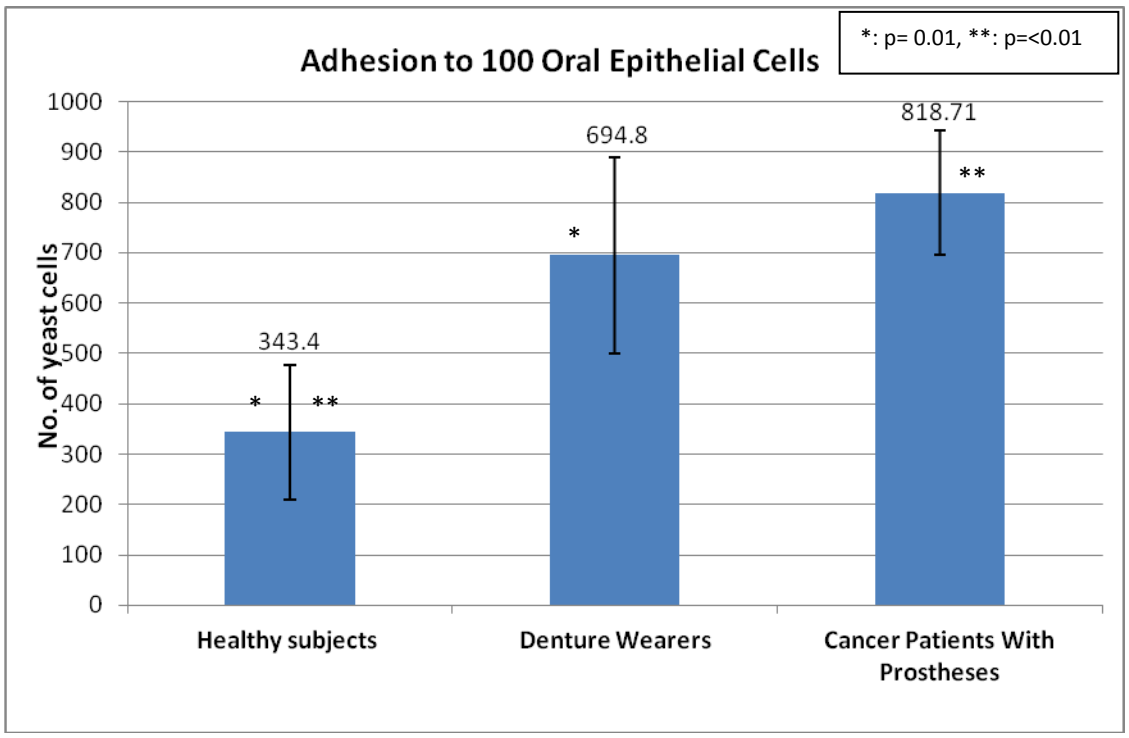

Figure 3.2 Adhesion to oral epithelial cells by *C. albicans* isolated from normal subjects, denture wearers and cancer patients with oral prosthesis

3.2.3 Phospholipase production

The mean of phospholipase production by the strains isolated from normal subjects, denture wearers and cancer patients with prostheses was 0.95±0.1, 0.8±0.16 and 1.0±0.18 respectively as

shown in Tables 3.11 and 3.13. The overall difference in the results was statistically significant ($p<0.01$).

Phospholipase was produced by 25%, 85% and 57.14% of the *C. albicans* isolated from normal subjects, denture wearers and cancer patients with prostheses respectively (Figure 3.5, Table 3.14).

Table 3.11 Phospholipase production by *C. albicans* isolated from Normal subjects, Denture wearers and Cancer patients with prostheses.

Subject no	*Pz	Subject no	*Pz	Subject no	*Pz
N2	1	D1	1	C1	1
N3	1	D3	0.77	C3	0.97
N8	1	D4	0.73	C4	0.55
N9	1	D5	0.91	C6	0.58
N11	0.97	D7	0.81	C7	1
N13	1	D9	0.96	C8	0.76
N15	1	D11	0.84	C9	0.74
N16	0.88	D16	1	C10	0.69
N22	1	D24	0.82	C11	0.75
N25	1	D25	0.82	C12	0.97
N30	1	D26	0.66	C14	1
N31	1	D27	0.62	C15	1
N33	1	D28	0.6	C16	1
N35	1	D29	0.91	C17	1
N40	0.68	D30	0.73	**Mean±SD**	**1±0.18**
N41	1	D31	0.8		
N43	0.83	D32	0.81		
N44	1	D33	0.69		
N45	1	D34	0.72		
N48	0.69	D35	1		
Mean±SD	**0.95±0.1**	**Mean±SD**	**0.8±0.16**		

***Pz: ratio of the colony diameter and the precipitation zone, therefore lower values indicate greater enzyme activity and 1 indicate no enzyme production.**

The results of phospholipase production were further analyzed using Scheffe pair wise comparison.

Phospholipase production of strains isolated from denture wearers was significantly high compared to the strains isolated from the normal individuals ($p<0.01$) as shown in Figure 3.3. However, there was no significant difference found between the results of strains isolated from normal subjects and cancer patients with prostheses ($p>0.05$) as well as between denture wearers and cancer patients with prostheses groups.

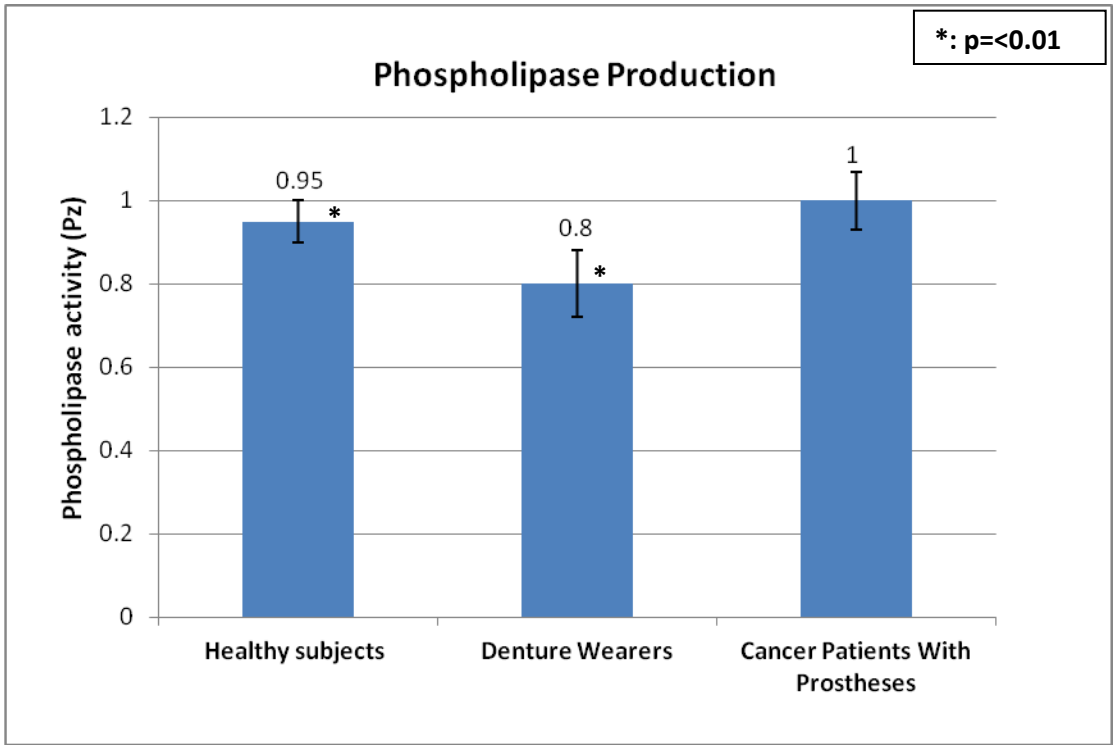

Figure 3.3 Phospholipase production by *C. albicans* isolated from normal subjects, denture wearers and cancer patients with oral prostheses Pz: ratio of the colony diameter and the precipitation zone, therefore lower values indicate greater enzyme activity and 1 indicate no enzyme production.

3.2.4 Proteinase production

The mean proteinase production by the strains isolated from normal subjects, denture wearers and cancer patients with prostheses was 0.92±0.09, 0.81±0.12 and 0.93±0.12 respectively (Table 3.12, Table 3.13). The overall difference in the results was statistically significant (p=0.02). Proteinase was produced by 60%, 80% and 64.29% of the *C. albicans* isolated from normal subjects, denture wearers and cancer patients with prostheses respectively (Figure 3.5, Table 3.14).

Table 3.12 Proteinase production by *C. albicans* isolated from Normal subjects, Denture wearers and Cancer patients with prostheses.

