THE CHARACTERISATION OF *CANDIDA* ISOLATES FROM THE ORAL CAVITIES OF A GROUP OF ACUTE LEUKAEMIA PATIENTS.

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DECLARATION

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

Date: 27/1/97

OPSOMMING

Die doel van hierdie studie was om die invloed van chemoterapie en antifungale terapie op *Candida* spesies in die mondholte van 29 leukemie pasiënte te ondersoek.

Smere en mondspoele was geneem van die pasiënte. *Candida* isolate was op Sabouraud dekstrose agar sowel as differensiële media gekweek. Isolate was geïdentifiseer met behulp van fenotipiese eienskappe asook koolhidraat assimilasie profiele. Genotipering van *C. albicans* stamme was gedoen met behulp van die *C. albicans* spesie-spesifieke peiler, Ca3.

'n Hoë persentasie (72%) van die pasiënte het klinies gemanifesteer met mondletsels, met kandidose (44.8%) as die letsel met die hoogste voorkoms. Ses en-tagtig *Candida* isolate was geïsoleer uit die mondholtes van 25 (86.2%) pasiënte. *C. albicans* (56.98%) was die mees prevalente spesie geïsoleer en was ook verantwoordelik vir die meeste van die infeksies. Agtien *C. glabrata* isolate was geïsoleer van 36% van die pasiënte. *C. krusei, C. tropicalis, C. guilliermondii* en 9 onbekende isolate was ook gekweek. Die meerderheid van die isolate was slegs geïnhibeer deur matige tot hoë dosisse (8- 32µg/ml) van ketokonasool, moontlik omdat die pasiënte vir lang tydperke aan die antifungale middel blootgestel was. In teenstelling daarmee, was die meeste isolate geïnhibeer deur lae dosisse van flukonasool, moontlik omdat die pasiënte glad nie blootgestel was aan die middel nie. Genotipering van *C. albicans* isolate het aangedui dat sommige pasiënte deurgaans identiese stamme gedra het, terwyl ander pasiënte meer as een stam gedra het. DNA analise dui ook aan dat 'n klein persentasie van isolate wat kiembuise en chlamydospore vorm, moontlik verkeerdelik as *C. albicans* geïdentifiseer was.

SUMMARY

The aim of this study was to investigate the effect of cytotoxic therapy and prolonged antifungal therapy on oral *Candida* species in a group of 29 acute leukaemia patients.

The presence of oral lesions was noted, and smears and saline rinses were collected from the oral cavities of the patients. *Candida* isolates were grown on Sabouraud dextrose agar and differential media. Isolates were identified with the aid of phenotypical characteristics and sugar assimilation profiles. *C. albicans* strains were genotyped with the *C. albicans* species-specific probe, Ca3.

A high percentage (72%) of the patients manifested with oral lesions during the study period, with oral candidosis as the most prevalent (44.8%) lesion. Eighty-six oral *Candida* isolates were obtained from 25 (86.2%) of the patients included in the study. *C. albicans* (56.98%) was the most prevalent species isolated and was also responsible for the majority of infections. Eighteen *C. glabrata* isolates were obtained from 9 patients in the study group. Other *Candida* isolates included *C.krusei*, *C. tropicalis*, *C.guilliermondii* and 9 unidentified isolates. The majority of isolates were inhibited only by intermediate to high (8 - 32µg/ml) concentrations of ketoconazole, possibly due to the prolonged exposure of the patients to the drug. It was further found that the majority of isolates needed low doses (4µg/ml and less) of fluconazole for growth inhibition, possibly because the isolates were not exposed to this antifungal drug.

Genotyping revealed that some patients carried the same strain of *C. albicans* throughout the study period, while multiple genotypes were identified in others. DNA analysis further showed that a small percentage of germ tube and chlamydospore positive isolates was possibly incorrectly identified as *C. albicans*.

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ABBREVIATIONS

µg	Microgram
µl	Microlitre
14DM	14 alpha demethylase
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AraC	Cytarabine arabinocide
CaMDR1	*Candida albicans* multidrug resistance
CDR	*Candida* drug resistance
CTAB	Cetyltriammoniumbromide
Cyt P450	Cytochrome P450
dCTP	deoxycytocine triphosphate
DEAE-cellulose	Dethylaminoethyl cellulose
DMSO	Demethylsulphoxide
DNA	Deoxiribonucleic acid
EDTA	Ethylenediaminetetra-acetate
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL	Interleukin
LB	Luria Bertani
$MgSo_4$	Magnesiumsulphate
MIC	Minimum inhibitory concentration
ml	Millilitre
MPO	Myeloperoxidase

NaCl	Sodium chloride
NaOH	Sodiumhydroxide
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nanogram
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RNA	Ribonucleic acid
SDA	Sabouraud Dextrose Agar
SDS	Sodiumdodecylsulphate
sIgA	Secretory Immunoglobulin A
spp.	species
TE	Tris-EDTA buffer
Th 1	T-helper 1
Th 2	T-helper 2
TNF - α	Tumor necrosis factor alpha
TTC	Triphenyltetrazoliumchloride
TTE	TrisHCl-taurine-EDTA buffer
UPGMA	Unweighted paired group method of association

CHAPTER ONE. LITERATURE REVIEW

1.1. HISTORICAL OVERVIEW

Candidosis is an opportunistic fungal infection, caused by yeasts belonging to the genus *Candida*. It varies from superficial mycoses of skin and mucosal areas, to invasive life-threatening disease (1, 2).

Candidosis[syn. Candidiasis or moniliasis (3)] of mucosal areas is known as thrush (4). Infection of the blood is called candidemia, while the term "systemic candidosis" refers to infection of the internal organs (kidneys, liver, brain). The term "moniliasis" is incorrect as *Monilia spp.* are fungi that cause rotting of fruit (2). "Candidiasis" and "candidosis" are both accepted, but Odds (2), prefer to use the term "candidosis" as all other mycoses are described by terms that end with the suffix "-osis", e.g. aspergillosis, histoplasmosis.

The disease was first recognised in 4 BC by Hippocrates, who described oral lesions resembling oral candidosis. The causative organism was first described by Langenbeck in 1839. Various names such as *Oidium*, *Sporotrichum* and *Saccharomyces albicans* were assigned to it before it became *Candida* in 1923 (2).

Candida infections increased during the last few decades (5, 6, 7, 8, 9). Candidemia now constitutes approximately 8-10% of all bloodstream infections (7) and is the fourth most common nosocomial infection in the United States of America (10). Mortality due to systemic candidosis remains higher than 50% (7). Various factors contributed to the increased incidence of candidosis. Medical procedures such as surgery and aggressive immunosuppression increase the survival rate of patients, but also add to the risk of developing opportunistic infections, like candidosis and aspergillosis (6).

Increased knowledge about the disease and its causative organisms, as well as better diagnostic procedures (e.g. computed tomography to identify internal organ infection, polymerase chain reaction to detect *Candida* DNA in blood, and immunoprecipitation) increase the number of

patients that are diagnosed with candidosis. Another reason for the observed increase in candidosis is the appearance of HIV and AIDS-related diseases (11, 12, 13, 14). Oral and oropharyngeal candidosis affect 43 - 93% of HIV-seropositive and AIDS patients and is used as a marker for the progression of HIV-seropositivity to full-blown AIDS (15). In a study by Fischer-Hoch and Hutwagner, these patients showed a tenfold increase of candidosis (8), compared to a twofold increase amongst patients with haematological malignancies.

Infections by *Candida spp.* other than C. *albicans* have increased in recent decades. These species now cause 45% of all *Candida* infections in oncology patients (16, 17, 18). In addition, some species that were previously regarded as non-pathogenic (such as *C. lusitaniae* and *C. zeylanoides*) are now recognised as human pathogens (18, 19, 20, 21, 22).

1.2. THE GENUS CANDIDA

1.2.1 The characteristics of *Candida*

Members of the genus *Candida* are classified as eukaryotic, unicellular organisms. Perfect states (where organism can reproduce sexually) are described for some species, but *Candida* is classified as *Deuteromyces*, or *Fungi Imperfecti* [no sexual reproduction] (23).

Only a few of the more than 150 known species of *Candida* occur in man and cause disease. These organisms live as commensals in the moist, warm areas of the body, i.e. oral cavity, vaginal cavity, gastrointestinal tract and skinfolds. The number of reported *Candida* carriers varies, due to different sampling techniques and study populations (2, 24). Healthy individuals carry lower numbers of organisms than those with predisposing conditions (25, 26). The numbers of healthy carriers are also less than individuals with predisposing conditions (27). Approximately 17 to 58% of hospitalised patients, healthy control subjects and health personnel carry *Candida* on their hands (28). Eight to 20% of healthy people carry *Candida* in the anorectal tract, compared to 1 to 53.1% for individuals

presenting with various diseases (2).

Candida albicans is the most prevalent yeast in humans. Other species of medical importance, are *C. glabrata* (previously *Torulopsis glabrata*), *C. krusei*, *C. parapsilosis*, *C. kefyr* (previously *C. pseudotropicalis*), *C. tropicalis* and *C. guilliermondii*. Species such as *C. lipolytica* and *C. viswanathii* are isolated occasionally from humans.

Candida spp. grow aerobically at temperatures ranging between 20 to 47°C (optimum 37°C). The yeast multiplies asexually by budding, forming oval to elongated blastospores (23). *Candida spp.* are dimorphic organisms and can exist in the yeast (blastospore) or mycelial phases (pseudohyphae). Blastospores are seen predominantly during the commensal state in humans, while pseudohyphae and hyphae predominate during the infectious state (29, 30, 31). All *Candida spp.*, except *C. glabrata*, are able to produce pseudohyphae. *C. albicans* is the only species that are able to form hyphae, i.e. elongated cellular forms that are separated by septa. *C. albicans* possesses unique qualities that distinguish it from the other species, such as the ability to form germ tubes (structures growing out of blastospores and representing the beginning of the hyphal phase) and chlamydospores. Chlamydospores are spherical structures formed at the tip of hyphae. These spores appear when the organism grows in nutrient poor media such as cornmeal agar (32). Recently a new group of clinically isolated *Candida* that are germ tube positive and produce chlamydospores, were described. These isolates do not produce carbohydrate assimilation profiles typical to *C. albicans* isolates. Subsequent genetic analysis led to the reclassification of these strains as *C. dubliniensis* (33).

Candida species are differentiated by colony morphology, colour on differential media (34, 35), as well as the carbohydrate assimilation and fermentation patterns of the species.

The cell wall of *C. albicans* contains mannoproteins, glucan, lipids and chitin (36). It consists of approximately three to five layers, consisting respectively of fibres, amorphous material and granules (37, 38). The inner layer forms invaginations into the cytoplasm (Fig.1).

Figure 1. **The cell wall of *C. albicans*.**

A. Layer with mostly electron-dense mannoproteins
B. Electron-dense area, mostly mannan
C Intermediate electron-dense area (? mannan and glucan)
D Transparent area, mostly glucan
E Electron-dense area (glucan, chitin, mannan)

Figure adapted from Odds (2)

The genotypical characteristics of *Candida* are not fully elucidated yet. Although controversy exists, it is thought that *C. albicans* is a diploid organism (36, 39). *C. albicans* possesses 9 - 10 chromosomes (40), *C. glabrata* 10 and *C. guilliermondii* 6 chromosomes. *C. stellatoidea*, which is considered to be a variant of *C. albicans*, possesses 10 - 12 chromosomes (41). Mitochondrial DNA from *Candida albicans* contains repeated sequences (42). These DNA repeats are utilised in hybridization experiments to detect DNA polymorphisms and, therefore, allow discrimination between different strains of *C. albicans* (43, 44).

1.2.2. Factors that play a role in virulency.

Adhesion: *Candida* isolates possess various adhesins (Table 2) that aid the organism in the establishment of colonisation, infection and evasion of the human immunological defence mechanisms. These adhesins are carbohydrates or proteins that are expressed on the cell wall of

blastospores or hyphae. Mannoproteins, a combination of mannan and proteins, are the most important adhesion molecules (45). Some adhesins are present on all cellular forms of the yeast, but others, like the laminin receptor (46) and complement binding receptors, are expressed exclusively on hyphae.

The fibronectin receptor of *Candida* is related to α5-β1-integrins (47). Fibronectin is a glycoprotein found on epithelial cells, as well as in plasma and body fluids (48,49). The fibronectin receptor is only found in *C. tropicalis* and *C. albicans*. Fibronectin-coated vaginal cells have higher numbers of adhering yeast cells than uncoated vaginal cells. Accordingly, the incidence of vaginal candidosis is higher during periods of the menstrual cycle when more fibronectin is expressed on vaginal cells (50).

Table 1. Adhesion molecules that help *Candida* to adhere to host cells.

Receptor in hosts	**Adhesins on *Candida***
Glycoprotein	Mannoproteins
Molecule on basement membrane of mucosal cells	Laminin receptor, possibly a protein (46)
Acrylic dental material, via mucin	Glycoprotein (51)
Lactocylceramide	Unknown (52)
Fibronectin	α5-β1- integrin
iC3b; C3b	Unknown

Evidence indicates that *C. albicans* binds the complement factors, iC3b and C3b (53,54, 55) through non-covalent bonding. For opsonisation to take place, binding to iC3b must be covalent. Gilmore *et al.* (55) suggested that the non-covalent binding of *Candida* might be a mechanism by which *Candida* evade opsonisation and neutralisation by human immune defence mechanisms.

C. albicans, *C. tropicalis* and *C. krusei* have the best ability to adhere to mucosal surfaces (56).

Invasive strains of C. *albicans*, as well as hyphae and germ tubes, are more adhesive than commensal strains (57) and germ tube-negative strains, thus pointing towards the role of adhesion in the invasion process.

Enzymes: Membrane-damaging phospholipase A, B and C are secreted by pathogenic *Candida* and the catalytic activity of these enzymes is optimum at an acidic pH (58). During infection of mice, invasive isolates produce more of these enzymes (59), causing a greater degree of membrane damage and death. Clinically, the optimum activity at low pH is possibly related to the development of denture-stomatitis (a form of oral candidosis), as the pH of the denture-bearing surfaces is similarly low (58, 60).

Another enzyme, aspartate proteinase, is either secreted by C. *albicans*, or is present in the cell wall. It hydrolyses secretory IgA, complement factor 3 (C3), albumin and keratin. Invasive isolates secrete higher concentrations of proteinases (61, 62) and adhere more readily to mucosal surfaces, thus indicating this enzyme as a virulence factor. This enzyme plays a role in the development of vaginal candidosis (63). Less pathogenic *Candida spp.* also possess a proteinase, termed 265. This proteinase has a lower level of hydrolytic activity than proteinases from more pathogenic species, such as C. *albicans* and *C. tropicalis* (64). Lastly, it was found that antifungal resistant *Candida* isolates (65), as well as those from diseased patients (66), produce more proteinases than antifungal sensitive isolates.

Hydrophobicity: *Candida* cells with hydrophobic surfaces are more adhesive than those with hydrophilic surface areas (67). In C. *albicans*, the degree of hydrophobicity of strains is determined by factors like temperature, the phase of growth (hyphae or blastospores) and changes in the surface exposure of cell wall proteins (68). The precise role that hydrophobicity plays as a virulence factor, is poorly defined, with varying and inconsistent reports. These inconsistent reports are the result of differences in culture methods and material (69).

Germ tube and hyphae formation This phenomenon apparently facilitates the invasion process, since hyphae (and pseudohyphae) can grow more easily between or through cells, than blastospores. Germ tube formation (beginning of hyphal stage) by *C. albicans* enhances the virulence of the organism (70, 71). Hyphae and germ tubes from *C. albicans* adhere better to cells, and hydrolytic enzymes are localised in the tips of hyphae during the invasion process (72). Fibril-like cell wall components, that are found on hyphae of *C. albicans*, appear to enhance the capability to adhere (73).

Switching of colonies: *C. albicans* can switch its colony phenotype from smooth, white colonies to various other forms such as star-shaped, opaque, and wrinkled. This phenomenon is called phenotypical switching (74) and are well described in laboratory strains such as C. *albicans* WO-1 and C. *albicans* 3153A (75). The WO-1 (white to opaque colonies) system are co-ordinated by the *OP4*, *PEP1*, and *Wh1* genes. *OP4* and *PEP1* are active during the opaque phase, coding for products which induce the phenotypical characteristics. The cells of white colonies exhibit normal blastospore characteristics, while the cells of opaque colonies are elongated and larger, with changes in the cell wall (76). Switching possibly plays a role in the pathogenicity of *C. albicans*, as strains from deepseated infections areas exhibit switching at higher frequency than strains from superficial infections (77).

Coagglutination with bacteria Certain piliated bacteria enhance yeast adhesion to epithelial surfaces in vitro (78, 79, 80). At least one paper reported that adherence to bacteria helped *C. albicans* to establish bladder infection (30).

1.3. AETIOLOGY OF CANDIDA INFECTION

Yeasts are acquired through person-to-person contact (81) e.g. a mother to her baby during birth, or from the environment to a person and from one bodily site to another. In the absence or breakdown of host-defence mechanisms, *Candida* invades the superficial surfaces of the host. Commensal

strains usually are the source of infection. Strain replacement can occur, usually by other commensal strains that have traits which help them to adapt better to the host (82).

