

A study at the Brooklyn Chest Hospital to assess the change in the oral carriage of *Candida* species in patients co-infected with HIV and TB, before and after antifungal therapy.

by

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Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Dental Sciences

at the University of Stellenbosch.



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March 2002

DECLARATION

I, JULIAN MARCUS FISHER hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

.....16th.....day of.....August.....2002

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ABSTRACT

The aim of this study at the Brooklyn Chest Hospital (BCH) was to assess the change in the oral carriage of *Candida* species in twenty-nine patients co-infected with the Human Immunodeficiency Virus (HIV) and Tuberculosis (TB), before and after anti-fungal treatment.

Each patient accepted onto the study underwent a comprehensive oral and peri-oral examination where the presence, site and clinical features of all oral and peri-oral lesions were recorded. The purpose of the examination was to provide a clinical diagnosis of oral candidosis.

Each patient was also asked to provide a sample of oral fluid for laboratory analysis. This was collected using an oral rinse. The results of a variety of laboratory investigations were used to identify the species of *Candida* obtained from the oral rinse. Both the oral and peri-oral examination and the oral rinse procedure were repeated after one month and at three months.

A sample from each oral rinse was inoculated on CHROMagar *Candida* chromogenic medium (CHROMagar *Candida*, France, Paris).

CHROMagar is used for the isolation and presumptive identification of *Candida* sp. from other yeasts on the basis of strongly contrasted colony colours, which are produced by the reactions of species-specific enzymes with a proprietary chromogenic substrate.

After forty-eight hours the CHROMagar plate was examined for growth, when a record of colony morphology and colour was made. A single sample from each different colour-coded colony was taken and streaked onto a Sabouraud plate (Oxoid, Basingstoke, England) and then incubated for forty eight hours at thirty-seven degrees centigrade.

A variety of laboratory investigations were subsequently carried out on a single colony taken from the Sabouraud agar plate (Oxoid). The results of these tests were used to identify the individual species of *Candida* isolated from each oral rinse.

Oral candidosis was the most prevalent oral lesion observed on admission and at three months.

Six different species of *Candida* were identified during this study, namely *Candida albicans*, *Candida dubliniensis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*. *C. albicans* was the most commonly identified species in study population. *Candida dubliniensis* was isolated and identified for the first time in a South African HIV population.

Each specimen of *Candida* sp. identified by laboratory analysis was tested for sensitivity to Nystatin, Amphotericin B and Fluconazole anti-fungal agents. An additional sensitivity test was performed using Ajoene and Allicin (extracts of garlic) to assess the comparative antifungal properties of these compounds.

OPSOMMING

Die doelwit van hierdie studie by die Brooklyn Borshospitaal (BCH) was om die verandering in orale draerstatus van die *Kandida* spesies in nege-en-twintig HIV/TB koinfekteerde pasiënte vas te stel, voor- en na antifungale behandeling.

Elke pasiënt in die studie het 'n volledige intra- en ekstra-orale ondersoek ondergaan. Die teenwoordigheid, area en kliniese voorkoms van alle letsels is noteer.

Die doel van die ondersoek was om 'n kliniese diagnose van orale kandidiase te verkry.

'n Monster orale vloeistof is geneem van elke pasiënt vir laboratorium analise. Die monster is in die vorm van 'n mondspoel geneem. Verskeie toetse is gedoen om die verskillende *Kandida* spesies in elke monster te identifiseer.

Die orale- en ekstra-orale ondersoek sowel as die mondspoelmonster is na 1 en 3 maande herhaal. Elke mondspoelmonster is op CHROMagar *Kandida* chromogene medium (CHROMagar *Candida*, France) inokuleer.

CHROMagar word gebruik vir die vermoedelike identifikasie en isolasie van *Kandida* spesies teenoor ander swamme. Dit word gedoen op die basis van kontrasterende koloniekleure, wat teweeggebring word deur spesie-spesifieke ensiemreaksies op 'n chromogene substraat.

Die CHROMagar plate is na 48 uur ondersoek vir groei en die kolonie-morfologie en -kleur is noteer.

'n Enkel monster is geneem van elke verskillende kolonie (geskei op kleur) en is uitgestreep op 'n Saboraud plaat (Oxoid, Basingstoke, England). Dit is dan vir 48 uur inkubeer teen 37°C.

Verskeie laboratorium ondersoeke is daarna uitgevoer op 'n enkel kolonie geneem vanaf die Saboraud agar plaat (oxoid). Die resultate van die ondersoeke is gebruik om individuele spesies van *Kandida* te identifiseer.

Orale Kandidiase was die mees algemene orale letsel geïdentifiseer by toelating en 3 maande ondersoeke. Ses verskillende spesies *Kandida* is identifiseer tydens die studie, naamlik: *Kandida albicans*, *K.dublinskiensis*, *K.Krusei*, *K.glabrata*, *K.parapsilosis* en *K.tropicalis*. *K.albicans* was die mees algemeen identifiseerde spesie in die studiepopulasie.

K.dublinskiensis is vir die eerste keer in Suid-Afrika in 'n HIV⁽⁺⁾ populasie isoleer en geïdentifiseer. Elke monster van identifiseerde *Kandida* spesies is getoets vir sensitiwiteit teenoor Nistatien, Amfotensien B en Flukonasool. Addisioneel is ook getoets vir sensitiwiteit teenoor Ajoene en Allicin (knoffelekstrakte).

Acknowledgements:

I would like to thank the following people and institutions:

- My wife Ilka for her trust, love and encouragement,
- Dr. N Basson, my supervisor for his enthusiasm, patience and guidance during this project
- Prof. A van Zyl for his time and energy, and also for his teaching during the clinical work at the Brooklyn Chest Hospital,
- Professor PR Donald (Dept of Paediatrics, Tygerberg Hospital) and Professor CW van Wyk (Oral and Dental Research Institute) for their support and suggestions during the writing on the project protocol,
- Dr P Morris and the staff of the Brooklyn Chest Hospital for their unstinting help and support,
- Professor Hunter (University of Cape Town) for supplying the ajoene and allicin used in the study,
- The Harry Crossley and Doris Crossley foundation for their financial support,
- a very personal thanks to the patients of the Brooklyn Chest Hospital who took part in the study.

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Chapter 1

INTRODUCTION

The earliest descriptions of human candidosis appears to be that of 'oral thrush', as noted in the writings of Hippocrates (600 BC), Galen (AD130) and later others such as Samuel Pepys (1665).

In 1839 Langenbeck made the connection between oral thrush and a yeast. Further work by Gruby (1842) defined the clinical picture of thrush and its aetiological agent, assigning the organism to the genus *Sporotrichum*. Debate continued with Robin (1853) reassigning it to the genus *Oidium*, where it was first given the name *Oidium albicans*. In the early 1900's Castellani and Chambers suggested that other yeasts might be aetiological agents in the disease process, and reassigned it to the genus *Monilia*. Eventually in 1923 Christine Berkhout gave the name 'candida' to this group so differentiating the 'medical' monilia infections from the plant and leaf rotting moulds (as reviewed by Odds)(1).

Candidosis* has gained prominence in the later half of this century, particularly with emergence of the HIV/AIDS epidemic in the 1980's (2,3,4,5). A greater understanding of Candidosis and its causative organisms, combined with more sensitivity diagnostic investigations (for example computed tomography, polymerase chain reaction and immunoprecipitation) have lead to a rise in the incidence of patients diagnosed with the disease. The prevalence of oral candidosis in patients with the HIV infection ranges from 43–93% (6,7).

HIV patients are also vulnerable to opportunistic infections such as tuberculosis, where both the immuno-suppression and drug therapy further increase the risk of candidal infections. Advances in drug therapy have been responsible for the remarkable success in oncology and organ transplantation therapies (8). Management of these complex drug regimes frequently requires patient hospitalisation. Researchers have reported a dramatic increase in susceptibility to oral and systemic candidosis in the nosocomial environment (6,9,10,11,12).

Studies found that AIDS patients who fail to respond to standard antifungal therapy for oro-pharyngeal candidosis show a median survival of 184 days after the onset of fluconazole resistant pseudo-membranous oral candidosis, and only 83 days after the onset of clinical resistance to Amphotericin B. Although oral candidosis is not a direct cause of death, evidence suggests that failure of antifungal treatment is most probably a co-morbidity factor in the rapid demise of AIDS patients.

*Oral candidosis is the collective name given to a group of clinical disorders caused by the yeast *Candida*. The term 'Candidosis' is adopted in this text rather than candidiasis, as the suffix 'osis' is consistent with the ending used in the vast majority of fungal infections, for example histoplasmosis, aspergillosis.

Candida species are generally regarded to be normal commensal micro-organisms of the oral cavity (13). *Candida* species can also be opportunistic pathogens (9,14,15,16,17). With the advent of HIV infection there has been renewed interest in the pathogenesis and sequelae of fungal infections, particularly in South Africa where oral and/or pharyngeal candidosis is present in a high percentage of HIV/AIDS patients (18). Although *C.albicans* has been cited as the most common pathogen in oral candidosis, there is growing evidence that non – *C.albicans* species, including *C.krusei* and *C.glabrata*, play a significant role in oral disease (19,20,21-25). In 1995 a new species, *Candida dubliniensis*, was identified (26,27,28). This species shares many phenotypical characteristics with *C.albicans*, which may account for its earlier misidentification (29,30,31,32). *C.dubliniensis* appears to be a clinically important species in HIV/AIDS patients (33,34,35,36). It has the potential to initiate oral disease, perhaps acting as an independent or symbiotic pathogen (37,38,39). *C.dubliniensis* is susceptible to existing anti fungal drugs but possesses the ability to develop stable resistance to fluconazole following direct exposure to the drug *in vitro* (40,41,42).

While *C. dubliniensis* has been isolated in Argentina, Australia, Brazil, Europe, Israel, North America and Switzerland, it has not been identified in Africa (29,43,44).

An extensive amount of research has been conducted into the incidence and prevalence of HIV infection / AIDS associated oral lesions (45). A review of the literature indicates that there has been no documentation or investigation of the oral cavity of HIV/AIDS and TB co-infected patients. The aetiology of any oral lesion in this patients group is likely to be extremely complex.

Chapter 2

Literature review

2.1 The genus *Candida*

The *Candida* species are classified as Fungi Imperfecti (Deuteromycetes) as the perfect or sexual phase of their reproduction has not been discovered (46). Fungi are eukaryotic, heterotrophic organisms that possess a rigid cell wall but lack chlorophyll. There are two basic morphological forms: yeasts and moulds. Yeasts are unicellular structures that reproduce by budding and less commonly internal subdivision of their cytoplasm. Moulds are multinucleate and grow in long filamentous patterns. These filaments are called hyphae and are subdivided by cross walls known as septae. *C.albicans* and *C. dubliniensis* also form a third morphological form called pseudohyphae. As with the yeast form, the pseudohyphae appendage arises by budding, with a characteristic stricture between mother and daughter cells. This appendage continues to elongate, giving the appearance of a hyphal form. Nutritional factors play the most important role in determining which of the three morphologies of *C. albicans* and *C. dubliniensis* is generated.

C. albicans and *C. dubliniensis* also possess a second quality that enables them to be distinguished from other *Candida* species. They are able under certain conditions to produce chlamydo spores. Chlamydo spores are spherical structures that form at the tip of the hyphae and appear when the organism grows in a nutrient poor environment (47).

Candida sp. have been reported in almost every tissue of the human body, living in the moist, warm areas i.e. for oral cavity, vagina, gastrointestinal tract and skin folds. Studies that have investigated the prevalence of *Candida* sp. in the general population have produced variable results due to different sampling techniques used and study populations investigated. (1,48,49). Healthy individuals appear to have a lower carriage of *Candida* sp. than those with predisposing conditions (18,50). Approximately 17 to 58% of hospitalised patients carry *Candida* sp. on their hands (51).

Although more than 150 species of *Candida* sp. have been identified only a relatively small number are human pathogens. Candidoses can be divided into superficial and systemic infections. Systemic candidoses are relatively uncommon, but have serious consequences (52). Superficial candidoses can affect the mucosa of the oral, vaginal and aural cavities, the genitalia, skin and nails.

C.albicans is the most prevalent yeast and commonly identified fungal pathogen in humans. Other species isolated from the human body included *C.dubliniensis*, *C.glabrata*, *C.krusei*, *C.guilliermondii*, *C.tropicalis*, *C.parapsilosis* and *C.kefyr* (previously *Candida pseudotropicalis* (17,19,20,26,53).

2.1.1 *Candida dubliniensis*

A new species of *Candida*, *C.dubliniensis* was identified in 1995 (26). The earliest isolate now known to have been *C. dubliniensis* was recovered from a post mortem lung specimen from a patient who died in the UK in 1957. This strain was misidentified as a strain of *Candida stellatoidea*. In the late 80's and early 90's atypical *Candida* isolates were identified in HIV and AIDS patients in Australia, Ireland and the United Kingdom. In 1995 it was decided that these isolates represented a new species of *Candida*. This new species (*dubliniensis*) was named after Dublin the capital of the Republic of Ireland where it was first proposed (26). Since then isolates have been reported in Argentina, Australia, Belgium, Canada, France, Finland, Germany, Greece, Ireland, Spain, Switzerland, Brazil, Israel the UK and the United States of America (27,28,29,33,43,44).

C.dubliniensis is a chlamydospore and germ tube positive yeast and was initially associated with HIV positive and AIDS patients (18,30,33,34,35). *C.dubiniensis* has also been recovered from HIV negative individuals with clinical signs of oral candidosis (54). *C.dubliniensis* is frequently isolated with other *Candida* species, the most common being *C.albicans*. Research results indicate that *C. dubliniensis* is a minor component of the normal oral microflora of humans. Studies from the USA have isolated *C.dubliniensis* in systemic candidosis (55).

Studies have demonstrated that *in vivo* populations of *C.albicans* can include a large number of subtypes, which differ in their relative susceptibility to antifungal agents. Exposure to fluconazole *in vitro* can give rise to stable resistance to this anti-fungal agent but such resistance has not been convincingly demonstrated *in vivo* (56). The development of such resistance may depend on the dosage of drug administered, the duration of therapy, or on the immune status of the patient. *C.dubliniensis*, unlike *C.albicans* possesses the ability to rapidly develop stable resistance to fluconazole following direct exposure to the drug *in vitro*. An Irish study has reported that *C. dubliniensis* encodes multi-drug transporters which facilitate efflux of fluconazole from the cell via specific multi-drug transporters (MDR1). However strains of *C. dubliniensis* that demonstrated reduced susceptibility to fluconazole remained susceptible to Amphotericin B and 5FC (40,41).

2.1.2 *Candida glabrata*

Until the early 1980's *C.albicans* was regarded as the only human pathogen of this genus. With the advent of the HIV infection and widespread use of immunosuppressive therapy, there has been an increase in the number of infections caused by species other than *C.albicans*. These *non-Candida albicans* species are reported to account for the dramatic rise in nosocomial transmission and candidal infections in immuno-suppressed patients (57-62). Several species that were regarded as non-pathogenic are now recognised as human pathogens (19,34,57,63-5).

The generally non-pathogenic nature of *C.glabrata* in animals would suggest it has few virulence factors. The high paediatric mortality rate and the rapidity with which candidosis caused by a *C.glabrata* is communicated within hospitals, indicates that it is an important human pathogen (19).

2.1.3 Distribution of yeasts in the mouth.

Many studies have attempted to report on the prevalence of *Candida* sp. in humans. Variations in the parameters used by the different investigators such as patients selection, sampling techniques and the technique of the operator, have not permitted proper comparison and interpretation of the results (1,48,49).

Yeasts are acquired in several ways; person to person contact, for example from a mother to baby at birth, from the environment to the individual, or from one body site to another (13,14,51,66). Colonisation of the oral cavity by *Candida* sp. is low during the first few days of life, then increases over the next eighteen months to a peak at two years after birth (13). Thereafter the carriage rate falls during childhood but appears to rise again later in life.

The oral carriage rate for *Candida* sp. in healthy individuals varies from between 2–71 %, where again sampling methodology and identification techniques play a significant role in quantification (1,48,49). Individuals sampled in hospital while attending for treatment or diagnosis of disease tended to show consistently higher values of *Candida* sp. carriage compared to their healthy counterparts (18,50,67).

There is broad agreement as to the distribution of *Candida* sp. in the healthy oral cavity. *C.albicans* is the single most commonly isolated species both in disease and in health, with *C. glabrata* and *C.tropicalis* next most frequently isolated group, and *C.parapsilosis*, *C.guilliermondii*, *C.krusei* and *C.kefyr* infrequently isolated.

Studies using the imprint sampling method have yielded results of 10–20 colonies per cm². The number of *Candida* cells per millilitre of oral fluid in normal healthy carriers ranges from 200 – 600 CFU / ml (49).

In dentate individuals yeasts are most commonly isolated on the dorsum of the tongue. The buccal mucosa, floor of the mouth and angles of the mouth are less common sites for yeasts.

Very little work has been done on *Candida* sp. distribution in patients infected with the HIV, or with other immune-compromised states. A study found that gingival biopsies from HIV sero-positive patients with gingivitis demonstrated the presence of pseudohyphae of *Candida* sp. in the parakeratinised oral epithelium (68). The sero-negative group showed no evidence of *Candida* sp. invasion.

Candida dubliniensis has been implicated in the aetiology of linear gingival erythema, which is found in patients with the HIV infection (37).

Although the presence of *Candida* sp. in dental plaque is contentious, one study suggested that *C.albicans* can invade dental hard tissue and thus act as a potential reservoir for disseminated candidal infection (69).

2.1.4 Morphogenesis of *Candida* sp.

Candida sp. grow aerobically in a temperature range of 20 to 47°C with an optimum growth at 37°C. The pH range for *Candida* sp. growth is between 2-8. Both *C.albicans* and *C.dubliniensis* respond to changes in the environmental pH with a change in cell shape and differential gene expression (70).

Candida sp. can exist as yeasts where they replicate by budding or as a hyphal form (46). It was previously thought that the yeast form was primarily seen in the commensal state whereas the presence of hyphal and pseudo-hyphal forms indicated an infectious state (71). *C.glabrata* only exists in yeast form and is now known to act as a human pathogen (19).

C.albicans exhibits several different morphological forms under different environmental conditions, including budding yeast cells (blastospores), pseudohyphae, true hyphae and chlamydospores. The yeast form is favoured at temperatures below 33°C, where as at elevated temperatures and near neutral pH mycelial growth is favoured. One study found that commensal and transient oral bacterial populations might selectively influence the differential expression of germ-tube-forming ability of *C.albicans* isolates (72). Chlamydospores are special structures at the tips of hyphae and appear under poor nutrient conditions (73). Pseudohyphae are encountered *in vivo* and *in vitro*. The morphogenic form of *Candida* sp. appears to be principally determined by alterations in cell metabolism.

Candida species are differentiated by colony morphology, colour on differential media, as well as carbohydrate assimilation and fermentation patterns of the species (46,47,74,75). Newer technology has allowed for more accurate identification (76,77,78).

2.1.5 *Candida* cell wall and antigenic structure

The structure and composition of the cell wall is an important feature, as it is involved in adhesion and colonisation of epithelium and mucosa (79,80). It is composed of beta-glucans, which are thought to provide its structural integrity, mannoproteins and a small quantity of chitin (81-4). Similar proportions of these polymers are found in yeast and mycelial states. Transmission electron microscopy has demonstrated that the wall is composed of five distinct layers with some species having an outer fibrillar layer. The main antigenic component of the cell wall is the mannoprotein fraction (79,85).

C.albicans possess a 'fuzzy' coat and the fibrillar layer is contained within this coat. This fuzzy coat is believed to be important in the overall virulence of the organism by affecting adherence and phagocytosis (86,87). Another important feature of the cell surface of *C.albicans* is the presence of receptors for the complement fragment C3b. *C.albicans* binds Cb3 non-covalently, impairing the phagocytic uptake of the yeast by human polymorphonuclear leucocytes (88).

Antibodies against certain cell surface antigens of *C.albicans* help the host resist disseminated candidiasis (89). The cell wall also offers targets for anti fungal agents; the beta-glucans and chitin of the cell wall are not present in the human host and therefore could offer potential targets for future generations of antifungal medication.

2.2. Virulence factors of *Candida sp.*

An increasing number of virulence factors are being identified in *Candida sp.* infections of the oral cavity.