Normal subjects		**Denture wearers**		**Cancer patients with prostheses**	
Subject no	***Pr**	**Subject no**	***Pr**	**Subject no**	***Pr**
N2	0.84	D1	1	C1	0.87
N3	0.87	D3	0.92	C3	1
N8	0.9	D4	0.91	C4	1
N9	0.93	D5	0.85	C6	1
N11	0.93	D7	0.91	C7	1
N13	1	D9	0.75	C8	0.88
N15	1	D11	0.85	C9	0.57
N16	1	D16	0.74	C10	0.74
N22	0.93	D24	0.81	C11	0.99
N25	0.89	D25	0.89	C12	1
N30	1	D26	1	C14	0.99
N31	0.87	D27	0.53	C15	0.99
N33	0.95	D28	0.57	C16	0.94
N35	1	D29	0.58	C17	0.98
N40	0.69	D30	1	**Mean±SD**	**0.93±0.12**
N41	1	D31	0.53		
N43	0.83	D32	1		
N44	1	D33	0.66		
N45	1	D34	0.78		
N48	0.68	D35	0.83		
Mean±SD	**0.92±0.098**	**Mean±SD**	**0.81±0.12**		

The results of proteinase production were further analyzed using Scheffe pair wise comparison.

Proteinase production of strains isolated from denture wearers was significantly high compared to the strains isolated from the normal individuals (p=0.03) as shown in Figure 3.4. However, proteinase production of strains isolated from cancer patients with prostheses was not significantly different compared to the strains isolated from the normal individuals. When the proteinase production by the strains isolated from the denture wearers and cancer patients with prostheses were compared, the results showed that strains isolated from the denture wearers produced significantly higher amounts (p=0.04).

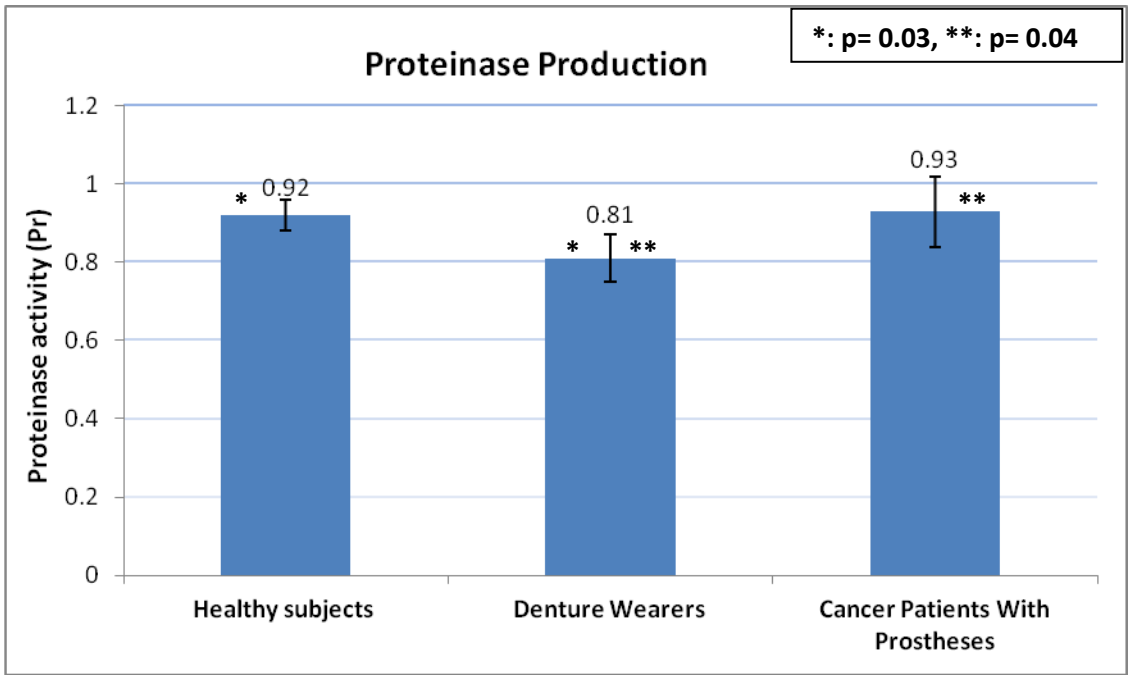

Figure 3.4 Proteinase production by *C. albicans* isolated from normal subjects, denture wearers and cancer patients with oral prostheses. Pr: ratio of the colony diameter and the clear zone, therefore lower values indicate greater enzyme activity and 1 indicate no enzyme production.

3.2.5 Summary of results of the pathogenic characteristics of *C. albicans*

The summary in Table 3.13 shows the quantitative analysis of germ tube formation, adherence, phospholipase and proteinase production by the strains isolated from the study groups. Overall there was a statistical difference between the results of study groups. However, when the results of the study groups were compared to one another, germ tube formation was found to be higher only by the strains from Cancer patients with prostheses compared to the strains from normal subjects. The adherence by the strains isolated from the denture wearers and cancer patients with prostheses were higher compared to the strains isolated from the normal subjects. However, phospholipase and proteinase production by only the strains isolated from the denture wearers was higher than the strains isolated from the normal subjects.

Table 3.13 Summary of results from Germ tube formation, adherence to oral epithelial cells phospholipase and proteinase production by *C. albicans*

Pathogenic characteristics	**Quantitative analysis Mean ± SD**			**Overall *P* value**
	Normal subjects n=20	**Denture wearers n=20**	**Cancer patients with prostheses n=14**	
Germ tube formation (%)	41.2 ± 21.85*	43.2 ± 15.11	57.17 ± 17.48*	0.03
Adherence to oral epithelial cells (Yeast/100 epithelial cells)	343.4 ± 267.6 **, §	694.8 ± 391.44 **	818.71± 286.1 §	<0.01
Phospholipase production (Pz)	0.95 ± 0.1¤	0.81 ± 0.16 ¤	1.0 ±0.18	<0.01
Proteinase production (Pr)	0.92 ± 0.09 #	0.81 ± 0.12 #	0.93 ± 0.12	0.02

*: p=0.05, **, §, ¤: p=0.01, #: p=0.03

3.2.6 Number of strains isolated from the study groups presented with pathogenic characteristics

The number of strains that produced germ tube, showed adherence ability and produced phospholipase and proteinase are shown in Table 3.14 and Figure 3.5. All the strains produced germ tube and showed adherence ability. Phospholipase was produced by 25%, 85% and 57.14% of the *C. albicans* strains isolated from normal subjects, denture wearers and cancer patients with prostheses respectively. Proteinase was produced by 60%, 80% and 64.29% of the *C. albicans* strains isolated from normal subjects, denture wearers and cancer patients with prostheses respectively. When the number of phospholipase producing strains was compared between the groups, significant difference was found ($p>0.01$). However, no difference was found in number of strains that produced proteinase ($p>0.05$).

Table 3.14 Number of strains isolated from the study groups presented with pathogenic characteristics

Pathogenic characteristics	**No. of strains (%)**			**Overall *P* value**
	Normal subjects n=20	**Denture wearers n=20**	**Cancer patients with prostheses n=14**	
Germ tube formation	20 (100)	20 (100)	14 (100)	>0.05
Adherence to oral epithelial cells	20 (100)	20 (100)	14 (100)	>0.05
Phospholipase production	5 (25)*,**	17 (85)*	8 (57.14)**	<0.01
Proteinase production	12 (60)	16 (80)	9 (64.29)	>0.05

*: $p<0.01$, **:$p=0.05$

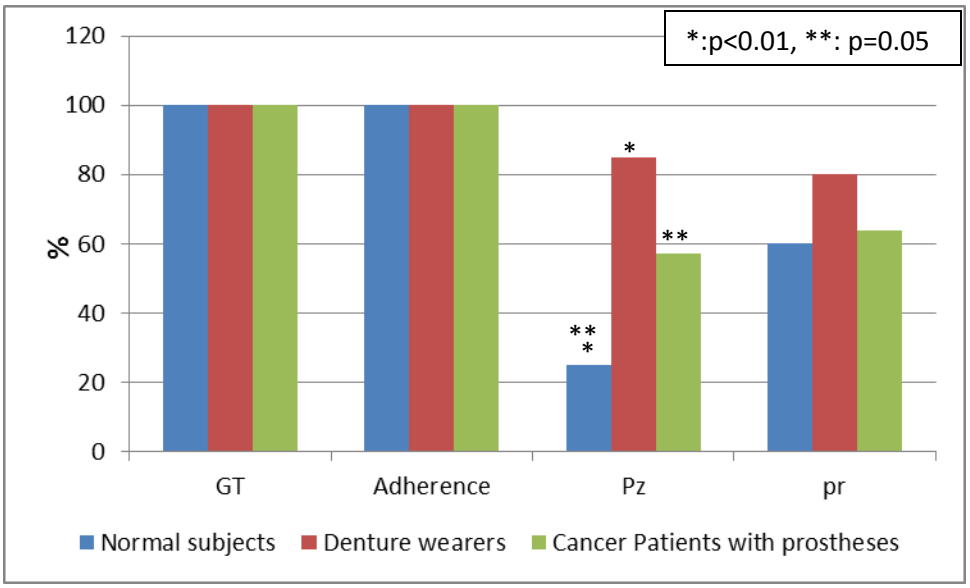

Figure 3.5 Number of strains isolated from the study groups produced virulence factors

3.3. *Candida* counts and virulence factors

Candida counts and the results of the pathogenic characteristics of *C. albicans* strains isolated from the study groups are presented in the Table 3.15.