The blastospores attach to host surfaces with the aid of cell surface adhesins (83). It was demonstrated, with *C. albicans*, that granule rearrangement of cytoplasmic and the cell wall components occur (84) as the blastospores convert to the hyphal phase (31, 37, 71), with the formation of germ tubes. Hydrolytic phospholipases in the tips (85) of hyphae are secreted into the contact area between the penetrating hyphae and the membranes of the host. *C. albicans* hyphae exhibit thigmotropism (contact sensing) and grow in areas where the least resistance to pressure occurs (86). Although hyphae enhance the ability to invade, it was observed that tissue destruction by *C. albicans* blastospores can also occur before the formation of hyphae (87).

Hyphae germinate to form buds and spores. Characteristic candidal lesions such as white plaques in the oral cavity; fungus balls in the brain, microabscesses in the liver, brain and valve endocardium are formed. A typical kidney lesion contains mycelial elements in the tubular lumina, as well as a polymorphonuclear neutrophil infiltrate. Macrophages and lymphocytes are seen on the periphery of micro-abscesses (30).

1.4. THE HOST

1.4.1 Predisposing factors that play a role in *Candida* infection.

Candidosis occurs in patients with debilitating diseases such as diabetes mellitus (88), AIDS (89), and haematological malignancies (90). Infection occurs as a result of the side-effects of therapy, e.g. cytotoxic therapy (91), or a due to the immunosuppressive effect of some diseases, such as AIDS. Oropharyngeal candidosis manifests clinically in AIDS patients when CD4+ lymphocytes are declining (92,93). It is hypothesised that changes in the host, due to treatment or disease, "select" for more virulent and adaptable commensal *Candida albicans* strains (94). This phenomenon is seen

in diabetic, pregnant women (95), whose isolates adhere better to vaginal mucosa than isolates from control patients. Similarly, *C. albicans* isolates from HIV-seropositive patients are more adhesive and secrete more proteinases than isolates from healthy patients (94).

Trauma and existing infections are contributory factors in the development of candidosis. *Candida* superinfection occur in burn wounds (96). Superficial infections caused by *Staphylococcus aureus*, *Serratia marcescens*, *Streptococcus faecalis* and *Herpes*, can become superinfected with *Candida* (97, 98). Disseminated candidosis frequently occur concomitantly with bacterial sepsis (91).

A carbohydrate-rich diet, malnutrition, vitamin (99), and iron deficiency is suspected to be contributory factors in the development of *Candida* infection (2). In vitro experiments demonstrated that an excess of carbohydrates might stimulate *Candida* growth (100), leading to overgrowth.

An intact immune system is vital for protection against *Candida*. *Candida* outbreaks in neonatal intensive care units (ICUs) are well described in the literature (101,102,103,104). Their immature immune systems cause neonates to suffer from oral candidosis, especially during hospitalisation. Supportive care mechanisms for neonates add to the risk of nosocomial infection, e.g. oxygen support, catheterisation and antibiotic administration.

At least one report described the nursing personnel, who were carriers of *Candida*, as the infectious source of *Candida* (101). As in the case of debilitation, it was shown that *C. albicans* adheres better to mucosa of preterm neonates (105) than those born on term. The role of the immune system is also demonstrated in patients with defective immune systems, e.g. patients with certain endocrine disorders and genetic immune defects (2), who suffers from chronic mucocutaneous candidosis (CMC).

About a third of all women develop vaginal candidosis (106). Pregnancy, oral contraceptives, the use of antibiotics, (107, 108) certain spermicidal preparations such as Nonoxynol-9 (108), tight-

fitting nylon underwear (109, 110) and hormonal changes are risk factors in the development of vaginal candidosis. The influence of hormonal changes is observed during the first and fourth week of the menstrual cycle. During this time, intermediate epithelial cells express more fibronectin, higher levels of glycogen are measured and adhesion to vaginal cells is higher (45). The species responsible for vaginal candidosis are *C. albicans*, *C. glabrata* and *C. tropicalis*.

Candidosis, as a side-effect of medical treatment, has increased in recent decades. Such is the risk caused by certain treatments that Marsh *et al.* (111) commented that "the price for increased survival is the propensity to develop unusual infections" (opportunistic infections). The use of antibiotics is one of the major factors that increase the risk of developing candidosis. Antibiotics (112, 113) eliminate normal bacterial flora from the gastro-intestinal tract, leading to yeast overgrowth (114, 115). These bacteria normally inhibit yeast growth, e.g. Gram-negative bacilli form a mucus layer on the gastro-intestinal epithelium of mice, effectively blocking adhesion of competitors like *Candida* (116). Surgical patients (117,118) develop candidosis, such as fungal endocarditis, due to contamination of wounds or catheters. *Candida* endocarditis (119, 120) is a complication in open heart surgery patients. The risk factors in these cases were identified as the use of central venous catheters (121), parenteral alimentation, antibiotic administration (112), corticosteroids (122), respiratory assistance, as well as possible transfer of commensals from health care workers (118) to patients. *C. parapsilosis* infections especially are associated with invasive prosthetic devices. In cases of infected catheter lines, candidosis is rapidly cured simply by the removal of catheters and subsequent antifungal treatment (123). The risk of infection is further enhanced by the fact that *C. albicans* can adhere well to areas that are hydrophobic, like plastic catheters (124). Other drugs that are implicated in the development of various forms of candidosis are psychotropic drugs (125), non-steroid anti-inflammatory drugs and steroids (2).

The nosocomial environment plays an important role in the increased incidence of candidosis. A review by Harvey et al. (5) found an eightfold increase in nosocomial (hospital acquired) fungaemia,

of which candidosis was the main infection. DNA analysis provided evidence that *Candida* can be transmitted to patients in the nosocomial environment (126). Transmission occurs through fomites (127), hospital personnel that are carriers of Candida (118, 128), visiting family of hospitalised patients (129) and contaminated food (127). The longer the period of hospitalisation and immunosuppression (130), the greater the risk for developing Candida infections.

1.4.2 Host immune response to *Candida*

Secretory immunoglobulin A (sIgA), present in saliva and breast milk, is produced in response to *Candida* antigen and protects against mucosal colonisation (131, 132). The inhibiting effect is probably achieved by specific sIgA molecules that block *Candida* adhesion sites on epithelial surfaces.

The host's response to invasion: The first line of host defence is the recruitment of phagocytes, such as polymorphonuclear leukocytes and macrophages, to the site of infection. Polymorphonuclear leukocytes are more successful than macrophages in the intracellular killing of *C. albicans* (133). Both opsonised and unopsonised cells are ingested by phagocytes, but opsonised cells are phagocytosed at a much higher rate than unopsonised cells (134). Phagocytosis and intracellular killing of yeast cells are enhanced in the presence of serum opsonins (135,136).

Killing in polymorphonuclear leukocytes is mediated by the myeloperoxidase-hydrogenperoxide-halide system (137), but also by the aid of a second, unidentified mechanism (138). Danley and Hilger (139) demonstrated that *Candida* mannan can initiate the myeloperoxidase-hydrogenperoxide-halide (MPO-H_2O_2-halide) system in neutrophils. Polymorphonuclear leukocytes also exert a fungistatic effect on yeast cells (140). This mechanism occurs when neutrophils lyse in areas of dense infiltrates of yeast cells. Through the lysis process a fungistatic cytoplasmic protein is released. This mechanism possibly controls the infection and prevents spreading to other areas.

Macrophages adhere to *Candida* mannose residues through their mannose receptor, MR (141). This process is calcium-dependent. Upon phagocytosis, macrophages are stimulated to produce H_2O_2 and oxygen radicals, resulting in the destruction of yeast cells (142).

Non-phagocytosable hyphae are killed by macrophages through direct contact between the macrophage and the hyphae (143). The macrophage attaches to the hyphus and spreads itself alongside the hyphus, which then fails to proliferate any further. The precise mechanism of killing is not known. It is postulated that this mechanism helps with destruction of hyphae that are too big to be phagocytosed. This hypothesis is supported by the observation that macrophages can distinguish between the hyphal and blastospore phases (144) and have a different response to each phase.

The candidacidal activity of unstimulated phagocytes are inefficient, while activation leads to increased killing of *Candida* (145, 146). Augmentation of the immune response occurs through two possible routes. (1) a T-cell independent route, and (2) a T-cell dependent route.

T-cell independent route: Macrophages are activated by myeloperoxidase which is released by polymorphonuclear leukocytes into the environment (147,148). Similar observations of anti-*Candida* macrophage-activation by *Candida*-mannan (149), avirulent C. *albicans* strains (150) and other microbes have been reported. Activated macrophages produce more reduced oxygen intermediates, they induces cytokine production and destroy higher numbers of *Candida* (151).

T-cell dependent route: Upon mucosal challenge with C. *albicans*, epithelial cells exhibit the human leukocyte antigens DQ and DR on its surface (152). These cells are phagocytosed by macrophages, which then present an antigen to T-lymphocytes. The latter proliferates and produce various cytokines, e.g. interferon (IFN-γ) and tumor necrosis factor (TNF-α). Secreted IFN-γ activates macrophage production of TNF-α, which then activates polymorphonuclear leukocytes to kill *Candida*. This stimulatory mechanism, proposed by Ashman and Papadimitriou (153), is very important, as unactivated polymorphonuclear leukocytes and macrophages destroy only

approximately 20 - 30% of ingested *Candida*.

Experiments with mice showed that IFN- γ and TNF- α induce an augmented polymorphonuclear leukocytes-response (154), while cell-growth stimulatory factor (C-GSF) induces the production of more leukocytes in the bone marrow (155).

Murine models demonstrated that mucosal colonisation by C. *albicans* induces a T-helper-1 (Th1) or T-helper-2 (Th2) lymphocyte response (156, 157, 158, 159). The Th1 response results in the generation of immunological protection against infection of mucosa and organs. The Th2 response fails to stop massive organ infiltration by *Candida*, leading to abscess formation and death in mice.

The induction of the Th-reponse is manipulated by various cytokines. The presence of interleukin-4 (IL-4) induces the Th2 response, while the Th1 response can be induced in an animal model by the neutralisation of this cytokine. Protective immunity in organs is conferred by macrophages, and is induced in mice when phagocytes are stimulated with avirulent C. *albicans* (156, 157, 160).

The role of the intact immune system in the protection against candidosis, is clearly demonstrated in AIDS-patients, where there is a definite correlation between declining numbers of CD4+ lymphocytes and the development of oral and oesophageal candidosis (161, 162). The importance of neutrophils in protection against dissemination is underlined by the fact that neutropenic patients (with less than 500 cells /cm^3) are highly predisposed to develop candidosis (163).

1.5. ANTIFUNGAL TREATMENT OF CANDIDOSIS

The polyenes (nystatin, amphothericin B) and the various azoles (imidazoles, triazoles) are the drugs of choice for the treatment of various forms of candidosis (164, 165).

Mechanisms of drug action. Polyenes are produced by soil *Actinomycetes* and consist of a macrolide ring that is closed by an ester or lactone (Fig 2). The target of the polyenes is ergosterol that is present in the cytoplasmic membrane of yeast cells. The drug forms a complex, possibly a

ring structure, with ergosterol. This ring structure results in pore-formation in the membrane, inducing a change in ion transport over the membrane. Eventually the cell contents leak out of the cell, resulting in cell lysis.

The azole drugs target the cytochrome P450 dependent enzyme, lanosterol 14 α-demethylase (Cyt $p450_{DM}$). This enzyme demethylates lanosterol in the pathway that leads to the formation of ergosterol, one of the building blocks of the cytoplasmic membrane.

The introduction of azoles into yeast cells causes the inhibition of the lanosterol demethylation step. No ergosterol is available to be built into the cell membrane, and undesirable sterols build up in the cell wall, with the result that membranes become more permeable, leading to lysis of the cell.

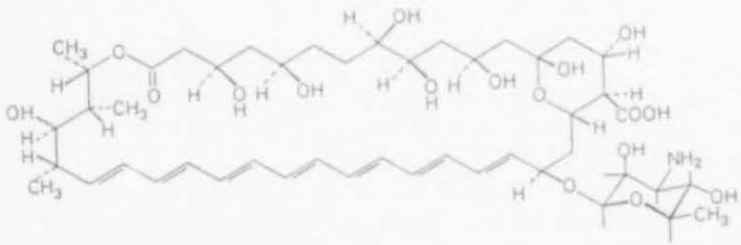

Figure 2. The structure of Amphothericin B (166)

Drug resistance: Drug resistance usually follows prolonged treatment with an antifungal, especially with fluconazole treatment (167, 168, 169, 170, 171). Azole treatment seems to contribute to the emergence of higher numbers of non-*albicans Candida spp.*(16). The mechanisms of resistance are not fully understood, but various mechanisms were proposed. Reduced permeability of the cell wall to fluconazole (172), higher numbers of non-esterified fatty acids in the cell membrane (173), reduced drug accumulation (174) and increased gene expression to compensate for drug-induced inhibition of enzyme activity can cause drug resistance. Van den Bosche *et al.* (175) observed that increased expression of *14DM*, the gene that codes for 14 α- demethylase, caused azole resistance in a *C. glabrata* isolate.

Recent studies revealed the presence of genes that codes for drug transporters that actively pump the antifungal drug out of yeast cells (176, 177). These genes, *CDR1* and *CaMDR1* (178, 179) produce well-characterised transporter molecules, with *CDR1* (*Candida* drug resistance) coding for a protein that belongs to the ABC transporter family and *CaMDR1* coding for a protein that causes benomyl resistance. Increased levels of *CDR1* and *CaMDR1* encoded messenger RNA was found in azole resistant C. *albicans* isolates (178).

1.6. THE ORAL CAVITY AND CANDIDA

C. *albicans* is the most prevalent species found in the oral cavity. Reports on C. *albicans* carrier rates in the oral cavity range between 47 - 75% (180). Oral *Candida* colony counts higher than 400 cfu/ml are associated with oral candidosis, while colony counts less than 400cfu/ml are normally associated with a healthy mucosa and thus carrier status (181).

The posterior dorsum of the tongue harbours the highest numbers of *Candida* in the oral cavity, followed by the palatum and the buccal mucosa of the cheeks (180). In denture wearers, the highest numbers of *Candida* are found on the denture bearing surfaces (182).

Innate protection against *Candida* infection of the mucosa is provided by the washing action of saliva, antimicrobial factors in saliva such as histatins (183) and lactoferrin (184), as well as a well-defined epithelial barrier (185). Competition for food and habitat between *Candida* and the other microbes in the oral cavity helps to maintain low numbers of oral *Candida*. Denture wearers have increased numbers of *Candida*, especially at the denture-bearing surfaces (186).

1.7. CLINICAL PRESENTATION OF ORAL CANDIDOSIS

Acute pseudomembranous candidosis manifests as white plaques on the oral mucosal surfaces. These plaques are scraped off to reveal a red, erythematous surface (187). Infants, the elderly and patients with debilitating disease are most commonly affected by this form of candidosis. Acute atrophic candidosis is associated with antibiotic therapy and present as an erythematous, painful lesion.

Chronic atrophic candidosis (denture stomatitis) is seen in more than 60% of denture wearers (188), especially elderly people. The clinical picture is represented by an extremely erythematous mucositis on the denture-bearing surfaces. The origin of this infection is usually endogenous, from a commensal in the oral cavity (189, 190).

Angular cheilitis is a condition where fissuring of the corners of the mouth occurs. *Candida spp.* are not the sole etiological agents and are frequently found concomitantly with *S.aureus*. The lesion is erythematous and painful (188).

Chronic hyperplastic candidosis (candidal leukoplakia) manifests as persistent white lesions on the oral mucosa. These lesions can be removed, but not as easy as in the case of pseudomembraneous candidosis. The presence of hyphae on smears is confirmation of the diagnosis. It is not known whether *Candida* is the primary cause of the lesion or a secondary invader.

Chronic mucocutaneous candidosis (CMC) involves, besides the oral mucosa, also the skin, nails and

other mucosal surfaces. Autosomal recessive tendencies, as well as familial factors such as endocrine disorders (such as diabetes mellitus, hypoparathyroidism, hypothyroidism) are the underlying conditions that predispose to the development of CMC. CMC lesions show massive inflammatory cells infiltration of lesions (191).

Median rhomboid glossitis manifests as an asymptomatic, diamond-shaped lesion at the junction of the anterior two-thirds and posterior one-third of the tongue. This area is depapillated and appears red.

Recently the pathological features of *Candida* oesophagitis were characterised by Wilcox *et al.* (192). *Candida* oesophagitis is the most common fungal infection in patients with AIDS, presenting as yellow to tan coloured plaques. The individual plaques coalesced (blended) when the disease presented more severely. Plaques extended towards the gastroesophageal junction.