2.2.1 Germ tube formation and contact sensing

Germ tubes, which mark the onset of hyphal growth of *C.albicans*, are suspected of being involved in the pathogenesis of candidosis (90,91). Besides *C.albicans* and *C.dubliniensis* germ tubes have also been observed in strains of *C.tropicalis*. In the case of minor immunosuppression the expression of virulence factors for adherence and germ tube formation plays an important role in the pathogenesis of candidosis (92).

Deeper penetration of keratinised epithelia is assisted by hyphal formation and by the presence of hydrolytic enzymes, which are located in the tips of the hyphae (93). *C.albicans* may use contact sensing (thigmotropism) as a guiding mechanism (94).

A hyphae-specific surface protein Hwp1 isolated from *C.albicans*, which has similarities with the mammalian small proline rich proteins, has been shown to serve as a substrate for mammalian transglutaminases. Researchers found that *C.albicans* strains, which lack the protein Hwp1, are unable to form stable attachments to human buccal epithelial cells. In mice, these strains have a reduced capacity to cause systemic candidosis (95).

2.2.2 Adhesion of *Candida sp.* to mucosa

There is good evidence that adherence of the fungus to the surface of the oral mucosa is a critical prerequisite for permanent colonisation or infection of a site (96-9). This is certainly true for the oral cavity, where the mechanical washing by oral fluid and the action of the oral musculature are very powerful defence mechanisms. Adherence values do appear to vary between individuals. There is evidence of a daily variation in the attachment affinity of the same yeast cell to human buccal epithelial cells. Fluctuations in the level of certain hormones also appear to affect the adherence of *C.albicans* to human buccal epithelial cells (100).

Research investigating HIV positive individuals appears to indicate that there is an association between the HIV infection and the selection of *C.albicans* strains, which have an increased ability to adhere to oral mucosa (101-3). A study found that the use of anti-bacterial medication and zidovudine were associated with increased adhesion of *Candida sp.* to buccal epithelial cells (101). These findings may explain the high levels of oral *Candida* carriage in HIV / TB co-infected patients.

Studies on paediatric burns patients reported no correlation between *C.albicans* adhesiveness and the site of isolation (autographs, blood, faeces,

throat swabs, tracheal aspirates, wounds, intravenous catheters) although isolates from catheters were generally less adhesive to epithelial cells (104). The same study using a model of a systemic candidal infection in mice found no correlation between the virulence of isolates and the biotype, serotype or the site of isolation (104).

C.krusei is emerging as an important pathogen of oral candidosis in immunocompromised patients. In a comparative study *C.krusei* showed significantly higher hydrophobicity than *C.albicans* isolates. The researchers noted a correlation between the surface hydrophobicity of *C.krusei* and its adhesion to epithelial cell surfaces (105).

Glycosides containing L-fucose or N-acetyl-D-glucosamine can act as epithelial receptors for *C.albicans* (106). In insulin-using diabetic patients the adhesion of *C.albicans* was influenced by the availability of sugars in the growth medium, as well as the strain of *C.albicans* present. In these patients palatal epithelial cells appeared to retain significantly more *C.albicans in vivo* (107).

2.2.3 Adhesins

Candida sp. adhesion molecules appear to be of three general types. In one type the protein moiety of a surface glycoprotein binds to arginine-glycine-aspartate sequences common to fibronectin, vitronectin, collagens, laminin and other extracellular glycoproteins (80). In the second type the protein moiety of a surface glycoprotein binds in a lectin-like manner to a sugar portion of host membrane glycoproteins. In the third and least well understood, the polysaccharide moiety of a *Candida sp.* surface mannoprotein binds to unknown host receptors (84).

Several potent adhesins are associated with the fibrillar coat and fimbriae of fungi (86,108). Mannoprotein appears as a component of the fibrillar outermost layer of the fungal cell wall (99,109). CaMNT1 is a gene involved in the synthesis of mannoproteins. This gene encodes for an enzyme required for the correct mannosylation of proteins that appear to be critical for adhesion of *C.albicans* (85).

Studies that have examined the nikkomycins (chitin synthetase inhibitors), demonstrated that *C.albicans* adhesion to buccal epithelial cells shows reduced adhesion and changed fungal morphology in the presence of these agents. This points to involvement of chitin in the adhesion of *C.albicans* to buccal epithelial cells (110).

In order to approach and to bind to mucosal epithelial cells *Candida sp.* must traverse the overlying mucus layer. *C.albicans*, *C.dubliniensis* and *C.tropicalis* all adhere well to mucin. *C.parapsilosis* and *C.lusitaniae* are moderately adherent, while *C.glabrata* and *C.krusei* are weakly adherent to mucin. Adherence of *C.albicans* to buccal epithelial cells is quantitatively inhibited by graded concentrations of mucin (111). A study found that *C.albicans* may adhere to and enzymatically degrade mucins through the action of secretory aspartyl proteinase. These properties may act to modulate *Candida sp.* populations in the oral cavity and gastrointestinal tract (111).

2.2.4 Propensity to switch phenotype

When grown *in vitro* *C.albicans* can exhibit a switch from smooth white colonies to other variant forms such as the star-shaped, opaque or rough/wrinkled colony forms. This ability is called phenotypical switching (112-4). Under certain conditions both *C.albicans* and *C.dublinsiensis* are able to switch colony phenotype (115). A variety of environmental conditions including a change in pH and low dose UV light will lead to differential gene expression and expression of variant colony form.

C.tropicalis and *C.glabrata* are also known to possess this type of switching potential.

A correlation exists between the phenotypic characteristics of the fungal strain and the adhesion potential of the blastospore to buccal epithelial cells. *Candida* strains that exhibit rough or coarse fringes demonstrated a greater potential for adhesion (86,116).

Phenotypical switching may enhance the pathogenic potential of *C. albicans* increasing its capacity to invade and proliferate in the different body environments. *C.albicans* strains isolated from deep-seated infections appear to display a higher frequency of phenotypical switching than strains isolated from more superficial infections (117).

2.2.5 Surface hydrophobicity

Surface hydrophobicity is a non-specific factor that can govern mutual adhesion of cell types via van der Waal forces. The exact role that hydrophobicity plays in *Candida* sp. virulence is poorly understood, but there is a strong correlation between cell surface hydrophobicity and adhesion (118). *Candida* sp. cells with hydrophobic surfaces are more adherent than those with hydrophilic surface areas (119). Hydrophobic interactions are involved in the adherence of *C.albicans* and *C. parapsilosis* to buccal epithelial cells and acrylic surfaces (120). *C.dublinsiensis* appears to lack the cell surface hydrophobicity entities, which could account for its limited ability to cause disseminated infections (121).

The degree of hydrophobicity of the different strains of *C.albicans* is determined by a variety of factors including temperature, the phase of growth and changes in the surface exposure to cell wall proteins (122). Anti-mycotics are believed to reduce cell surface hydrophobicity.

2.2.6 Lytic enzymes

The majority of candidal proteinases are glycoproteins of approximately 45 kDa molecular mass and are related to pepsin, rennin and cathepsin D. Fungal proteinases degrade serum albumin into small fragments and this indicates low substrate specificity. Very few proteins were found to resist degradation by candidal proteinases at acidic pH. All the noted enzymic effects of candidal proteinases were found to require a low pH. Physiological pH at 37°C inhibits the activity of candida proteinases and causes alkaline denaturation. *Candida* sp. isolates that are antifungal resistant produce more proteinases than antifungal sensitive isolates (123).

Secreted aspartic proteinases (SAPs) are important virulence factors involved in adherence mechanism of some *Candida* sp. to the oral mucosa

(124,125,126). Aspartic proteinase is either secreted by *C.albicans* or present in the cell wall and is able to hydrolyse secretory IgA, complement factor 3, albumin and keratin. Secreted aspartic proteinases facilitate tissue penetration and may possess auxiliary roles as adhesins acting singly or synergistically to enhance the adhesiveness of *C.albicans* (125). Invasive isolates of *C.albicans* secrete higher concentrations of proteinases and adhere more readily to mucosal surfaces (127,128).

Pathogenic *Candida* sp. also secrete membrane damaging phospholipase A, B and C (129). Studies have found that phospholipase-producing strains adhere most strongly to epithelial cells. The production of phospholipases by *Candida* sp. correlates with pathogenicity and is predicative of mortality in animals (130). These phospholipases are produced by virulent strains but not commensal organisms.

A study of the pathogenic traits of *C.parapsilosis* isolates found a relationship between the phosphatase activity, as measured with p-nitrophenol phosphate, and the adhesion of the yeasts to the buccal epithelial cells. The research implied that both alkaline and acid phosphatases of *Candida* sp. might play a crucial role in potentiating the virulence of *Candida* sp. (131).

2.2.7 Mitogen Activated Protein Kinases (MAP kinases)

MAP kinases in eukaryotic cells are important for the transduction of a variety of extracellular signals that regulate cell growth and differentiation. MAP kinases homologous to yeast Fus3/Kss1 MAP kinases have been identified in several fungal pathogens and found to be important in fungal pathogenesis i.e. invasive hyphal growth, as well as differentiation and survival (132).

2.3 Host factors and oral candidosis

2.3.1 Predisposing factors for *Candida* infection

The specific oral profile of *Candida* sp. in a healthy individual may be modified by a variety of host factors including nutritional state, presence of disease, use of medication, and the composition of the oral environment (133). Malnutrition, a carbohydrate rich diet, vitamin and iron deficiency can all contribute to the development of candidosis (1,134). The use of antibiotics is an important factor in increasing the risk of developing *Candida* sp. infection (135,136). Broad-spectrum rather than narrow spectrum antibiotic therapy leads to *Candida* sp. overgrowth and is a predisposing factor for deep candidosis (137-139). The weight of available data implies that although antibiotics can influence the oral carriage of *Candida* sp. the extent of affection would appear to be dependant on additional local and host factors, together with the strain of *Candida* sp. in question (140-145). The basic mechanism by which antibiotics enhance candidal growth is by reducing the commensal bacterial population.

Local host factors of the oral cavity are crucial determinants of *Candida* sp. survival, and for the development of the disease state. These factors include the integrity of the oral mucosa, the expression of micro-structural protein receptors of the oral epithelial cells, anti-candidal factors, the glucose concentration of oral fluid, other competitive oral microflora, the pH and the

temperature of the oral cavity. Trauma and existing infections may contribute and precipitate the development of candidosis. For example super-infection with *Candida* sp., which occurs in severe burn injuries and in superficial infections caused by *Staphylococcus aureus*, or the *Herpes* virus (146-48). Pregnancy, oral contraception, some spermicidal preparations (Nonoxynol-9), tight fitting stockings, and hormonal changes are all reported as being risk factors for the development of vaginal candidosis, a common affliction of women (109,149-153).

A fully developed and intact immune system is critical for protection against *Candida* infection. Neonates who have an immature immune system are prone to developing *Candida* sp. infections (154-157). The importance of the immune system with regard to *Candida* sp. is illustrated in patients with defective immune systems, such as those with certain endocrine disorders and genetic immune defects, who suffer from chronic mucocutaneous candidosis (1).

Candidosis has been described as the "disease of the diseased", as it frequently occurs in patients with debilitating illness such as diabetes mellitus, AIDS and haematological malignancies (158-160).

2.4 Host response

The initial host defences are the non-specific barriers including intact skin and mucosal epithelium, as well as indigenous bacterial flora that compete for binding sites. The body is protected from microbial invasion by a variety of factors, two of the most important anti-candidal defences are epithelial proliferation and the cellular immune response. Observations and histological examination of the epidermal proliferation encountered in chronic mucocutaneous candidosis shows massive hyperkeratosis at a rate equal to or greater than the rate of fungal penetration of the epidermis (161). There is little evidence to support that this proliferation occurs during *Candida* sp. invasion of the vagina or oral mucosa (162).

Evidence indicates that cell-mediated immunity is a key factor in the primary host defence of superficial candidosis, whereas neutrophils and possibly macrophages are important in deep candidosis (163-4). The evidence for the importance of cellular immunity in defence against mucosal and cutaneous *Candida* sp. infections comes from observations of patients with definable T-cell defects (165). Patients with AIDS often suffer from oral candidosis but less commonly have deep *Candida* sp. infections, while patients with haematological and other malignancies who become neutropenic during chemotherapy are at more risk for deep *Candida* sp. infections (166-9). Langerhans cells may play a role in the generation of T-lymphocyte responses to cutaneous *Candida* sp. infection (170).

Various aspects of cell-mediated immunity involved in oral candidosis and chronic mucocutaneous candidosis have been examined. Defects in cell-mediated immunity to *Candida* sp. antigens (as manifest by negative delayed hypersensitivity reactions) and abnormal *in vitro* assays of lymphocyte function (showing impaired cytotoxic or migration inhibition factor responses)

support the important role cell-mediated immunity plays in these types of candidal infection.

The major cell surface antigen of *Candida* sp. is mannin (171). *C.albicans* is a constituent of the normal gastrointestinal flora and the majority of humans will show evidence of low levels of antibodies to *Candida* sp. mannin. In infected patients, elevated levels of *Candida*-specific antibodies have been found representing the IgG, IgM and IgA immunoglobulin classes. Secretory immunoglobulin A (sIgA) levels in oral fluid are elevated in *Candida* sp. stomatitis. It is produced in response to *Candida* sp. antigen and protects against mucosal colonisation (172-3). The role of *Candida*-specific antibodies in host defence against disseminated candidosis is unclear, as patients who develop disseminated candidosis frequently have *Candida*-specific antibodies in their serum (174).

Although antibodies titres to various *Candida* sp. antigens are raised during infection, it has been demonstrated that the humoral response does not appear to play an important role in host defence against *Candida* sp. infection.

2.4.1 Pathogenesis of oral candidosis

A range of different host and fungal factors play a critical role in the pathogenesis of *Candida* sp. infection. Initial colonization will be determined by the surface adhesive components of the fungus as well as local host defences and competing oral microflora. Researchers suggest that unconventional immune cells have some role in host defence (170). Epithelial cells inhibit the growth of *C.albicans* and endothelial cells are able to phagocytose *C.albicans*. *C.glabrata* does not appear to induce endothelial cell phagocytosis, which suggests that endothelial cell phagocytosis may be species-specific or restricted to *C.albicans* alone. Keratinocytes are capable of phagocytosing *C.albicans* cells (175). Other cell lines, monocytes and eosinophils, are also capable of anti-candidal activity but play a far smaller role. Human natural killer cells are non-phagocytic large granular lymphocytes, which are cytotoxic to certain tumour cell lines and to virally infected cells. Although large granular lymphocytes themselves do not have any antifungal activity, studies have reported when stimulated by *C.albicans* these cells secrete a potent activator of polymorphonuclear leucocytes.

Research suggests there is a complex relationship between *C.albicans* and natural killer cells.

The major cellular defence mechanism against *Candida* sp. consists of phagocytosis by polymorphonuclear leucocytes and macrophages (176). Polymorphonuclear leucocytes (PMNL) are the cells of foremost importance in this initial response (177,178). The phagocytosis and intracellular killing of yeast cells is enhanced by the presence of opsonins, with opsonised cells ingested by phagocytes at a faster rate than non-opsonised cells (179-81). Neutrophilic granulocytes themselves may allow yeast phase *C.albicans* to evade intracellular killing. The internal milieu of the phagosomes is acidic and therefore competition between acid fungal hydrolases and acid hydrolases of the phagosome may determine the outcome of the phagocytosis.

There are two mechanisms by which PMNL can destroy *Candida* sp. One is mediated by the myeloperoxidase-hydrogenperoxide-halide system and the second is still as yet unidentified (182-3). Researchers demonstrated the myeloperoxidase-hydrogenperoxide-halide system in PMNL could be initiated by *Candida* sp. mannan (184). PMNL's exert a fungistatic effect on yeast cells (185). This effect is seen when PMNL's lyse in areas of dense infiltrates of yeast cells. A fungistatic cytoplasmic protein is released during the lytic process, which may possibly control the infection and prevents further spread. PMNL's can be considered to be equally efficient against both *C. albicans* and *C. dubliniensis* (176).

Macrophages adhere to the mannose residues of *Candida* sp. via their mannose receptor (186). This process is calcium dependant. Phagocytosis by the macrophage stimulates the production of H₂O₂ and oxygen radicals and the killing of ingested *Candida* organisms (187).

As well as phagocytosing blastospores macrophages are also capable of damaging non-phagocytosable pseudohyphae and hyphae (188). Although the method of destruction is not fully understood, it appears that this mechanism may play a role in the destruction of pseudo-hyphae and hyphae that are too large to be phagocytosed. This hypothesis is supported by the observation that macrophages are able to differentiate between yeasts and hyphae and respond to each selectively (189). In addition macrophages may further be stimulated by myeloperoxidase released by PMNL's, to produce more reduced oxygen derivatives and induce cytokine production (190).

T-cell enhancement is important for the body to mount an effective immune response (191-2). Unstimulated phagocytes (PMNL's and macrophages) provide a relatively ineffective immune response, eliminating approximately 20-30% of ingested *Candida* (193). The immune response may be enhanced via two mechanisms; a. T-cell-independent route, or b. T-cell dependant route.

a. T cell independent route:

Macrophages can be activated by myeloperoxidase, which is released into the environment by PMNL's (194-5). Anti-*Candida* macrophage activation has been observed with *Candida*-mannan, avirulent *C. albicans* strains and other microbes (196-99). Studies have found that activated macrophages produce more reduced oxygen derivatives, induce cytokine production and destroy higher numbers of *Candida* organisms (190).

b. T cell dependant route:

Candida sp. adherence induces mucosal cells to presentation of MHC II class antigens (DQ and DR)(200). These cells are phagocytosed by macrophages, which present an antigen to T-lymphocytes and possibly Langerhans cells. Activated T-cells release cytokines, interferon-gamma and tumour necrosis factor-alpha (TNF-alpha). Interferon gamma activates macrophage production of tumour necrosis factor alpha, which in turn activates PMNL's to enhanced killing of *Candida* sp. (201). The concurrent release of cell-growth stimulatory factor (C-GSF) also induces the production of more leucocytes in the bone marrow (269).

Research using murine models have demonstrated that colonisation by *C.albicans* induces a T-helper-1 (Th1) or T-helper-2 (Th2) lymphocyte response (203-06). Studies on mice have shown that Th-1 type response characterized by cytokines interleukin-2, Interferon gamma and interleukin-12 is associated with protection against systemic infection whereas Th-2 type responses characterized by cytokines interleukin-4, interleukin-5 and interleukin-10 and antibody production, IgA and IgE is associated with susceptibility to systemic *Candida* sp. infections.

2.4.2 Inhibition of the immune system

Inhibition of parts of the immune defences of the host by *C.albicans* has been observed by a number of researchers with regard to chronic mucocutaneous candidosis.

A polysaccharide fraction of *C.albicans* has been shown to inhibit the proliferation of human T-lymphocytes and the production of interleukin-1 and 2.

2.4.3 Prostaglandins

Enhanced prostaglandin production during fungal infection could be an important factor in promoting fungal colonization and chronic infection (207). Host cells are one source of prostaglandins, however another potential source of prostaglandins is the fungal pathogen itself.

Most cells *in vivo* seem to produce prostaglandins, most commonly prostaglandin E2 (PGE2) and prostaglandin F2alpha. Both these prostaglandins play a part in inflammation by intensifying the effects of chemical mediators of inflammation such as histamine. A study isolated a PGE series lipid from *C.albicans* and *Cryptococcus neoformans*, which was biologically active on both fungal and mammalian cells. The fungal PGE (x) enhanced the yeast to hyphae transformation in *C.albicans*. In mammalian cells fungal PGE (x) down regulated chemokine production, TNF alpha production and splenocyte production while up-regulating interleukin-10 production.

Prostaglandins-like compounds are produced only by pathogenic fungi, which are critical for the growth of the fungi and can modulate host immune functions (207).

2.4.4 Interference with complement (C)

The protection mechanism of complement appears to be associated with enhanced phagocytosis and killing of the fungus even in the presence of the HIV infection (208-09). The presence of the HIV infection may promote fungal virulence by inducing hyphae formation, but may also be active in reducing virulence by augmenting phagocytosis (210-11).

2.5 Clinical presentation and classification of oral candidosis

Clinically the oral candidal lesion can present in a variety of forms. In 1966 Lehner proposed a concise classification for oral candidosis. However with the advent of the HIV infection and the accompanying increase in research on

Candida, various authors have suggested modifications to his work. Holmstrup and Bessermann in 1983 suggested a clinico-pathological classification, which would for instance more accurately distinguish between erythematous and atrophic forms of oral candidosis.