Table 3.15 Comparison of counts and the virulence factors of *C. albicans* strains isolated from normal subjects with denture wearers and cancer patients with prostheses

Study groups	**Comparison of parameters of normal subjects to study groups**				
	***C. albicans* counts**	**Germ tube formation**	**Adherence ability**	**Phospholipase production**	**Proteinase production**
Denture wearers (35)	High (15 with >1000 cfu/ml)	*	High (p=0.01)	Quantity: High (p<0.01)	Quantity High (p=0.03)
				No. of strains: High (p<0.01)	No. of strains: *
Cancer patients with prostheses (19)	High (12 with >1000 cfu/ml)	High (p=0.05)	High (p<0.01)	Quantity: *	Quantity: *
				No. of strains: High (p=0.05)	No. of strains: *

*: p=>0.05 (not significant)

The *C. albicans* counts were higher (>1000 cfu/ml) in the denture wearers (15/35 patients) and cancer patients (12/19 patients) with prostheses compared to the normal subjects 0/49 patients). Even though all the strains isolated from all the study groups produced germ tube, strains isolated from the cancer patients with prostheses produced greater quantities of germ tubes compared to the strains isolated from the normal subjects (p=0.05).

All the strains isolated from all the study groups showed adherence ability, but strains isolated from the denture wearers and cancer patients with prostheses showed greater adherence ability to the epithelial cells ($p=0.01$) compared to the strains isolated from the normal subjects.

Many strains isolated from the denture wearers produced higher quantities of phospholipase compared to the strains isolated from the normal subjects ($p<0.01$). Although many strains of *C. albicans* isolated from cancer patients with prostheses produced phospholipase ($p=0.05$), the quantities of the enzyme was not significantly different from the strains isolated from normal subjects.

Strains isolated from the denture wearers produced higher amounts of proteinase compared to the strains isolated from the normal subjects ($p=0.03$). The quantities of the enzyme produced by the *C. albicans* isolated from cancer patients with prostheses were not significantly different from the strains isolated from normal subjects.

CHAPTER 4

DISCUSSION

4.1 Normal subjects and *Candida species*

Candida species, particularly *C. albicans* is a commensal carried by many normal healthy individuals in their gut, skin, female vagina and oral cavity as shown in this study as well. If opportunity is given, these commensals can cause infections. . In our study, the prevalence of *C. albicans* in normal subjects was 42.86% (Table 3.4). Similar results of 40-50% in healthy individuals were reported in the studies of Chavasco et al., 2006, Martinez et al., 2002, Ariyawardana et al., 2006. A previous South African study found prevalence of 63% from 100 normal healthy subjects (Patel et al, 2006). The prevalence of *Candida* count was also found to be low in this study. In this study 42.86% of normal subjects had*Candida* count of >1000 cfu/ml as compared to the previous study, where 71.4% of normal subjects carried >1000 cfu/ml of *Candida*. In addition, only *C. albicans* strains were isolated from our normal subjects whereas in other studies,*Candida* species other than *C. albicans* (*C. dubliniensis, C. parapsilosis, C. tropicalis*) were observed (Ariyawardana et al, 2006, Patel et al, 2006).

4.2 Denture wearer and *Candida species*

It is generally known that the presence of dentures increases the number of microorganisms in the oral cavity. In addition to the dentures, many etiological factors contribute towards this colonization. They are co-morbidity such as diabetes and HIV infection, systemic illnesses, high carbohydrate intake and the actual denture hygiene (Samaranayake et al, 2002). Among these microorganisms many denture wearers carry *Candida* in their oral cavity. Therefore they are predisposed to oral candidiasis i.e. denture-associated denture stomatitis (Samaranayake et al,

2002). It occurs due to accumulation of microbial plaque with yeasts and bacteria on the fitting surfaces of the denture and the underlying mucosa. In this study, the factors that influence the oral colonization with *Candida* in the denture wearers were not considered because prevalence of *Candida* was not our primary objective. However, since the strains of *C. albicans* required for this study had to be isolated from denture wearers, many patients were screened which generated the reported data. The results showed that 74.3% of the denture wearers carried *Candida* in their oral cavity compared to 42.9% of normal subjects without any prostheses in their mouth (Table 3.4) and the difference was significant. C*andida* carriage in our study population of denture wearer was slightly higher than C*andida* carriage of other studies. The study conducted by Daniluk et al., (2006), showed the prevalence of *Candida* carriage in the oral cavity of denture wearers to be 66.7% whereas Chopde et al., (2012), reported 55.4%. The increase in the prevalence of *Candida* carriage in our study group could be due to the other contributing factors such as HIV, diabetes, nighttime wearing of dentures and systemic illnesses (Gendreau and Loewy, 2011) that were not considered in this study. Furthermore, the mean age of denture wearers in our study population was 75 years which might have influenced the carrier rate (Table 3.2). *Candida* carriage is generally higher in elderly people (Akpan and Morgan, 2002).

The results of this study showed not only the high prevalence of multiple *Candida* carriage but also the very high (mean count of 18616 cfu/ml) *Candida* counts which was performed on oral rinses collected while patients were wearing dentures. Abaci et al., (2010) reported that when the count of *Candida* species in saliva is $\geq$ 400 cfu/ml, then frequency of development of denture related stomatitis increased (Abaci et al, 2010). In our study group, 25 (71.63%) colonized

patients carried ≥100 cfu/ml of which 15(42.86%) patients carried >1000 cfu/ml which suggest that these patients are at high risk of oral candidiasis development.

Candida albicans was the predominant species isolated from the denture wearers (46%). Similar results have been reported by Cavaleiro et al., (2013). Patients with locator-retained overdenture and bar-retained overdentures also carry predominantly *C. albicans* in their oral cavity (Kilic et al., 2014). *Candida albicans* is known to be more virulent than other *Candida* species because of its ability to produce virulence factors including production of destructive hydrolytic enzymes, hyphae formation and ability to adhere to the oral epithelial cells (Silva et al., 2012). However, more recently pathogenesis of *Candida* other than *C. albicans* is emerging. Yeasts such as *C. glabrata, C. tropicalis, C. krusei and C. parapsilosis* have been isolated from blood stream and urinary tract infections (Giri and Kindo, 2012, Kauffman et al., 2011).

Candida glabrata (26.5%) was the second most common *Candida* species isolated from denture wearers (Table 3.6), similar to the results obtained by Coco et al., (2008) and Loster et al., (2012). These authors also reported that mixed infections with *C. albicans* and *C. glabrata* were correlated to the degree of inflammation suggesting the role of other *Candida* subspecies in the pathogenesis. In addition, *C. glabrata* has developed resistance to widely used antifungal fluconazole and relatively newly developed antifungal echinocandins (Pfaller et al., 2012, Silva et al., 2012). *Candida glabrata, C. tropicalis, and C. parapsilosis* which were also isolated in this study are capable of producing biofilms on medical implants and no doubt on dentures

(Cuèllar-Cruz et al., 2012). They also produce pseudohyphae and some of the hydrolytic tissue destructive enzymes (Silva et al., 2012). These properties can be attributed to pathogenesis of these non-*Candida* species. In our study, 46% of denture wearers, (Table 3.5) carried multiple yeasts which suggest that detection of all types of C*andida* is important in the management of patients particularly patients who do not respond to the antifungal agents.

4.3 Cancer patients with prostheses

Since oral candidiasis is considered a disease of the diseased, cancer patients most certainly fall into this category. The patients with cancers carry *Candida* in their oral cavity and during cancer treatment they develop oral candidiasis. The carriage rate was 49.4%, 64.6% and 36.9% in hematological malignancy, solid tumor and head and neck cancers respectively (Schelenz et al., 2011). However, carriage can increase with the age, duration of illness and treatment, and presence of dentures in the mouth (Gammelsrud et al., 2011, Davies et al, 2002). It is known that the mere presence of prostheses in the oral cavity predisposes patients to oral infections, cancer patients with prostheses are highly vulnerable to oral infection. Furthermore, the prevalence of oral candidiasis is normally high in head and neck cancer patients due to the development of xerostomia (Bensadoun et al., 2011, Mañas et al., 2012).