Histological tests revealed four, instead of the normal three layers (basal, prickle, functional) of the oesophageal epithelium. The basal (zone I) and parabasal or prickle (zone II) layers were hyperplastic. An acanthotic layer of mature squamous epithelium, mixed with fibrin, cellular debris and bacteria was visible above the parabasal layer (zone II). Variable numbers of inflammatory cells, mostly neutrophils, are found in zones III and IV. The superficial zones III and IV appear to be sloughing (shedding) from the underlying zones. Extensive infiltration of *Candida* hyphae and filamentous bacteria are visible in the sloughing tissue. Hyphae rarely extended into zone II.

1.8. THE LEUKAEMIA PATIENT

Leukaemia is a haematological malignancy with devastating direct and indirect effects on the afflicted body. Acute leukaemia is classified as acute lymphocytic leukaemia (ALL), or acute myelocytic leukaemia. This classification is based on the kind of haemopoietic cell that becomes malignant. AML has a worse prognosis than ALL, and the associated manifestations are much more severe

(193). Leukaemia cells can invade almost all the tissues of the body, with serious sequelae, e.g. invasion of bone causes severe pain in children with ALL. Healthy haemopoietic cells can be completely replaced by cancer cells, due to the fast leukaemia cell turnover. This results in an impaired immune system.

Anti-leukaemia treatment cause further immune suppression (194). Treatment focuses on the eradication of leukaemia cells (Table 2), and usually consists of a combination of drugs (91, 195, 196). These drug protocols are aggressive and lead to severe immunosuppression. Some drugs have unfavourable side-effects (Table 3), such as nausea and vomiting.

Recent cytotoxic protocols (91) result in more severe bone marrow suppression, longer periods of neutropenia and side effects are usually visible within days of the commencement of therapy (198, 199, 200, 201, 202, 203). New therapies increased the number of leukaemia survivors. Patients in remission are monitored carefully to ensure that relapses are diagnosed early. Certain patients can undergo bone-marrow transplantation (BMT), with a good chance of complete remission.

More than 60% of all leukaemia patients eventually develop oral complications during the course of their disease. The incidence of complications are higher in patients with pre-existing, low-grade or asymptomatic infections.

Oral complications include painful enlargement of gingiva due to the invasion of oral tissue by leukaemia cells, gingival ulceration, infections and alveolar bone and periodontal destruction. Petechial bleeding occur as a result of depletion of platelets in blood (196, 197). Ulceration occur when cytotoxic drugs inhibit all cells having a high turnover, including oral epithelial cells, leading to thinning of the barrier. Mucositis and ulceration of oral mucosa are visible within hours after drug administration. Ulceration usually starts as discrete lesions and occurs more commonly on the non-keratinised surfaces of the mouth (buccal mucosa, soft palate etc.) (198).

Table 2. Antileukaemia drugs and its mechanisms of action.

Classification	Example	Mode of action
Antimetabolites	Methotrexate	Folic acid antagonist, inhibiting the action of FH_2- reductase. The result is inhibition of nucleic acid synthesis.
	Cytorabine arabinocide	A pyrimidine analogue, interfering with the synthesis of pyrimidine, and therefore inhibits DNA-synthesis
	Thioguanine	Purine analogue, interfering with purine synthesis, and therefore inhibiting DNA-synthesis
Alcylating agents	5-fluoro-acyl	Form bonds with DNA, resulting in prevention of replication.
Natural products	Vincristine; vinblastine	Inhibits mitosis by interrupting the spindle apparatus.
	L-asparaginase	Limiting the essential amino acid that is available to cancer cells, leading to starvation and cell death.
Hormones (Adrenocorticosteroids)	Prednisone	Unknown action

Data from: Bachur (199)

Table 3. Antileukemia drugs and their side-effects on the body.

DRUG	SIDE EFFECTS
Methotrexate	Bone marrow suppression, ulceration of gastrointestinal tract, renal failure
Cytarabine	Nausea, vomiting, bone marrow suppression
Prednisone	Hypertension, diabetes, increased susceptibility to infection, osteoporosis
Thioguanine	Slight bone marrow suppression, but generally well tolerated
Mitoxantrone	Alopecia, slight mucositis, bone marrow suppression
Adriamycin	Severe ulcerative stomatitis
Vincristine	Muscle weakness, alopecia, usually no oral ulceration
Daunorubicin	Stomatitis, bone marrow suppression, cardiotoxicity

Xerostomia occurs in *acute leukaemia* patients who receive irradiation (200), because this treatment affects the salivary glands, leading to impaired saliva secretion. The normal turnover of the oral epithelial cells is also inhibited by radiotherapy, resulting in the thinning of the mucosal layer, as well as ulceration. The decreased flow of saliva with its antimicrobial substances such as lactoferrin, lysosyme and immunoglobulin, creates an increased microbial load in the oral cavity and, therefore, an increased risk to develop infection (201, 202, 203).

Any infection present in an acute leukaemia patient on chemotherapy is regarded as a medical emergency. It is therefore essential that these patients are carefully monitored during chemotherapy and that antimicrobial drugs are administered prophylactically (204).

About fifty percent of all oral infections in leukaemia patients are caused by *Candida*, especially C *albicans* (202, 203). Usually candidosis presents clinically as acute pseudomembranous or erythematous candidosis, with 50 - 60% of cancer patients manifesting with oropharyngeal candidosis during the course of the disease (205). Incidents of oral candidosis are related to periods

of neutropenia, especially when neutrophil cell counts drop to less than 500/mm^3. Other risk factors are prolonged hospital stay and periods of long-lasting fever.

It is already established that bacterial oral infections can precede systemic disease (206). Although no definite evidence exists that oral candidosis leads to systemic infection, De Gregorio *et al.* (207) demonstrated that oropharyngeal candidosis usually preceded systemic candidosis in a group of patients. Another study showed that genetically identical *C. albicans* isolates were responsible for oral candidosis and disseminated candidosis in at least one patient (208).

The need for prophylactic measures to prevent oral infections have long been acknowledged (209, 210, 211). Antimicrobial rinses (Chlorohexidine; Nystatin), as well as topical and systemic antifungal drugs (fluconazole, nystatin) seem to be relatively successful to minimise the incidence of oral candidosis (212).

Systemic candidosis (infected liver, kidney, spleen, lungs) remains a serious complication in cancer patients (7, 9, 17). The development of systemic candidosis might be associated with specific antileukemic protocols used to treat patients. In a study by Bow *et al.* (91) it was shown that the so-called protocol AML-87 (high doses of AraC and etoposide, followed by daunorubicin) increased the incidence of systemic candidosis.

In spite of improved detection methods for systemic candidosis, it remains difficult to diagnose (213, 214, 215). Cultures (blood, organ biopsies) are frequently negative (216, 217), symptoms are non-specific and often masked by overt bacteremia (218,219). Most cases of systemic candidosis are identified only after death (220,220,221, 223). Pizzo *et al.* (222) predicted that 33% of leukaemia patients who do not respond to antibiotic therapy and remains febrile, probably would have a systemic fungal infection (either by *Aspergillus* or *Candida*). Additionally, Antilla *et al.* (214) and Talbot *et. al* (223) suggested that systemic fungal infection should be suspected in neutropenic, febrile patients who have elevated C-reactive protein and arabinitol levels in the blood, and present

with *Candida* endophtalmitis. A patient with hepatosplenic candidosis typically manifests with a fever that is unresponsive to antibiotics. In spite of systemic therapy, more than 50% of patients usually die as a result of this infection (217- 219, 222, 224).

Oral and systemic candidosis are major problems for the leukaemia patient as well as the clinicians who treat these patients. Morbidity and mortality associated with this opportunistic disease remain high.

1.9. THE AIM OF THE STUDY

The aim of this study was to investigate the effect of chemotherapy and antifungal prophylaxis on the phenotypical and genotypical properties of oral *Candida* from acute leukaemia patients. These results were correlated with the clinical manifestation of oral candidosis during immunosuppression.

CHAPTER TWO. MATERIALS AND METHODS.

2.1. SAMPLE GROUP

Twenty-nine acute leukaemia patients were seen at Tygerberg Hospital, Cape Town, South Africa from August 1994 to April 1996. These patients were newly diagnosed or relapsed cases of leukaemia. Two patients additionally were treated for diabetes mellitus and cirrhosis of the liver respectively. Participants entered the study before or soon after the initiation of chemotherapy. Therapy for acute myelocytic leukaemia patients consisted of cytarabine arabinocide or adriamycin in combination with daunorubicin, vincristine or idarubicin. Acute lymphoblastic leukaemia patients received cytarabine arabinocide in combination with vincristine, VP16-epipoxiphyliotoxin or daunorubicin.

The patients received methylprednisolone (an anti-inflammatory agent) for immunosuppression, as well as allopurinol to diminish the overproduction of uric acid. The patients received antibiotics and systemic ketoconazole prophylactically. Betadine oral rinses were used by patients who were suspected to suffer from infectious complications. Topical antifungal drugs (amphothericin B lozenges) were administered to treat patients with oral candidosis, and acyclovir to treat oral ulcerations of viral origin (*Herpes simplex*). One patient received systemic amphothericin B, instead of ketoconazole. Two patients did not receive any antifungal prophylaxis.

Clinical examination and sampling: Patients were evaluated every alternative week, except when they were not hospitalised. During each visit, the medical status of the patient was noted, including the presence or absence of fever, the presence of oral candidosis and other oral lesions. Oral candidosis was reported as pseudomembranous, erythematous, mixed pseudomembranous and erythematous candidosis, angular cheilitis and/or rhomboid glossitis. Haematological results were obtained from hospital records.

Smears were obtained from the posterior dorsum of the tongue and the palate of each patient, by scraping the respective surfaces with a wooden spatula. These smears were fixed on slides with a cytological fixative. In cases of suspected oral candidosis, the infected area was also swabbed with a sterile cotton swab. An oral rinse was obtained from each patient, by letting the patient rinse for one minute with 5 ml of phosphate-buffered saline (PBS).

2.2. PROCESSING OF THE SAMPLES

2.2.1. Microscopical investigation of smears

All smears were stained with the periodic acid Schiff method (PAS) and examined microscopically (40x magnification) for the presence of hyphae and spores.

2.2.2. Oral *Candida* isolation

The oral rinses were vortexed and 200 µl of each rinse was plated onto Sabouraud dextrose agar plates (Oxoid) by spreading the liquid over the surface of each plate with a sterile glass rod. Chromogenic media, i.e. Chromagar *Candida* (Mast diagnostics) plates and Pagano-Levin plates (Difco), were also inoculated with 200µl of rinse to ensure the detection of multiple species of *Candida* (225, 226, 227). During the course of the study it was found that rinses from patients presenting clinically with candidosis yielded confluent growth of *Candida* on all the plates. The rinses from these patients were therefore diluted and 200 µl aliquots of the 10^{-2} and 10^{-4} dilutions were plated onto media.

The plates were incubated aerobically at 37°C and assessed for the presence of yeast colonies after 2 - 3 days of incubation. *Candida* colonies were counted on Sabouraud Dextrose agar plates and reported as colony-forming units per millilitre (cfu/ml) of rinse.

2.3. THE PHENOTYPIC CHARACTERISATION OF *CANDIDA* ISOLATES.

Colony morphology on plates, as well as colony colours on the differential media were noted (Tables 4 and 5). Single colonies of different colour were selected on Chromagar and streaked onto Sabouraud dextrose agar-plates to obtain pure cultures. These plates were incubated for 48 hours at 37 °C. Colonies from these pure cultures were subsequently frozen in the Microbank System (Mycoplasma International) at -86 °C. The pure cultures were also used for identification of the isolates.

Table 4. Colony characteristics of *Candida* isolates.

Colour on Sabouraud Dextrose Agar	White, light cream, or dark cream colonies
Colour on Pagano-Levin agar	Pale pink, dark pink, or orange
Texture	Smooth, rough, or dry
Colony borders	Smooth, wrinkled, rough, or mycelial

Table 5. Colour of yeast isolates on Chromagar

SPECIES	COLOUR OF COLONIES ON CHROMAGAR
C. albicans	Green
C. glabrata	Dark pink, pale edges
C. krusei	Pale pink
C. parapsilosis	White to pale pink
C. tropicalis	Blue to grey
C. kefyr	Pink to purple

2.4. GERM TUBE TEST

The germ tube test was done according to the method described by Buckley (32). Five-hundred microlitres of human serum was inoculated with a small fraction of a *Candida* colony and incubated at 37°C. After 2 to 3 hours of incubation, a drop of the serum was examined at 40 times' magnification under the light microscope. The presence of germ tubes identified the isolate as *C. albicans*.

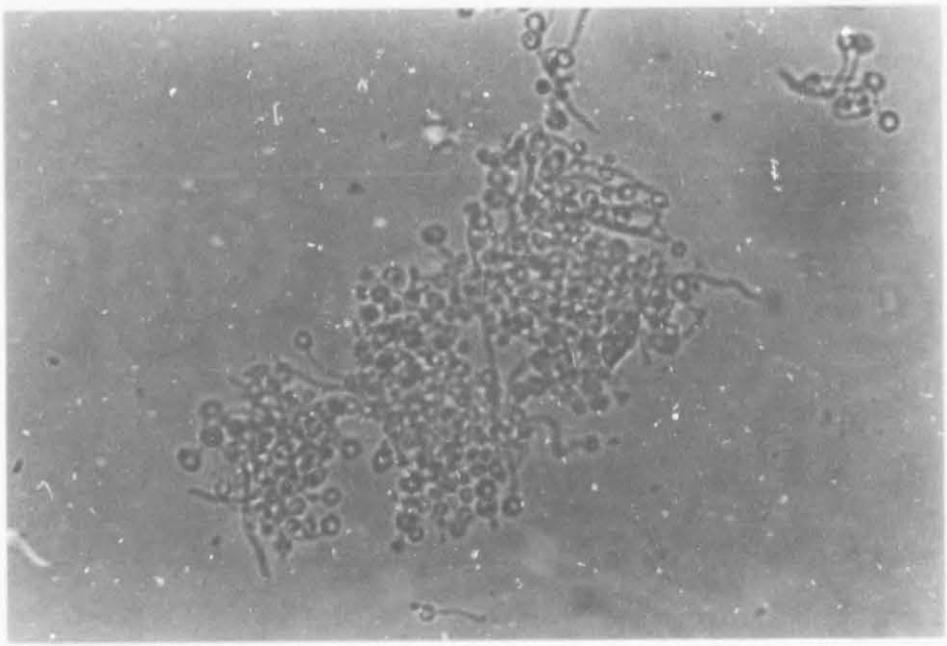

Figure 3. Germtubes of *C. albicans*

2.5. THE DISK-DIFFUSION METHOD FOR THE IDENTIFICATION OF YEASTS.

The disk-diffusion method of Sobczak (228, 229) is based on the ability of certain chemical dyes to inhibit growth of *Candida spp*. Each species exhibits a distinct pattern of sensitivity to these chemicals. The dyes are impregnated onto 6 disks, each containing one of the following: 150µg of

janus green, 37.5µg of ethidium bromide, 150µg of 2,3,5 triphenyltetrazoliumchloride, 1.5µg of brilliant green, 15µg of cycloheximide or 60µg of rhodamine 6G.

A barely turbid saline (NaCl, 0.85% w/v) suspension was prepared with a fraction of a yeast colony. The liquid was spread over the surface of a Sabouraud dextrose agar plate with a sterile swab. The respective disks were placed onto the inoculum in a specific numerical order (Fig. 4). Plates were incubated at 37°C for 16 to 48 hours. The presence or absence of a zone of inhibition around each disk were recorded. Isolates were classified as sensitive or resistant to each dye, depending on the measured zone of inhibition. Identification codes were assigned to each isolate according to the method of Sobczak (228).

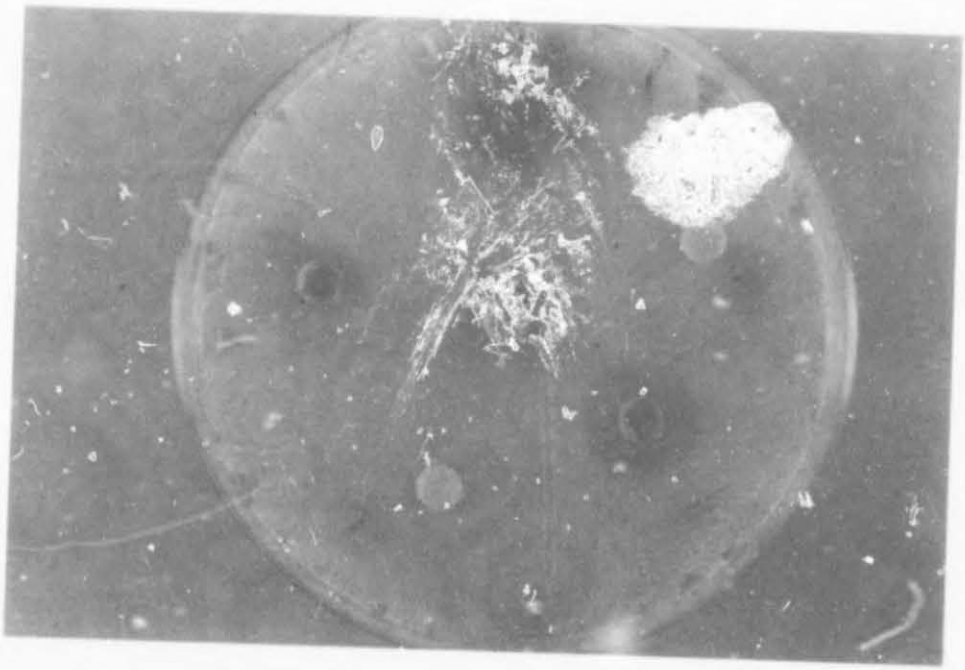

Figure 4. *Candida albicans* isolate identified with the disk-diffusion method.