Candida sp. can produce a wide variety of human infections but can broadly be classified into superficial and deep infections. Superficial candidosis involves the skin, nails and mucous membranes of the oral cavity and the vagina. Deep candidosis include candidaemia, localised infections of various deep tissues and disseminated candidosis in immuno-suppressed patients. This classification is significant as the host defence involved in the two types of infection may be different.

2.5.1 Classification of oral candidoses

Classification taken from Handbook of Oral Disease by C.Scully (1999)

<u>Acute/chronic</u>	<u>Current nomenclature</u>
Subgroup	
Acute	Acute pseudomembranous
Chronic	Chronic pseudomembranous
Acute	Acute erythematous
Chronic	Chronic atrophic (denture-induced sore mouth)
Chronic	Chronic mucocutaneous
Chronic	Chronic hyperplastic
Acute/chronic	Angular cheilitis

In 1962 Newton proposed that chronic atrophic lesions of the palate could be further categorised:

- Newton's type 1: pin-point lesions- Localised simple inflammation
- Newton's type 2: diffuse erythema - Generalised simple inflammation
- Newton's type 3: granular - Papillary hyperplasia

In 1985 Ohman suggested expanding on the subdivisions of angular cheilitis to define the degree of clinical involvement including the depth and number of folds (rhagades)

- type 1 : single fold or rhagades limited to the corner of the mouth.
- type 2 : the lesion is more extensive in depth and length than type 1 lesions

- type 3 : several fissures / rhagades radiating from the angle of the mouth into the adjacent skin with the skin redness limited to the vicinity of the rhagades
- type 4 : no rhagades evident but extensive erythematous area of the skin adjacent the vermillion border

The pseudomembranous form of candidosis is prevalent in patients who are infected with HIV or diagnosed with AIDS. This form of candidosis, if left untreated may persist for several months, so can be classified as a chronic form of the disease. The erythematous form of oral candidosis may be related to sloughing of pseudomembranous lesions or may be a separate entity. It appears that the erythematous form may affect any part of the oral cavity.

Linear gingival erythema is a clinical description for a prominent well-demarcated, fiery red band that appears to follow the limits of the physiological sulcus common in patients infected with HIV or in patients with AIDS. Recent research has implicated *Candida* sp., in particular *C. dubliniensis*, in the aetiology and pathogenesis of this condition.

Candida oesophagitis is a diagnostic characteristic for AIDS, presenting as thick tan covered plaques, which can extend towards the gastro-oesophageal junction.

Another characteristic feature of oral candidosis in AIDS patients is that different forms of the disease can appear at different sites in the oral cavity at the same time. These lesions can also be of a chronic nature often termed chronic multi-focal oral candidosis.

2.6 Diagnosis and Laboratory identification techniques

Oral candidosis can be diagnosed from the patient history and clinical picture alone. However a clinical diagnosis is best augmented with a laboratory tests specific for fungal identification.

There are a variety of methods for isolating yeasts from oral samples:

- Smear – direct microscopy smears taken from lesional tissue (phase contrast or PAS stain techniques).
- Swab – obtained by rubbing a sterile cotton-tipped swab over lesional tissues. *C.albicans* can survive at least 24 hours on a moist swab without loss of viability, however rapid transportation to the laboratory decreases the risk of desiccation.
- Imprint culture technique – Sterile plastic foam pads are dipped in Sabouraud's broth and then applied to the surface under investigation for 60 seconds. This is a reliable and sensitive method of sampling.
- Impression culture technique – this technique involves taking maxillary and mandibular alginate impressions, transportation to a laboratory, for casting in 6% fortified agar with incorporated Sabouraud's dextrose broth. The agar models are then incubated in a wide necked sterile screw topped jar for 48-72 hours at 37° C and the colony forming units of yeasts estimated.

- Salivary culture technique – involves requesting the patients to expectorate 2ml of mixed unstimulated saliva into a sterile universal container, which is then vibrated for 30 seconds to achieve adequate disaggregation. The number of *Candida* is estimated by counting the resultant growth on Sabouraud's agar and expressed as colony forming units/ml of saliva.
- Oral rinse technique – consists of requesting the patient to rinse the mouth for 60 seconds with a quantity of phosphate buffered saline, which is returned into a universal container. A small sample is then plated onto Sabouraud medium and the growth expressed as colony forming units/ml of rinse.

2.6.1 Primary culture media

In 1896 Sabouraud described a peptone-glucose or peptone-maltose medium as suitable for the growth and isolation of *Candida* sp. Sabouraud's medium has a pH of less than 6, so it suppresses many of the commensal oral bacteria. Additional suppression can be obtained by adding an antibiotic such as chloramphenicol or streptomycin. Once inoculated Sabouraud culture plates are then incubated at 37° C for 2-3 days after which time positive cultures will show cream coloured, smooth or rough convex colonies.

A variety of other mediums with chromogenic components have been used to help distinguish between the various species of *Candida* on primary isolation. CHROMagar is a new commercial chromogenic medium, which is suitable for initial differentiation between *C.albicans*, *C.krusei*, *C. tropicalis* and *C. parapsilosis*. The addition of an antibiotic by the manufacturers suppresses bacterial growth.

2.6.2 Morphological tests

- Direct microscopy – A sample is stained with the Gram stain which if positive will show the characteristic rounded or oval budding cells of yeast blastospores (yeast form) with or without hyphal phase. The blastospores can be distinguished from bacteria by their greater size (3-6 micrometres), oval shape and tendency to produce pseudohyphae.
- Water mount/wet film – A water emulsion of the sample is mounted on a slide and the blastospores examined for their average size, shape and type of budding.
- Potassium hydroxide – Potassium hydroxide is an effective cleansing agent, eliminating most debris and intensifying the contrast of fungal structures.
- Germ tube test - this is a rapid screening procedure for differentiating *C.albicans*, *C.dubliniensis* and some strains of *C.tropicalis* from other *Candida* sp. The yeast is lightly inoculated into approximately 1 ml of sterile serum and incubated for 2-2.5 hours at 37°C. After incubation suspensions are placed on a glass slide mounted under a cover slip and examined for the presence of germ tubes. A germ tube is filamentous, cylindrical outgrowth from the yeast cell with no constriction at its base.

- Chlamydospore formation - Chlamydospore formation is a property peculiar to *C.albicans* and very rare in isolates of *C.tropicalis* and *C.stellatoidea*. The chlamydospore has been defined as a thick-walled non-deciduous intercalary or asexual spore formed by a rounding off of a cell or cells. They are thought to be dormant growth forms produced under conditions of nutrient depletion.

2.6.3 Physiological tests

Carbon and nitrogen assimilation tests –This test assesses the ability of a particular yeast isolate to assimilate a specific carbohydrate as its sole carbon source.

Carbohydrate fermentation –This test is useful for differentiating between *Candida*. However it is less sensitive and therefore less dependable than the assimilation test.

There are rapid commercial systems, which offer a high accuracy of identification. The API 20C system is the one most widely used.

2.6.4 Strain differentiation within *Candida*

- Serotyping – typing according to the antigenic characteristics of their cell walls. There are two serotypes of *C.albicans*, serotype A and B.
- Biotyping – typing according to biochemical characteristics, where a variety of proteinases and lipases are used to distinguish between the biotypes. In 1980 Odds described a set of nine tests that together permitted identification of 512 potential biotypes. These tests comprised of; acid and salt tolerance test, proteinase production, two-resistance tests and four assimilation tests.

2.7 Histological appearance or oral candidosis

Histologically the pseudomembranes seen in oral candidosis consist of a thick surface zone of sloughed epithelial cells, fibrin, food debris, leucocytes, bacteria and *Candida* sp. mainly in the hyphal phase. The pseudomembranous plaques are caused by the invasion of the superficial epithelial cells by candidal hyphae along with a proliferative epithelial response. Neutrophils can be observed throughout the epithelium in particular at the junction with the glycogen rich zone where microabscesses form. In chronic atrophic candidosis the epithelium is acanthotic rather than atrophic and the oedema of the superficial epithelium has a chronic cellular infiltrate. Ultramicroscopic preparations shows that yeast cells are often partially enclosed within phagocytic invaginations of epithelia cells. Six hours after epithelial infiltration, hyphae and yeast cells can be found in submucosal tissue.

Some reports have described a granulomatous response with *non-C.albicans* species including *C.glabrata*.

These histological features are significantly altered in immunodeficiency where there is frequently a diminished or even absent cellular infiltrate but with increased oedema and tissue necrosis.

2.8 Antifungal agents

A range of anti-fungal medication is available for the treatment of topical and systemic fungal infections. Although anti-fungal agents such as Amphotericin B and Nystatin were developed in the early 1950's, the pharmacological principles of anti-fungal agents are still not fully understood. In addition *in vitro* susceptibility testing of anti-fungal agents do not provide consistent and reproducible results. These factors do not allow for accurate therapeutic values of anti-fungal agents to be determined.

There are three main categories of antifungal agents available for the treatment of oral candidosis: polyenes, azoles and disinfectants.

2.8.1 The polyenes

Two polyenes are commonly used for the treatment of oral candidosis; Amphotericin B and Nystatin. Since their discovery (Amphotericin B in 1956 and Nystatin (1950)], there have been very few recorded instances of fungal resistance to these agents. Amphotericin B and Nystatin are frequently used as *Candida* infection prophylaxis for AIDS patients.

2.8.1.1 Mode of action:

The precise mechanism of the polyenes on *C.albicans* is still unclear. They appear to bind to sterol components in the cell wall of *C.albicans* forming a ring complex. This complex prevents the biosynthesis of ergosterol making the cell wall more permeable. This leads to the impairment of barrier function, leakage of cellular components, metabolic disruption and as a result the cell becomes non-viable. In addition the polyenes affect chitin synthetase, which is required for the secondary cell wall formation.

2.8.1.2 Nystatin

Nystatin has significant anti-fungal properties, although it is only suitable for the treatment of topical candidosis (212-14). It is highly toxic when administered parentally. The biological activity of Nystatin is expressed in international units (IU) as pharmaceutical preparations contain a heterogeneous mixture of compounds in addition to the major ingredient Nystatin A₁. The anti-fungal effect of Nystatin is compromised by the diluting effect of oral fluid and the cleansing action of the oral musculature. It also has a very unpleasant taste so many preparations (suspension & pastille forms) contain flavouring and sucrose to make the medication more palatable. A suspension form is commonly used for paediatric patients where compliance is often difficult to obtain. The effectiveness of topical Nystatin is often reduced by rapid clearance. Nystatin tablets have no flavouring or sucrose. Nystatin pastilles do contain these additives and as they are sucked ensures a longer anti-fungal effect.

2.8.1.3 Amphotericin B

Despite newer anti-fungal medications Amphotericin B remains the choice for the treatment of invasive mycoses (215-16). It is available as an intravenous injection (Fungizone) and as a topical application. Amphotericin B, unlike Nystatin, can be used for the management of both topical and systemic *Candida* infections. It is extremely active against *Candida* sp. and resistance is rare.

Amphotericin B binds avidly to ergosterol a major sterol component of the fungal but not human cell membrane. Low concentrations result in permeability of the cell with leakage of intracellular components, most commonly measured as a loss in intracellular potassium.

Amphotericin B is not metabolised but accumulates in lipid rich tissues throughout the body. For several months small amounts of amphotericin B are excreted into the urine and small amounts also appear in the bile.

Conventional amphotericin B has many side-effects, the mechanism may be related to the ability to release prostaglandins or tumour necrosis factor from phagocytes.

2.8.2 Azoles

The azole antifungals are a diverse group of compounds administered by topical and intravenous routes. They act by inhibiting the synthesis of ergosterol. They target the cytochrome P450 dependant enzyme, lanosterol 14 alpha demethylase. This enzyme demethylates lanosterol in the pathway that leads to the formation of ergosterol, one of the building blocks of the cytoplasmic membrane. They are divided on the basis of their chemical structure into imidazoles (clotrimazole, miconazole and ketoconazole) and the triazoles (fluconazole and itraconazole)

2.8.2.1 Fluconazole

Fluconazole is a water-soluble triazole derivative. A key feature of this drug is its high absorption from the gastrointestinal tract regardless of stomach acid or food content. In contrast to ketonazole and itraconazole most of the drug is not protein bound and is excreted in an inactive form in the urine. Children have a more rapid clearance of fluconazole than do adults.

Azoles are considered to be teratogenic and birth defects following administration of fluconazole have been reported.

2.8.3 Ajoene and Allicin

These are natural compounds found in the herb garlic. The fungistatic and fungicidal properties of garlic have been recognised for many decades (217-226). The growth of a micro-organism, synthesis of proteins, nucleic acids and lipids is hampered by garlic, as is cell respiration. Garlic has been shown to have synergism with Amphotericin B.

2.8.4 Antiseptics

A variety of antiseptic agents are available and licensed for anti-microbial use in the oral cavity. Chlorohexidine gluconate, either as a mouthwash (0.1 % or 0.2%) or spray is used in the treatment of oral candidosis. The mechanism of anti-fungal activity is not clearly understood however it appears to interfere with cell wall synthesis and organisation (227-31).

2.8.5 Anti-fungal resistance

Fluconazole resistance has been identified in *Candida* infections, primarily in AIDS patients with oropharyngeal candidosis (232-4). There are several mechanisms for this resistance. With fluconazole therapy the susceptible population is initially eradicated and the more resistant population fills the void. The resistant organisms may be inherently resistant to fluconazole (e.g. *C.krusei*) or resistance may develop during therapy (57,235-38). In this latter instance several reasons can explain the decrease in susceptibility of the fungus. There may be an alteration in the target enzyme, cytochrome P450 so that the azole cannot bind to the enzyme resulting in the lack of inhibition. Fungi exhibiting this feature usually demonstrate cross-resistance to all azoles. Another potential explanation for this resistance is either decreased influx or increased efflux so that intracellular concentrations of the drug are decreased. Finally a general increase in the amount of cytochrome P450 in the cell can compensate for the inhibitory effects of the antifungals and ergosterol synthesis sufficient to maintain growth.

Studies have revealed the presence of genes that code for multi drug transporters, which actively pump the antifungal drug out of yeast cells (239,240). These genes CDR1 (*Candida* drug resistance) and CaMDR1 produce well-characterised transporter molecules coding for a protein that belongs to the ABC transporter family (241-42). Increased levels of CDR1 and CaMDR1 encoded RNA have been reported in azole resistant *C.albicans* isolates (242). Also the sterol composition of the fungal plasma membrane is altered thus reducing the uptake of the antifungal agent into the cell.

2.9 HIV and Tuberculosis

2.9.1 Tuberculosis

In developed countries adult and paediatric tuberculosis (TB) is a relatively minor problem, both in terms of absolute numbers and the proportion that TB represents in terms of the national caseload (rates for children 0-14 years will usually be less than 5/100,000). In developing countries childhood rates may exceed 200/100,000. The case load of paediatric TB may rise to more than 40%, particularly high incidence areas such as the Western Cape region of South Africa. The incidence of TB has been exacerbated both directly and indirectly by the HIV/AIDS pandemic. In the Western Cape the 2001 figure is 500/100,000 with some commentators predicting a national rate of 2500/100,000 by the year 2005.

2.9.2 Microbiology

The causative organism of TB is a member of the genus *Mycobacterium*. This genus consists of 60 species, which can be classified either according to growth rates (fast or slow) or their ability to be pathogenic in man and animals. Some species are obligate pathogens (e.g. *M. leprae* or *M. tuberculosis*) while others are environmental saprophytes which may cause specific / non-specific opportunistic diseases in animals and man. Others, also environmental saprophytes, may rarely or never cause disease.

The *Mycobacterium* organism is a non-motile, non-sporing rod. It is an obligate aerobe, grows and metabolises slowly and can survive in macrophages. Despite investigation no single determinant of virulence has been discovered in the mammalian tubercle bacilli. It appears that the virulence is in part due to the ability of *Mycobacterium* to survive within the macrophages and that some more virulent strains are able to modify the immune response of the host, so damaging the host rather than the *Mycobacterium* itself. It has the ability to remain dormant in apparently healthy individuals and while the precise mechanism is unknown may be due to metabolic dormancy or a slow rate of replication.

The primary characteristic of the genus *Mycobacterium* is the complex, lipid rich, hydrophobic cell wall. Another property resulting from the unusual cell wall is its ability to retain colour imparted by arylmethane dyes following treatment with weak acids. For this reason *Mycobacterium* are often referred to as 'acid fast bacilli'.

2.9.3 The HIV infection

History

The acquired immunodeficiency syndrome (AIDS) was first recognized in 1981 and has since become a major worldwide pandemic. AIDS is caused by the human immunodeficiency virus (HIV). By leading to the destruction and/or functional impairment of cells of the immune system, notably CD4⁺ T cells, HIV progressively destroys the body's ability to fight infections and certain cancers.

An HIV-infected person is diagnosed with AIDS when his or her immune system is seriously compromised and manifestations of HIV infection are severe. The U.S. Centre for Disease Control and Prevention (CDC) currently defines AIDS in an adult or adolescent age 13 years or older as the "presence of one of 26 conditions indicative of severe immunosuppression associated with HIV infection, such as *Pneumocystis carinii* pneumonia (PCP), a condition extraordinarily rare in people without HIV infection". Most other AIDS-defining conditions are also "opportunistic infections" which rarely cause harm in healthy individuals. A diagnosis of AIDS also is given to HIV-infected individuals when their CD4⁺ T-cell count falls below 200 cells/cubic millimetre (mm³) of blood. Healthy adults usually have CD4⁺ T-cell counts of 600-1,500/mm³ of blood. In HIV-infected children younger than 13 years, the CDC definition of AIDS is similar to that in adolescents and adults, except for the addition of certain infections commonly seen in paediatric patients with HIV.

HIV epidemiology

At the end of 2000 an estimated 36.1 million people worldwide, 34.7 million adults and 1.4 million children younger than 15 years, were thought to be living with HIV/AIDS. Through 2000, cumulative HIV/AIDS-associated deaths worldwide numbered approximately 21.8 million, 17.5 million adults and 4.3 million children younger than 15 years.

Among the many criteria used over the years to prove the link between putative pathogenic (disease-causing) agents and disease, perhaps the most often quoted are Koch's postulates, which were developed in the late 19th century.

Aetiology of HIV

HIV is an RNA virus and is more specifically part of the lentivirus subgroup. The virus is more than 100nm in diameter and both HIV-1 and HIV-2 have cone shaped cores consisting of p25 protein, which makes up the capsid. Inside this capsid lie two identical strands of RNA. The Gp120 protein, referred to as the external envelope protein, lies on the outer surface of the virus particle and contains the binding site for immune cells as well as the domain for the neutralising antibodies.

Infection with the human immunodeficiency virus type 1 (HIV-1) results in progressive loss of immune function marked by depletion of the CD4+ T-lymphocytes, leading to opportunistic infections and malignancies characteristic of AIDS. Although both host and viral determinants influence the rate of disease progression, the median time from initial infection to the development of AIDS among untreated patients, ranges from 8 to 10 years. The clinical staging of HIV disease and the relative risk of developing opportunistic infections historically relied on the CD4+ T-lymphocyte counts. Although more recent studies have shown the importance of viral load quantification in determining the rate of disease progression, it is still useful to categorize HIV disease stage on the basis of the degree of immunodeficiency: early disease (CD4+ > 500 cells/ml), mid-stage disease (CD4+ between 200 and 500 cells/ml), and end-stage disease (CD4+ < 50 cell/ml).

Pathogenesis of HIV

It is generally agreed that HIV is the primary causative agent of AIDS initiating and propagating the pathogenic process.

Various mechanisms of transmission have been identified including sexual contact, intravenous drug use, blood transfusions, vertical transmission, and more contentiously transmission via oral fluid.

Cells that are infected by the HIV include CD4+ helper cell, macrophages / monocytes, dendritic cells and CD4- cells. The host reacts to the HIV infection via a humoral response; neutralising antibodies, antibody dependant cytotoxicity, enhanced antibodies, complement-fixing antiviral antibodies, and also via a cell mediated response; cytotoxic Natural Killer cells, CD4+ cells and CD8+ cells.

Clinical aspects of HIV

There are a number of systems to categorize the HIV infection. One is based on clinical symptoms, while another preferred by the CDC in Atlanta USA, uses the immunopathogenesis of HIV, classifying the disease according to the CD4+ levels in the blood.