Although the study groups were not matched (age, gender, HIV status etc.) in this study, the result showed that the C*andida* carriage rate was significantly high in cancer patients with prostheses (84%) compared to the 42.85% of normal subjects (Table 3.4). However, this was not

significantly higher than patients with denture (74%) which suggests that in patients with oral prostheses, cancer does not increase the carriage rate of *Candida.* However, Epstein et al., (1993) reported that, in cancer patients in the presence and use of oral prostheses were shown to correlate with oral colonization of *C. albicans* before radiation therapy.

The mean counts of *Candida* in the cancer patients (59084 cfu/ml) compared to the denture wearers (18616 cfu/ml) was much higher (Table 3.7) and 63% of cancer patients were carrying more than 1000 cfu/ml of *Candida* (Table 3.8), which suggests that the immune response, environmental and physiological factors may have played a significant role. Although the salivary flow was not measured in the samples of these patients, xerostomia is known to occur and can influence the *Candida* counts. Improvement in the hygiene of prostheses may decrease the number of organisms. However, cancer patients are often too ill to care for their prostheses. In addition, secreted IgA is very important in host defense against oral candidiasis and may regulate the oral *Candida* population (Coutinho, 2009). And cancer patients are known to have reduced secretory immunoglobulin A (Shpitzer et al., 2007).

Candida other than *C. albicans* such as *C. glabrata, C. tropicalis and C. krusei* were isolated from cancer patients with prostheses. These non - *C. albicans Candida* species were also isolated from cancer patients by Dahiya et al., 2003, Thaweboon et al., 2008. The role of non - *C. albicans* yeasts in the pathogenesis of oral candidiasis has been identified in recent years and has become clinically important. In addition, they may be resistant to many antifungal agents (Silva

et al., 2011). When Dahiya et al., (2003) investigated oral candidiasis in cancer patients; they found that 3 of 37 patients were infected with non-*C. albicans Candida*. Redding et al., (2002) added that *C. glabrata* colonization is common in patients receiving radiation treatment for head and neck cancer. These results suggest that isolation and identification of *Candida* species in cancer patients is very important for the appropriate treatment which can be instituted without a delay. Oral prostheses including dentures are mostly prepared using acrylic resin which also allows adherence of *C. glabrata* and this may have been the reason why this species was isolated from both study groups (Zamperini et al., 2012).

Oral cancer patients may undergo a series of treatment including oral prostheses due to the anatomical abnormalities created by surgical resections. Material such as silicon or polymethyl methacrylate, titanium and AO/ASIF stainless steel are used in the reconstructions of patients with maxillary and mandibular defects (Harsha et al., 2012, Depprich et al., 2008). Some of these materials may increase the risk of colonization of microorganisms which can result in local or systemic infections. Not much is known about the bacterial contaminants present on these prostheses. Depprich et al., (2008) studied colonization of 36 obturators used by patients and found high level of oral and gut flora as well as *C. albicans*. They also showed that titanium-based obturators harbored fewer microorganisms than silicon/PMMA (polymethyl methacrylate) obturators (Depprich et al., 2008).

It is clear that many denture wearers and cancer patients carry high numbers of *Candida* including *C. albicans* in their oral cavity. These high counts may predispose these patients to oral candidiasis. However, those colonized by *C. albicans* may be exposed to infection with the aid of

virulence factors such as hyphae formation, adherence ability and the production of hydrolytic enzymes. Therefore, these virulence factors produced by the strains isolated from the 3 study groups were measured and compared.

4.4 Pathogenic characteristics of *C. albicans* isolated from the study groups

The virulence factors expressed by *Candida* may vary, depending on the type, stage and site of infection such as superficial or deep sited and the nature of the host immunity (Mohan das and Ballal, 2008). Germ tube formation, the production of hydrolytic enzymes and ability to adhere to epithelial cells are known to contribute in the pathogenesis of *C. albicans* (Haynes, 2001, Calderone and Fonzi, 2001). Host-Candidal interaction is best studied in the host which can be an animal. However, some of these virulence factors are measurable in the laboratory environment (Pinto et al., 2008, Lyon and de Resende, 2006, Ramla et al., 2015) and therefore virulence factors of *C. albicans* isolated during commensal state were measured in this study and compared between the study groups.

4.4.1 Germ Tube formation and Adhesion

Germ tube formation is the initial stage in the yeast-hyphal transition. The presence of hyphae seems to facilitate the candidal colonization on the mucosal surfaces and raises the *C. albicans* counts (Bilhan et al., 2008). All the strains of *C. albicans* isolated from all the study subjects produced germ tube (Figure 3.5). However quantitative analysis showed that higher number of yeast cells isolated from cancer patients with prostheses produced germ tubes compared to the

strains isolated from the normal subjects (Figure 3.1). High number of *C. albicans* isolated from HIV positive patients have shown to produce germ tubes (27.5%) compared to the 14.7% of the healthy individuals (Wibawa et al., 2015). These results are contradictory to those reported by Ramla et al., (2015) who found no difference in the germ tube formation between the strains isolated from normal subjects and cancer patients either on radiotherapy or chemotherapy. Chemotherapy has been reported to increase the hyphal formation (Moussa et al., 1990) and decrease the germ tube formation (Land et al., 1980). Based on the germ tube and *Candida* counts (Table 3.15) results obtained in this study suggest that the strains isolated from the cancer patients have a greater potential to cause infection.

Although germ tube formation is important in the disease process, all the virulence factors are not required at all the stages of infection (Naglik et al., 2003). Nevertheless, germ tube formation is important in the oral and vaginal infections and in some other invasive infections.

In the invasive candidiasis, germ tube antibodies are detectable using the Immunofluorescent IgG test. Germ tube cell wall surfaces contain 230-250 kDa mannoprotein which acts as an antigen and during infection, patients develop antibodies against this antigen. However, often in very ill patients early detection of this antibodies and treatment with antifungal agents can reduce mortality (Perman et al., 2011). This suggests that it would be interesting to investigate antibody levels in denture patients with and without stomatitis as well as cancer patients with and without mucositis.

The adhesion of *Candida* cells to host mucosal surface is a vital prerequisite for successful colonization and infection (Cannon et al., 1995). *C. albicans* adheres to host cells more than any other species of *Candida.* In this study, *C. albicans* strains isolated from the denture wearers as well as cancer patients had significantly higher level of adherence to buccal epithelial cells than strains isolated from normal subjects (Figure 3.2). Similarly *C. albicans* isolated from the HIV positive patients and patients with diabetes mellitus have shown increased adherence ability (Sweet et al., 1995, Manfredi et al., 2006). Adherence allows yeast cells the chance to proliferate and colonize the host, with or without signs or symptoms of infection (Calderone and Fonzi, 2001). The balance between colonization and overt infection may be tipped toward infection in compromised individuals by changes in the expression of adherence ligands and receptors. This explains the high counts of *Candida* and the greater ability to adhere in the denture wearers and cancer patients with prostheses (Table 3.7 and Table 3.10).

Wearing prostheses was the common factor in denture wearers and the cancer patients for *Candida* carriage. Acrylic dentures are known to act as reservoir for microorganisms including *Candida* and therefore play an important role in the development of Candidiasis. Silicone and PMMA made prostheses obturators are also known to become colonized with *Candida* (Depprich et al., 2008). In addition, pores, cracks and structural defects allow penetration and therefore persistence of *Candida* into these prostheses (Glass et al., 2001). This explains the high counts found in the oral cavities of these two groups.

Adherence of *C. albicans* to plastic medical devices (medical implants, prostheses and catheters) allows the organism to propagate and establish biofilms to release microorganisms from these biofilms which contributes to or initiates acute disseminated nosocomial infections. Several components of the microfibrillar surface layer of *C. albicans* are implicated in plastic binding and plastic adherence and appear to be mediated by cell surface hydrophobicity (CSH) through hydrophobic bonds between the plastic surface and the peptide moieties of the mannoproteins. These mannoproteins may play other significant roles in cell physiology rather than specifically bind to plastic surfaces. Salivary proteins, adsorbed to plastic material of dental or voice prostheses may also help colonization by fungal cells (Mishra et al., 2007).

In the oral cavity, saliva mechanically clears food debris and microorganisms. In addition, adhesion and colonization of *C. albicans* is generally suppressed by saliva from healthy persons due to the antimicrobial compounds such as lactoferrins, lactoperoxidases and specifically antifungal compounds histatins and defensins (Yamagishi et al., 2005, Umazume et al., 1995). In cancer patients, salivary secretion is greatly suppressed by cancer therapy which might also be responsible for the higher counts of *Candida.* In addition, whole salivary glycoproteins and released glycans also inhibit the interaction of *C. albicans* with buccal epithelial cells (Everest-Dass et al., 2012).