Table 6. Reference Codes of the disk diffusion method to identify *Candida*.

Species	Codes for species		
C. albicans	100406	*C. krusei*	023456
	103406		023056
	123406		023456
	00040		123456*
	120406		
C. glabrata	000050	*C. tropicalis*	123456
	020050		
	023050	*C. kefyr*	123406
	023056		
	023450	*C. parapsilosis*	020450
	123050		120450

Data from Sobczak (228) and Stead and Neil (229). * Code identical to *C. tropicalis*, but *C. krusei* distinguished by characteristic dry colonies.

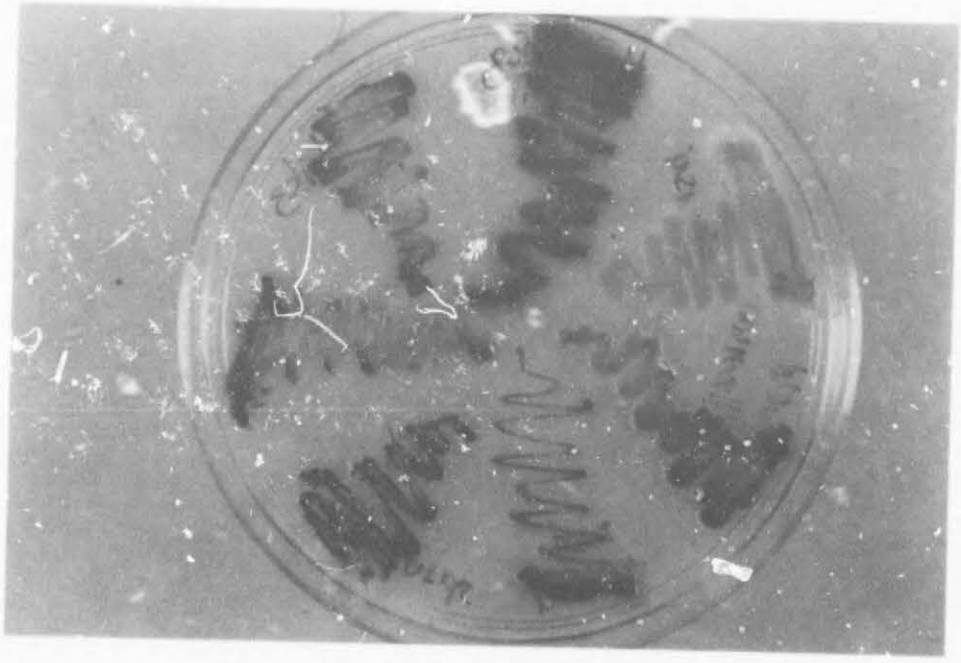

Figure 5. *Candida* isolates on Chromagar *Candida*. (*C. albicans* = green.; *C. glabrata* = smooth, pink; *C. krusei* = flat, dry, pink; *C. tropicalis* = bluegreen)

2.6. THE IDENTIFICATION OF CANDIDA WITH AUXANOGRAMS

The API 20C and ID 32C systems (Bio Merieux, Marcy L'etoile, France) were used to determine carbohydrate assimilation patterns of yeasts. A barely turbid suspension of yeast cells was made with saline and a fraction of a yeast colony. One hundred microlitres of this suspension were used to inoculate an ampoule of prepared API 20C suspension medium. The 19 cupules, each with a different sugar, on the API strip were inoculated as described by the manufacturers and incubated in a moist chamber at 37°C. Results were read after 24 hours, 48 hours and 72 hours. Turbid growth, equal to or more than the positive control, was regarded as a positive result. An identification code, based on the sugar assimilation patterns of each species, was assigned to each isolate. The isolates were identified according to the API reference tables.

2.7. BIOTYPING OF ISOLATES WITH THE API 20C SYSTEM

A biotype was assigned to each isolate of *C. albicans* according to the method of Williamson *et al* (230) (Table 7).

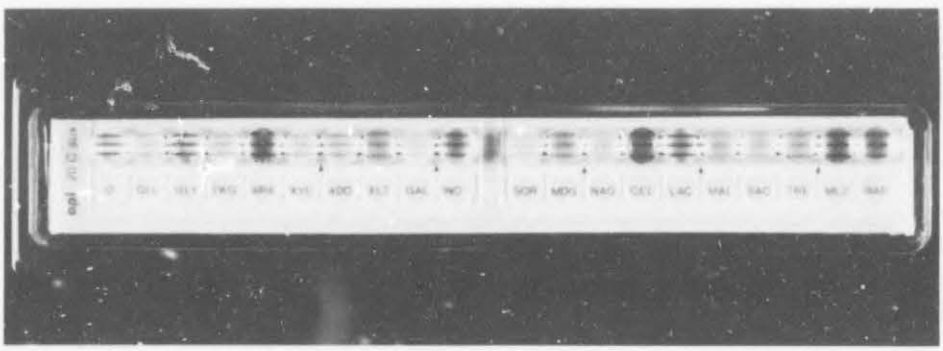

Figure 6. Auxanogram of *C. albicans*

Table 7. Biotypes of C. *albicans* according to API 20C profiles

	Assimilation of sugar in wells										
BIOTYPE	***3**	**5**	**6**	**7**	**8**	**11**	**12**	**13**	**17**	**18**	**19**
1	-	-	+	+	+	+	+	+	+	+	-
2	-	-	+	-	+	+	+	+	+	+	-
3	-	-	+	+	+	+	-	+	+	+	-
4	+	-	+	+	+	+	+	+	+	+	-
5	-	-	-	+	+	+	-	+	+	+	
6	-	-	+	+	+	+	+	+	+	-	-
7	-	-	+	+	+	+	+	-	+	+	-
8	-	+	+	+	+	+	+	+	+	+	-
9	-	-	+	+	+	-	+	+	-	+	-
10	-	-	-	+	+	+	+	+	+	+	+
11	-	-	+	-	+	-	+	+	+	+	-
12	-	-	+	+	+	+	+	+	+	+	+
13	-	-	+	+	-	-	+	+	+	+	-

Table adopted from Williamson *et al.* (16)
*3 - glycerol; 5 - L-arabinose; 6 - xylose; 7 - adonitol; 8 - xylitol; 11 - sorbitol;
12- methyl-D-glucoside; 13 - Naccetyl-D-glucosamine; 17-sucrose; 18 - trehalose;
19 - melezitose
+positive ; - negative

2.8. DNA ANALYSIS OF *C.ALBICANS* ISOLATES.

2.8.1. Culturing and purification of bacteriophage Lambda Ca3

Escherichia coli LE392 was used as the host for the amplification of lambda bacteriophage Ca3.

This hybrid phage contains a fragment of DNA from C. *albicans* 3153A (42, 43, 44). A Ca3 probe was prepared by random priming with radiolabeled deoxycytosine triphosphates (dCTP) and was applied to analyse the genetic relatedness of clinically isolated C. *albicans* strains. Probe Ca3 hybridises to approximately 15 to 25 bands of DNA restricted with EcoR1 (231).

Phage stock solutions of high titre were prepared as described by Sambrook *et al.* (232). A modification of the method of Manfioletti and Schneider (233) was used to obtain pure DNA from bacteriophage lysates.

2.8.2. Preparation of the bacterial host

Escherichia coli LE392 was grown overnight, at 37°C, in 10ml of Luria-Bertani broth (Sigma), containing 0.2% maltose and 10mM $MgSO_4$. One millitre of the overnight culture was used to inoculate 50ml of Luria-Bertani broth (LB broth), supplemented with 0.2% maltose and $MgSO_4$. (The presence of maltose stimulates the formation of a maltose receptor molecule on *E.coli* cells, by which bacteriophage particles attach to the bacteria). The culture was incubated at 37°C until the culture reached an optical density of 0.5 (i.e. is approximately 2.5×10^8 cells/ml) at 600nm. The bacterial cells were pelleted by centrifugation, for 20 minutes at 3 000g in a Beckman centrifuge (Model TJ-6). The supernatant was discarded and the pellet resuspended in 0.01M $MgSO_4$. The bacterial cell concentration of the suspension should preferrably be approximately 8×10^8 cells/ml.

2.8.3. Preparation of phage stocks of high titre.

Approximately 10^5 plaque-forming units (pfu), of a plaque stock solution in SM buffer (0.1M NaCl, 8mM $MgSO_4 \cdot 7H_2O$, 10mM Tris-HCl (pH 7.5), 1% gelatin) were added to 100µl of the bacterial suspension in a sterile tube. The solution was mixed and incubated for 15 minutes at 37°C, allowing the attachment of the phage to the host cells. Three millilitres of molten 0,7% agarose, prepared in LB broth, were added to the solution. The agarose was mixed and immediately poured onto a plate

of LB-agar (Sigma). All plates were incubated in an inverted position at 37°C until lysis occurred. Harvesting of the phage was done by pouring 3ml of SM solution the agar surface. The liquid, which contained the phage, was harvested with a sterile Pasteur pipette after 3 to 4 hours of incubation at 4°C. A drop of chloroform was added to the harvest and cellular debris removed by centrifugation at 5 000 revolutions per minute (rpm). The phage solution, containing approximately $1x10^{9-11}$ phage particles, was stored at 4°C in the presence of a few drops of chloroform, which inhibited bacterial growth in the stock solution.

2.8.4. Preparation of bacteriophage lysates from LB broth

Approximately 10^{8-10} pfu of the phage stock solution, 500µl of SM, as well as approximately 10^{10} of bacterial cells were added together, mixed well and incubated for 15 - 20 minutes at 37°C. This mixture was added to 100ml of prewarmed LB broth, containing 0.5M $CaCl_2$. The cells were incubated in a shaking waterbath at 37°C, until lysis occurred (4 - 7 hours). Two millilitres of chloroform was added to the lysate and the broth incubated for a further 15 minutes at 37°C. The lysate was centrifuged at 5000 rpm and the supernatant transferred to clean Sorvall centrifuge tubes.

An equal volume of a solution, containing 20% (w/v) polyethylene glycol (PEG 8000) and 2M NaCl, was added to the lysate and the solution incubated at 4°C for 16 hours to precipitate the phage particles. (The presence of NaCl promoted the dissociation of bacteriophage particles from bacterial debris). The phage particles were pelleted by centrifugation at 14 000 rpm in a Sorvall centrifuge (Model RC-5) for 30 minutes. The supernatant fraction was discarded and all of the remaining liquid removed from the pellet with a pipette. The precipitate was resuspended in 15 ml of SM buffer, containing 2M NaCl. Twenty micrograms of DNase I was added and the suspension incubated at room temperature for 30 minutes. Thereafter the solution was centrifuged at 7 500 rpm, for 10 minutes and the supernatant transferred to a clean tube.

An aliquot of 255 µl of a 0.3% gelatine, 0.1% sodium azide solution was added to the phage suspension, followed by an equal volume of a 75% DEAE cellulose (DE-52) slurry, which was prepared in LB broth. This mixture was rotated at room temperature for 30 minutes to allow the polyanions in the lysate to bind to the anion-exchanger (DE-52). The slurry was spun down twice, at 15000 rpm, 4°C for 10 minutes and the supernatant was retained.

Disodium ethylenediaminetetra-acetate (EDTA) and proteinase K were added to final concentrations of 20mM and 50µg/ml respectively, and the mixture incubated at 45°C for 20 minutes to disrupt the phage and digest proteins. Cetyltriammoniumbromide (CTAB) was added to a concentration of 0.1% from a 5% CTAB, 0.5% NaCl solution, and incubated at 68°C for 6 minutes. The lysate w cooled on ice for 30 minutes and then spun down for 10 minutes, at 15 000 rpm. The resulting pellet of DNA, bound to the cationic CTAB, was resuspended in 3ml of NaCl (1.2M) and

precipitated with 2.5 volumes of cold ethanol (100%). The DNA, which was now bound to Na, was left at -70°C for 30 minutes, and then pelleted by centrifugation at 10 000 rpm, for 10 minutes. All salts were removed from the DNA with 70% ethanol, the DNA was air-dried and dissolved in 250µl of TE (pH 8). Quantitation of DNA was done spectrophotometrically.

2.8.5. Preparation of the radiolabeled probe

The undigested DNA isolated from the bacteriophages was radiolabeled with α^{32}P-dCTP by random priming with the Prime-It RMT labelling kit (Stratagene) according to the manufacturer's instructions. Water and 25 to 50 ng of DNA were added together to a final volume of 42 µl in a microfuge tube. The DNA was denatured by heating at 100°C for 5 minutes. Five microlitres of radiolabeled dCTP and 3 µl of DNA polymerase (4U/µl) were added to the reaction tube. The solution was mixed well and incubated for an hour at 37 °C. The labelling reaction was stopped with the addition of 2 µl of the Prime-It stop solution. Labeled probe were desalted by centrifugation

through a Sephadex G50 spin column (232). The purified probe was stored frozen at -20 °C and used within two weeks after the preparation thereof.

2.8.6. The isolation of yeast DNA from isolates of *C. albicans*.

DNA was prepared from *C. albicans* by a modification of the method described by Scherer and Stevens (43). Twenty millilitres of yeast extract-peptone-dextrose (YPD) broth were inoculated with a colony of *C. albicans* and incubated overnight at 37°C in a shaking waterbath. The cells were pelleted twice by centrifugation at 5 000 rpm for 5 minutes and washed with 3ml of a cold buffer containing 25 mM Trischloride and 50mM EDTA (pH8). The cells were weighed and resuspended in a buffer containing 1.4 ml of a 25mM Trischloride, 50mM EDTA solution per gram of wet yeast cells.

Beta-mercaptoethanol was added to the suspension to a final volume of 17,5µl per gram of wet cells. Water was added to the suspension, to a final volume of 3.5ml. This suspension was incubated for 20 minutes at 30°C to remove the outer mannan layer of the yeast cell wall.

The cells were pelleted and then resuspended in 1ml of a solution containing 1M sorbitol (for osmotic balance), 50mM potassium phosphate (pH 7.5), 0.1% B-mercaptoethanol and 50 mM EDTA. The yeast cell suspension was incubated at 30°C in the presence of 100µg of a β-glucanase (Zymolyase 20T). This enzyme digests the cell wall and leads to the formation of spheroblasts. Spheroblast formation was monitored by adding 10µl of the yeast suspension to 90µl of water. A clear solution indicated that the formed spheroblasts were lysed in the absence of an osmotic buffer. Cells were pelleted when 90% of cells formed spheroblasts, by centrifugation at 5 000 rpm for 5 minutes (Beckman centrifuge). The pellet was resuspended in 3ml of TE (pH 8), followed by the addition of 300 µl of 10x concentrated Proteinase K buffer (0,1M Trischloride, 0,05M EDTA and 5% SDS) and 150µl of proteinase K (10 mg/ml). The lysate was incubated for 16 to 18 hours at 45°C to digest proteins. The centrifugation step was not done if lysis of spheroblasts occurred,

because it is difficult to resuspend the lysed mixture of cells and DNA after centrifugation. In these cases the Proteinase K buffer and Proteinase K were added directly to the lysed mixture.

Protein debris were removed by a phenol extraction as described by Sambrook *et al.* (232). An equal volume of a phenol-chloroform-isoamylalcohol (25:24:1, V/V) solution was added to the DNA mixture and mixed until an emulsion formed. The organic and aqueous phases were separated by centrifugation at 3 000 rpm for 30 minutes. The aqueous layer was transferred to a clean tube and mixed with an equal volume of chloroform-isoamylalcohol (24:1) to remove traces of phenol. After centrifugation for 20 minutes at 3 000 rpm the aqueous layer was transferred to a clean tube and sodium acetate (3M, pH 5.2) was added to a final concentration of 0.3 M. The DNA was precipitated by the addition of 1 volume isopropanol, spooled around a glass rod and washed once with an excess of 70% ethanol to remove salts from the DNA. The spooled DNA was air-dried and dissolved in 500 µl of TE, pH 8.

Contaminating RNA was digested by incubating the DNA solution in the presence of 2µl of pancreatic RNAase (5mg/ml) for 30 minutes at 37°C. The DNA was phenol extracted and ethanol precipitated as described above. After two subsequent alcohol precipitations to ensure that all the impurities were removed, the DNA was air-dried and dissolved in 200µl of TE (pH 8). The DNA concentration was determined with a spectrophotometer, as described by Sambrook *et al.* (232).