Oral manifestations of HIV infection.

Oral lesions were amongst the earliest lesions described in patients with AIDS. In 1992 a classification of oral lesions associated with HIV was published after a meeting of the EC clearing house on Oral problems related to HIV infection. The classification divides the lesions into three groups. Group 1 are those strongly associated with HIV infection, group 2 those less commonly associated with HIV infection, and group three those lesions seen in HIV infection. Oral lesions are often early features of the HIV infection and are thought to predict progression.

Treatment of HIV

The management of HIV is aimed at delaying the progression of HIV, prevention of opportunistic infections and early recognition and treatment of infections and neoplasms. The primary focus of HIV medication and treatment is to delay the progression of immunodeficiency, by means of antiretroviral therapies. Antiretroviral therapy is based on a number of different drugs, which affect different areas of HIV development. Vaccines against HIV are also being investigated.

2.9.4 Tuberculosis / HIV co-infection

The mutually deleterious interaction between HIV/AIDS and TB was recognised in adults soon after the scope of the HIV epidemic became evident (243). The interaction between TB and the HIV infection / AIDS has been responsible for substantial increases in the incidence of TB in all the sub-Saharan African countries and has appropriately been termed the 'cursed duet' (244-45).

In populations where there is a high incidence of infection due to *M.tuberculosis*, the clinical appearance of TB disease is one of the first signs of a deteriorating immune status in patients in HIV-infected individuals (246). There is also a fundamental difference in the development of TB disease between HIV-infected adults and children. In developing countries adults will frequently have been infected with TB before acquiring the HIV infection. Evidence suggests that the picture is different in children where the children are almost always acquire the HIV infection neo-natally and are only later be infected with TB.

It is now apparent that the development of TB precipitates a more rapid decline in cellular immunity than might otherwise occur and accelerates the course of the HIV infection. The converse situation of this scenario is also true i.e. that that newly acquired TB infection will progress more rapidly in the presence of the HIV infection.

The presence of multiplying *M. tuberculosis* in monocytes and macrophages enhances HIV-1 multiplication and is associated with increased concentrations of TNF alpha and beta-2 microglobulin. The associated destruction of the CD4 subset of lymphocytes in its turn promotes the development of AIDS complex and further susceptibility to opportunistic infections.

2.9.5 The Interaction between Nutrition, Tuberculosis and the HIV infection

The relationship between nutrition and TB is well documented. This relationship is now further complicated by the interaction between the HIV infection / AIDS and TB as both these diseases are closely associated with the development of various forms of protein-energy malnutrition (247-50).

There are differing opinions as to the importance of nutrition in the pathogenesis of TB. There are several mechanisms by which TB could influence nutrition. Firstly TB will often be associated with increased circulating concentrations of TNF, which will contribute to increased catabolism in order to provide the materials to combat the infection. Secondly a significant number of children with primary TB will also have involvement of the abdominal lymph nodes as a result of retrograde spread of the *mycobacteria*. This development will not lead to the development of overt abdominal TB in most cases but could, none the less, compromise the lymphatic drainage of the gut and so lead to malabsorption of drugs and nutrients. Thirdly, overt abdominal TB in its various forms may be associated with the malabsorption of nutrients and drugs as well as loss of nutrients.

Deficiencies in cellular immunity are probably the commonest cause of increased susceptibility to TB but protein-energy malnutrition together with deficiencies of individual vitamins and micronutrients may also be implicated. Vitamin D and its metabolites fulfil an important role in cellular immunity.

2.9.6 Nutrition, the HIV infection and Opportunistic infection

Malnutrition is one of the major complications of HIV infection / AIDS and is a result of many factors including the HIV infection itself, HIV/AIDS associated complications and opportunistic infections especially those affecting the gut. Studies suggest that nutrition may be an important determinant of the progression of AIDS associated disease.

The oral manifestations of HIV have been well documented with oral candidiasis one of the most common and significant findings (251). Interestingly studies conducted into pulmonary TB in HIV positive patients have also reported a high incidence of oral and oro-pharyngeal candidiasis (251).

2.9.7 The Oral Cavity, Tuberculosis and the HIV infection /AIDS.

Tuberculous lesions of the oral cavity are an uncommon manifestation of the disease, usually co-existent with pulmonary TB (252-6). It is estimated that oral lesions are seen in 0.05% to 1.5% of the patients with TB. Direct inoculation of the oral mucosa is thought to be rare as the intact squamous epithelium of oral mucosa is relatively resistant to penetration by

Mycobacterium tuberculosis. Oral tuberculous lesions usually present as non-specific lesions. These can take the form of irregular ulceration or a discrete granular mass, most commonly on the tongue or gingiva.

Chapter 3

Aim of the study

The aim of the study was to:

- a. assess the change in the oral carriage of *Candida* species in patients co-infected with HIV and TB before and after antifungal treatment,
- b. to record the sensitivity of *Candida* sp. isolated in the study population against a variety of antifungal agents.

Chapter 4

Methods and Materials

4.1 Sample Group

Twenty-nine patients co-infected with HIV and TB were studied at the Brooklyn Chest Hospital (BCH), Cape Town, South Africa from July 2000 to March 2001.

The patients had been referred to the BCH from satellite health clinics and other hospitals in the Western Cape for the treatment and management of TB. The sample group included cases of both pulmonary and extra-pulmonary TB. The subjects were between 16 and 40 years of age, had laboratory-confirmed HIV sero-conversion, and a diagnosis of TB made by a qualified medical consultant, using chest X-rays, sputum microscopy (smear positive test for Acid Alcohol Fast bacilli) and bacterial culturing (257). All patients selected for this study had been diagnosed as being HIV sero-positive prior to admission. Details of the place and date of HIV testing were included in each patient's referral to the BCH.

On admission to the BCH the patients were examined by the resident medical doctor who requested further tests where required and prescribed the appropriate TB treatment regime as detailed in the government national TB guidelines.

4.2 Oral Medicine clinical examination and sampling

The cohort for this study was selected from consecutive new admissions to the Brooklyn Chest Hospital. The examiner outlined the objectives and purpose of the study to each prospective patient. He also gave a detailed verbal explanation as to what participation the study would involve (*translation where appropriate). In the presence of a witness and with the patient's verbal agreement, a written information sheet and separate consent form was provided and signed by the patient (English, Afrikaans, Xhosa forms used as appropriate).

A qualified specialist in Oral Medicine carried out all the clinical examinations. The examiner had a standardized sterile examination kit for each patient, which included a dental mirror, dental and CPITN probe, tweezers, cotton wool, gauze. Each examination took place in the surgical theatre of the BCH to ensure the highest levels of cross infection control.

The mouth and related structures of each study participant were examined. A descriptive record was made of all oral and peri-oral lesions. A lesion was any pathological or traumatic discontinuity of tissue or loss of function of a part. Diagnosis of the oral lesions was based on their clinical appearance and each lesion classified according to E.C. Clearing House criteria (258).

The medical history of each volunteer was obtained from the BCH patient records, which included details of current and past illnesses and medication, social history giving details of previous and present use of tobacco, alcohol and other drugs, and details of whether the patient was taking part in any other clinical studies.

The findings of the oral / perioral examination were recorded on the Study Examination Form (see Appendices). The Periodontal Index of Treatment Needs (CPITN – WHO 1982) was used to assess the periodontium (259). Photographs of unusual oral lesions were also taken.

After the oral examination each subject was asked to rinse their mouth with 5 millilitres (ml) of sterile phosphate buffered saline solution from a sterile universal container. Each subject rinsed for one minute before expectorating the oral fluid-buffer solution into the same container, which was immediately sealed and placed in a cooler-box for transportation. The same examination and rinse procedure was followed at the one month and three month inspections.

The diagnosis of oral candidosis in this study included the presence of clinical changes consistent with oral candidosis, the presence of either the yeast form, hyphae or pseudohyphae in the oral samples from the affected area, and a high yield (>400cfu/ml) of *Candida sp.* grown in cultures from the lesion (260-1).

4.3 Processing of Samples

4.3.1 Oral *Candida sp.* isolation

An initial trial carried out by Dr Basson at the Oral and Dental Research Institute, found that it was necessary to dilute the oral fluid rinse sample by 10^2 to ensure adequate separation of colonies for enumeration after 48 hours.

After transportation to the laboratory 100 microlitre (μ l) of each rinse was added to a sterile glass pot containing 900 μ l of sterile phosphate buffered saline (PBS). This solution was gently mixed for one minute and a 100 μ l aliquot was plated onto CHROMagar. The oral fluid/buffered phosphate saline mixed liquid was spread over the plate using a sterile right-angled glass rod and incubated aerobically for 48 hours (hr) at 37°C.

The plate was assessed for the presence of yeast colonies after 48 hrs. *Candida* colonies were counted on CHROMagar plates and reported as colony-forming units per millilitre (cfu/ml) of rinse (Fig 1).

4.3.2 The phenotypic characteristics of *Candida sp.* isolates

Each morphologically and chromogenically distinct colony identified on the CAC plates was photographed and described (Fig 2 – Fig 12). A single sample from each different colour-coded colony was taken and streaked onto Sabouraud's dextrose agar plate to obtain single species culture. Each Sabouraud plate (Oxoid) was then incubated for 48 hrs at 37°C. These sub-cultured colonies were used for all subsequent isolate identification tests. These tests included sugar assimilation profile, germ tube test, chlamydospore production, growth at 45°C, beta glucosidase test and anti-fungal sensitivity tests (262).

All the original CHROMagar plates were kept refrigerated at 4 °C for three weeks, and at which time a further inspection and examination of the colonies was carried out.

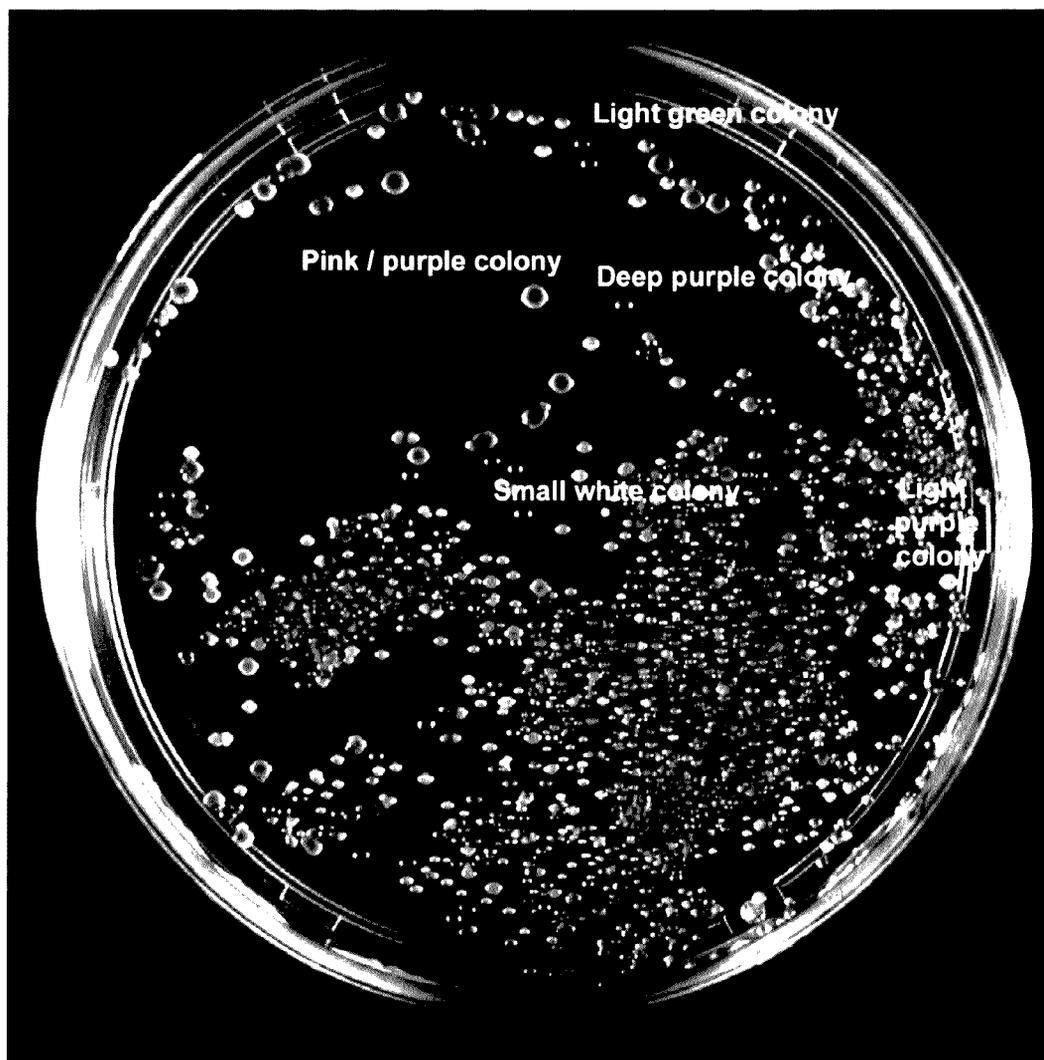


Figure1. A CHROMagar plate showing five *Candida* species isolates: Green colony; *Candida albicans*, Small white colony; *Candida parapsilosis*, Pink/purple colony; *Candida krusei*, Light purple colony; *Candida glabrata* and deep purple colony; *Candida tropicalis*

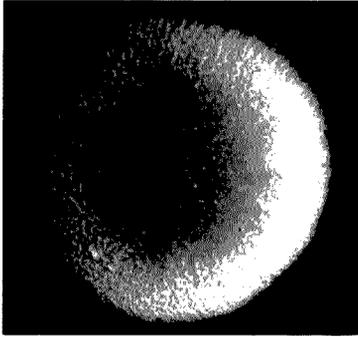


Figure 2. *Candida krusei*



Figure 3. *Candida tropicalis* (left);
showing its distinctive 'halo'
in CHROMagar

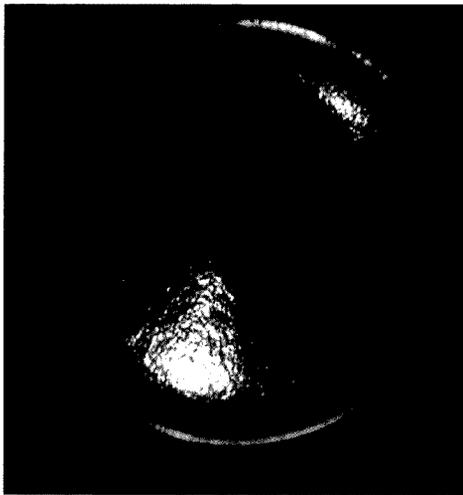


Figure 4. *Candida albicans*
(light green colony)

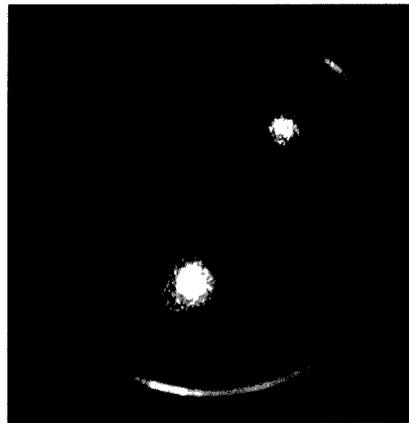


Figure 5. *Candida dubliniensis*
(dark green colony)



Figure 6. *Candida parapsilosis*

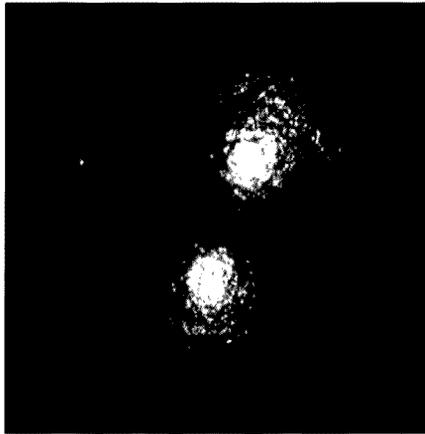


Figure 7. *Candida parapsilosis*

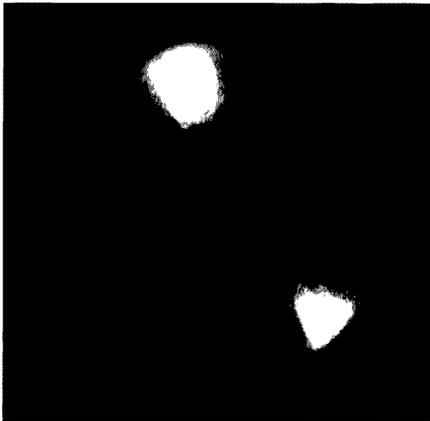


Figure 8. *Candida Parapsilosis*

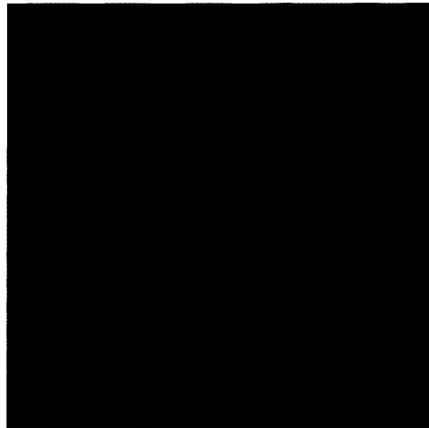


Figure 9 *Candida Parapsilosis*

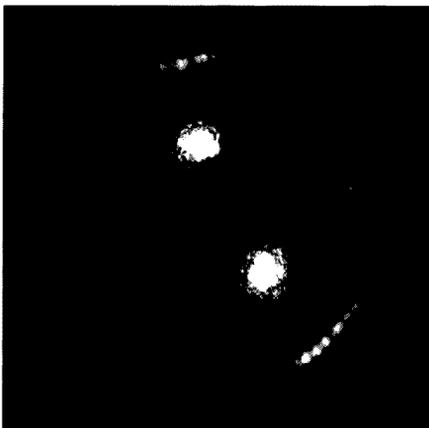


Figure 11. *Candida glabrata*

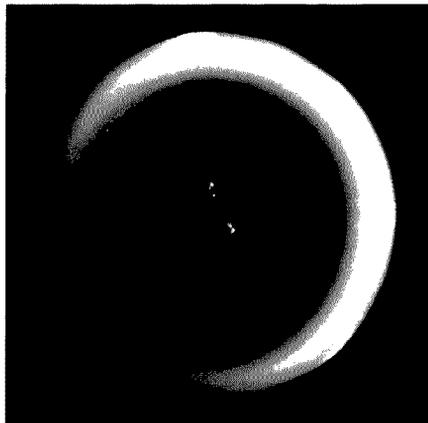


Figure 12. *Candida glabrata*

4.3.3 Identification of *Candida sp.*

4.3.3.1 Germ tube test

The germ tube test was performed according to the method described by Buckley (263). 500µl of fetal calf serum was inoculated with a small sample from the subculture of each morphologically and chromogenically different colony and incubated at 37°C. After three hours a drop of the inoculum was examined under a light microscope using phase contrast microscopy. The presence of germ tubes identified the isolate as *C.albicans* or *C.dublinsiensis*.

4.3.3.2 Identification of *Candida sp.* using a sugar assimilation - disc diffusion test

A small sample from each morphologically and chromogenically different colony was plated on a yeast nitrogen base medium (Sigma, St Louis, MO, USA). A concentrated sterile solution of dextrose, mannose, maltose, raffinose, lactose and sucrose was prepared.

A quantity of 5 mm discs of absorbent paper were sterilised in a dry oven at 160 ° C for two hours. Each 5 mm disc was impregnated with one of the selected concentrated sterile sugar solutions and placed a suitable distance apart on the medium. The prepared plates were incubated for 48 hours at 37°C and then examined for evidence of sugar assimilation.

4.3.3.3 The identification of *Candida sp.* with Auxanograms

Each morphologically and chromogenically different colony was tested for its substrate assimilation profile using the API ID32C yeast identification system (bioMerieux, France). The API ID 32 C is an identification system for yeasts using standardised and miniaturised assimilation tests with a specially adapted database. The ID32C system consists of 32 cupules, each containing a dehydrated carbohydrate substrate. A semi solid chemically defined, minimal medium is inoculated with a suspension of the colony to be tested. After 24-48 hours of incubation at 37°C, growth in each cupule is detected by the ATB instruments or by visual reading. Identification is obtained by referring to the Analytical Profile Index or with the corresponding identification software.