Increased adherence ability of the strains isolated from these two groups suggests that even during the colonization state these strains shows readiness to cause infection. In recent years, a

novel approach of therapy, targeting virulence factors rather than the conventional antifungal approach has been suggested (Gauwerky et al., 2009). Therefore, development of chemical agents that can prevent the adherence mechanisms may be a better therapeutic agent in the maintenance of reduced quantity of C*andida* in the oral cavity rather than the use of fungicidal agents.

4.4.2 Phospholipase and proteinase production

Phospholipases are a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids. They can effectively degrade the membrane of the host cells leading to cell lysis and death. Both the adherence of *Candida* to receptor sites and its subsequent penetration of damaged tissue can be facilitated by this process (Williams and Lewis, 2011). They are considered putative virulence factors because they are associated with adhesion to epithelial cells, host cell penetration, invasion of epithelial cells, and interaction with host signal transduction pathways (Schaller et al., 1999, Mishra et al., 2007). Experiments involving the disruption of a gene that encodes for a phospholipase showed a decrease in virulence and a reduced ability of *C. albicans* to penetrate host cells. This suggests that phospholipases plays a role in causing damage to host cell (Leidich et al., 1998, Khan et al., 2010).

In this study, phospholipase production was significantly high in strains of *C. albicans* isolated from denture wearers compared to the normal subjects (Figure 3.3). There was no significant difference in phospholipase production by strains isolated from cancer patients and the normal

subjects. These results with cancer patients are contradictory to the results obtained by Ramla et al., (2015). They found that many strains of *C. albicans* isolated from the cancer patients produced high quantities of phospholipase compared to the strains from normal subjects. Although the production of hydrolytic enzymes is one of the mechanisms in the pathogenesis, not all clinical strains necessarily produce these enzymes (Kantarcioglu and Yucel, 2002). In this study, 85% of strains isolated from denture wearers, 57.14% from cancer patients and 25% from normal subjects produced phospholipase (Table 3.14). When Abaci (2011) compared the phospholipase production by strains of *C. albicans* isolated from denture wearers with and without stomatitis, they found no significant difference in phospholipase producing strains. Similar results were also found by Kadir et al., (2007) but they found higher quantities of phospholipase in the strains isolated from the patients with stomatitis. In contrast Marco-Arias et al., (2009) reported that phospholipase producing strains were significantly higher in patients with Newton's type II and III classified denture stomatitis compared to the commensal state. Similarly *C. albicans* isolated from HIV positive and HIV negative patients have shown no difference in the phospholipase production (Mane et al., 2012, Patel et al., 2009).

Phospholipases are also produced by the strains isolated from normal individuals as also shown in our study (25%). Strains of *C. albicans* isolated from the anatomical sites of healthy humans produce different amounts of phospholipases. Oksuz et al., (2007) reported that phospholipase activity is higher in oral and fecal isolates of *C. albicans* compared to other anatomical sites. Despite these contradictory results, it is clear that during invasive infection and serious illnesses the amount of phospholipase production increases and it increases the mortality rate (Ibrahim et

al., 1995). This suggests that the role of extracellular phospholipase production by *Candida* in pathogenic processes cannot be ignored and requires further research.

Secreted aspartic proteinases (SAPs) are secreted by pathogenic species of *Candida* including *C. albicans* during infection. They are responsible for the adhesion, tissue damage and invasion of host immune responses. Proteinases fulfill a number of specialized functions during the infective process. They include digesting molecules for nutrient acquisition, digesting or distorting host cell membranes to facilitate adhesion and tissue invasion, and digesting cells and molecules of the host immune system to avoid or resist antimicrobial attack by the host (Mohan das and Ballal, 2011). These researchers postulated that proteinases may act on the epithelial tissues whereby it may uncover sites on the tissue in order for the fungal cells to adhere and hence penetrate (Kwon-Chung et al., 1985). As phospholipases and aspartyl proteinases of *C. albicans* are considered important virulence factors, the absence or lowered expression of these enzymes may indicate the less virulent nature of *Candida* species, when compared to *Candida* species with higher expression of these enzymes, particularly in immunosuppressed patients, and the degree of virulence and pathogenicity are correlated with the level of secreted proteinases, (Mohan das and Ballal, 2008 and Akçağlar et al., 2011).

In the present study, strains isolated from denture wearers produced significantly higher amount of proteinase compared to the normal subjects and cancer patients (Figure 3.4). This suggests that these strains have greater potential to cause infection. In the literature results of proteinase

activity are controversial. It has been reported that strains of *C. albicans* isolated from patients with denture stomatitis produce higher amounts of proteinase compared to the colonizing strains isolated from the denture wearers (Koga-Ito et al., 2006, Abaci, 2011). In contrast, Marcos-Arias et al., (2009) showed no difference in the proteinase production by strains of *C. albicans* isolated from the denture wearers with and without stomatitis. Similarly, strains of *C. albicans* isolated from patients with type 2 diabetes mellitus produce increased quantities of proteinase compared to the strains isolated from normal healthy individuals (Tsang et al., 2007). Manfredi et al, (2006) showed no difference in the proteinase production by the strains isolated from diabetic patients and normal individuals. Furthermore similar controversial results have been reported in HIV positive patients (Mane et al., 2012, Ollert et al., 1995, Patel et al., 2009).

Although the drugs used in chemotherapy and radiation (in vitro study) has shown to increase the production of proteinase by *C. albicans*, no difference in the proteinase production was found between the strains isolated from normal subjects and cancer patients on either cancer therapy (Ueta et al., 2001, Ramla et al., 2015). Similarly in this study no difference was found in the production of proteinase between the strains isolated from normal subjects and cancer patients with oral prostheses.

In *C. albicans*, 10 SAPs have been identified but their role in the commensal state is not known. These SAPs are produced at different stages of infections and the production is also associated with the morphological state of *C. albicans*. In vitro studies have reported that SAPs 1, 2 and 3

are expressed by the yeast phase, only while SAPs 4, 5 and 6 are expressed in the hyphal phase. In addition, environmental factors and host factors also influence the production of these enzymes (Naglik et al., 2003). Proteinase detection tests performed in the laboratory environment are not uniform across the laboratories and they not very accurate which could be the reasons for the controversial results in the literature. Molecular techniques with the detection of gene expression may prove to be more accurate.

4.5. Role of Immune response

Candidiasis may occur due to immunological dysfunction of the host as well as due to the expression of infection-associated genes. The products of these genes contribute to fungal pathogenicity and are described as virulence factors. These virulence factors are required at different stages of infection (Khan et al., 2010). Therefore the virulence factors of *C. albicans* isolated from the two susceptible groups were measured and compared to the normal healthy subjects. The summary of these results are shown in Table 3.15.

The host defense against *Candida* infection depends on the early activation of innate immunity cells such as neutrophils, followed by a specific immune response that is mediated by lymphocytes (Gasparoto et al., 2010, Netea and Marodi, 2010). This process is initiated by the recognition of chemical structures called pathogen-associated molecule patterns (PAMPs), present on the invading pathogen by pattern recognition receptors (PRRs), such as CD206, TLR2, TLR4 and Dectin-1 (Gasparoto et al., 2010, Netea and Marodi, 2010, Tessarolli et al,

2009). Neutrophils have been shown to influence the innate immune response in the oral mucosa (Netea et al., 2008, Schaller et al., 2004). Adherence of *C. albicans* to the oral epithelial cells is the initial stage of proliferation, colonization and the infection. The counts and the adherence ability were increased in the *C. albicans* isolated from denture wearers and cancer patients with prostheses suggest that this early activation of innate immunity may have been compromised (Table 3.15). In cancer patients, the cancer therapy causes myelosuppression and damage to the mucosal barrier which is known to increase the adherence property of these organisms (Bensadoun et al., 2011). Furthermore, secreted IgA is important in regulation of *Candida* colonization and host defense against oral candidiasis (Coutinho HDM, 2009) and the reduced secretory IgA in cancer patients, (Shpitzer et al., 2007), may have been responsible for the high *Candida* counts in these patients.

The activation of neutrophils and phenotypic modulation which is the first defense is impaired by age (Ginaldi et al., 2001) which may increase the chances of *Candida* infections in older people. In this study, the average age of denture wearers and cancer patients with prostheses was 75 and 70 respectively (Table 3.2 and 3.3). This suggests that both groups were possibly defective in the early activation of innate immunity and may have reduced expression of salivary CD66b, CD64 and blood, and salivary TLR2 (Gasparoto et al., 2012).