2.8.7. DNA Fingerprinting of *C. albicans*.

Six micrograms of *C. albicans* DNA, 5µl of EcoR1 (12U/µl), water and 20µl of incubation buffer were added together to a final volume of 200µl. This mixture was incubated overnight at 37°C. To asses if DNA samples were completely digested, a 7µl aliquot of each sample was mixed with 3µl of loading buffer (0.25% bromophenolblue, 15% Ficoll 400) and loaded onto a small 0.8% agarose gel (Seakem FMC). Electrophoreses was performed at 95V for approximately 120 minutes in 1 x concentrated Trischloride-taurine-EDTA (TTE) buffer (89.8mM TrisCl, 288mM taurine, 5 mM

EDTA, pH 8). The gel was stained in ethidiumbromide (0.5µg/µl) and digested DNA visualised with ultraviolet light.

Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M to the digested DNA solution. Two volumes of ice-cold 100% ethanol were added and the DNA precipitated by overnight incubation at -20°C (or for 30 minutes at -86°C). The precipitated DNA was pelleted by centrifugation at 15 000 rpm for 30 minutes, at 4°C. After it was washed in 70% ethanol, the DNA was air-dried and dissolved in loading buffer, containing Marker 10 DNA (Boehringer Mannheim) as the internal marker. The volume of loading buffer was determined by the intensity of the stained DNA on the minigel, and ranged from 10µl to approximately 25µl of buffer. This was done to ensure that equal concentrations of DNA were loaded on the fingerprinting gel.

DNA fragments were separated in a 0.8% agarose gel by gel-electrophoreses, for 18 hours, at 1 volt per centimetre (V/cm). Electrophoreses was performed in 1x concentrated TTE buffer (pH 8). The gel was stained in ethidiumbromide (0.5µg/µl) for 30 minutes, allowing the visualisation of DNA bands with ultraviolet light.

2.8.8. The Southern hybridisation of the DNA to probe Ca3.

The DNA -fragments were denatured by incubating the gel in 500ml of 0.4M NaOH for 20 minutes. Minute drops of the orientation markers, containing denatured *C.albicans* (12.5µg/µl) and Marker X DNA (1.25µg/ml) were applied to the four corners of a nylon membrane (Hybond N+, Amersham). The gel was washed with water and 20 X SSPE buffer and the DNA fragments transferred to the nylon membrane by capillary blotting as described in Sambrook *et al.* (232).

DNA was fixed to the nylon filter by baking at 80°C for 2 hours. Thereafter it was incubated for at least 3 hours in the hybridisation solution [5xSSPE, 5xDenhardt's reagent, 0.1% SDS, 50% formamide and salmon sperm DNA (100µg/ml)], at a temperature of 42°C. Ten microlitres (approximately 0.5 ng/µl stock solution) of the prepared radiolabeled probe was denatured by

heating at 100°C for 5 minutes. The probe was added to 20 ml of hybridisation solution. Hybridisation was performed overnight at 42°C. The membrane was initially washed twice at room temperature in 500 ml of a solution containing 2xSSC (0.3M NaCl, 0.03M sodium citrate) and 0.1% SDS for 30 minutes. Thereafter it was washed twice, in a similar buffer, at 55°C for 30 minutes each.

The filter was sealed in a clean plastic bag and exposed to an x-ray film for 16-24 hours at -70°C. The autoradiograph was developed in a dark room, by immersing it in the developing solution for 3 minutes, the stop solution (3% acetic acid) for 20 seconds and fixing solution for 1 minute. Thereafter it was washed with water and left to dry.

2.8.9. Non-radioactive labelling of the internal marker with the enhanced chemiluminescence (ECL) detection system.

The radioactive probe Ca3 was stripped from each blot by incubating it for 20 minutes in 0.4N NaOH at 45°C. Each stripped blot was washed in neutralising buffer (0.2 x SSC, 0.2% SDS, 10mM Trischloride, pH 8) for 15 minutes.

Labelling, hybridisation of labeled Marker X and visualisation of bands were done with the non-radio-active ECL detection system (Amersham). The ECL Gold buffer was prepared as described by the manufacturer. The buffer was preheated at 42°C before 25ml was added to a new hybridisation bag containing a previously stripped blot. The blot was incubated in the hybridisation buffer for at least three hours at 42°C.

Marker X was labeled according to the ECL protocol (Amersham). One microlitre of Marker X DNA (0.25µg/µl) and 14µl of water were mixed in a microfuge tube. The DNA was denatured by heating it at 100°C for 5 minutes, followed by immediate cooling on ice. Equal volumes of the ECL labeling reagent and glutaraldehyde were added to the tube. The solution was mixed well and

incubated for 10 minutes at 37°C. The probe was added to the hybridisation bag and hybridisation performed overnight at 42°C.

Two subsequent washes of the blot were done in primary wash buffer (0.5xSSC, 0.4% SDS, 6M urea) at 42°C for 20 minutes, followed by two brief washes (5 minutes) at room temperature in 2 x SSC. All liquid was removed from the blot and the latter was placed in a new plastic bag. Equal volumes of the ECL detection reagents 1 and 2 were mixed and added to the hybridisation bag. The liquid was evenly spread over the blot for one minute, before it was removed from the bag. X-ray films were exposed to the blot for short periods of time (a few seconds to minutes) before it was developed as discussed above.

2.8.10. Computer assisted analysis of *Candida albicans* DNA digests.

Autoradiographs were scanned (HP IIcx/T; Hewlett Packard) and analysed with the Gelcompar software (Windows Version 4; Applied Mathematics, Kortrijk, Belgium). Banding patterns were normalised by the superimposition of the autoradiographs of the Ca3 hybridisation patterns (of isolate DNA digests) over that of the internal marker. Banding patterns of the isolates' digested DNA were compared with the unweighted paired group (UPGMA) clustering method, using the Dice coefficient. Similarity matrices were generated to determine the degrees of relatedness between oral *C. albicans* strains as discussed above (234).

2.9 THE DETERMINATION OF ANTIFUNGAL SENSITIVITY OF *CANDIDA* ISOLATES.

The broth macrodilution method was performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (235). *C. albicans* NCPF 90029, *C. parapsilosis* NCPF 90018 and *C. glabrata* NCPF 90030 were used as reference strains. The

minimum inhibitory concentrations (MIC) of various antifungal drugs needed to inhibit growth of these reference strains are known and these strains were therefore included in each test batch. The MIC tests results of clinical isolates are regarded as reliable if the MIC values of the reference strains for ketoconazole are between 0.03 - 0.125 µg/ml and 0.25 - 1 µg/ml for *C. albicans* and *C. glabrata* respectively. The MIC values of the reference strains for fluconazole should be between 0.125 - 0.5 µg/ml and 8 - 16 µg/ml for *C. albicans* and *C. glabrata* respectively.

Ketoconazole was a kind gift from Jansen Research Foundation (Beerse, Belgium) and fluconazole from Pfizer Inc. (New York). Antifungal stock solutions were prepared in the appropriate solvents (dimethylsulphoxide (DMSO) or water) and stored at -20°C. Twofold drug dilutions were prepared in RPMI 1640 medium (Sigma), which was buffered to pH 7 with 0.165M morpholinepropanesulfonic acid (MOPS). Concentrations of drugs ranged from 0.03125 to 32µg/ml.

The yeast isolates were grown on Sabouraud-dextrose agar plates for 24 hours. Yeast cell suspensions with an optical density, at 620nm, of 0.08 - 0.1, were prepared in saline (0.85%). An aliquot of 900µl of each yeast suspension was used to inoculate sterile tubes, containing 100µl of the antifungal solution at the various dilutions. The mixture was mixed well and incubated aerobically at 30°C. The MIC endpoints were determined as the minimum concentration of drug where growth inhibition of yeast cells were observed. MIC's were read after 48 hours.

CHAPTER THREE. RESULTS

3.1. THE CHARACTERISTICS OF THE STUDY GROUP.

Fifteen (52 %) of the 29 acute leukaemia patients included in this study presented with acute lymphoblastic leukaemia and 14 (48 %) with acute myeloblastic leukaemia (Table 1). The ages of the patients ranged from 14 to 74 years (median 25 years).

Table 8. The prevalence of the types of leukaemia in the study group

	AML (%)	ALL (%)
	n=29	
Female	9 (31.03%)	6 (20.7%)
Male	5 (17.24%)	9 (31.03%)

Table 9. The age distribution of the patients.

Age (years)	AML (n=14)	ALL (n=15)
10 - 19	2	4
20 - 29	5	6
30 - 39	3	1
40 - 49	0	2
50 - 59	4	0
60 - 69	0	1
70 79	0	1

3.2. HAEMATOLOGICAL EVALUATION OF PATIENTS

Available haematological results were analysed and showed that patients became severely immunosuppressed during cytotoxic therapy. The white blood cells level in the blood dropped as low as 200 cells/mm^3. The lowest concentration of lymphocytes recorded during the study period was 30 cells/mm^3 in AML patients and 90 cells/mm^3 in ALL patients. Polymorphonuclear leukocyte levels dropped to 10 cells/mm^3 in AML patients and 90 cells/mm^3 in ALL patients.

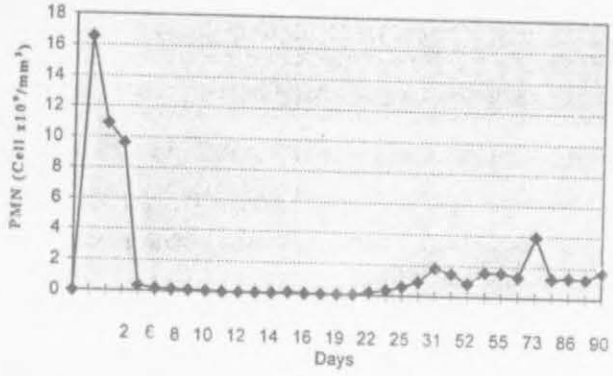

Figure 7. The neutrophil profile of a patient on cytotoxic therapy.

3.3. ORAL LESIONS

Twenty - one (72.4%) of the patients in this study group presented with oral lesions during the course of the study, with oral candidosis (44.8%) and gingivitis (34.5%) as the most prevalent lesions. Five patients (17.24%) exhibited ulcerations due to *Herpes* virus infection, one developed severe pericoronitis and one palatal petechiae (Table 10). Eight patients (27.6%) in the group never presented with any oral lesions.

The clinical manifestation of oral candidosis. Oral candidosis was clinically diagnosed in 8

(57.14%) patients with AML and 5 (33.33%) patients with ALL (Table 10). Erythematous candidosis (45.8%) was the most prevalent clinical manifestation, affecting nine patients in the study group (Table 11).

Table 10. The incidence of oral lesions in acute leukaemia patients.

	AML Patients (%) n=14	ALL Patients (%) n=15	Total number of patients (%) n=29
Patients with oral lesions	9 (64.3)	12 (80)	21 (72.4)
Patients with no oral lesions	5 (35.7)	3 (20)	8 (27.6)
Type of lesion			
Herpes infection	2 (14.3)	3 (20)	5 (17.24)
Gingivitis	5 (35.7)	5 (33.33)	10 (34.5)
Pericoronitis	1 (7.14)	0	1 (3.45)
Oral candidosis (OC)	8 (57.14)	5 (33.33)	13 (44.8)

Table 11. Clinical manifestations of oral candidosis.

Clinical manifestation	Nr of incidents of oral candidosis (%), n=24	Nr of patients involved
1. Pseudomembranous candidosis	1 (4.17)	1
2. Erythematous candidosis	11 (45.83)	9
3. Mixed pseudomembranous candidosis and erythematous candidosis	6 (25)	4
4. Mixed pseudomembranous candidosis, erythematous candidosis, with angular cheilitis	4 (16.7)	4
5. Pseudomembranous candidosis with rhomboid glossitis	1 (4.17)	1
6. Rhomboid glossitis	1(4.17)	1

Four patients presented with mixed pseudomembranous and erythematous oral candidosis, with three incidents in a single patient. The same patient also presented with pseudomembranous candidosis, in combination with rhomboid glossitis. Three patients presented with mixed pseudomembranous and erythematous candidosis, together with angular cheilitis.

3. 4. THE PREVALENCE OF *CANDIDA* ISOLATES IN THE ORAL CAVITY

Twenty-five (86.2%) patients in this study group carried *Candida* spp. with a total of 86 isolates. The majority of the isolates (56.98%) were phenotyped as *C. albicans* (Table 13), based on their ability to form germ tubes, a green pigment on Chromagar *Candida*, and their sugar assimilation and disk diffusion patterns. Eleven of these isolates presented with atypical API code, but were classified as *C. albicans* on grounds of their ability to produce germ tubes. Eighteen (20.93%) isolates were identified as *C. glabrata* and were obtained from nine patients (36%). Five (5.81%) isolates, from 3 patients, were identified as *C. krusei* while 4 (4.65%) *C. tropicalis* isolates were obtained from a single patient. A single isolate of *C. guilliermondii* (1.16%) was isolated.

Sixty percent of the patients carried a single species throughout the course of the study. Two species were isolated from 16% of the group and three species from 20% of the group (Table 14). These species were isolated concomitantly, or during subsequent visits. *C. albicans* was isolated alone, or in combination with *C. glabrata*, *C. krusei* and/ or *C. tropicalis*.

Thirteen patients each had only one isolate of *Candida* during the study period. Three of these patients were seen once only, due to death or unavailability. The rest of the isolates were obtained from the remaining 10 patients, either at the start of chemotherapy (5 patients), or much later (5 patients).

Table 12. Oral *Candida* isolates in the study group.

	Nr of patients (%)
Total patients	29
Patients with oral *Candida*	25 (86.2)
Patients without oral *Candida*	4 (13.8)

Table 13. The prevalence of oral *Candida* spp. in the study group

Isolate	Number of patients (%) n=25	Number of isolates (%) n=86
*C. albicans**	18 (72%)	49 (56.98)
C. glabrata	9 (36%)	18 (20.93)
C. krusei	3 (12%)	5 (5.81)
C. tropicalis	1 (4%)	4 (4.65)
C. guilliermondii	1 (4%)	1 (1.16)
No ID	6 (24%)	9 (10.47)

* All isolates that produced germ tubes and green pigment on Chromagar, including those not identified with the API 20C Aux.

Table 14. The pattern of *Candida* colonisation in the oral cavity

Number of species isolated	*Number of patients (%) n=25*
One species	15 (60)
Two species	4 (16)
Three species	5 (20)

Candida albicans biotype 1 (46.94%) was the most prevalent strain found in this group of patients (Fig 8). Biotypes 4 (8.16%), 6 (4.08%), 8 (4.08%), and 5 (2.04%) (Fig 8). were also isolated from the patients. Seventeen (34.7%) of these isolates were classified as atypical biotypes. This group included eleven isolates that were germ tube positive, but produced atypical API profiles.

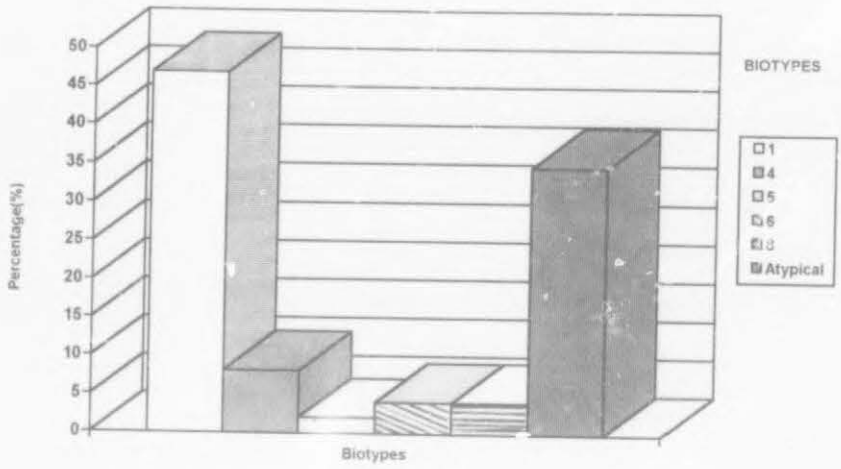

Figure 8. Biotypes of *C. albicans* in the study.

3.5. IN VITRO ANTIFUNGAL SENSITIVITY.

Most of the *C. albicans* isolates (73.46%) were sensitive to fluconazole and were inhibited by a concentration of 4µg/ml or less of this antifungal agent. Seven isolates (14.28%) were inhibited by a slightly higher concentration of this antifungal drug (8 - 14µg/ml), while 10.2% of these isolates had a MIC of more than 16µg/ml. The sensitivity pattern of *C. albicans* to ketoconazole differed, with most of the isolates (53.06%) showing MIC values higher than 16µg/ml. A percentage of 44.44% of the isolates needed concentrations between 8 - 16µg/ml to be inhibited. Ten isolates (20.4%) were inhibited by 4µg/ml or less of ketoconazole (Table 15).

Eight (33.33%) *C. glabrata* isolates were inhibited by 4µg/ml or less of fluconazole, 4 (22.22%) by concentrations between 8 - 16µg/ml and 5 (27.78%) had MIC values higher than 16µg/ml. Eight of these *C. glabrata* isolates (44.44%) were only inhibited by ketoconazole concentrations higher than 16µg/ml, while the remaining nine isolates were inhibited by ketoconazole concentrations lower than 16µg/ml.