4.3.3.4 Tests for *C.dublinsiensis*

Those isolates that tested positive for germ tube formation were each examined for chlamyospore production, growth at 45°C and beta glucosidase activity.

One sample was incubated on 1% rice-cream-1% Tween 80 agar (Sigma, St Louis, MO, USA) at 25 °C and examined for the presence of chlamyospore after 48h. A second sample was sub-cultured as a single streak onto two separate plates of Sabouraud dextrose agar (Oxoid). One plate was incubated at 37 °C and the other at 45 °C for 48 hr. The streak on each plate was assessed after 24h and graded according to percentage growth of the inoculated sample (Fig 13).

A group of intracellular enzymes called glucosidases are found in great number of bacteria and fungi. One enzyme, Beta-D-glucosidase permease, is

located on the cell wall and is responsible for the transport and accumulation of specific substrate to within the cell.

C.dubliniensis unlike *C.albicans* does not produce intracellular beta glucosidase. To test for beta glucosidase activity, a third sample from each isolate that tested positive for germ tube formation was inoculated onto Brain Heart Infusion medium (Difco Laboratories, Detroit, MI) and incubated for 24 hours at 37°C. A good loop of the resultant colony was taken and suspended in two ml of sterile PBS. One ml of this culture was centrifuged at 16,000-x g in a micro-centrifuge. The supernatant was extracted and the remaining cells were re-suspended in 100µl of 0.1M sodium acetate at pH 5.5, containing 0.1% methylumbelliferyl-beta-glucosidase (Sigma, St Louis, MO, USA). Glass beads were added, followed by mixing twice on a bench vortex mixer for one minute and then centrifuged again for two minutes at 16,000-x g in a micro-centrifuge. The supernatant was transferred to a new Eppendorf tube and once more centrifuged at 16,000-x g. The resulting supernatant was transferred to the wells of a micro-dilution plate, allowed to stand for fifteen minutes at room temperature, and then examined on a UV transilluminator of 302 nanometers. Strains positive for beta glucosidase produced a bright fluorescence indicating *C.albicans*. The remaining strains that did not fluoresce were classified as *C.dubliniensis*.

4.4 Antifungal sensitivity tests on *Candida* sp. isolates

This study used two methods to measure the sensitivity of antifungal agents (listed below) against all the *Candida* sp. identified from the patients participating in this study.

The anti-fungal agents used were:

1. Fluconazole
2. Amphotericin B
3. Nystatin
4. Ajoene
5. Allicin

The E-Test antifungal sensitivity test kit is produced by AB Biodisc of Sweden and is accredited for research and investigational use. E-Test strips were used to measure sensitivity for Fluconazole and Amphotericin B. Studies have shown that the Minimum Inhibitory Concentrations (MIC) obtained with the E-test kit correlate with both reference agar and dilution broth procedures. Since no commercial test for Nystatin sensitivity is available, the disc diffusion method was used to quantify antifungal sensitivity (Fig 14,15,16).



Figure 13. Growth of *Candida dubliniensis* at 45°C (left) and 37°C (right)

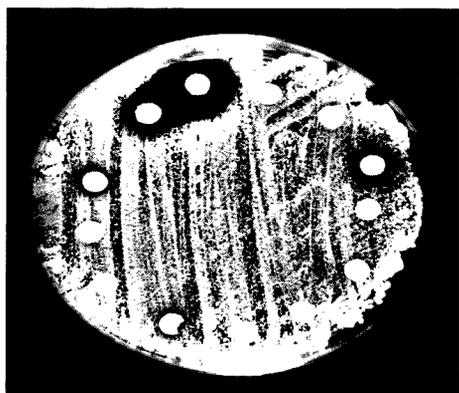


Fig 14. Ajoene dissolved in sterile water

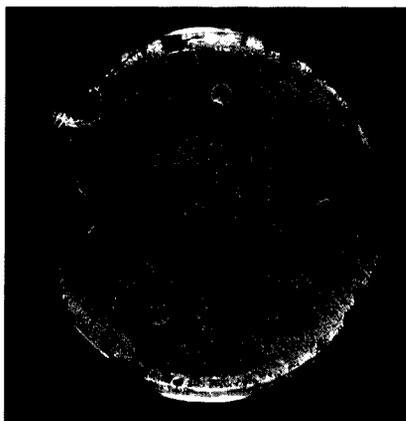


Figure 15 shows anti-fungal activity of 100,000 and 10,000 units of Nystatin.

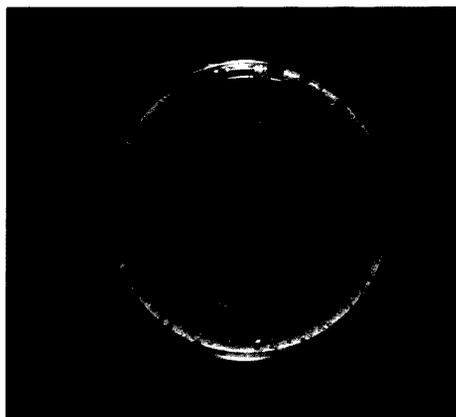


Figure 16 shows anti-fungal activity of 100,000 and 10,000 units of Nystatin, allicin and 1mg/ml of Ajoene.

Ajoene and Allicin are compounds found in garlic that have been identified as possessing anti-fungal properties. Professor Roger Hunter of the Department of Physical Chemistry at the University of Cape Town has determined the structure and composition of these two compounds. The Ajoene used for antifungal sensitivity testing was a chemically pure sample of Ajoene synthesised by Prof Hunter. The Allicin used in the antifungal sensitivity tests was obtained from the natural herb. Prof. Hunter provided both samples of Ajoene and Allicin used for the anti-fungal sensitivity testing in this study. A disc diffusion method was used to quantify antifungal sensitivity (Fig 14,15,16).

4.4.1 Antifungal tests

One operator prepared all the items required for the E-tests and disc diffusion measurements. The same operator interpreted the results and noted the Minimum Inhibitory Concentration (MIC) values

The instructions and procedures for the E-test kit were followed and allowed for straight-forward interpretation of results.

4.4.2 E-Test

The construction of a Nephelometer was required as part of the E-test investigation.

To prepare a Nephelometer (0.5 MacFarland solution), 0.05 ml of a 1% solution of chemically pure barium chloride (Merck) was put into a tube and a quantity of a 1% chemically pure sulphuric acid solution (Merck) sufficient to make a total volume of ten ml was added. This tube was then sealed.

Ten ml of sterile saline (0.85% sodium chloride) was added to a new tube, which had the same size and dimensions as that used for the Nephelometer. An amount of each isolate was added to this tube, which produced the same turbidity as that of a 0.5 MacFarlands. Both the tube containing the isolate and the 0.5 MacFarland tube were shaken well prior to comparison. The resultant tube of a 0.5 MacFarland inoculum suspension was sealed.

A sterile plate of Sabouraud dextrose medium (Oxoid) with a depth of 4.0 +/- 0.5 millimetres was prepared. A sterile non-toxic swab (not too tightly spun) was dipped into the inoculum suspension and rolled against the base of the plate. The entire base of the plate was swabbed evenly and in three directions. Ten to fifteen minutes was allowed for the excess moisture to be absorbed.

Using forceps the E-test strips for Amphotericin B and for Fluconazole were placed on the dry surface of the plate ensuring the MIC scale was facing upwards and the concentration maximum facing towards the periphery of the plate.

4.4.3 Anti-fungal sensitivity testing for Nystatin, Ajoene and Allicin

A disc diffusion technique was used to assess the anti-fungal susceptibility of the commercial anti-fungal agent Nystatin and natural derived garlic components Ajoene and Allicin. A preliminary sensitivity test was first performed using Nystatin, Ajoene and Allicin to gauge anti-fungal sensitivity ranges (Fig 15).

As described for the E-tests, solutions of Nystatin, Ajoene and Allicin were prepared in a range of concentrations. One five mm discs was impregnated with each of the solutions and the discs were placed a suitable distance apart on the medium. The prepared plates were incubated for 48 hours at 37°C and then were examined for evidence of anti-fungal activity.

The resultant values were used to determine the sensitivity range of each anti-fungal agent to be used in the subsequent test.

Chapter 5

Results

5.1 Sample group

A total of 50 HIV positive patients who were admitted to the Brooklyn Chest Hospital (BCH) between July and December 2000 for treatment of Tuberculosis (TB) were eligible for this study. There were 29 females (58%) and 21 males (42%).

Only 29 patients remained as inpatients at the BCH for the three-months assessment period. This group of patients were used for the study. Of the 21 patients who were excluded from the study six were discharged or referred to other institutions and 15 (8 female and 7 male) died. The author was unable to establish if there was any common cause of death as in many cases hospital records only noted time of death. Various factors may have contributed to the demise of some patients including nutritional status, HIV viral load and severity of the TB. The study therefore included 18 (62%) females and 11 (38%) males. The average age was 33.9 years with the female average 33 years and the male average 35.3 years.

5.2 Oral lesions

5.2.1 Extra-oral lesions

The most common site of lymphadenopathy was in the cervical node group, predominantly on the right side (Fig 17). Right and bi-lateral cervical lymphadenopathy showed a constant prevalence on admission and at three months. There was a decrease in the prevalence of left side cervical node lymphadenopathy at three months (Fig 17). A statistically significant number of patients presented with only right-sided cervical lymphadenopathy. No other extra-oral signs of the HIV infection / AIDS such as parotid swelling / uni-lateral or bi-lateral swelling of the major salivary glands were observed. No lesions indicative of lupus vulgaris, scrofuloderma or orificial tuberculosis were observed.

Figure 18. shows an example of a peri-oral lesion seen on admission. The lesion was asymmetrical, discrete, localised and crusted, indicating it could possibly be a recurrent herpes simplex virus infection. None of the study patients showed perioral lesions at the three months examination. Thiacetazone is occasionally used in TB therapy, and is associated with an adverse reaction leading to skin lesions. This drug was not used in the TB management of the study population.

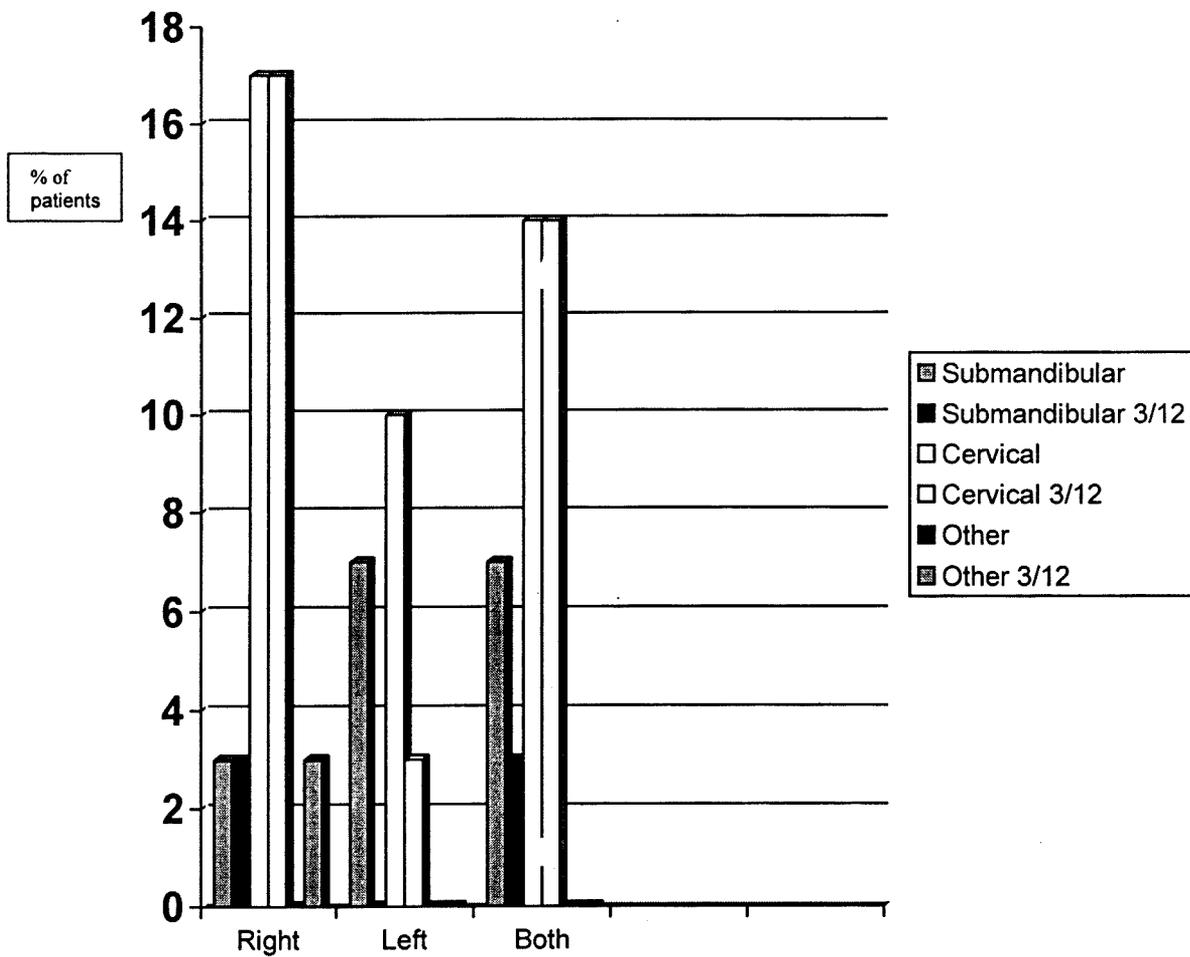


Fig 17. Frequency and location of lymphadenopathy.



Figure 18. Recurrent HSV infection involving the vermillion border and skin of peri-oral tissue.

5.2.2. Intraoral lesions

Intra-oral lesions were observed in 96% of patients included in this study. All categories of oral lesions showed a decrease over the three months study period. The results (Fig 19) indicate that oral candidosis was a significant and persistent oral lesion, both on admission (96%) and after three months (93%). Linear gingival erythema (LGE, Figs 20,21) was the second most common lesion observed both on admission (31%) and at three months (24%). Angular cheilitis was observed in 24% of patients on admission but had decreased to 7% at the three months (Figs 22,23).

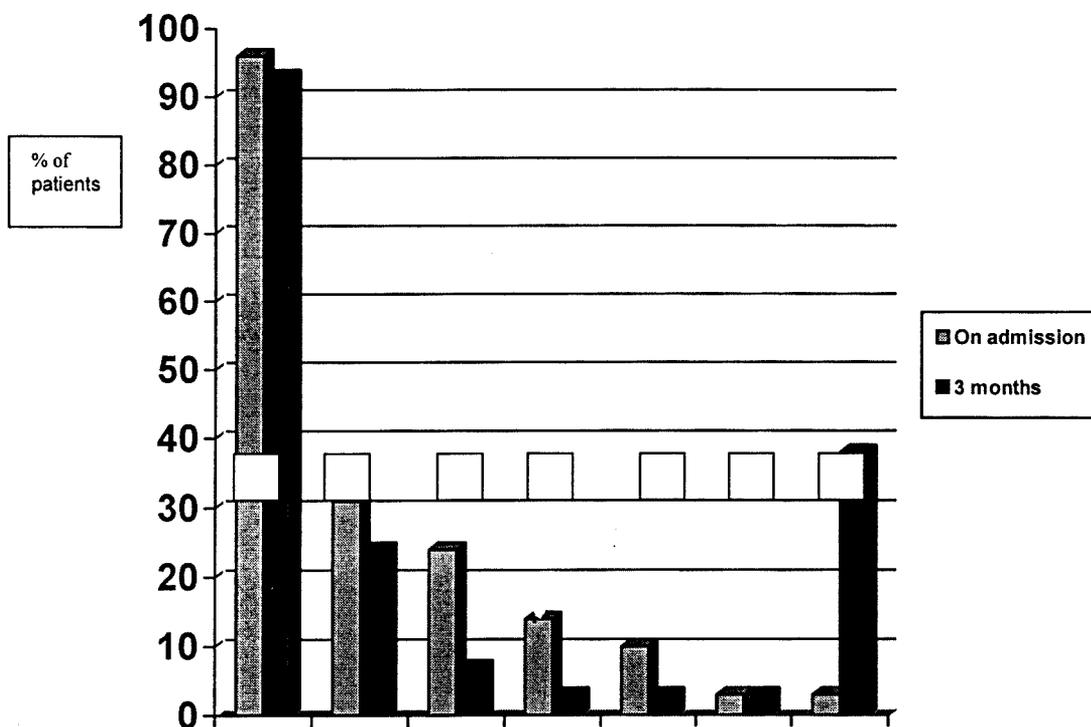
Lip ulceration including the vermillion border was present in 13% of patients on admission decreasing to 3% at the three months assessment. Severe haemorrhagic crusting of the lower lip was observed in 14% of patients both on admission and at the three months (Fig 24).

At the initial oral examination one patient presented with an unusual lesion on the dorsal surface of the tongue (Figs 25,26). When first observed on admission it was a well-circumscribed, flat, homogenous lesion with a smooth surface exhibiting no surface fissuring or pigmentation. The borders of the lesion were well demarcated by a prominent deep fissure. Subsequently the lesion was noted to have 'fractured' with several fissures appearing to run through the dorsal surface, dividing it into smaller islands, all of similar texture and appearance. No biopsy or additional investigations could be carried out due to the patient's medical status. A smear was taken from the dorsal aspect of the lesion. The pathologists report described the presence of abundant fungal hyphae. The patient also presented as a complex management case with regard to TB, suffering from several episodes of drug hypersensitivity and exhibited a poor response to TB medication.

5.2.3 Oral candidosis

The most common form of oral candidosis on admission was the pseudomembranous type, which occurred in over 50% of the study population (Fig 27). At three months an increase in the erythematous type and reduction in pseudomembranous type of oral candidosis was observed (Fig 27). There appeared to be little significant change in the prevalence of the mixed pseudomembranous & erythematous type of oral candidosis (Fig 27).

Figures 28 and 29 show the distribution of types of oral candidosis on five surfaces of the oral cavity. There was a marked increase in subjects presenting with the pseudomembranous type of oral candidosis associated with the floor of the mouth over the study interval. All other surfaces of the oral mucosa examined showed a reduction in the pseudomembranous type of oral candidosis. There was an increase in the erythematous type of oral candidosis associated with all surfaces of the oral mucosa examined over the study period. No significant change was observed in the mixed pseudomembranous / erythematous type of oral candidosis on the surfaces of the oral cavity over the study period.



Column 1: Oral candidosis (all clinical forms) = 96 / 93%
 Column 2: Linear gingival erythema = 31 / 24%
 Column 3: Angular cheilitis = 24 / 7%
 Column 4: Lip ulceration = 13 / 3%
 Column 5: Hairy leucoplakia = 10 / 3%
 Column 6: Kaposi sarcoma = 3 / 3%
 Column 7: Lip with 'moth eaten' appearance = 3 / 38%

Fig 19. Percentage of patients with specific oral lesions identified at examination; on admission (blue) and at three months (red). See Fig 27 for further subdivision of oral candidosis.



Figure 20. Linear Gingival Erythema



Figure 21. Generalised Linear Gingival Erythema



Figure 22. Angular Cheilitis



Figure 23. Angular Cheilitis



Figure 24. Severe haemorrhagic crusting of the lower lips

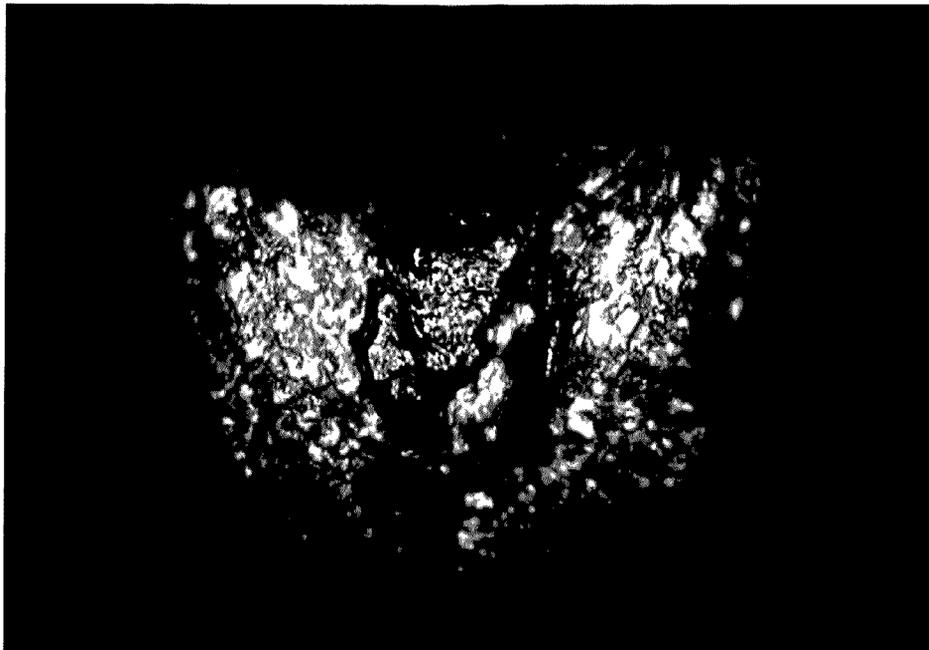


Fig 26 . An unusual, long standing lesion associated with the dorsal surface of the tongue.