Many strains of *C. albicans* isolated from denture wearers produced high quantities of phospholipase and proteinase. Whereas, only the number of phospholipase producing strains

isolated from cancer patients with prostheses were high and they produced low quantities of this enzyme. The reason for the difference in the results of denture wearers and cancer patients cannot be explained. Nevertheless, in the denture wearers, high quantities of enzymes produced by many of the strains collectively may accelerate the development of infection. It also suggests that hygiene of the oral cavity, dentures and the prostheses is important in the reduction of number of *Candida* and hence prevention of oral candidiasis. Proper cleaning and regular use of mild antimicrobial mouth rinses can keep the number of *Candida* low. Triclosan containing mouth rinses are known to reduce the number of *Candida* in the oral cavity (Patel et al., 2008).

Both the groups were aged and carried prostheses. Therefore, age related factors such as low salivary flow and medications for hypertension etc. and factors related to the prostheses such as hygiene could have been the same in the two groups. Larger sample size, data regarding risk factors and more sensitive techniques for the proteinase production may have explained these results.

4.6 Future Research

1 In cancer patients often mucositis and candidiasis is not differentiated which causes delay in the appropriate treatment. The use and the efficacy of the germ tube antibody test in the cancer patients with mucositis could be researched. This test is much quicker than the culture technique.

2 Antifungal drugs are frequently used by the denture wearers and cancer patients. *C. albicans* is developing resistance towards antifungal drugs (Perlin, 2010). Not much data is available in the strains isolated from South Africa. Therefore antifungal susceptibility tests can be performed to detect any drug resistance.

3 *Candida species* other than *C. albicans* are often isolated from the oral cavities of denture wearers and cancer patients and they are generally resistant to antifungal drugs. However. Their ability to produce virulence factors and their role in the development of oral infection is not known which can be studied.

4 Using a large sample size, multiple risk factors for the colonization and development of infection can be assessed and compared to the *Candida* counts, types of *Candida* and their virulence factors.

5 More sensitive and accurate tests for the production of hydrolytic enzymes by *C. albicans* can be developed

4.7 Limitations of the study

1 It is known that quantity of saliva affects *Candida* counts and therefore collection of saliva in these patients in a specific time period would have been ideal. However, many of these patients had xerostomia and the collection of saliva was not possible. Therefore, oral rinse was collected and the *Candida* counts could not be compared with quantity of saliva.

2 Many factors such as age, HIV status, systemic illnesses, denture-stomatitis, related medications, type of cancer and prostheses, and oral hygiene can influence the carriage rate in patients with dentures and cancers as well as normal subjects. However, the primary objective of this study was not "the comparison of carriage rate or prevalence of *Candida*" in these study groups. The average age of denture wearers and cancer patients with prostheses was 70-75 years and it was difficult to match with normal healthy subjects with that age. There were many issues with the HIV status of the study population. For example, if the patient is HIV positive, they are not obliged to reveal their status. If the patient did not know their HIV status there were consenting, costing for the test and counselling implications. This would have created 3 groups of results such as HIV positive, HIV negative and unknown within a small sample of 20 patients.

3 Molecular techniques applied directly to the oral rinse collected from the study subjects may have partially answered the research question regarding the production of germ tube and hydrolytic enzymes. They would have been accurate and sensitive but in this project it would have been disadvantageous in many aspects. They would have increased the cost of study tremendously; they would not have given the quantities of enzymes (Pz & Pr), types of *Candida* and their counts. Probably for these reasons techniques applied in this study are widely used (Mane et al., 2012, Ueta et al., 2001, Ramla et al., 2015, Kadir et al., 2007, Manfredi et al., 2006).

4.8. Conclusions

Many denture wearers and cancers patients with prostheses carried higher numbers of *Candida* compared to normal subjects. *C. albicans* was the predominant species however, *Candida* other than *C. albicans* such as *C. glabrata* and *C. tropicalis* were also isolated from the oral cavities of denture wearers and cancer patients with prostheses. Although the germ tube formation was significantly (weak) increased in the strains isolated from cancer patients with prostheses compared to the isolates from normal subjects, all the strains isolated from the study groups produced germ tube which suggest that germ tube is not a good factor for the measurement of virulence.

Adherence to epithelial cells was significantly increased in strains isolated from denture wearers and cancer patients with prostheses compared to the isolates from normal subjects. Adherence is the initial stage in the proliferation, colonization and the infection. The counts and the adherence ability was increased in the *C. albicans* isolated from these two groups suggest that this early activation of innate immunity may have been compromised. Many strains of *C. albicans* isolated from denture wearers produced phospholipase and proteinase and the quantities of these enzymes were also high compared to the strains isolated from the normal subjects. These results suggest that strains isolated from denture wearers had more potential or readiness to cause infection. Due to the high quantities and many enzyme producing strains, collectively these enzymes may accelerate the development of infection in this study group.

CHAPTER 5

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CHAPTER 6

APPENDICES

6.1 Composition and preparation of media and reagents

ChromAgar plate (CHROMagar, Paris, France)

65g Chromagar powder

1000ml dH_2O

Preparation Method: Dissolve Chromagar powder in sterile distilled water; bring it to boil while stirring. Let it cool to 45-50°C and pour plates under laminar flow. Do not autoclave.

0.5% Coomassie brilliant blue (Pierce Biotechnology)

0.5g Coomassie blue

45ml Ethanol

10ml Acetic acid

Preparation method: Weigh Coomassie blue powder; mix it with ethanol then acid. Keep it at room temperature.

Destaining solution

45ml Ethanol

45ml dH_2O

10ml Acetic acid

Preparation method: Mix distilled water with ethanol then acetic acid. Keep it at room temperature.

Egg yolk media

13g Sabouraud agar

11.7g NaCl

0.11g CaCl2

20ml Egg Yolk Emulsion

184ml dH_2O

Preparation Method: Dissolve SAB agar, NaCl, $CaCl_2$ in dH_2O and autoclave. Cool to 45^oC, add egg yolk emulsion, mix gently and pour plates.

Saboraud (SAB) Agar

65g Saboraud agar

1000ml distilled H_2O

Preparation Method: Suspend SAB agar in distilled water. Heat half the water to a boiling point. Dissolve the powder in water and add to the boiling water. Add the rest of the water and heat to boiling point. Pour into large bottles and sterilize at 121b for 15min in autoclave

SAB broth (pH 5.6)

4% Dextrose

1% Peptone

5.0mg Chloramphenicol

Preparation method: Weigh 65g of SAB powder, dissolve in 1000ml of dH_2O. autoclave at 121 °C for 15 min.

YCB-SAB plate

140 ml of 1.5% Colombia agar

1.17% Yeast Carbon Base Powder

0.2% BSA

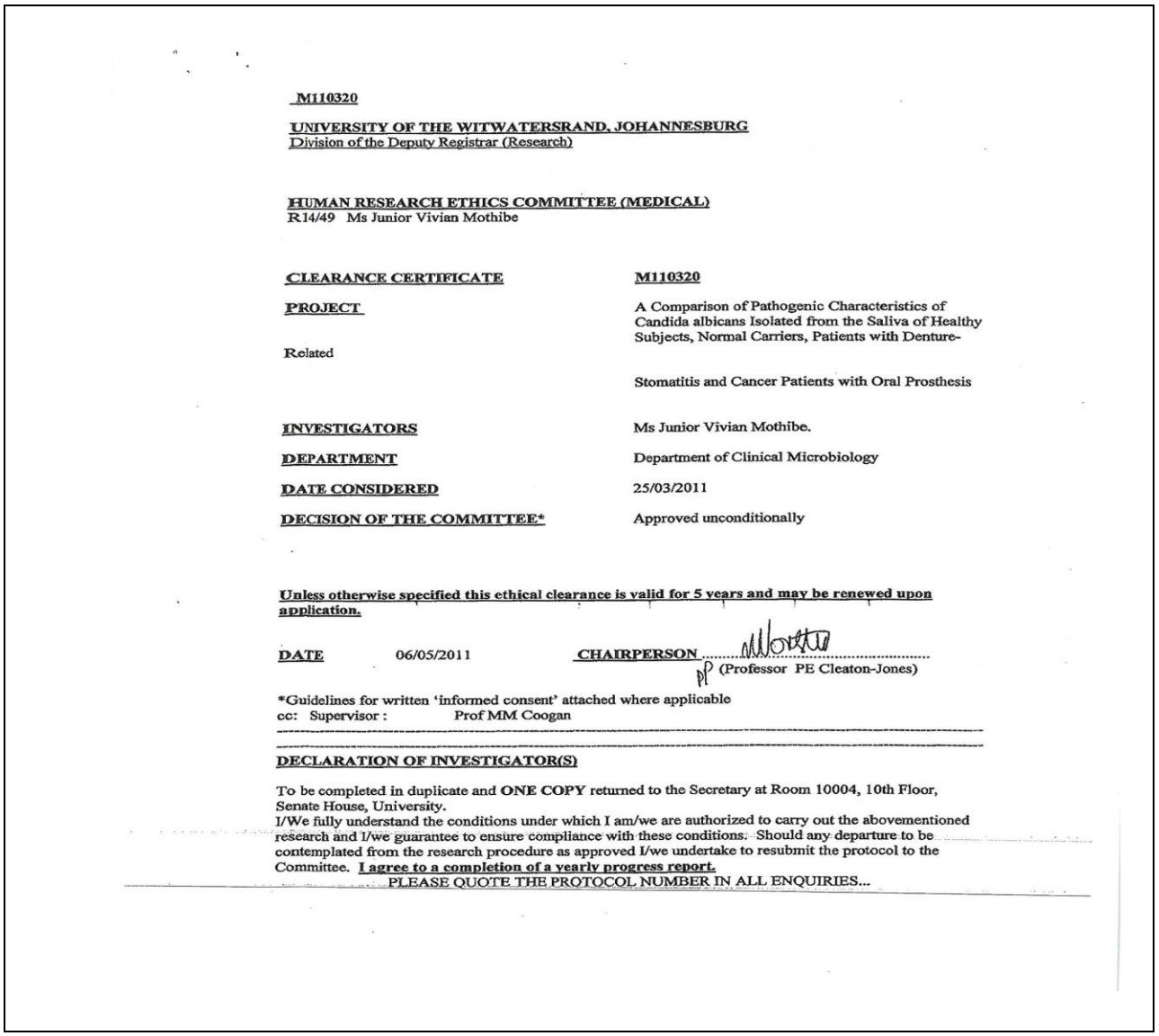
0.2% Glucose

0.5g K_2HPO_4

0.04g $MgSO_4.7H_2O$

Preparation Method: Dissolve above dry ingredients in 60ml distilled water, adjust pH to 3.5 and filter sterilize. Add this mixture to 140ml sterile Columbia agar medium. Pour plates.

6.2 Ethical clearance certificate



M110320

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Junior Vivian Mothibe

CLEARANCE CERTIFICATE	**M110320**
PROJECT	A Comparison of Pathogenic Characteristics of Candida albicans Isolated from the Saliva of Healthy Subjects, Normal Carriers, Patients with Denture-
Related	Stomatitis and Cancer Patients with Oral Prosthesis
INVESTIGATORS	Ms Junior Vivian Mothibe.
DEPARTMENT	Department of Clinical Microbiology
DATE CONSIDERED	25/03/2011
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06/05/2011 **CHAIRPERSON** pp (Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof MM Coogan

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

6.3 Statistical analysis

Carriage comparison between groups:

```
1 . tabi 21 27\26 9,row chi2
```

Key
frequency *row percentage*

row	col 1	2	Total
1	21 43.75	27 56.25	48 100.00
2	26 74.29	9 25.71	35 100.00
Total	47 56.63	36 43.37	83 100.00

Pearson chi2(1) = 7.6843 Pr = 0.006

```
2 . tabi 21 27\16 3, row chi2
```

Key
frequency *row percentage*

row	col 1	2	Total
1	21 43.75	27 56.25	48 100.00
2	16 84.21	3 15.79	19 100.00
Total	37 55.22	30 44.78	67 100.00

Pearson chi2(1) = 9.0118 Pr = 0.003

```
3 . tabi 21 23\23 12, row chi2
```

Key
frequency *row percentage*

row	col 1	2	Total
1	21 47.73	23 52.27	44 100.00
2	23 65.71	12 34.29	35 100.00
Total	44 55.70	35 44.30	79 100.00

Pearson chi2(1) = 2.5559 Pr = 0.110

4 . tabi 21 23\15 4, chi2 row

Key
frequency *row percentage*

row	col 1	2	Total
1	21 47.73	23 52.27	44 100.00
2	15 78.95	4 21.05	19 100.00
Total	36 57.14	27 42.86	63 100.00

Pearson chi2(1) = 5.2814 Pr = 0.022

-
-> enzyme = Pz

Key
frequency
row percentage

Group	Production 0	1	Total
Normal	15	5	20
	75.00	25.00	100.00
Denture	3	17	20
	15.00	85.00	100.00
Cancer	6	8	14
	42.86	57.14	100.00
Total	24	30	54
	44.44	55.56	100.00

likelihood-ratio chi2(2) = 15.6687 Pr = 0.000

P2 P = <0.01 Sig.

-
-> enzyme = Pr

Key
frequency
row percentage

Group	Production 0	1	Total

Normal	8	12	20
	40.00	60.00	100.00
Denture	4	16	20
	20.00	80.00	100.00
Cancer	5	9	14
	35.71	64.29	100.00
Total	17	37	54
	31.48	68.52	100.00

likelihood-ratio chi2(2) = 2.0874 Pr = 0.352

P = > 0.05

not sig..

```
. bysort  group: tab enzyme production, row lrchi2
```

-> group = Normal

Key
frequency
row percentage

Enzyme	Production 0	1	Total
Pz	15	5	20
	75.00	25.00	100.00
Pr	8	12	20
	40.00	60.00	100.00
Total	23	17	40
	57.50	42.50	100.00

likelihood-ratio chi2(1) = 5.1345 Pr = 0.023

-> group = Denture

Key
frequency
row percentage

Enzyme	Production 0	1	Total
Pz	3	17	20
	15.00	85.00	100.00
Pr	4	16	20
	20.00	80.00	100.00

Total	7	33	40
	17.50	82.50	100.00

likelihood-ratio chi2(1) = 0.1737 Pr = 0.677

-> group = Cancer

Key
frequency
row percentage

Enzyme	Production 0	1	Total
Pz	6	8	14
	42.86	57.14	100.00
Pr	5	9	14
	35.71	64.29	100.00
Total	11	17	28
	39.29	60.71	100.00

likelihood-ratio chi2(1) = 0.1499 Pr = 0.699

Stats for Virulence factors:

```
. *VIRULENCE
. table category, c(mean gt mean adh mean pr mean pz) format(%9.2f)

------------------------------------------------------------
 Category |   mean(gt)   mean(adh)    mean(pr)    mean(pz)
----------+-------------------------------------------------
   Normal |      41.20      367.50        0.92        0.95
  Denture |      43.20      694.80        0.81        0.81
   Cancer |      57.00      818.71        0.93        0.86
------------------------------------------------------------

.
. bysort category:sum gt, detail

------------------------------------------------------------------------------------------
------------------------------------------------------------
-> category = Normal

                            gt
------------------------------------------------------------
      Percentiles      Smallest
 1%           12             12
 5%           12             12
10%           12             12       Obs                  20
25%           22             18       Sum of Wgt.          20

50%           40                      Mean                41.2
                        Largest       Std. Dev.       21.85502
75%           60             66
90%           73             68       Variance        477.6421
95%           79             78       Skewness        .2794933
99%           80             80       Kurtosis        1.955886

------------------------------------------------------------------------------------------
------------------------------------------------------------
-> category = Denture

                            gt
------------------------------------------------------------
      Percentiles      Smallest
 1%           20             20
 5%           22             24
10%           24             24       Obs                  20
25%           29             24       Sum of Wgt.          20

50%           44                      Mean                43.2
                        Largest       Std. Dev.       15.11221
```

```
75%           51           60
90%           62           62       Variance       228.3789
95%           69           62       Skewness       .2266828
99%           76           76       Kurtosis       2.446377

-------------------------------------------------------------------------------
-> category = Cancer

                            gt
-------------------------------------------------------------
      Percentiles      Smallest
 1%           28           28
 5%           28           28
10%           28           32       Obs                  14
25%           50           50       Sum of Wgt.          14

50%           62                    Mean                 57
                      Largest       Std. Dev.       17.44883
75%           68           68
90%           78           76       Variance       304.4615
95%           80           78       Skewness      -.5189462
99%           80           80       Kurtosis        2.1732


. bysort category:sum adh, detail

-------------------------------------------------------------------------------
-> category = Normal

                            adh
-------------------------------------------------------------
      Percentiles      Smallest
 1%           64           64
 5%           64           64
10%           72           80       Obs                  20
25%          132          103       Sum of Wgt.          20

50%          246                    Mean                367.5
                      Largest       Std. Dev.       317.4063
75%          504          584
90%          960          940       Variance       100746.8
95%       1041.5          980       Skewness       1.153901
99%         1103         1103       Kurtosis       3.181711

-------------------------------------------------------------------------------
-> category = Denture

                            adh
-------------------------------------------------------------
      Percentiles      Smallest
 1%          152          152
 5%          192          232
10%          252          272       Obs                  20
25%          420          360       Sum of Wgt.          20
```

```
50%          646                      Mean              694.8
                        Largest       Std. Dev.      391.4364
75%          908            980
90%         1216           1120       Variance       153222.5
95%         1531           1312       Skewness       .9877353
99%         1750           1750       Kurtosis       3.831629

-------------------------------------------------------------------------------
-------------------------------------------------------------
-> category = Cancer

                              adh
-------------------------------------------------------------
      Percentiles      Smallest
 1%          246            246
 5%          246            508
10%          508            568       Obs                  14
25%          640            640       Sum of Wgt.          14

50%          879                      Mean           818.7143
                        Largest       Std. Dev.      286.1034
75%          968            968
90%         1230           1102       Variance       81855.14
95%         1278           1230       Skewness      -.1744836
99%         1278           1278       Kurtosis       2.509747

. bysort category:sum pr, detail

-------------------------------------------------------------------------------
-------------------------------------------------------------
-> category = Normal

                              pr
-------------------------------------------------------------
      Percentiles      Smallest
 1%          .68            .68
 5%         .685            .69
10%          .76            .83       Obs                  20
25%          .87            .84       Sum of Wgt.          20

50%          .93                      Mean              .9155
                        Largest       Std. Dev.      .0982197
75%            1              1
90%            1              1       Variance       .0096471
95%            1              1       Skewness      -1.191902
99%            1              1       Kurtosis       3.670551

-------------------------------------------------------------------------------
-------------------------------------------------------------
-> category = Denture

                              pr
-------------------------------------------------------------
      Percentiles      Smallest
 1%          .53            .53
 5%          .53            .53
10%          .55            .57       Obs                  20
```

5%	.61	.62		
10%	.64	.66	Obs	20
25%	.725	.69	Sum of Wgt.	20
50%	.81		Mean	.81
		Largest	Std. Dev.	.1238845
75%	.91	.96		
90%	1	1	Variance	.0153474
95%	1	1	Skewness	.083499
99%	1	1	Kurtosis	2.053012

-> category = Cancer

pz

Percentiles		Smallest		
1%	.55	.55		
5%	.55	.58		
10%	.58	.69	Obs	14
25%	.74	.74	Sum of Wgt.	14
50%	.97		Mean	.8578571
		Largest	Std. Dev.	.1713377
75%	1	1		
90%	1	1	Variance	.0293566
95%	1	1	Skewness	-.6210996
99%	1	1	Kurtosis	1.818905

```
.
. kwallis gt, by(category)
```

GT

Kruskal-Wallis equality-of-populations rank test

category	Obs	Rank Sum
Normal	20	470.00
Denture	20	501.50
Cancer	14	513.50

```
chi-squared =      6.534 with 2 d.f.
probability =      0.0381

chi-squared with ties =      6.551 with 2 d.f.
probability =      0.0378

. oneway gt category, tab sch
```

Category	Summary of gt Mean	Std. Dev.	Freq.
Normal	41.2	21.855025	20
Denture	43.2	15.112212	20
Cancer	57	17.448826	14

```
------------+---------------------------------------
      Total |   46.037037   19.271607          54

                        Analysis of Variance
    Source              SS         df      MS            F     Prob > F
------------------------------------------------------------------------
Between groups       2311.52593      2   1155.76296      3.39     0.0414
 Within groups         17372.4     51   340.635294
------------------------------------------------------------------------
    Total            19683.9259     53   371.394829

Bartlett's test for equal variances:  chi2(2) =   2.5634  Prob>chi2 = 0.278
```

Comparison of gt by Category (Scheffe)

```
Row Mean-|
Col Mean |     Normal     Denture
---------+----------------------
 Denture |          2
         |      0.943
         |
  Cancer |       15.8       13.8
         |      0.058      0.110
```

```
. kwallis adh, by(category)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | category | Obs | Rank Sum |
  |----------+-----+----------|
  |   Normal |  20 |   342.00 |
  |  Denture |  20 |   623.00 |
  |   Cancer |  14 |   520.00 |
  +---------------------------+

chi-squared =     15.077 with 2 d.f.
probability =      0.0005

chi-squared with ties =     15.079 with 2 d.f.
probability =      0.0005

. oneway adh category, tab sch

           |          Summary of adh
  Category |       Mean   Std. Dev.       Freq.
-----------+------------------------------------
    Normal |      367.5   317.40635          20
   Denture |      694.8   391.43644          20
    Cancer |  818.71429   286.10338          14
-----------+------------------------------------
     Total |   605.7037   384.07805          54

                        Analysis of Variance
    Source              SS         df      MS            F     Prob > F
------------------------------------------------------------------------
Between groups       1928812.2      2   964406.101      8.35     0.0007
```

```
 Within groups      5889533.06     51    115481.04
-----------------------------------------------------------------------------
    Total           7818345.26     53   147515.948

Bartlett's test for equal variances:  chi2(2) =   1.6498  Prob>chi2 = 0.438
```

Comparison of adh by Category (Scheffe)

Row Mean-| Col Mean	Normal	Denture
Denture	327.3	
	0.014	
Cancer	451.214	123.914
	0.002	0.582

Pr

```
. kwallis pr, by(category)

Kruskal-Wallis equality-of-populations rank test
```

category	Obs	Rank Sum
Normal	20	625.00
Denture	20	401.00
Cancer	14	459.00

```
chi-squared =     7.202 with 2 d.f.
probability =     0.0273

chi-squared with ties =     7.439 with 2 d.f.
probability =     0.0243

. oneway pr category, tab sch
```

Category	Summary of pr Mean	Std. Dev.	Freq.
Normal	.9155	.09821968	20
Denture	.8055	.15981816	20
Cancer	.925	.12690093	14
Total	.87722222	.14022108	54

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	.164143331	2	.082071666	4.77	0.0126
Within groups	.877940063	51	.017214511		
Total	1.04208339	53	.019661951		

```
Bartlett's test for equal variances:  chi2(2) =   4.2577  Prob>chi2 = 0.119
```

Comparison of pr by Category

(Scheffe)

Row Mean- Col Mean	Normal	Denture
Denture	-.11	
	0.037	
Cancer	.0095	.1195
	0.979	0.041

P37

```
. kwallis pz, by(category)

Kruskal-Wallis equality-of-populations rank test
```

category	Obs	Rank Sum
Normal	20	726.50
Denture	20	392.50
Cancer	14	366.00

```
chi-squared =    11.409 with 2 d.f.
probability =     0.0033

chi-squared with ties =    12.512 with 2 d.f.
probability =     0.0019

. oneway pz category, sch
```

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	.20873742	2	.10436871	6.11	0.0042
Within groups	.870810706	51	.01707472		
Total	1.07954813	53	.020368833		

```
Bartlett's test for equal variances:  chi2(2) =   4.2878  Prob>chi2 = 0.117
```

Comparison of pz by Category
(Scheffe)

Row Mean- Col Mean	Normal	Denture
Denture	-.1425	
	0.005	
Cancer	-.094643	.047857
	0.126	0.579

```
.
. set more on

. log close
      name:  <unnamed>
       log:  /Users/Egbon/Documents/Stata/Vivian study3 Virulence.log
```

```
------------+------------------------------------
      Total |   46.037037   19.271607          54

                    Analysis of Variance
    Source              SS         df      MS            F     Prob > F
------------------------------------------------------------------------
Between groups      2311.52593       2   1155.76296      3.39     0.0414
 Within groups        17372.4      51   340.635294
------------------------------------------------------------------------
    Total           19683.9259      53   371.394829

Bartlett's test for equal variances:  chi2(2) =   2.5634  Prob>chi2 = 0.278
```

Comparison of gt by Category (Scheffe)

```
Row Mean-|
Col Mean |     Normal    Denture
---------+--------------------
 Denture |          2
         |      0.943
         |
  Cancer |       15.8       13.8
         |      0.058      0.110
```

```
. kwallis adh, by(category)

Kruskal-Wallis equality-of-populations rank test

  +---------------------------+
  | category | Obs | Rank Sum |
  |----------+-----+----------|
  |   Normal |  20 |   342.00 |
  |  Denture |  20 |   623.00 |
  |   Cancer |  14 |   520.00 |
  +---------------------------+

chi-squared =     15.077 with 2 d.f.
probability =      0.0005

chi-squared with ties =     15.079 with 2 d.f.
probability =      0.0005

. oneway adh category, tab sch

           |          Summary of adh
  Category |        Mean   Std. Dev.       Freq.
-----------+------------------------------------
    Normal |       367.5   317.40635          20
   Denture |       694.8   391.43644          20
    Cancer |   818.71429   286.10338          14
-----------+------------------------------------
     Total |    605.7037   384.07805          54

                    Analysis of Variance
    Source              SS         df      MS            F     Prob > F
------------------------------------------------------------------------
Between groups      1928812.2       2   964406.101      8.35     0.0007
```