All the *C. krusei* isolates were inhibited by a fluconazole concentration of 16µg/ml. Four isolates of this species were inhibited by a ketoconazole concentration higher than 16µg/ml. One isolate (20%) was inhibited by 1µg/ml of ketoconazole. The *C. tropicalis* isolates (100%) were all inhibited by ketoconazole concentrations higher than 16µg/ml. Two of the *C. tropicalis* isolates were inhibited by fluconazole concentrations of 4µg/ml or less and one at 16µg/ml. The remaining isolate was not inhibited by fluconazole concentrations within the range tested.

Table 15.The antifungal sensitivity of *Candida* isolated during the study.

	C. albicans (%) n=49		*C. glabrata* (%) n=18		*C. krusei* (%) n=5		*C. tropicalis* (%) n=4	
Concentration (µg/ml)	**FCZ**	**KCZ**	**FCZ**	**KCZ**	**FCZ**	**KCZ**	**FCZ**	**KCZ**
0 - 4	36 (73.47)	10 (20.4)	8 (44.44)	6 (33.33)	3 (60)	1 (20)	2 (50)	0
8 - 16	7 (14.28)	12 (23.48)	4 (22.22)	3 (16.67)	2 (40)	0	1 (25)	0
≥32	5 (10.204)	26 (53.06)	5 (27.78)	8 (44.44)	0	4 (80)	1 (25)	4 (100)

FCZ - fluconazole, KCZ - ketoconazole

3. 6. THE CORRELATION BETWEEN THE CLINICAL DIAGNOSIS OF ORAL CANDIDOSIS AND THE LABORATORY DIAGNOSIS.

Thirteen patients (44.8%) from the study group were clinically diagnosed with oral candidosis (Table 16), presenting with 24 episodes of oral candidosis. Eighteen (75%) of the smears from the 24 incidents of clinically diagnosed oral candidosis were positive for hyphae and/ or spores (Table 16).

In 58.3% of the cases of oral candidosis, *Candida spp.* were isolated at a concentration of at least 400 cfu/ml of rinse, and in 29.2% of the cases at a concentration of less than 400 cfu/ml of rinse. In eight (75%) of the patients the clinical diagnosis was confirmed with positive smears and the presence of at least 400 cfu/ml of rinse.

3.7. THE PREVALENCE OF *CANDIDA* SPP. IN ORAL CANDIDOSIS.

Thirty-two (37.21%) of the 86 isolates were involved in infection (Table 17). *C. albicans* was responsible for 54.17% of the infections, followed by *C. glabrata* (29.17%), and *C. tropicalis* (16.67%). Other species that were isolated from individuals with oral candidosis included *C. krusei* which was involved in 8,33% of infections, *C. guilliermondii* in 4.17% of the infections, and unidentified *Candida spp.* for 12.5% of the infections. These isolates were either involved as solitary etiological agents, or in combination with other species.

Multiple *Candida spp.* were isolated from 4 patients presenting with oral candidosis. One of these patients had multiple episodes of oral candidosis due to a combination of *C. albicans*, *C. glabrata* and *C. tropicalis*. Two patients suffered from oral candidosis due to a combination of *C. albicans* and *C. glabrata*. *C. albicans*, in combination with *C. krusei*, was responsible for infection in one patient.

Table 16. The relationship between clinical manifestation of oral candidosis, positive smears and *Candida* numbers in rinses.

	Nr. of incidents of oral candidosis (%) n=24	Nr. of positive smears during oral candidosis (%) n=24	Cfu/ml of rinse n=24		
Clinical variants			>400	1-400	0
Pseudomembranous	1 (4.17)	1 (4.17)	1 (4.17)		
Erythematous	11 (45.83)	7 (29.2)	6 (25)	4 (16.67)	1 (4.17)
Rhomboid glossitis	1 (4.17)	0	0	0	1 (4.17) *
Mixed pseudomembranous and erythematous	6 (25)	5 (20.83)	3 (12.5)	2 (8.33)	1 (4.17)*
Mixed pseudomembranous and erythematous, with rhomboid glossitis	1 (4.17)	1 (4.17)	1 (4.17)	0	0
Mixed pseudomembranous and erythematous, with angular cheilitis	4 (16.67)	4 (16.67)	3 (12.5)	1 (4. 17)0	0

* - Rinses were not available.

A single species of *Candida* was responsible for candidosis in 5 patients. Of these, *C. krusei* was responsible for infection in one patient, an unidentified isolate caused candidosis in another patient, while *C. albicans* was responsible for oral candidosis in the remaining 3 patients. No *Candida* isolates were obtained from 4 patients who presented clinically with oral candidosis.

Table 17. The prevalence of oral *Candida* isolates in infection.

Species	Number involved in infection (%) n=32	Number of infections# (%) n=24
C. albicans	15 (46.875)*	13 (54.17)
C. glabrata	7 (21.875)	7 (29.17)
C. tropicalis	4 (12.5)	4 (16.67)
C. krusei	2 (6.25)	2 (8.33)
Not identified	3 (9.375)	3 (12.5)
C. guilliermondii	1 (3.125)	1 (4.17)

* Include two atypical, germ-tube positive isolates; # total more than 24, because some incidents of candidosis caused by 2 or more species.

C. *albicans* Biotype 1 was involved in 33.33% of all the infections. Biotypes 4 (4.76%), 6 (4.76%) and 8 (4.76%) were also responsible for the development of oral candidosis (Table 18).

Table 18 *C. albicans* biotypes involved in oral candidosis

Biotype	Number of incidences of candidosis* (%) n=21*
1	7 (33.33)
4	1 (4.76)
6	1 (4.76)
8	1 (4.76)
Atypical	3 (12.5)

* No *Candida* isolated in 3 out of 24 incidents of oral candidosis.

* Total = 13 and not 24, because *C. albicans* not involved in all incidents of candidosis

3.8. THE RELATIONSHIP BETWEEN IMMUNOLOGICAL STATUS AND THE CLINICAL MANIFESTATION OF ORAL CANDIDOSIS.

Available results of cell counts (on dates when oral candidosis manifested clinically), were analysed. Fifty percent of the incidents of oral candidosis occurred when the white blood cell counts were less than 3000/mm^3 (Table 19). Single incidents of oral candidosis occurred during the treatment period when the WBC were more than 6 000 /mm^3.

Table 19. The association between oral candidosis and low levels of WBC.

WBC Concentration (x 10^3 cells/mm^3)	Number of incidents of clinical manifestation of oral candidosis (%); n= 16*
0 - 2,9	8 (50)
3 - 5.9	5 (31.25)
6 - 8.9	1 (6.25)
9 - 11	1 (6.25)
Above 20	1 (6.25)

WBC = White blood cells, * 16 results of WBC were available.

Seventy percent of the incidents of oral candidosis occurred while absolute polymorphonuclear leukocytes (PMN) levels were below 2 000 /mm^3, which indicates a state of neutropenia in the patients. Four incidents of oral candidosis occurred when the levels of neutrophils of the relevant patients were within the normal range (i.e. 2 - 7.5 x 10^3) (Table 20)

Sixteen (84.21%) incidents of oral candidosis occurred when lymphocyte levels in blood were below 1500 /mm^3. A few incidents of oral candidosis occurred when lymphocytes levels were within or higher higher than the normal counts of 1500 to 4 000 /mm^3 of blood (Table 21).

Table 20. The association between oral candidosis and low levels of neutrophils

Neutrophils (x 10^3 cells/mm^3)	Number of incidents of clinical manifestation of oral candidosis (%); n=20*
0 - 2	14 (70)
2.1 - 4	2 (10)
4.1 -6	3 (15)
> 6	1 (5)

* 20 results of PMN counts were available

Table 21. The association between oral candidosis and lymphocyte cell numbers.

Lymphocytes (x 10^3 cells/mm^3)	Number of incidents with clinical manifestation of oral candidosis (%): n=19*
< 1.59	16 (84.21)
1.6 - 2.99	1 (5.26)
3.1- 4.5	0
4.6 - 6	0
> 6	2 (10.53)

* 19 results of lymphocyte counts were available

3.9. GENETIC ANALYSIS OF *C. ALBICANS* ISOLATES.

Twenty-nine *C. albicans* isolates from 9 patients were fingerprinted with the *C. albicans* specific probe, Ca3. Additionally, 11 isolates, which closely resembled *C. albicans*, but could not be identified by their carbohydrate assimilation patterns, were also fingerprinted. Differences in band positions and the number of bands of strains were used to determine the degree of relatedness amongst strains. Dendrograms of the 29 strains, based on these differences, were constructed with the aid of calculated similarity indexes. Strains were regarded as identical if they shared a similarity index of 1, while non-identical strains were scored between zero and 0.99. Identical strains were grouped into clusters. The name of the group, as well as a distinguishing number, were assigned to each cluster. Isolates were designated with the prefix LC.

Three groups, A, B and C, of closely related strains were identified. Group A comprised of 2 clusters and 2 unique strains (Table 22, Fig. 9). Cluster A1 comprised of 4 isolates from patient R, and cluster A2 contained 2 isolates from patient D. Group A also contained two unique strains from patients P and R, respectively.

Group B contained 3 clusters. The isolates of clusters B1 and B2 were closely related to each other, sharing a similarity index of 0.96, indicating minor differences between the strains of the two clusters. Cluster B1 consisted of 2 isolates from patient VS and 1 isolate from patient R. Cluster B2 consisted only of isolates from patient D. This group also contained 2 unique strains LC 193 and LC 003. Cluster B3 consisted of 2 strains from patient VDW, as well as strain LC 40 from patient D, whose isolates were mainly found in cluster B2.

Table 22. Cluster analysis of *C. albicans* isolates from a group of acute leukaemia patients.

Classification	Nr. of samples	Nr of Unique strains	Source of unique strains (Patient)	Cluster name	Nr of samples in clusters	Source of samples in cluster(patient)
Group A	9	2	P	A1	5	R
			R	A2	2	DV
Group B	13	4	S, P, R, D	B1	3	VS, D
				B2	4	D, R
				B3	2	VDW
Group C	7	4	D, R, R, B	C1	3	K

Group C consisted of cluster C1, whose isolates were isolated from one patient only (Patient K), as well as 4 unique strains from patients D and R respectively.

Analysis of isolates from the respective patients.

The isolates from 4 patients (DV, K, VDW and VS) remained unchanged throughout the study period (Table 23). The isolates of the remaining 3 patients (D, P and R) exhibited different Ca3 hybridisation patterns.

Patient P: The two isolates LC 93 and LC 100, were distinctly different, with a similarity index of 0.76.

Patient R: The 10 isolates from this patient (Table 24, Fig 11b) were grouped into 5 genotypes. Five of these strains (50%) belonged to one genotype (cluster A1). The remaining strains were distributed amongst genetic groups A, B, and C. All of the remaining strains, except for LC 6, were classified as atypical biotypes of *C. albicans*. These atypical biotypes, LC 2, 3 and 82, yielded a normal 15-25 band Ca3 hybridisation profile.

Table 23. *C. albicans* strains in the study group.

Patient	Strains from patient	Nr. of identical strains	Nr of unique strains
By	LC 148	0	1
D	LC 18, 26, 38, 40, 55 and 63	4	2
DV	LC 1 and LC 9	2	0
K	LC 125, 139 and 161	3	0
P	LC 93 and 100	0	2
R	LC 2, 3, 6, 8, 42, 82, 83, 99, 130, 143	6	4
S	LC 193	0	1
VDW	LC 173 and 190	2	0
VS	LC 75 and 97	2	0

Strains are classified as unique or identical in relation to other strains of the same patient.

Table 24. Analysis of characteristics of isolates from Patient R.

Nr	Date of isolation	Biotype	Genotype	Antifungal treatment	MIC for KCZ (µg/ml)	Involved in infection?
LC99	7/9/94	1	Cluster A1	Systemic KCZ	16	No
LC6	14/9/94	1	Unique	Systemic KCZ	2	No
LC3	28/9/94	Atypical	Unique	Systemic KCZ	2	No
LC8	25/10/94	1	Cluster A1	Systemic KCZ	8	No
LC 2	29/11/94	Atypical	Unique	Systemic KCZ	2	Yes
LC 42	28/3/95	4	Cluster A1	None	4	Yes
LC 82	25/4/95	Atypical	Unique	None	16	No
LC 83	30/5/95	4	Cluster A1	None	16	Yes
LC 130	3/10/95	1	Cluster A1	Systemic KCZ; topical amphotericin B	>16	No
LC 143	25/10/95	1	Cluster A1	Systemic KCZ	16	Yes

KCZ =Ketoconazole, MIC = minimum inhibitory concentration

The MIC (minimum inhibitory concentration) values of the isolates of cluster A1 were intermediate (4-8 µg/ml) to high (16 µg/ml) MIC for ketoconazole. Three of the 4 unique strains (LC 2, 3, 6) had MIC values of 2µg/ml for ketoconazole. The remaining unique strain, LC 82, which were the least related to the rest of patient R's isolates, had a MIC-value of 16µg/ml for ketoconazole. The most prevalent genotype of this patient, i.e. those isolates from cluster A1, was isolated during 3 (75%) of the 4 episodes of oral candidosis in this patient. These isolates were either biotype 1 or 4 and had MIC values of 4, 16 and 16µg/ml respectively. Only one episode of oral candidosis occurred when a unique genotype was isolated.

Patient D: Isolates from patient D belonged to 4 different genotypes. The first three isolates of patient D were identical and belonged to cluster B1. Different genotypes were isolated during the remaining 3 visits. Five (83.33%) of the 6 isolates belonged to *C. albicans* biotype 1, except strain LC 38, which belonged to *C. albicans* biotype 8. LC 38, although belonging to a different biotype, were a member of cluster C1. The MIC values of the strains varied (Table 25, Fig 11a). Two isolates exhibited MIC values of 16 and higher, when oral candidosis manifested clinically. The MIC values of the isolates varied according to the presence and absence of systemic ketoconazole, with MIC's increasing with exposure to the drug, and decreasing in the absence thereof.

Table 25. The analysis of isolates from Patient D.

Date of isolation	Nr. of isolate	Genotype	Biotype	Antifungal given to patient	MIC for KCZ (µg/ml)	Involved in infection?
14/2	LC 18	Cluster B2	1	Ketoconazole	2	No
21/2	LC26	Cluster B2	1	None	16	Yes
10/3	LC 38	Cluster B2	8	None	1	Yes
23/3	LC 40	Unique	1	None	0.5	No
4/4	LC 55	Unique	1	Ketoconazole	> 16	Yes
24/4	LC 63	Unique	1	None	>16	No (Pas + T.P.D)

KCZ = ketoconazole

Six isolates were obtained from this patient, but only one (LC 148) of these isolates exhibited a normal Ca3 hybridisation profile (Fig. 12). The 5 atypical isolates of this patient were therefore not included in the dendrogram analysis, but grouped with the atypical *C. albicans* isolates. This group comprised of 11 germ tube positive isolates whose identity could not be confirmed with the API method, hybridised weakly to the *C. albicans* specific probe (Fig 12). It would appear as if the Ca3 repetitive fragment, which is known to be dispersed throughout the *C. albicans* genome, is not as prevalent in the genome of these atypical isolates, suggesting that these isolates are not *C. albicans*.

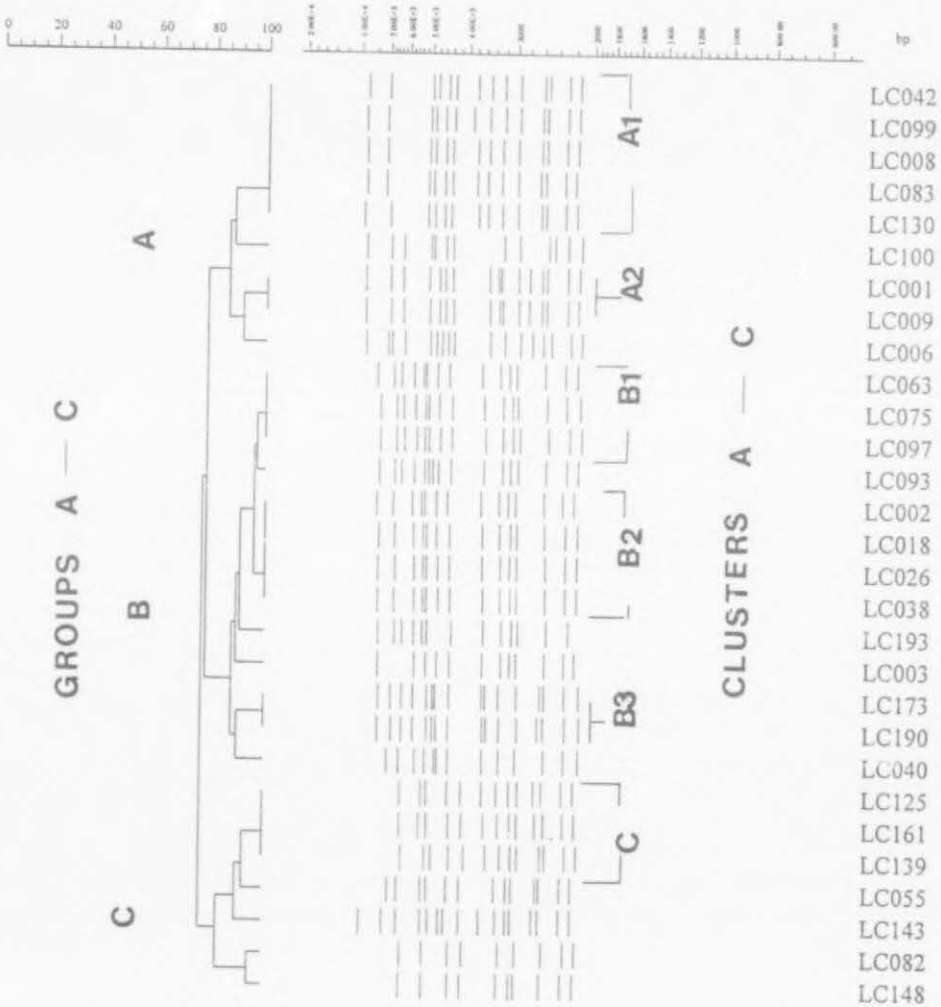

Figure 9. Dendrogram analysis of *C. albicans* EcoR1 restricted DNA.