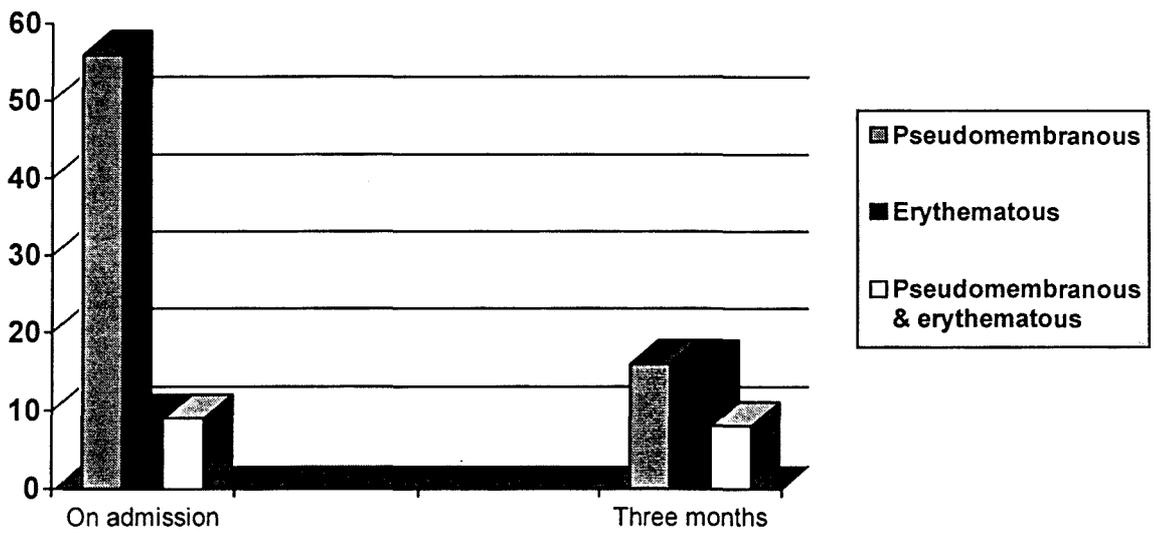


Fig 27. Percentage clinical type of oral candidosis found in oral cavity.

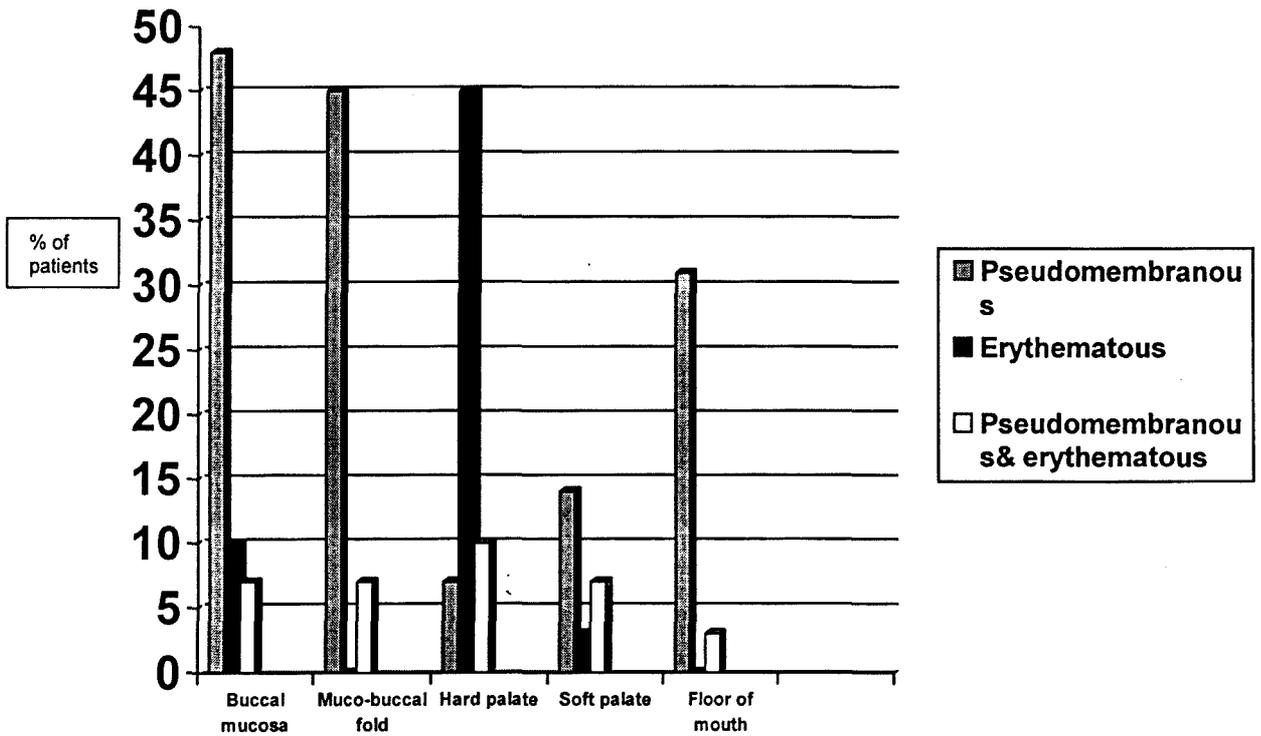


Fig 28. The distribution of clinical type of oral candidosis within the oral cavity on admission.

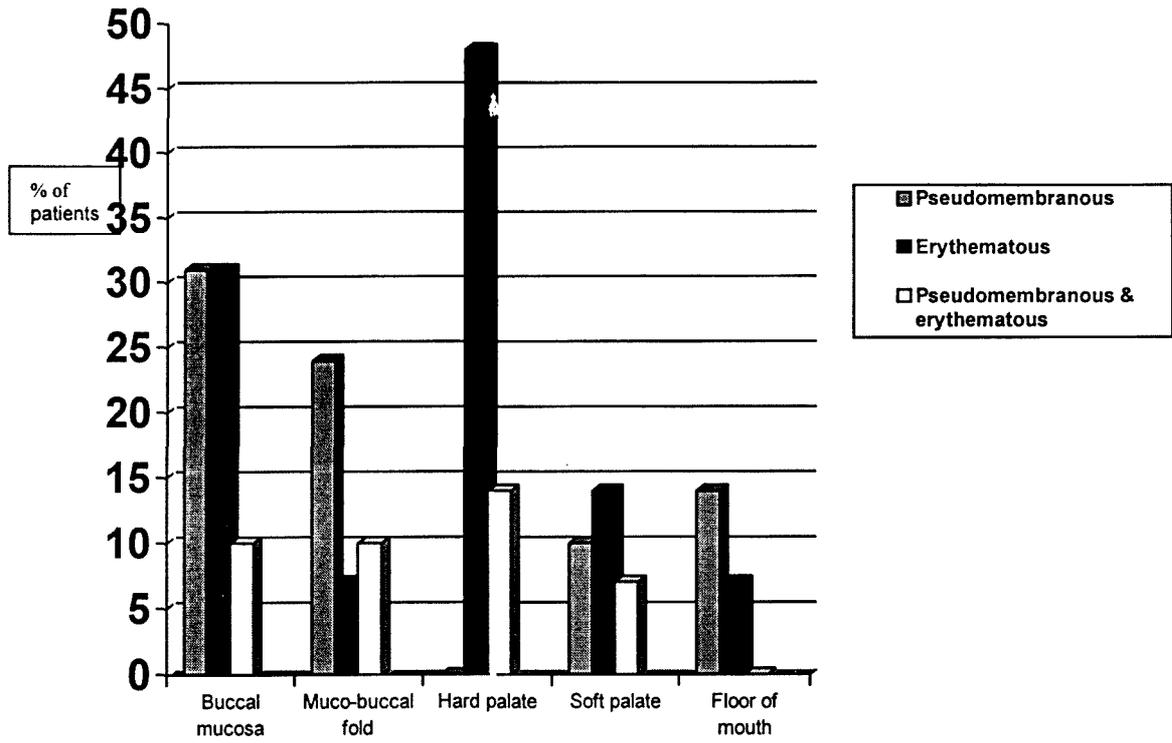


Figure 29. The distribution of clinical type of oral candidosis within the oral cavity at three months.

Over 80% of subjects presented with atrophy of the dorsal aspect of the tongue, both on admission and at three months (Fig 30).

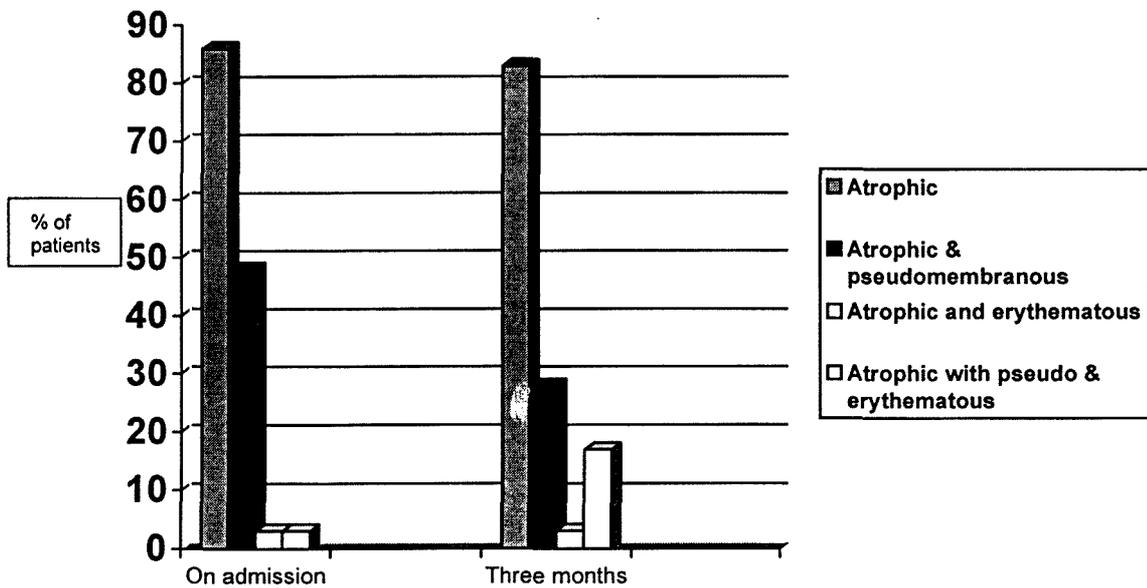


Figure 30. The distribution of the different clinical forms of oral candidosis observed On the dorsal surface of the tongue on admission and at three months.

5.3 Identification of *Candida* sp.

All light and dark green colonies isolated using CHROMagar medium tested positive for germ tubes (Fig 4,5). Three white colonies also tested positive for the germ tube test and further identification using the two sugar assimilation tests identified these isolates as *C.tropicalis* (Fig 31).

After the initial examination all the CHROMagar culture plates were kept in a refrigerator at 4°C for three weeks. At this time a variation in the uptake of chromogenic medium by the green colonies was noted. The majority of the light green colonies identified as *C. albicans* after 48 h had deepened to a dark green colour. Several of the dark green colonies identified as *C. dubliniensis* after 48 h had deepened to an inky black colour (Figs 32,33).

All but one of the isolates tested for beta D-glucosidase activity demonstrated fluorescence. Several isolates demonstrated poor fluorescence but only those isolates that showed no evidence of fluorescence were reported as negative for Beta D-glucosidase activity.

C.albicans isolates that had tested positive for both germ tube formation and beta D-glucosidase activity grew well at both 37 °C and 45 °C. The majority of isolates showed 100% growth, with only a few isolates exhibiting between 100% and 75% growth. The *C.dubliniensis* isolate was positive for germ tube formation but negative for Beta D-glucosidase activity showed no or poor (i.e. less than 25%) growth at 45 °C (Fig 13).

The isolates identified as either dark or light green did not generate hyper production of chlamydo spores in a consistent enough manner to allow for species identification.



Figure 31. Green colony: *Candida albicans*
White colony: *Candida tropicalis*

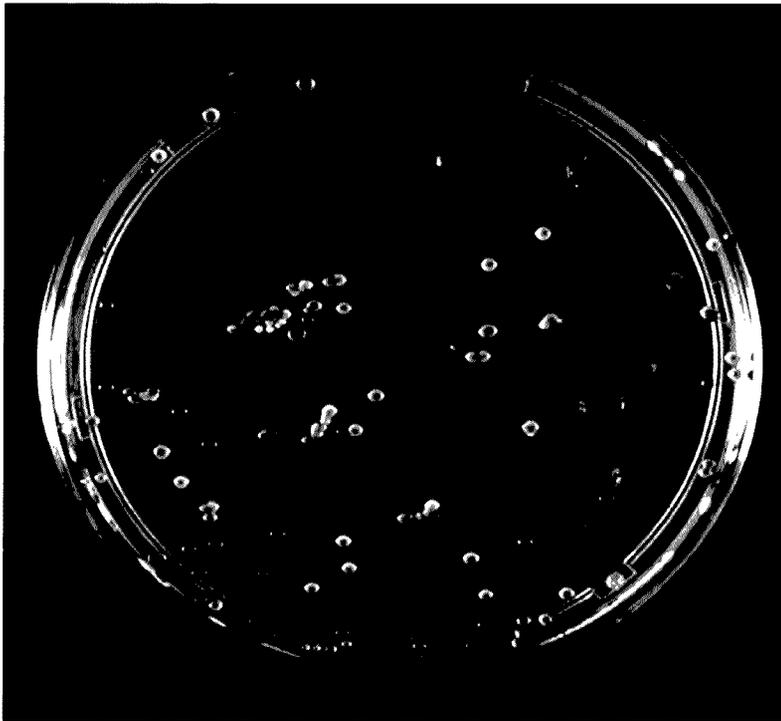


Figure 32.
A CHROMagar plate after incubation at 4°C for three weeks demonstrates the differential uptake of chromogenic agent by *C. albicans* (light green) and *C.dublinsiensis* (inky blue colony)

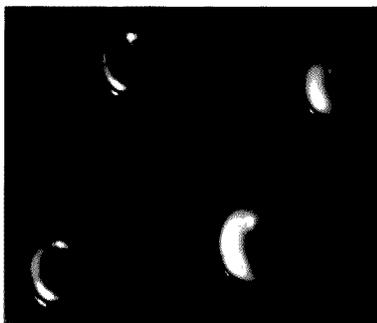


Figure 33 Dark blue *C.dublinsiensis*

5.3.1 *Candida* species

On admission *C.albicans* was the most common *Candida* sp. isolated with *C.tropicalis*, *C.glabrata*, *C.parapsilosis*, *C.dublinsiensis* and *C.krusei* isolated in descending order of frequency (Fig 34). At three months *C.albicans* was again the most common *Candida* sp. isolated, followed in descending order by *C.parapsilosis*, *C.glabrata*, *C.tropicalis* and *C.dublinsiensis*. These last two species were identified in equal amounts. *C.krusei* was not isolated at three months (Fig 34). The chromogenic characteristic of each *Candida* sp. isolate is recorded in Table 1.

The study population demonstrated a general shift towards fewer combinations of isolates at three months. 50% of subjects had only one species of *Candida* isolated from the oral rinse on admission and at three months (Table 2). There was a 6% increase in subjects showing only one species at three months (Table 3).

No comment could be passed on the relationship between combinations of *Candida* sp. isolated and clinical type of oral candidosis observed.

There was a decrease in the total number of colony forming units over the three-month study period (Fig 35). Female subjects had the highest *Candida* sp. load on admission (expressed as colony forming units per millilitre, cfu/ml) however they showed the largest decrease over three months (Fig 36). All *Candida* sp. isolates demonstrated a decrease in the number of colony forming units over the three months study period. *C.albicans* and *C.tropicalis* showed the largest decrease over the three months (Fig 37). Subjects presenting with severe and extensive oral candidosis had values of over 2500 for colony forming units per millilitre (cfu/ml).

It was not possible to establish any correlation between the clinical type of oral candidosis observed and the *Candida* sp. isolated from the oral rinse. No comment could be made on whether a specific species of *Candida* was responsible for linear gingival erythema.

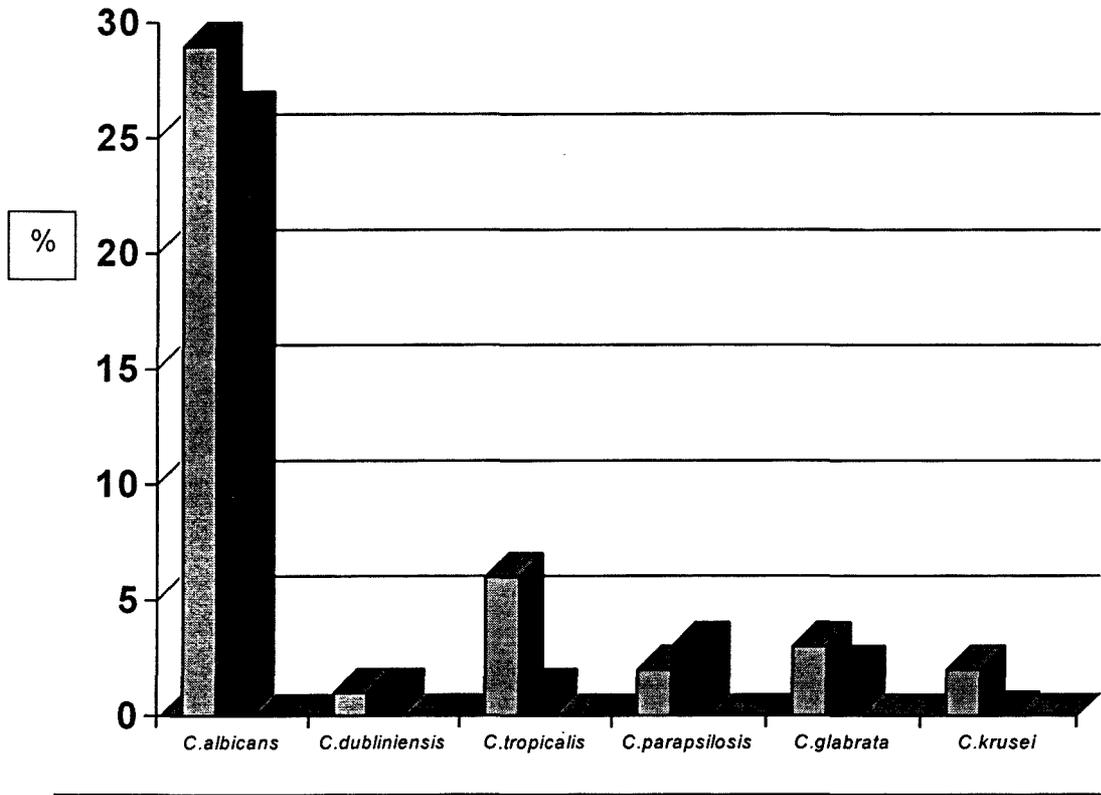


Figure 34. Prevalence of *Candida* species identified in study population on admission (blue) and at three months (red).

Table 1. Colony Characteristics of *Candida* sp. isolated on CHROMagar medium.

Species	Colour of Colonies on CHROMagar	Appearance
<i>C.albicans</i>	Green	Smooth, regular outline
<i>C.dubliniensis</i>	Smaller than <i>Candida albicans</i> Dark green or blue / green	Smooth, regular outline
<i>C.glabrata</i>	Dark pink / purple with paler edges	Pimpled surface, regular outline
<i>C.krusei</i>	Light / pale pink	Fluffy, pimpled surface,
<i>C.parapsilosis</i>	White / pale pink	smooth surface
<i>C.tropicalis</i>	Blue / deep purple with halo	Smooth surface with distinct bluish halo in the agar

Table 2. The pattern of *Candida* species colonisation in the oral cavity on admission.

Number of species isolated	Number of patients (%)
One species	17 (59%)
Two species	9 (31%)
Three species	2 (7%)
Four species	1 (3%)

Table 3. The pattern of *Candida* species colonisation in the oral cavity at three months.

Number of species isolated	Number of patients (%)
No species	2 (7%)
One species	22 (76%)
Two species	3 (10%)
Three species	2 (7%)

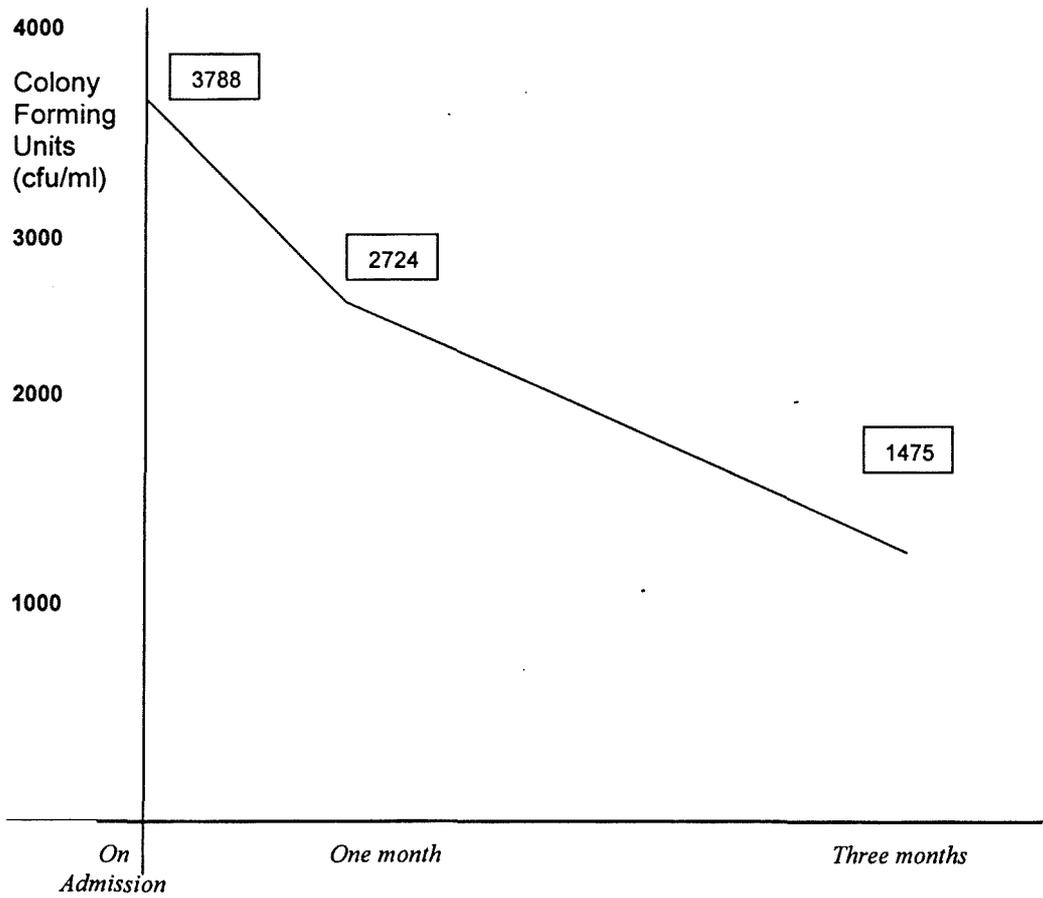


Fig 35. Mean value of *Candida* isolates expressed as colony forming units per millilitre of oral rinse (cfu/ml) identified in study population.

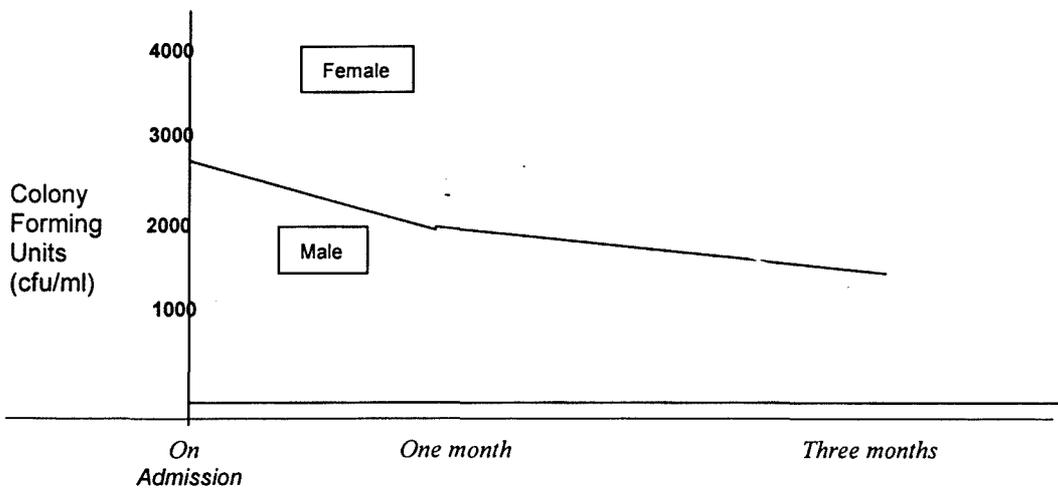


Fig 36. Mean value of *Candida* isolates expressed as colony forming units per millilitre (cfu/ml) identified in male and female patients of the study population.

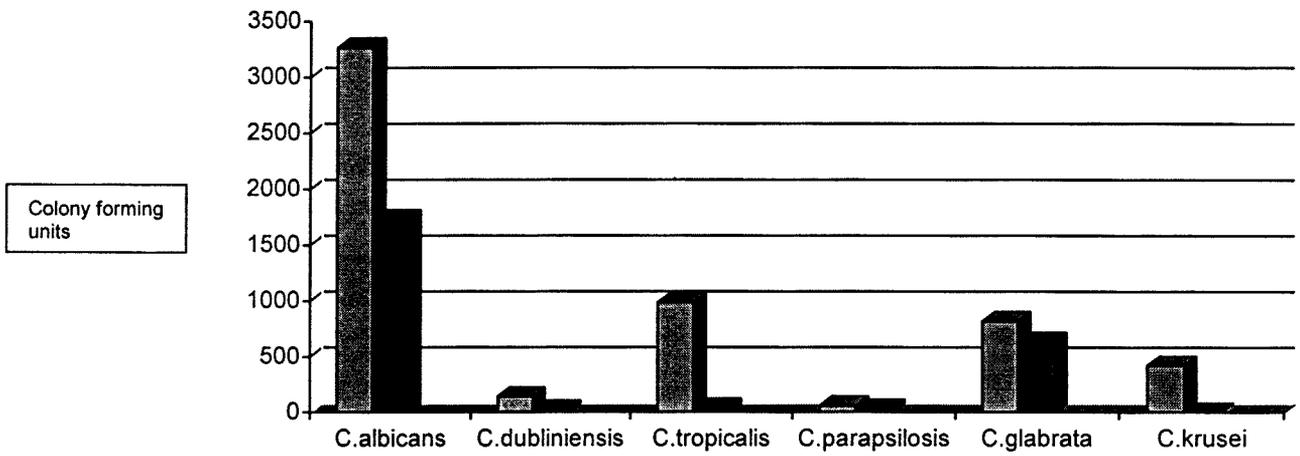


Fig 37. The relationship between *Candida* species and colony forming units (cfu/ml) for each *Candida* species on admission (blue) and at three months (red).

5.4 Antifungal sensitivity tests of *Candida* sp. isolates

53.5% of isolates identified on admission had a minimum inhibitory concentration (MIC) for fluconazole of less than 64 ug / ml, whilst 46.5% had a MIC of 64 ug/ml or more. At three months 45% had a MIC for fluconazole of less than 64 ug / ml whilst 55% had MIC's of 64ug/ml or more (Table 4).

All isolates identified on admission had a minimum inhibitory concentration of 64 ug/ml or below to Amphotericin B. One isolate identified as *C.glabrata* had an MIC of above 64 ug/ml at three months. This isolate recorded an MIC of >256 ug/ml indicating 100% resistance (Table 5).

Nystatin was effective against all of the isolates at concentrations between 10,000 and 100,000 IU and a high percentage of isolates at less than 10,000 IU both on admission and at three months. No response was recorded below 1000 IU either on admission or at three months (Table 6).

Allicin showed antifungal activity between 0.1 mg / ml and 1mg/ml. The results of the anti-fungal sensitivity testing with Allicin (1mg/ml) showed that only two isolates were resistant; both those of *C.glabrata*. Ajoene demonstrated variable response to some of the isolates but was not active below 1mg/ml. Seven isolates identified as *C.albicans* were susceptible to 1mg/ml of ajoene. Ethanol showed no antifungal activity against any of the *Candida* sp. isolated from the study (Table 7).

Table 4. Sensitivity of *Candida* sp. isolates to Fluconazole anti-fungal agent on admission / three months.

	<i>Candida species</i>					
	<i>C.albicans</i>	<i>C.dubliniensis</i>	<i>C.tropicalis</i>	<i>C.parapsilosis</i>	<i>C.glabrata</i>	<i>C.krusei</i>
Concentration (ug/ml)						
0 – 4	3 / 1	-	-	-	-	-
8 – 16	3 / 2	-	1 / 0	0 / 1	-	-
24	1 / 1	-	1 / 0	-	-	-
32	10 / 7	1 / 0	1 / 0	-	-	-
48	2 / 3	-	-	-	-	-
64	7 / 10	-	1 / 0	1 / 1	-	-
96	2 / 0	-	-	1 / 0	-	-
128	1 / 1	-	-	-	-	-
>256	0 / 1	0 / 1	2 / 1	0 / 1	3 / 2	2 / 0

Table 5. Sensitivity of *Candida* sp. isolates to Amphotericin B on admission / three months.

	<i>Candida species</i>					
	<i>C.albicans</i>	<i>C.dubliniensis</i>	<i>C.tropicalis</i>	<i>C.parapsilosis</i>	<i>C.glabrata</i>	<i>C.krusei</i>
Concentration (ug/ml)						
0 – 0.5	16 / 15	-	3 / 0	2 / 1	1 / 0	-
0.5 – 1	5 / 6	-	2 / 1	0 / 2	1 / 0	-
1 – 1.5	3 / 4	-	1 / 0	-	1 / 1	-
1.5 – 3	3 / 0	-	-	-	-	-
3 – 6	2 / 1	1 / 1	-	-	-	2 / 0
>256	0 / 0	-	-	-	0 / 1	-

Table 6. Sensitivity of *Candida* sp. isolates to Nystatin on admission / three months.

	<i>Candida species</i>					
	C.albicans	C.dubliniensis	C.tropicalis	C.parapsilosis	C.glabrata	C.krusei
Concentration (IU/ml)						
100,000	12 / 9	1 / 1	2 / 1	2 / 1	0 / 1	1 / 0
< 10,000	17 / 17	-	4 / 1	0 / 2	3 / 1	1 / 0

Table 7. Concentrations of antifungal agents Nystatin, Ajoene, and Allicin dissolved in water and their antifungal effectiveness on admission/at three months. The symbol +++ denotes a clear zone of 5mm or more around the impregnated disc, ++ a zone of between 1-5mm and +/- denotes a unclear but possible zone of 1mm.

Anti-fungal agent	Solvent	Concentration	Antifungal effect
Nystatin	sterile water	100,000 units/ml	+++
Nystatin	" "	10,000 units/ml	+++
Nystatin	" "	1000 units/ml	-
Allicin	" "	1 mg/ml	+++
Allicin	" "	10µl / ml	+ / -
Ajoene	" "	1mg / ml	+ / -
Ajoene	" "	10µl / ml	-
Ajoene	" "	0.1µl / ml	-
Ajoene	" "	50µl / ml	-
Ajoene	" "	20µl / ml	-

Table 8. Different concentrations of Ajoene dissolved in ethanol* / water solution and their antifungal effectiveness. The symbol +++ denotes a clear zone of 5mm or more around the impregnated disc, ++ a zone of between 1-5mm and +/-denotes a unclear but possible zone of 1mm.

Anti-fungal Agent	Solution agent	Concentration	Antifungal effect
Ajoene	Ethanol / water	1mg / ml	+ / -
Ajoene	" "	10µl / ml	-
Ajoene	" "	0.1µl / ml	-
Ajoene	" "	50µl / ml	-
Ajoene	" "	20µl / ml	-

* An additional test to assess the antifungal potential of ethanol against each of the Candida species identified on the study, found that ethanol alone possessed no antifungal properties.

Chapter 6

Discussion

6.1 Oral lesions

Oral lesions occurred in a high proportion of the subjects examined in this study, both on admission and at three months (Fig 19).

Oral candidosis was the most frequently identified oral infection (Fig 38, 39). This is consistent with the findings of other studies on the oral manifestations of HIV (165,264-73). In this study 96% of subjects on admission and 93% at three months presented with oral candidosis.

Candida sp. and *Staphylococcus sp.* infection, immune deficiency, mechanical factors and pernicious anaemia are all implicated in the aetiology of angular cheilitis (Figs. 22,23). The decreased prevalence of angular cheilitis over the study may be a result of rapid and effective treatment of infections by hospital staff and also improved patient nutrition.

Lip ulceration was present in 13% of patients on admission decreasing to 3% at the three months assessment (Fig 40,41).

Various studies have quoted the prevalence of oral ulceration in HIV infected individuals as between 2 - 4% (264). The design of the patient assessment form did not allow for a detailed clinical description of peri-oral and oral ulceration. Biopsy of the lesions was not possible hence more detailed comment is not available (Fig 42,44,45). The presence of atypical ulceration observed on the tongue may also be due to TB (226,227) (Fig 46).

Severe haemorrhagic crusting of the lower lip was observed in 14% of patients, both on admission and at three months (Fig 43).

A range to the clinical appearance of linear gingival erythema was noted in the study population (Fig 20,21). Recent reports have suggested *Candida sp.* may play role in the aetiology and pathogenesis of LGE (37,268). *Candida dubliniensis* has been identified in the gingival crevice of paediatric patients with LGE (37). An increased prevalence of *Candida dubliniensis* in HIV positive patients with poor oral hygiene and the co-aggregation of *C. dubliniensis* with *Fusobacterium nucleatum* have been reported (29,35,38).

To date there has been no specific research into the oral manifestations of patients co-infected with HIV and TB. The administration of antibiotics causes a change in the oral microflora leading to greater numbers of *Candida sp.*, and an increased risk of *Candida* infection. (135,137,274-5). Medical researchers investigating TB in HIV / TB co-infected patients have reported a high incidence of oral and pharyngeal candidosis in these patients (244,276).

A change in the prevalence of clinical type of oral candidosis was noted in the study group. There was a shift from the pseudomembranous type of oral candidosis (prevalent on admission) to the erythematous type of oral candidosis (prevalent at three months). This shift was common to all the

surfaces of the oral mucosa (Fig 38 & Fig 39). The parameters of this investigation preclude any further comment as to whether the change observed in this study population was a feature of HIV infection / AIDS alone, or whether other factors such as medication, malnutrition and immunosuppression due to TB, also played a role.

Atrophy of the dorsum of the tongue is commonly seen in patients undergoing prolonged antibiotic therapy (148). The oral microflora of these patients is altered, allowing resistant organisms such as *C. albicans* to flourish. On admission 86% of patients had varying degrees of atrophy associated with the dorsal surface of the tongue (Figs. 30&39). In addition to atrophy, 54% of patients on admission had clinical evidence of oral candidosis. At the final three months examination a high proportion of patients (83%) still showed atrophic changes to the dorsum of the tongue. A smaller percentage (48%) of patients had clinical evidence of oral candidosis.

Atrophy of the tongue has a multi-factorial aetiology (148). It is most likely a combination of ongoing vitamin deficiency / malabsorption, *Candida* infection and antibiotic related mucosal changes. It has not been reported in the medical literature as a significant clinical finding in HIV / AIDS patients and may be related to the specific circumstances of the study population.



Figure 38. An example of oral candidosis associated with the lower lip



Figure 39. Atrophic form of oral candidosis associated with the dorsum and dorso-lateral aspect of the tongue



Figure 40 Lip ulceration

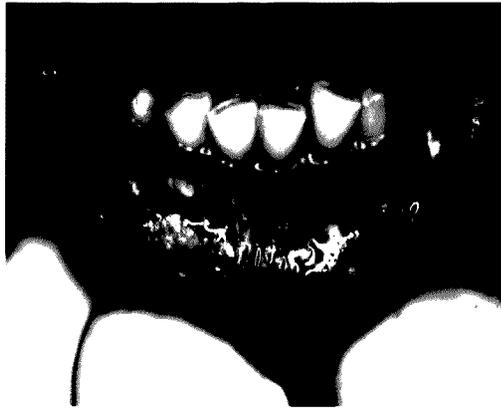


Figure 41. Lip ulceration



Figure 42. Oral ulceration / white patch



Fig 43. Acute generalised oral candidosis and haemorrhagic crusting of the lower lip.

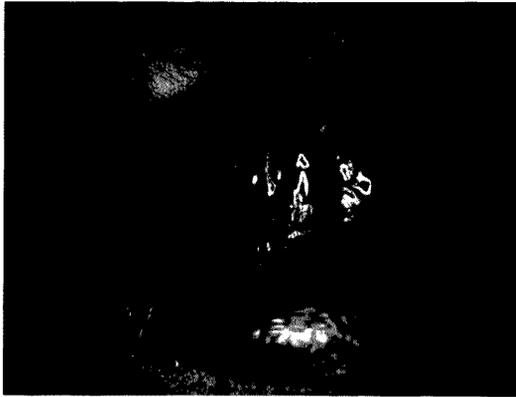


Figure 44. Large irregular ulcer



Figure 45. The same patient with a large ulcer displaying an inflammatory halo.

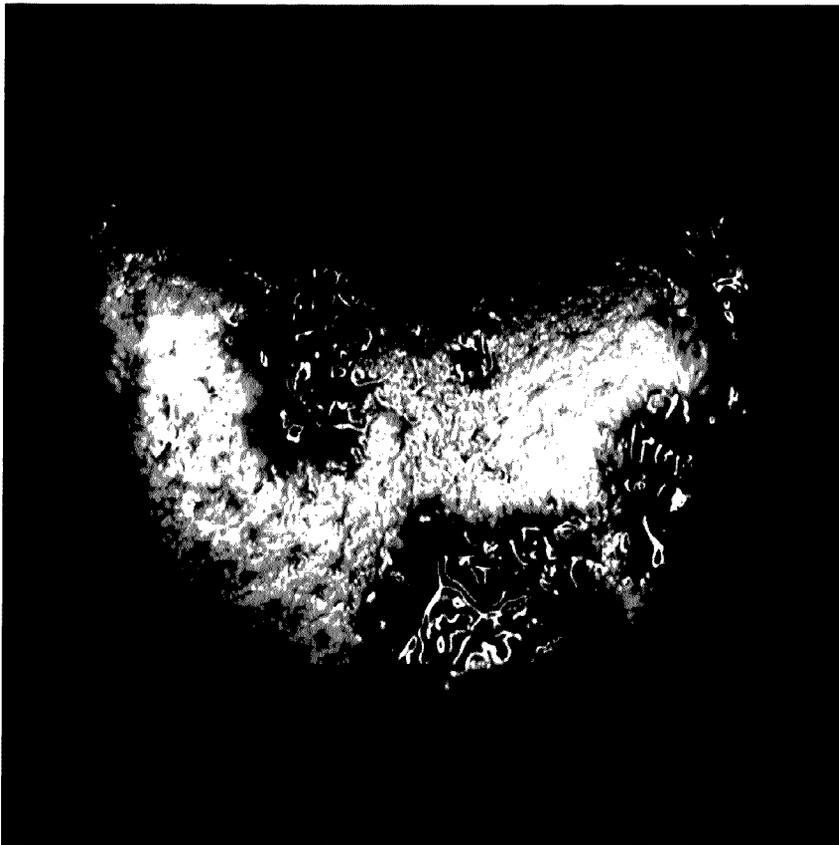


Figure 46. Ulcerative lesions associated with the dorsal surface of the tongue.