Figure 11a. Dendrogram analysis of *EcoR1* restricted DNA from Patient D.

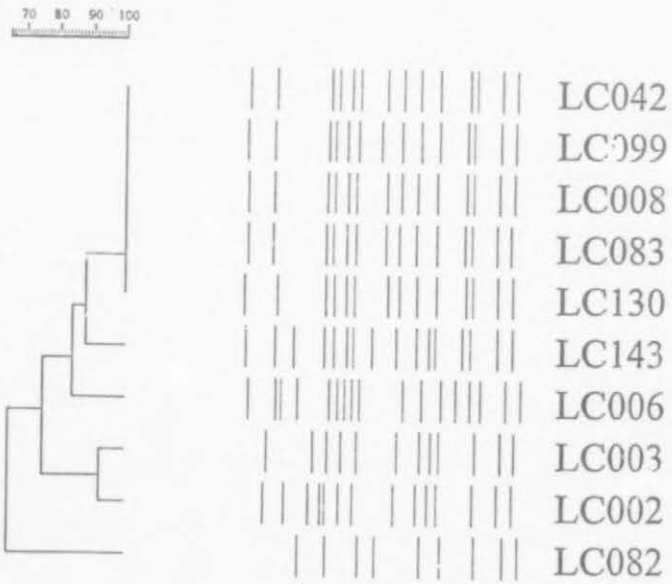

Figure 11b. Dendrogram analysis of isolates from Patients R

3.10. CHARACTERISATION OF A GROUP OF ATYPICAL *C. ALBICANS* ISOLATES.

The phenotypical characteristics of 11 strains, which only partially hybridised to probe Ca3 were investigated. These isolates did not assimilate 2-ketogluconate (2KG), L-arabinose (Ara), D-xylose (Xyl), α-methyl-D glucoside (MDG), cellobiose (CEL), lactose (LAC) melezitose (MLZ), and raffinose (RAF). All of the isolates were germ-tube positive, dark green on Chromagar, and produced chlamydospores upon culturing on Cornmeal agar (with added 1% Tween 80). These characteristics are typical of *C. albicans* isolates. The disk-diffusion method yielded codes 100406, 000406 or 103406, which identified these isolates as *C. albicans*. The isolates were therefore identified as *C. albicans*, based on germ tube production, the colour on Chromagar and disk-diffusion codes, although the API codes did not conform to known species of the genus *Candida* (Table 26). The *C. albicans* species specific probe Ca3 hybridised weakly to approximately 6 bands of DNA (Fig. 12).

Table 26. Phenotypical characteristics of a group of atypical *C. albicans* isolates.

ISOLATE	API CODES [$]		DD CODES	Colour on Chromagar
LC 49	6377134		120406	Dark green
LC 89	6576134	6172134	103406	Dark green
LC 71	2172134		103406	Dark green
LC 96	6572 134	6572 134	000406	Dark green
LC 104	2572 134	6176 134	100406	Dark green
LC 116	6172 174	2576 174	100406	Dark green
LC 132	6172174		100406	Dark green
LC 145	2172 134		100406	Dark green
LC 14	6172 134	6572134	000406	Dark green
LC 183	6142 134	2152 [illegible]	100406	Dark green
LC 192	6172134		100406	Light green

[$] Isolates were tested in duplicate. API assimilation profiles are unstable.

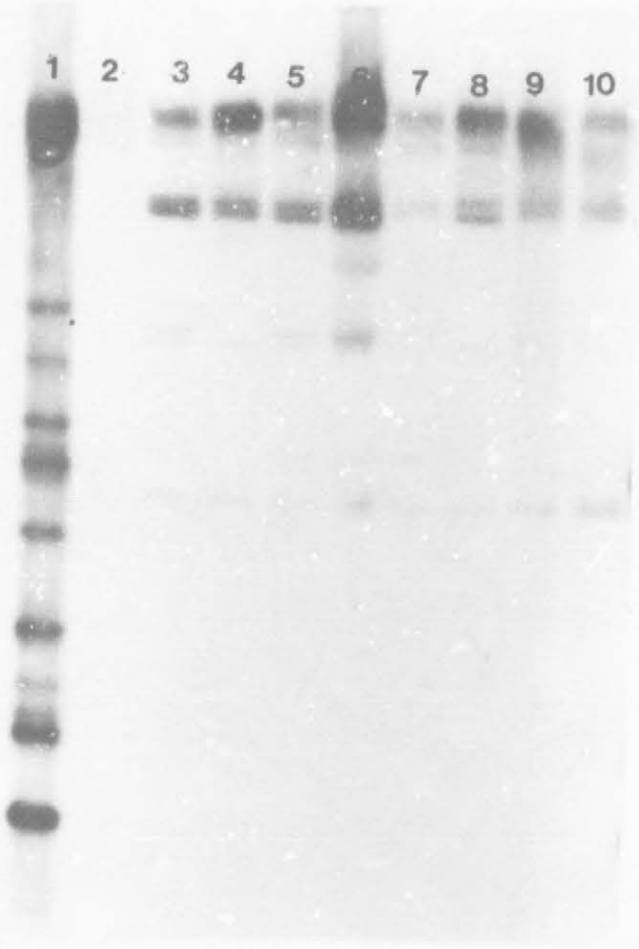

Figure 12. DNA profiles of a group of atypical *C.albicans* isolates.

Lane 1. *C. albicans* 3153A, with a typical Ca3 hybridization pattern of *C. albicans* isolates. Lanes 2-10: LC 14, 71, 96, 104, 132, 148, 183, 49, 80.

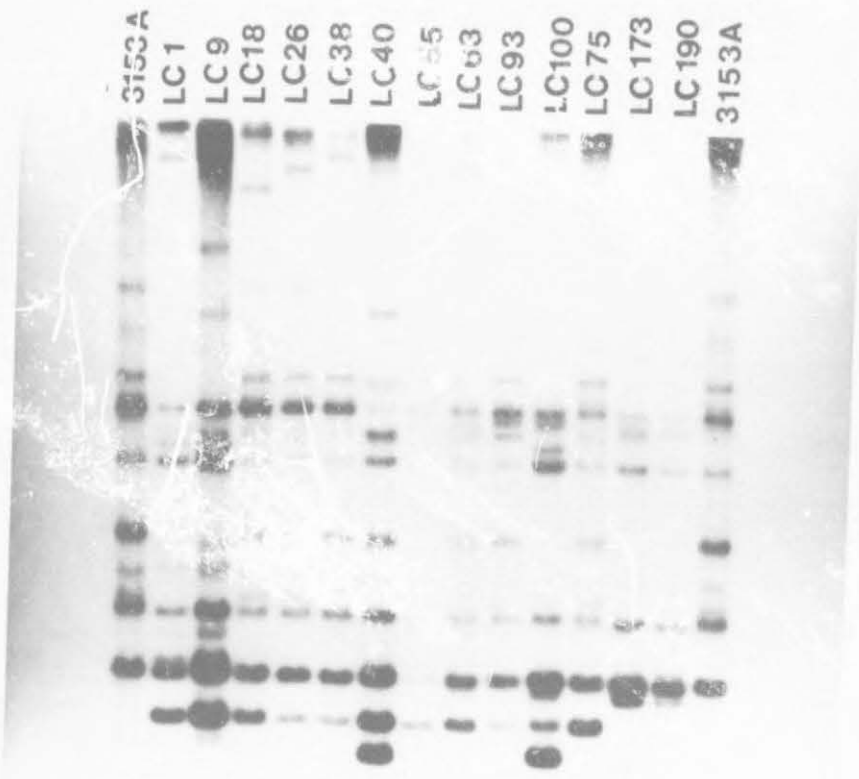

Figure 10. Southern blot of *C. albicans* DNA probed with Ca3.

CHAPTER FOUR. DISCUSSION

Oral lesions occurred in a high percentage (72%) of the patients included in this study. Candidosis, which has been reported as the most frequently occurring oral infection in patients with haematological malignancies (203), represented 44.8% of the oral lesions in this study group (Table 10). Results from other studies showed a similar incidence, with 50% and 58.6% reported by Dreizen *et al.* (203) and Wahlin *et al.* (236) respectively. Erythematous candidosis was the most prevalent (45.83%) clinical manifestation of oral candidosis in our study, while 45.87% of the incidents of oral candidosis manifested as combinations of erythematous, pseudomembranous candidosis, angular cheilitis and rhomboid glossitis (Table 11). Results from a study by Childers and co-workers (237) showed that the incidence of oral candidosis was significantly higher in AML patients than ALL patients. The authors, therefore, suggested that AML should be considered a risk factor for the development of oral candidosis. They ascribed the higher incidence of oral candidosis to the poor prognosis of AML and the more severe cytotoxic treatment regimens of these patients. In the present study, the number of AML patients (8) who developed oral candidosis, was just slightly higher than the number of ALL patients with oral candidosis (5). A higher incidence of oral candidosis also occurred amongst AML patients (16) than ALL patients (8) (Table 10).

Results from previous studies (238, 239, 240) proved that oral infections in patients receiving cytotoxic therapy can be reduced by proper oral care in the form of antimicrobial oral rinses, as well as systemic and topical antifungal prophylaxis. The patients included in the present study did not receive specific topical regimens during the study for the prevention of oral candidosis. A large number (13 patients or 44.8%) of the patients still manifested with oral candidosis in spite of systemically administered ketoconazole (Table 10).

Cytotoxic therapy increases the risk to develop oral infections due to the loss of salivary IgA,

reduced salivary flow (241,242) and the impairment of the protective salivary peroxidase system (243). The salivary peroxidases itself are not affected, but the system cannot function properly due to decreased levels of thiocyanate during cytotoxic therapy. The administration of antibiotic prophylaxis during chemotherapy leads to a change in the oral microflora, with increased numbers of organisms such as Gram-negative bacilli, *Staphylococcus aureus*, and *Candida albicans* (244, 245), thus increasing the risk for the development of infection.

Oral *Candida* species were isolated from the oral cavities of 86.2% of the patients included in this study (Table 12). This is comparable with the prevalence of between 70 - 92% found in other studies (26, 27, 241). Some of the patients in the present study carried high numbers of oral *Candida*, with yeast concentrations as much as 1×10^6 cfu/ml of rinse. Epstein *et al.* (181) proposed that yeast numbers higher than 400 cfu/ml indicate active infection, rather than just a carrier state. In the present study, it was found that only 58.3% of oral rinses obtained from patients presenting clinically with candidosis, had oral *Candida* concentrations higher than 400 cfu/ml (Table 16). The rinses (29.2%) that contained less than 400 cfu/ml of rinse (or no *Candida* at all) could have been influenced by the fact that these patients also had oral lesions other than oral candidosis and, therefore, were not eager to rinse with the saline rinse.

Various *Candida* species (Table 13, 14) and *C. albicans* biotypes (Fig 8) were found in this study group. *C. albicans* was the most commonly isolated species and was also involved in the majority of infections (Table 17). The success of *C. albicans* as a commensal and a pathogen is related to various virulence factors, such as the ability to adhere to epithelial cells (62), the ability to form germ tubes and hyphae (71), the presence of membrane-damaging proteases (62, 63, 65) and morphotype switching mechanisms (75). Previously, it was found that persistent *Candida* infection is associated with the presence of multiple biotypes of *C. albicans*, some of which are more dominant (190, 245) than others. Biotype 1 was the most prevalent strain of *C. albicans* in this study, representing 46.94% of *C. albicans* isolates (Fig 8), as well as 33.33% of the *C. albicans* isolates from infections

(Table 18). It is known that more prevalent *C. albicans* strains are more adaptable and more virulent than less prevalent strains (246).

Other *Candida* spp. isolated from the patients included *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. guilliermondii*. The increasing involvement of *Candida* spp., other than *C. albicans*, was first noted after the appearance of the azole drugs and increased ever since (16). It was thus not surprising to find that *C. glabrata* represented 21.8% of the isolates from infections. More than one species of *Candida* were isolated from approximately one-third of the patients with oral candidosis (Table 14).

A small percentage of isolates exhibited atypical substrate assimilation patterns with the API 20C Aux identification system (Tables 13), a finding that has been described before (247). Some of these isolates closely resembled *C. albicans* in its ability to produce germ tubes, chlamydospores and the appearance of green colonies on Chromagar *Candida* (Table 26). These findings suggest that the API 20C Aux system is limited in its ability to identify all yeasts isolates.

It has been reported that prolonged exposure to antifungal drugs such as fluconazole leads to drug resistance (169, 170, 172). Goff *et al.* (168) described the development of fluconazole resistance without prior exposure of clinical isolates to the antifungal drug. The majority of isolates in the present study exhibited intermediate to high MIC values for ketoconazole (Table 15). As systemic ketoconazole was given prophylactically to most of the patients, one can assume that the prolonged exposure to the drug was responsible for the high MIC values. However, as the clinical significance of in-vitro antifungal sensitivity testing is still a matter of controversy, we should be careful to draw any conclusions from these results. A large number of isolates in this study were inhibited at low doses of fluconazole, possibly because the patients were not exposed to the drug. Some of the isolates exhibited high MIC values for fluconazole . As cross-resistance to azoles by *Candida* was previously reported, especially between fluconazole and ketoconazole (170, 171, 177), it should be considered as a possibility for the observed in vitro resistance to fluconazole.

The present study investigated the genotypical relatedness of 29 oral *C. albicans* isolates. Dendrogram analysis divided these isolates into 3 genetic groups of closely related strains, containing 2, 3 and 1 clusters of identical isolates respectively. Additionally, ten unique *C. albicans* genotypes, which were distributed amongst the different genetic groups, were identified.

We have found that the genotypes of *C. albicans* isolates from 4 patients were identical to each other and remained unchanged during the study period, while the isolates from 3 other patients differed genetically from each other. Two of the patients with identical isolates manifested with oral candidosis at each visit (two and four times respectively). The ketoconazole MIC values of their isolates were 16µg/ml or higher, indicating in vitro resistance to these drugs. These two patients were also treated for diabetes and cirrhosis of the liver, respectively. The patient who suffered from liver cirrhosis, did not receive antifungal prophylaxis during this study period. It was therefore surprising to find such high MIC values in the isolates from this patient. Unless the patient was exposed to ketoconazole before he was included in the present study, one will have to assume that resistance developed without prior exposure to ketoconazole, as was previously described for fluconazole (168).

The diabetic patient received systemic ketoconazole therapy throughout the study period, as well as topical nystatin prior to the last visit. In spite of the treatment the patient still presented with combinations of pseudomembranous, erythematous and angular cheilitis during the study period, suggesting that the drugs were inefficient in the complete prevention and curing of the infection. The patient with liver cirrhosis carried *C. glabrata* and *C. albicans*, while the diabetic patient carried *C. albicans*, *C. glabrata* and *C. tropicalis*. The *C. glabrata* isolates of these patients were all, with the exception of one, sensitive to ketoconazole, while the *C. tropicalis* isolates were resistant, with MIC values of 16µg/ml and higher for ketoconazole.

Multiple genotypes of *C. albicans* were found in another two patients (Table 24, 25). Both patients suffered from multiple episodes of oral candidosis. One patient received systemic ketoconazole

therapy, topical amphothericin B treatment and, on a few occasions, also chlorohexidine. The other patient only received systemic ketoconazole. Various factors might have played a role in the isolation of different genotypes. Genetic changes could have occurred due to exposure to the antifungal agents. Alternatively, the antifungal therapy could have been responsible for strain replacement of ketoconazole sensitive strains by more adaptable (possibly more virulent?) strains. It is also possible that multiple genotypes were present in the oral cavity, but because they appeared as identical phenotypes they were eliminated by the selection of a single colony during the primary subculturing process. Lastly, it is also possible that variation in the degree of *EcoR1* digestion of DNA might lead to the appearance of identical isolates as different genotypes. The clonal origin of strains, i.e a single strain that undergoes evolution to produce a genetically different progeny (248), as well as strain replacement (249) have been described previously. Although more experiments are needed to confirm the source of the different genotypes in the present study, it would seem that the different genotypes from patient R can be ascribed to the fact that multiple strains were present in the patient. It is possible that the one strain at a time were selected during the primary subculturing process, thus explaining the inconsistent presence of strains. The fact that 3 of the 4 unique strains had a completely different MIC profile compared to the identical strains, suggest that more than one *C. albicans* strain variant was present. Three of these unique strains were also classified as different biotypes.

In the case of patient D (Table 25), it is possible that evolution of the strain, strain replacement, or elimination of the isolates during the subculturing process could have occurred. Possible strain replacement is suggested by the fact that a new ketoconazole sensitive genotype (MIC of 0.5µg/ml) appeared only after the termination of antifungal therapy in the mentioned patient. It is possible that ketoconazole therapy caused the disappearance of the ketoconazole sensitive genotype, thus explaining its earlier absence. It was further noticed that the MIC values of the first genotype varied in the presence and absence of ketoconazole, indicating that the strain adapted to survive.

Alternatively, one can argue that the observed changes in ketoconazole sensitivity could be related to the appearance of a new genotype. It is important to note that patient D had no isolate of *C. albicans* during the first visit. It is thus possible that *C. albicans* was acquired during the study period and adapted to survive in the presence of ketoconazole, eventually changing its MIC profile and genotype as a result of the environmental pressure. As the last 3 strains of patient P differed genetically from each other, it is possible that more than one strain developed from the original isolate.

The presence of multiple *C. albicans* genotypes in a specific body location has been described before by Mathaba *et al.* (190) and Soll *et al.* (250). In the study by Mathaba *et al* (190), it was found that genetic groups of closely related oral isolates contained strains from more than one patient, similar to the results of the present study (Table 22). Schmid *et al* (82, 126), found that *C. albicans* isolates from a group of immunosuppressed, hospitalised patients were closely related, in contrast to samples from non-hospitalised control patients. According to them, this similarity could be due to nosocomial transfer of *Candida spp*. Although the isolates in the present study were related to each other, a more detailed study needs to be undertaken before we can come to a conclusion similar to that of Schmid *et al*. Such a study will address influencing factors such as hospital personnel and their contact with patients, periods of patient hospitalisation and surveillance cultures of the hospital environment in the close proximity of the patients.

Eleven isolates hybridised weakly to the *C. albicans* species-specific Ca3 probe, even though the isolates were phenotyped as *C. albicans*, due to the ability to produce germ tubes, chlamydospores and a green pigment on Chromagar *Candida*. Five of these isolates were from a single patient. A sixth isolate from this patient presented with the normal 15 - 25 band Ca3 profile of *C. albicans*. This "normal" *C. albicans* isolate was only found once, during the patient's second period of hospitalisation. It disappeared again after a few weeks of systemic ketoconazole prophylactic therapy.

Various research workers described similar isolates which hybridised weakly to Ca3 (82, 251, 252, 253). These authors differed in their subsequent classification of the isolates. Scmid *et al.*(82) described their isolates as non-albicans *Candida*, while Boerlin *et al.* (253) classified their isolates as C. *albicans* on grounds of their ability to produce germtubes and chlamydospores. In the present study, these atypical isolates, as described above, presented with API codes that started with 6142, 2172, 6172, 6337, or 2572. The disk-diffusion codes were either 100406, 103406, or 000406, all of which are typical C. *albicans* codes. These isolates did not assimilate 2-ketogluconate, L-arabinose, D-xylose, α-methyl-D glucoside, cellobiose, lactose melezitose, and raffinose. Some of the isolated did not assimilate trehalose as well.

Recently a new species, which resemble C. *albicans*, was described by Schoofs *et al* (253) and Colemans *et al* (254). These isolates, named *C. dubliniensis*, present with typical C. *albicans* germ tubes and are chlamydospore positive. *C. dubliniensis* isolates are distinguished from C. *albicans* on Chromagar *Candida* by the production of a dark green colony and atypical API profiles. These isolates hybridise weakly with the C. *albicans* species-specific probes Ca3 and 27A. Although the atypical isolates in our study have similar characteristics to *C. dubliniensis*, more tests need to be done to identify these isolates as *C. dubliniensis*.

Results from the present study showed that laboratory identification of isolates as *C. albicans*, based solely on its ability to produce germ tubes and chlamydospores, could lead to the incorrect identification of *C. dubliniensis* and isolates similar to the ones as discussed above. The use of Chromagar *Candida* and methylblue Sabouraud dextrose agar (253, 254) could aid in the discrimination of these atypical isolates from C. *albicans*.

Previous studies reported the absence of pseudohyphae in smears of a small percentage of patients with oral candidosis (255,256, 257). In the present study, a few smears from patients with clinically diagnosed candidosis, contained no pseudohyphae or spores (Table 16). One of these negative smears was obtained from a patient with a well-defined rhomboid glossitis. This patient received

treatment with topical amphotericin B prior to sampling. It is possible that the antifungal treatment successfully removed *Candida*.

It is known that healthy people without oral candidosis can have *Candida* pseudohyphae in smears from the oral cavity (24, 258). However, the presence of numerous pseudohyphae in smears of immunosuppressed patients should be investigated as these patients are highly predisposed to the development of oral candidosis. In the present study it was found that smears from two patients had persistently had numerous hyphae and/ or spores in smears, even in the absence of oral candidosis. These patients each had multiple episodes of oral candidosis. One can hypothesise that oral candidosis was present in some subclinical form when smears were positive in the absence of clinical symptoms.

CHAPTER FIVE. CONCLUSION

1. A relatively high percentage of patients manifested with oral candidosis, even in the presence of systemically administered ketoconazole.
2. A high percentage of the patients was culture positive for oral *Candida*, with *C. albicans* as the most prevalent species, followed by *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. guilliermondii* and a number of unidentified isolates.
3. *C. albicans*, specifically Biotype 1, was responsible for the majority of infections.
4. The emerging pathogen, *C. glabrata*, was involved in a large number of infections.
5. Approximately a third of the patients presenting with oral candidosis, carried two or three *Candida spp.* in their oral cavities.
6. A high percentage of patients carried isolates which exhibited high ketoconazole MIC values, probably due to prolonged exposure to the drug.
7. The majority of isolates were sensitive to fluconazole, probably due to the fact that these patients were not exposed to the drug.
8. The identification methods employed in the present study were not sufficient to correctly identify all clinical isolates.
9. Three genetic groups of *C. albicans* were identified. Some patients had identical strains of *C. albicans*, while others presented with multiple genotypes in the oral cavity.
10. The presence of genetically different strains in one patient can possibly be ascribed to strain replacement of ketoconazole sensitive strains by more resistant strains, or alternatively, to genetic changes that occurred in the original isolate as a result of antifungal therapy. Additionally, it is possible that patients carried more than one strain of *C. albicans*, as is suggested by the different

antifungal profiles.

11. Isolates that produce germ tubes and chlamydospores, dark green colonies on Chromagar *Candida*, as well as atypical API profiles, are not *C. albicans*, as these isolates' DNA hybridise weakly with the *C. albicans* species specific probe, Ca3.

REAGENTS AND MEDIA

1. ISOLATION AND IDENTIFICATION OF CANDIDA FROM CLINICAL MATERIAL.

Phosphate buffered saline (Merck)

Prepared as described by the manufacturer.

Sabouraud-Dextrose agar (Oxoid)

Ingredients:

Mycological peptone	10g
Glucose	40g
Agar	15g

Dissolve 65g of prepared dry media in sufficient water, fill up to 1 litre. Sterilise and pour in Petri dishes.

Pagano- Levin media (Difco)

Ingredients:

Bacto Peptone	10g
Bacto Yeast extract	40g
Bacto Agar	15g

Dissolve 66g of the Pagano - Levin basal media in water. Fill up to 1 litre. Sterilise by autoclaving the media at 121°C for 15 minutes. Add 10ml of 1% triphenyltetrazoliumchloride to the media, when it is sufficiently cooled down. Pour in Petri

plates.

Mast ID Chromagar *Candida*

Obtained from Mast Diagnostics

One sachet of prepared media is dissolved in 100ml of distilled water. The media is heated to 100 °C, with constant stirring, until the agar is completely dissolved. Pour into plates. NB. This media must not be autoclaved, because it contains an antibiotic.

Ingredients:

Peptone	10g
Agar	15g
Chloramphenicol	0,5g
Chromogenic mix	22g

Human serum

Obtained from the Dept. of Medical Microbiology, Tygerberg Hospital

The serum was aliquoted into 500µl quantities and stored at -20°C until needed.

Disk Diffusion Kit for the identification of yeasts

Obtained from the South African Institute of Medical Research

API 20C Aux and ID32C

Obtained from Bio-Merieux, Marcy L'Etoile, France

Microbank

Purchased from Mycoplasma International.

2. MEDIA FOR THE EXTRACTION OF DNA FROM YEASTS

NB. All media were prepared in deionised water.

YPD Broth (Difco)

Dissolve 50g of the media in 1000 ml of water. Divide into 20ml aliquots in 50ml polypropylene tubes. Autoclave at 121 °C for 15 minutes.

Ingredients:

Bacto yeast extract	10g
Bacto peptone	20g
Bacto dextrose	20g

Sorbitol, 2M

Dissolve 36,434g of sorbitol (Mr = 182,17g/mol) in 100ml of deionised water. Sterilise by filtration with a 0.45 micron filter.

1M EDTA stock solution

Add 372.2g of EDTA to approximately 800ml of water. Adjust the pH to the appropriate pH, fill up to 1litre with water and autoclave to sterilise.

Zymolyase buffer for digestion of the cell wall

Add together

50ml of 2M Sorbitol (final c =1 M)

5ml of 1 M Potassium phosphate, pH 7.5 (final c = 50mM)

5ml of .M EDTA, pH 7.5 (final c = 50mM)

100µl of beta mercapto ethanol (final c= 0.1% w/v)

Fill up to 100ml with sterile water

1M Trischloride, pH 8

Dissolve 121,1g of Tris base (2-amino-2- (hydroxymethyl)-1,3-propandiol) in water. Adjust the pH of the solution to 8 by the addition of HCl. Fill up to 1 litre with water. Sterilise by autoclaving.

TE, pH 8

Contains 10mM Trischloride and 1mM EDTA

Add together 10ml of Tris-chloride (1M, pH 8) and 1 ml of 1M EDTA,

pH 8. Adjust to 1 litre with water.

Zymolyase 20T, 10 mg/ml

Obtained from ICN Biochemicals.

Dissolve 10 mg of the powder in 1 ml of 1M sorbitol. Use within two weeks time.

10 % (w/v) Sodium Dodecyl Sulphate (SDS)

Dissolve 100g of SDS in 1 000 ml of water.

10 mg/ml Proteinase K (Sigma)

Dissolve 50 mg in 5ml of water. Store at -20 °C.

Proteinase K buffer, 10 x concentrated (0,1M Trischloride, 0,05M EDTA and 5% SDS)

10 ml of 1M Trischloride solution, pH 7,8

5ml of 0,5M EDTA

50ml of 10% SDS

Fill up to 100 ml with water. Sterilise by autoclaving.

Pancreatic RNAase, 10 mg/ml (Sigma)

Dissolve 10 mg of RNAase in 1 ml of Trischloride, pH 7,5.

Boil at 100 °C for 15 minutes to inactivate the associated DNAase activity.

Store at -20 °C.

Sodium acetate (3M, pH 5.6)

Dissolve 24.612g of anhydrous sodium acetate in approximately 70 ml of water. Adjust pH to 5.6 with glacial acetic acid. Dilute to 100ml. Filter through a 0.45μ filter. Store at room temperature.

3. MEDIA FOR THE ISOLATION OF BACTERIOPHAGE AND BACTERIOPHAGE DNA.

20% Maltose (Saarchem)

Dissolve 20g of maltose in 100 ml of water. Filter sterilise with a 0,22 micron Whatman filter.

Luria-Bertani Broth (Difco)

Ingredients:

Bacto Tryptone	10g
Bacto yeast extract	5g
NaCl	10g

Broth prepared as described by manufacturer.

Luria- Bertani agar

Add 15g of agarose to unsterilised LB broth. Autoclave and dispense into plates.

0.7% Agarose in LB (top layer)

Add 0.7g agarose to 100ml of LB broth. Sterilise by autoclaving.

SM buffer

Ingredients:

NaCl 5.8g

$MgSO_4$ 2g

50ml of 1M Trischloride, pH 7,5

5ml of 2% gelatine solution

Dissolve and make up to 1litre. Sterilise by autoclaving.

$CaCl_2$, 1M

Dissolve 54g of $CaCl_2 6H_20$ in 200 ml of water. Sterilise by filtration through a 0.22 micron filter.

75% DE-52 Ion exchanger

Stir 15g of the pre-swollen DEAE cellulose ion-exchanger into hydrochloric acid until the pH is 4,8. Slowly add NaOH until the pH is 6,8. Add LB broth to the slurry and stir for 15 minutes. Allow the slurry to settle. Decant all excess fluid. Stir once again with LB broth until the pH of the buffer and the ion-exchanger are identical. Let the slurry settle, decant enough liquid off in order that the remaining liquid and resin constitutes a 75% slurry.

2M NaCl, 20% Polyethylene glycol (PEG)

Dissolve 20g of PEG (Sigma) in enough 2M NaCl, fill up to 100 ml with NaCl.

0,1% Sodium azide, 0,3% gelatine(w/v)

Dissolve 100 mg of azide and 300 mg of gelatine in 100 ml of water. Sterilise by autoclaving

5% Cetyltriammoniumbromide (CTAB), 0.5% NaCl (w/v)

Dissolve 5g of CTAB (Merck) and 500 mg of NaCl in 100 ml of water.

1,2M NaCl

Dissolve 69,2g of NaCl in 1litre of water. Sterilise by autoclaving

4. REAGENTS FOR RESTRICTION FRAGMENT ANALYSIS AND HYBRIDISATION.

10 x Trischloride-taurine-EDTA (891.8mM, 288mM, 5 mM)

Dissolve in water

Tris base	108g
Taurine (Mr 125.1)	36g
EDTA	2g

Fill up to 1 litre. Sterilise.

0.8% agarose (NuSieve)

Mini-gel: 0.8g of agarose are added to 100ml of 1x TTE.

Fingerprinting gel: 2.4g of agarose are added to 300ml of TTE.

Melt and cast gel in the appropriate gel trays.

Ethidium bromide (10mg/ml)

One gram of ethidium bromide powder is added to 100ml of water

Loading buffer

0.25g of bromophenolblue

15g Ficoll 400

100 ml Water

Loading buffer with Marker X

Add together 3.3µl of Marker X stock solution (0.25µg/ml) and 996 µl of loading buffer (previously diluted 1.3 (v/v) with TE)

Orientation marker

Add together 5µl of stock Marker X solution, 12.5 µg/ml of *C albicans* DNA and 7µl of TE. Mix thoroughly and then denature the DNA by adding an equal volume of NaOH (0.4N)

20 X SSPE (3 M NaCl; 0.2 M $NaHPO_4 7H_20$; 0.02 M EDTA)

Dissolve the following media in approximately 800ml of water.

NaCl	175.3g
$NaH_2PO_4.7H_20$	27.6g
EDTA	7.4g

Adjust the pH to 7,4 with 10N NaOH. Fill up to 1litre and sterilise by autoclaving.

20xSSC (3M NaCl; 0.3 M Trisodium citrate)

NaCl	175.3g
Sodium citrate	88.2g

Dissolve in enough water. Adjust the pH to 7 with 10N NaOH. Fill up to 1litre of water.

2 x SSC, 0.1% SDS (wash buffer)

50ml of 20x SSC

5ml of 10% SDS

Fill up to 500 ml with water. Autoclave. Store at room temperature

Denhardt's reagent

Ficoll 400	2g
Polyvinylpyrrolydine	2g
BSA	2g

Dissolve in 100 ml of water. Filter sterilise. Store at -20°C.

Hybridisation buffer

To prepare 80 ml of buffer, add together:

1.6 ml of 10% SDS (final c of 0.2%)

20 ml of SSPE

4 ml of Denhardt's reagent

1.6 ml of 100µ/ml Herring sperm DNA

40 ml of formamide

Dilute to 100 ml with sterile water.

CONSENT FORM

The following is an example of the consent forms given to patients included in the study. This form was also available in Afrikaans and Xhosa.

Consent form:

Acute leukaemia patients receiving chemotherapy

We would like to perform a thorough oral examination of your oral cavity prior to chemotherapy. This will enable us to determine whether any infection or condition present in the oral cavity can be affected by the treatment.

We also need you to rinse your oral cavity with saline (salt water) and to spit back into the container. We shall test it in the laboratory for the presence of microoorganisms. Lastly, we shall scratch the back of your tongue with a wooden spatula. These procedures will be done on a weekly basis and none will be hurtful or harmful.

I hereby agree to the abovementioned examination.

Signature: .....

Date:.....

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