6.2 *Candida* sp. identification

This study used an oral rinse sampling technique to assess *Candida* sp. carriage in the oral cavity. Studies investigating yeasts have employed a variety of methods (31,33,35,277). The oral rinse method to quantify yeast carriage was found to be simple to perform and as sensitive as other techniques (49).

CHROMagar is a commercial chromogenic culture medium that facilitates the isolation and presumptive identification of *Candida* sp. (Fig 1.)(32,75,278,). A study found that *C.albicans*, *C.tropicalis*, and *C.krusei* have a distinctive appearance on primary CHROMagar isolation (Fig 2,3,4). These three species can be recognised quickly and accurately by trained operators (75). This study confirmed that *C.tropicalis* and *C.krusei* could be identified from the primary isolation on CHROMagar with a high degree of accuracy.

Recent studies indicate that CHROMagar alone should not be used for definitive *Candida* sp. identification. Although the author could not corroborate the findings, other studies have reported that *C.albicans* can produce two chromogenic forms; light green colonies with paler edges and smaller dark blue/green colonies (Fig 4,5). The latter description has also been ascribed to *C.dublinsiensis* (Fig 5.).

This study found that a range of additional investigations including carbohydrate fermentation and assimilation, growth at various temperatures, Beta-D-glucosidase activity, chlamyospore and germ tube formation allowed for accurate species differentiation (73,261,262,277,279,280-2)

Three isolates that appeared as homogenous white colonies at initial CHROMagar isolation, tested germ tube positive. Subsequently sugar assimilation profiles using both the disc diffusion method and the API ID 32C system identified the species as *C.tropicalis* (Fig 31.). This species of *Candida* has strains that are positive for the germ tube test. It is also possible that the sample colony contained *C.albicans* thus accounting for the positive germ tube test result.

Although it has been sufficient to use bio-chemical tests to identify *Candida* sp. in the past, current medical knowledge demands research differentiates to strain level.

6.3 *Candida* species

C.albicans is the most common cause of opportunistic fungal disease in humans (283). *C.albicans* was the most frequently isolated species of *Candida* both on admission and at three months (Fig 34). The prevalence of *C.tropicalis*, *C.glabrata* and *C.parapsilosis* found in this study population all lay within the isolation rates quoted in the research literature (284-5). There was no significant change in the prevalence of *C.albicans* observed over the study period (Fig 34). *C.tropicalis* showed a significant decrease over the study period. *C. parapsilosis* showed a small increase, while other non-*Candida albicans* species showed little variation (Fig 34). Antibiotic use has been documented as a predisposing factor for *C. parapsilosis* infections (53,286).

Researchers studying candidoses have reported an epidemiological shift towards infections caused by non-*C.albicans* sp. (284). One hypothesis that may account for this shift is that these latter species, unlike *C.albicans*, can

persist in the oral cavity following antifungal treatment (287). *C.glabrata* and *C.krusei* are less susceptible to antifungals such as fluconazole. *C.dubliniensis*, while susceptible to the common antifungals, has a reduced susceptibility to fluconazole in AIDS patients who have previously been treated with the drug. Although various authors have speculated as to the underlying causes of this shift, no firm evidence has yet been advanced.

The traditionally held view is that the hyphal form of the *Candida sp.* is associated with the disease state and blastospheres are present in the commensal / non-infective state. Research has indicated that the hyphal form can occur at healthy sites within the oral cavity and the yeast form, for instance *C. glabrata*, can cause infection (19,284-5).

Candidosis as a side effect of medical treatment has been increasing over the last two decades. The use of antibiotics is one of the major factors that increase the risk of developing candidosis. The nosocomial environment is an important factor in the increased incidence of candidosis. One study indicated an eight-fold increase in nosocomial fungemia, the main source of which were *Candida sp.* (9). Researchers from the USA noted that the number of nosocomial bloodstream infections due to *Candida* species is increasing, with non-*C.albicans* species, in particular *C.glabrata*, an important nosocomial pathogen (288 –92). Hospital studies found that 17 to 58% of hospitalised patients and health personnel carry *Candida sp.* on their hands, with *C.parapsilosis* the most common pathogen isolated (286,293-95). One study found nursing personnel were a potential infectious source of *Candida sp.* (296). Analysis of DNA shows that *Candida sp.* can be transmitted to patients in the nosocomial environment (297). There are a number of vehicles for transmission including hospital personnel, visiting family of hospitalised patients and contaminated food (298-9). One study found that the longer the period of hospitalisation and immuno-suppression, the greater the risk of developing a candidal infection (300).

For a number of reasons Brooklyn Chest Hospital has a large turnover of both in- and out-patients undergoing treatment for TB. Medical and nursing staff moves between paediatric and the adult wards throughout the working day. This study did not investigate the possibility of cross-infection between patients and also between patients and staff. It is possible that cross infection may have had a dramatic effect on the oral *Candida sp.* profile of the study population, particularly in terms of anti-fungal agent resistant species. This nosocomial transmission is of crucial importance to neonates and young infants in the BCH paediatric wards as studies have reported this group to be at particular risk from candidal infections (292,301-04)

6.4 *C. dubliniensis*

This is the first study to identify *C. dubliniensis* in the South African HIV/AIDS population. This study concurs with the findings of other researchers, that is the differential diagnosis between *C. albicans* and *C. dubliniensis* using the colour and morphological characteristics of colonies on CHROMagar is unreliable (31). Several studies have reported that *C. dubliniensis* more often produced colonies of dark green appearance on primary isolation on CHROMagar (32,278)(Fig 5). In this study some dark green colonies initially labelled as *C. dubliniensis* after isolation on CHROMagar demonstrated good growth at 45 °C and demonstrated a positive result for beta-D-glycosidase activity. These results indicated that *C. albicans* could also form dark green colonies on CHROMagar. This study did not establish whether the converse was also true. Other studies have reported that the light green colonies can also correspond to *C. dubliniensis* (31). It has been reported that the colour of the *Candida* colonies on CHROMagar deepens with time (75). Due to this phenomenon the manufacturer recommends that optimum time for identification should be after 48 h. This study found that if the CHROMagar plates were kept for three weeks in a refrigerator at 4°C, the light green colonies identified as *C. albicans* (using the tests outlined in the methods) deepened to a dark green, whilst the darker green colonies identified as *C. dubliniensis* deepened to an inky black colour (Fig 25,27). This adds another useful marker for *C. dubliniensis*.

This study used a variety of tests to positively identify *C. dubliniensis* (30,32,73,78,136,262,277). Previous studies have indicated that *C. dubliniensis* differs phenotypically from *C. albicans* by its production of abundant chlamydo spores and its failure to grow at 45 °C (73,262). Results from this study show that poor growth or an inability to grow at 45 °C can be used as a marker for *C. dubliniensis* (262)(Fig 13).

One study found that chlamydo spore formation on Staib agar was a species-specific characteristic for *C. dubliniensis* (73). This study could find no consistent evidence of hyper production of chlamydo spores.

For the beta-D-glycosidase activity test only those isolates that showed no evidence of fluorescence were reported as negative (27,277). Only one isolate from the study population demonstrated clear non-fluorescence. The author noted that there was a marked variation in the intensity of fluorescence amongst those isolates demonstrating beta-D-glycosidase activity. One possible explanation for the variation in fluorescence is the presence of both *C. albicans* and *C. dubliniensis* in the sample examined.

Substrate assimilation profile using the ID32C and other yeast identification systems did not consistently identify any common pattern in those isolates identified as *C. dubliniensis* (281). A report outlining a new product VITEK 2 ID-YST suggests that this system will be able to provide an accurate specific assimilation profile for the species (279). Molecular and gas-liquid chromatography technology can also permit accurate, single procedure identification of *C. dubliniensis* using a species-specific molecular beacon (76-8,305).

C. dubliniensis is reported to be more adherent to human buccal mucosa epithelial cells than *C. albicans* isolates, which suggests that this species may

be particularly adapted to colonisation of the oral cavity (36). *C.dubliniensis* may operate as a lone pathogen or in symbiosis with other oral microorganisms (38). These facts are particularly significant in the light of the role that other non – *C. albicans* species play in diarrhoea of hospitalised paediatric patients and the attendant risk of the nosocomial spread (6,9,10,11,12).

This preliminary data suggests that the prevalence of *C. dubliniensis* in HIV/AIDS patients in South Africa (3%) is lower than in other studies. An Irish study found that *C. dubliniensis* was recovered from 27% of the oral cavities of HIV-infected individuals and 32% of AIDS patients suffering from oral candidosis (27). This variation may be due to several factors such as inter-study variation in performing the identification tests, geographic variance in the oral profile of *Candida* species and the availability of anti-fungal medication in South Africa. Further research in HIV sero-positive and HIV sero-negative individuals is required to establish a baseline for *C. dubliniensis* in the South African population.

6.5 *Candida* sp. load

A 50% reduction in the *Candida* load was observed over the three months study period (Fig 10). The female study population demonstrated a greater reduction in *Candida* load than their male counterparts (Fig 11).

A complex series of factors may account for these changes;

- A regular, balanced and supervised diet,
- Vitamin and iron supplementation where required,
- Use of prophylactic nystatin
- Treatment of severe recurrent *Candida* infections with fluconazole
- Use of anti-microbial mouthwashes to control and treat secondary oral infections
- The prohibition of smoking and drinking in the hospital,
- The employment of the DOT system (Directly Observed Treatment) for the in-patient Tuberculosis therapy,
- Concomitant infections treated and sensitivities obtained where required,
- Encouragement with personal and dental hygiene measures.

The instruments used in this study do not allow for a more detailed analysis in the variation of *Candida* load between the male and female populations of the study.

6.6 Anti fungal regimes at the Brooklyn Chest Hospital

The scale and variety of patient management at the BCH necessitate that all medications, including oral topical anti-fungal preparations, be administered before breakfast. Researchers found that many of the anti-mycotics available for the treatment of oral candidosis are diluted by saliva and by the cleansing action of the oral musculature. This reduced the availability of the antifungal agents below that of effective therapeutic concentration. These studies

indicate that sub-therapeutic levels of topical antifungals may modulate candidal colonisation of the oral mucosa (306-08).

95% of the study population were placed on long term prophylactic Nystatin anti-fungal medication for varying periods of their in-patient stay. Medical residents are rotated from Tygerberg Hospital to the Brooklyn Chest Hospital on a three monthly basis. This factor could account for the wide variation in provision of Nystatin medication. Brooklyn Chest Hospital does not have a protocol for the provision of anti-fungal agents for their HIV positive patients. There was little change observed in the prevalence of *C.albicans* over the study period (Fig 34). Other *non-Candida albicans* species showed a decrease in prevalence (Fig 34). This might be accounted for by the fact that Nystatin is more affective at suppressing the adhesion of *non-C.albicans* species than *C.albicans* (306,309-10).

Fluconazole was prescribed for severe cases of oral candidosis. Prior approval had to be obtained from the Hospital Administrator for each 10-day course of fluconazole. Again variations in prescription protocols were observed among patients. A total of 8 patients received one or more 10-day courses of fluconazole for treatment of oral candidosis. One patient received fluconazole for the duration of her inpatient stay.

6.7 Resistance to anti fungal agents in the study population

Antifungal resistance can be divided into two categories; clinical resistance and in vitro resistance.

Clinical resistance signifies a lack of clinical response to the antifungal agent used which may denote low levels of the agent in the serum, or in / around the tissues. Two of the most important reasons for low levels are, non-compliance and incorrect application. In AIDS patients antifungal agents even a very high doses may not completely eradicate the fungi from the host.

In vitro resistance can be subdivided into primary and secondary resistance. Primary resistance is where the organism has an innate or intrinsic resistance to the agent, for example *C.krusei* to fluconazole (57). Secondary or acquired resistance is described when the isolate producing the infection becomes resistant to the antifungal agent. This form of resistance was rare in the past, however it is now the most frequently reported form in AIDS patients who suffer recurrent azole resistant oropharyngeal or oesophageal candidosis (232-35). Prior exposure to antifungal medication may affect subsequent responsiveness to antifungal therapy (57,232-36,311-14). Studies have detailed the development of fluconazole resistance to clinical isolates of *Candida sp.* not previously exposed to the antifungal medication (210,315).

The author was not able to discover any records of previous antifungal medication exposure for the study population prior to admission to the BCH. It is possible that multiple genotypes are present in the oral cavity. Primary identification of *Candida sp.* was performed using the phenotypical characteristics of the isolates on CHROMAgar. Subsequent selection and sub-culturing of a single colony may have resulted in the elimination of certain genotypes and so may not have allowed for consistent selection of genotypes for antifungal sensitivity testing. Hence the author cannot comment on any

relationship between antifungal regimes and decreased anti-fungal agent susceptibility.

None of the study population exhibited resistance to Nystatin. However resistance to Nystatin has been reported in the literature (311). All but one isolate had levels of Amphotericin B susceptibility below 6 ug/ml (Table 5). Susceptibility to Fluconazole decreased by 10% over the three months study (Table 4). There was an increase of 1% in the number of patients who showed fluconazole susceptibility of greater than 128 ug/ml. There are a number of factors that may have influenced these results.

Research into the treatment and management of non-*C.albicans* oral candidosis indicate that important susceptibility patterns are emerging for these yeasts (2). Amphotericin B is extremely active against most *Candida sp.* and general resistance is rare. However Amphotericin B may not be the agent of choice for infections caused by *C.parapsilosis*, *C.glabrata* and *C.kefyr* (Table 5). An oral rinse taken on admission from one male patient gave an isolate of *C.glabrata* resistant to Amphotericin B (Table 5)(316). Although *C.glabrata* isolates give higher minimum inhibitory concentration values for Amphotericin B, it is generally sensitive to the antifungal agent (316). The antifungal agent flucytosine is reported to have greater action against *C.glabrata* (19).

Both isolates of *C.krusei* identified in this study were resistant to fluconazole. This *Candida sp.* is innately resistant to fluconazole (Table 4)(19,57). All *C.glabrata* species isolated in this study demonstrated a low sensitivity to fluconazole (19, 236).

C.dubliniensis was isolated in one patient both on admission and at three months. The isolate of *C.dubliniensis* at three months displayed an MIC greater than 256 ug/ml indicating fluconazole resistance. This patient received one 10-day course of fluconazole during the study period. Although this *C. dubliniensis* isolate displayed a lack of sensitivity to fluconazole, it remained susceptible to Amphotericin B (Table 4)(40). Studies have reported that the development of fluconazole resistance by *C.dubliniensis* may depend on the dosage of drug administered, the duration of therapy, or the immune status of the patient. These aspects of fluconazole resistance have also been documented for *C.albicans* (317). Investigations *in vivo* suggest that *Candida* populations including that of *C.dubliniensis* may have the ability to respond in a dynamic fashion to antifungal therapy and may involve changes in gene expression. Studies have suggested that *C.dubliniensis*, unlike *C.albicans* appears to possess the ability to rapidly develop stable resistance to fluconazole following direct exposure to the drug *in vitro*. An Irish study has reported that *C. dubliniensis* encodes multi drug transporters which facilitate efflux of fluconazole from the cell via specific multidrug transporters (MDR1)(41).

6.8 Garlic

Studies have suggested that the herb garlic has antibacterial and anti fungal properties (217 – 225). Allicin and Ajoene are constituents of the herb garlic (226). Unpublished data from a study using Ajoene distilled from fresh garlic suggests that it possesses potent anti-fungal properties. This study made use of a synthetic Ajoene synthesised by Professor Hunter of the University of Cape Town. This preparation showed poor anti-fungal properties. A possible explanation may be that samples of naturally distilled Ajoene contain impurities that are essential for potentiating its anti-fungal properties. The Allicin used in the study was distilled from the fresh herb source and demonstrated significant anti-fungal properties. It showed weak or no anti-fungal action against the resistant isolates of *C.krusei* and *C.glabrata*. This adds strength to the proposition that fresh garlic possesses significant anti-fungal properties. Unfortunately the unpleasant smell and long after taste of garlic prevent its widespread use as a treatment modality.

Chapter 7

Conclusions

A large percentage of patients presented with oral lesions, both on admission to the BCH and at the end of the study period.

Oral candidosis was the most commonly diagnosed oral lesion in the study population. The author could find no literature source that specifically investigated the oral lesions of HIV/TB co-infected patients. However oral candidosis is the most commonly observed oral lesion in HIV/AIDS patients.

The clinical type of oral candidosis found in the study population changed, from the pseudomembranous type prevalent on admission, to the erythematous type prevalent at three months.

The erythematous type of oral candidosis is associated with AIDS patients.

C. albicans was the most commonly isolated species of *Candida* in the study population, both on admission and at three months. There was no evidence of a significant change in the prevalence of non-*Candida albicans* species over the study period

C.dubliniensis was identified for the first time in South Africa.

Fewer species of *Candida* were isolated from the oral rinses at three months. There was a significant reduction in *Candida* load, as defined by colony forming units per millilitre, over the study period. This reduction was observed in both the male and female study population.

Resistance to fluconazole increased over the study period. One isolate obtained at three months and identified as *C.glabrata* was resistant to Amphotericin B. None of the *Candida* species isolated during the study exhibited resistance to Nystatin.

Allicin, a component of natural garlic, appeared to possess good antifungal properties. The synthetic ajoene demonstrated poor antifungal properties. Factors present in the natural garlic may play an important role in potentiating the antifungal properties of these compounds.

In conclusion, there was a significant change in the oral carriage of *Candida* species, both in terms of *Candida* species identified and *Candida* sp. load.

Chapter 8

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Chapter 9

Appendices

Patients oral examination for

DATE : / /

Patients Details

Patients Name :

.....

Address :

.....

.....

Hospital Number :

Date of Birth : / /

Male / Female : M F

Social History:

Ethnic Origin :

Occupation :

Marital Status

Children

Alcohol:

Current..... Past..... Never..... How long stoppedyrsmths

Current/Past: Age startedYrs Age stopped yrs Average units/week.....
Drinker

Tobacco:

Current Past Never..... How long stoppedyrsmths

Current/Past Cigarettes Pipe Other

Numbers smoked/day Age startedYrs Age stoppedyrs

Intravenous drug use

History/signs of IVDU:

General Medical History:

Relevant Medical History:

Current medication / Drug treatment:

Date of commencement

Relevant medical history (cont'd)

When diagnosed HIV positive:

Where diagnosed HIV positive

CD4 count (if known):

Viral load (if known):

When diagnosed TB positive:

Where diagnosed TB positive

Details of previous episodes of TB and
outcome:.....
.....
.....
.....

Extra oral examination

Clinical Details

Swelling	Y / N
Sinus	Y / N
Lymphadenopathy	Y / N

Lips Normal / Abnormal

Facial tissues Normal / Abnormal

Muscles of Mastication Normal / Abnormal

Intra oral examination

Lips and vermillion border & Labial mucosa Normal / Abnormal

Commisure of mouth Right Normal / Abnormal

Left Normal / Abnormal

Buccal mucosa Right Normal / Abnormal

Left Normal / Abnormal

Mucobuccal fold Right Normal / Abnormal

Left Normal / Abnormal

Tongue Ventrum Normal / Abnormal

Lateral Normal / Abnormal

Dorsum Normal / Abnormal

Floor of mouth Anterior Normal / Abnormal

Posterior Normal / Abnormal

Hard Palate Normal / Abnormal

Soft Palate Normal / Abnormal

Pillar of Fauces	Right	Normal / Abnormal
	Left	Normal / Abnormal
Gingival tissues	Free	Normal / Abnormal
	Attached	Normal / Abnormal

CPITN Score

Additional clinical findings

.....

.....

Candidal infection

Clinical Details

Present Y / N

Lesion – single / multiple / wipes off

Site

Surface Appearance

Size

Consistency

Other associated lesions

Pseudomembranous Y / N

Erythematous Y / N

If palate involved	
Pin point hyperaemia	Y / N
Diffuse hyperaemia	Y / N
Granular	Y / N
Hyperplastic	Y / N
Plaque like	
Nodular type	
Median Rhomboid glossitis	Y / N
Acute / chronic atrophic	Y / N
Candida associated Angular cheilitis	Y / N

Extra information:

Patient info sheet given

History and examination taken by:

Name

Signature:

Date:

Location: