

Declaration

The work described in this thesis was carried out by me under the supervision of Dr. Sirimali Fernando, Prof. L.P. Samaranayake and Prof. M. T. M. Jiffry and a report on this has not been submitted to any university for another degree.

Signature

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Declaration by the supervisors

I/We certify that the above statement made by the candidate is true and this thesis is suitable for submission to the University for the purpose of evaluation.

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**THE PHENOTYPIC AND GENOTYPIC DIVERSITY OF ORAL *Candida albicans*
ISOLATES IN SRI LANKAN COHORTS OF VARYING AGE GROUPS**

By

Manjula Manoji Weerasekera

**Thesis submitted to the University of Sri Jayewardenepura for the award of the degree of
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THE PHENOTYPIC AND GENOTYPIC DIVERSITY OF ORAL *Candida albicans* ISOLATES IN SRI LANKAN COHORTS OF VARYING AGE GROUPS

By Manjula Manoji Weerasekera

ABSTRACT

Information on phenotypes and genotypes of *C. albicans* isolates obtained from Asian populations is sparse. Hence, the main aim was to determine the proportion of people colonized with *C. albicans* in their mouths, among children, adult and elderly volunteers selected from the Colombo district and to find out if there are any phenotypic and genotypic variations in *C. albicans* in three different age groups. We applied randomly amplified polymorphic DNA (RAPD) genetic typing technique, and a (commercially available) phenotyping technique for strain delineation of *C. albicans*.

A total of 120 healthy individuals in three different age groups (<6 years, 30-50 years and >70 years) were sampled by oral rinse method. Twenty-nine isolates of *C. albicans* were identified by Gram staining, 'germ tube' and API 20CAUX test. Biotypes were determined using their ability to assimilate a variety of different sugars, and their resistance to boric acid. For genotyping, DNA was extracted and fingerprinted with the RAPD technique using two random primers. This technique is versatile, simple and reproducible and has been previously used for typing candida.

Forty one percent of the volunteers carried yeast in their mouths. The proportion of *C. albicans* carriage was greater (23%) than that for other *Candida* species. There was a significant difference ($p < 0.01$) between the recovery rate of yeast and *C. albicans* within the three age groups.

On biotyping, 7 types were identified while RAPD analyses revealed 10 different genotypes. When the genotyping and the biotyping profiles were combined, a total of 18 composite types emerged.

Both the RAPD and the phenotyping system we have established to type *C. albicans* yielded reproducible results. Further investigation using the above methods and other epidemiologic typing techniques will be necessary to enhance the understanding of the epidemiology and pathogenesis of candidiasis.

CHAPTER 1

**THE PHENOTYPIC AND GENOTYPIC
DIVERSITY OF ORAL *Candida albicans*
ISOLATES IN SRI LANKAN
VOLUNTEERS OF VARYING AGE
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1.1 INTRODUCTION LITERATURE REVIEW

Candidiasis is a collective name given to disorders, caused by the yeast, candida. The oral and vaginal forms of candidiasis were originally referred to as thrush. In the 19th century, thrush was described as a disease of the newborn and was mostly associated with malnutrition and carried a high morbidity if untreated.

Yeasts were first observed microscopically in the latter part of the 17th century by Leeuwenhoek (Samaranayake, 1990). Bennet, in 1844, identified the fungal etiology of thrush (cited by Odds, 1988). Subsequently in 1846, Bergn established a relationship between the 'thrush fungus' and the mouth lesions.

Candida species is considered as an innocuous oral commensal. It may cause disease under certain conditions. 'Candidiasis is a disease of the diseased' is an often-used aphorism. The transition of this innocuous commensal to the disease causing 'opportunistic pathogen' may be associated both with the virulence attributes of the organism and the host factors.

Among the members of the genus candida, *C. albicans* is the predominant species causing candidiasis in man, followed by *C. parapsilosis*, *C. tropicalis*, *C. stellatoidia*, *C. guilliermondii*, *C. krusei* and *C. pseudotropicalis*.

The increasing importance and interest shown in oral candida infections and their causative organisms have been matched with the increasing number of immune suppressed population in the world.

Age related susceptibility to *C. albicans* has been observed by a number of scientists. They found that the *C. albicans* is more common in older adults and in children than in the young adults.

Whether *C. albicans* strains vary in different age groups and if so, whether it could account for the varying susceptibility to candidiasis is not studied so far. Hence, we decided to study the strain variation of *C. albicans* in different age groups.

If a variation could be observed it may be linked to the pathogenesis of oral candidiasis and one may be able to predict the strains, which are "more virulent".

It will also assist to evolve a typing system for *C. albicans* with good discriminatory power. This typing system may be applicable to other epidemiologic and taxonomic studies and could be used to investigate nosocomial outbreaks of candida infections particularly in neonatal units.

1.1.1. Taxonomy of candida species

The taxonomic position of the 'thrush fungus' was subjected to much confusion, and different terms were used to identify the genus candida. Gruby in 1842 assigned the thrush fungus to the genus sporotrichum. Ten years later in 1853 this organism was reassigned to the genus oidium, and gave the first specific name as *Oidium albicans* by Charles Robin.

At the end of 19th century Plaut was able to isolate yeast like fungus from lesions of oral thrush in throats of chicken. Zopf in 1890 named this thrush fungus as *Monilia albicans*. Christine Berkout in 1923 was the first to suggest the term candida to distinguish the medical monilia infections from the botanical diseases. Inability to form a sexual stage in life let this organism being classified under fungi imperfecti and the class deuteromycetes (Lodder, 1970).

The genus candida is a collection of some 150 asporogenous yeast species (Samaranayake, 1990). Among these, *C. albicans* is the most frequently isolated species from humans both in the health and disease.

1.1.2 Epidemiology of candida species with special reference to *C. albicans*

Candida species are widely distributed in nature and have invaded a diverse habitat (MacFarlane, 1990). They primarily live as saprophytes and also as opportunistic pathogens under favorable conditions. Candida species have been isolated frequently from humans and animals and occasionally from the environmental sources. Candida species have been recovered from certain foodstuffs too (Wade, 1993).

C. albicans has been found to be the predominant species of candida in man in virtually all the surveys conducted on candida carriage (Odds, 1988).

C. albicans, found in the normal flora of human gastrointestinal tract and female genital tract and also, it colonizes the normal skin.

The prevalence of yeast tends to be lower in healthy individuals than in any hospitalized patients (Odds, 1987). Health care workers significantly contribute to the spread of nosocomial candidiasis (Pfaller, et al., 1988). Candida species have been isolated from the hands of health care workers. In one study, almost 80% of the nurses were shown to harbour yeasts on their hands (Strausbaugh, et al., 1996), and candida species were recovered from 63% of them.

Reports from National Nosocomial Infections Surveillance in United States of America showed *C. albicans* in the seventh rank among hospital pathogens isolated from major infection sites (from 1990 through 1992 in hospitals) (Fridkin & Jarvis, 1996).

Hence, it is important to identify the source of *C. albicans* in a hospital outbreak to implement effective control measures.

There are now at least seventeen species of candida that have been shown to cause disease in humans (Rinaldi, 1993). With further developments in medical intervention and with the increasing number of patients with immunodeficiencies or those who have undergone transient long-term immunosuppression, the list of yeasts that can cause diseases would continue to grow (Hazen, 1995). A survey done on oncology patients with candida infections has shown that 46% of cases were infected by non- albicans species (Wingard, 1995). The most common non-albicans species was *C. tropicalis* among the cancer patients (Wingard, 1995). *C. glabrata*, *C. parapsilosis*, *C. krusei* were also isolated from these groups of patients (Salkin, 1979).

C. stellatoidia was found to be associated with vaginal candidiasis and also with systemic candida infections (Salkin, 1979).

The relative pathogenicity of *C. krusei* and *C. albicans* has been studied using rat oral mucosa model (Samaranayake, 1998). *C. parapsilosis* is now considered as an emerging opportunistic pathogen, which cause systemic and superficial infections in immunocompromised hosts (Panagoda, 1998).

C. lusitaniae was also found to be similar to the other candida species, in its ability to colonize individuals and cause opportunistic infections in immune-compromised patients (Merz, 1984).

A novel species associated with oral candidiasis in HIV-infected individuals is identified within the genus candida. This species that is closely related to both *C. albicans* and *C. stellatoidia* species, is named as *C. dubliensis* (Sullivan , 1995).

1.1.3 Age related variation of oral carriage of *C. albicans*.

C. albicans is a harmless commensal, and assumes a pathogenic role due to other factors.

Carriage of candida by humans has been extensively reviewed by Odds (Odds, 1988).

Yeast and candida species have been recovered from the surface of the mucous membranes from the healthy and the diseased. The mouth has been identified as the site, from which candida is most commonly isolated (Arendorf & Walker, 1979). Colonization of *C. albicans* on cleaned human dental hard tissues have been studied and it has been suggested that dental hard tissues may be invaded by *C. albicans* and thus can potentially present a reservoir for disseminated candida infections (Sen, et al., 1997). *C. albicans* has been identified as a common inhabitant of the oral cavities of children and adults (Russell & Lay, 1973).

Investigators looking at the age related carriage of candida have been able to isolate candida species from newborns in their very first day of life (MacFarlane, 1990) and identified a dramatically increased recovery rate with age (MacFarlane, 1990).

In the elderly population many workers have found that the recovery rate of *C. albicans* and other candida species was higher (Kuc, 1999) than that in the middle aged adults. Several researchers who have studied the carriage rate had documented the median as 16% in first few days of life and increases during the next 18 months (median 44%) (Kuc, 1999). The carriage rate falls during childhood (median 6%) but tend to rise again during middle age and in the elderly (Matthews & Burnie, 1989). The median frequency of

carriage of candida has been shown to be higher (55%) for hospitalized patients when compared with that of the healthy volunteers (34%) (Matthews & Burnie, 1989).

1.1.4 Predisposing factors for colonization by candida and oral candidiasis

All forms of oral candidiasis are considered opportunistic, and the epithet 'diseases of the diseased' has been applied to these infections. Nevertheless, it is often difficult to identify an exact predisposing factor for the disease. A variety of local and systemic factors have been identified and extensively studied by a number of workers.

Very old and the very young populations have been identified as more susceptible to oral candidiasis than the middle aged people (Samaranayake, 1990). In the young age this condition may probably be due to the immature immune system, while in the elderly, debility, malnutrition, use of dentures may outweigh the immune mechanisms (Hay, 1993).

In the elderly population high prevalence of the candida species is due to the ill-fitting dentures, and poor oral hygiene. Wearing dentures increases the oral carriage rate of *C. albicans* in normal subjects. It has been estimated that 60% of all full denture wearers aged 65 or above suffers from oral candidiasis (Samaranayake, 1986).

Research carried out to see the carriage of yeast in oral cavities of infants revealed that 5.7% of babies harbour candida species on the first day of life (MacFarlane, 1990). Babies may acquire the organism from the birth canal, or horizontally from the health care workers. Dramatically the prevalence has been shown to increase to 14.2% for candida species and 16.2% for yeast by the seventh day of life (Russell and Lay, 1973). This may

be due to the change in the diet particularly the increase in sugar intake (Russell and Lay, 1973). The *C. albicans* isolation rate, increases still further, and by the end of fourth week it has been shown to be >50% (Russell and Lay, 1973). In the same study, they also investigated the antibody titers of the infants and found that 29 of them showed antibody titers specific for *C. albicans* upto 1: 8 due to transplacental passage from the mother. The changes in the prevalence of oral candida with age of the infants may also be linked with the simultaneous changes in the rest of the oral flora.

Cawson was the first to describe the apparent relationship between iron deficiency and oral candidiasis (Cawson, 1963). It was found that the role of carbohydrates in the pathogenesis of mucosal candidiasis would be in favour of the parasite rather than the host, by providing conditions, which support the virulence of the resident candida species (Samaranayake & MacFarlane, 1985).

Several workers have looked at the relationship between diabetics and oral candidiasis. It is now established that uncontrolled diabetes predispose to superficial and systemic candida infections such as oral candidiasis (Samaranayake, 1990).

Dysfunction of patient's immune system is a major predisposing factor for candidiasis. Mainly in acute leukemia, candida followed by aspergillus shown to play an important role in the morbidity and mortality of patients (Rajendran, et al., 1992; Thanos, et al., 1996). Scientists have identified a relationship between aggressive cancer therapy and the resulting adverse effect on the candida colonization. They found that antineoplastic agents and X-irradiation therapy in cancer patient have a positive relationship with the colonization of candida (Ghannoum, et al., 1988). Low CD4⁺ cell counts, pregnancy, and

postpartum antibiotic use were found to be associated with vaginal colonization of *C. albicans* and candidiasis (Burns, et al., 1997).

The impact of corticosteroid therapy on oral candidiasis has been widely studied, and a significant relationship between the uptake of the corticosteroids and the development of the subsequent candidiasis has been shown (Rajendran, et al., 1992).

Arendorf and Walker (1980) have suggested that smoking may also lead to localize epithelial alterations that may facilitate candida colonization (Arendorf & Walker, 1980).

1.1.5 Infection with candida with special reference to oral candidiasis

Candidiasis is usually restricted to the skin and mucus membranes but may, occasionally spread to deep organs. Oral candidiasis is one of the commonest fungal infections of man. The wide spread use of antibiotics, immunosuppressant drugs and increasing elderly population are the main reasons for the high incidence of oral candidiasis seen in Western world (Samaranayake & Lamey, 1988). Systemic forms of candidiasis may affect only one organ or may be disseminated hematogenously to several organs.

According to imprint -culture studies on the distribution of yeast in the mouth, tongue was found to be the most prevalent site, followed by the palate and the buccal mucosa. It is important to note that oral candida infections can occasionally manifest as a result of systemic complications. These complications are rare with the exception of acquired immunodeficiency syndrome (AIDS), where candidiasis remains superficial and patients usually do not die from disseminated candidiasis.

1.1.5.1 Pseudomembranous candidiasis

Oral candidiasis in the form of thrush is an acute infection, but may recur as a chronic infection in patients using corticosteroids, in HIV infected individuals, and in other immunocompromised patients (Alejandro, et al., 1996).

Thrush is characterized by white patches on the surface of the oral mucosa, and tongue. The lesions develop and form confluent plaques that resemble milk curd and when wiped off leaves, a raw, erythematous and sometimes bleeding base.

Esophageal candidiasis or candidiasis of the upper respiratory tract is a possible complication of oropharyngeal candidiasis. This is particularly seen in HIV-infected patients and also in patients on long term steroid inhalers (Holmstrup & Samaranayake, 1990).

1.1.5.2 Erythematous (atrophic) candidiasis

According to Lehner (1967), erythematous candidiasis is associated with corticosteroids, broad-spectrum antibiotics or HIV disease. It may arise as a consequence of persistent acute pseudomembranous candidiasis when pseudomembranes are shed, may develop *de novo*, or in HIV infection may precede pseudomembranous candidiasis (Samaranayake, 1992).

Clinically erythematous areas are seen on the dorsum of the tongue, palate, or buccal mucosa. Lesions on the dorsum of the tongue present as depapillated areas (Hannula, et al., 1997).

1.1.5.3 Hyperplastic candidiasis (Candida leucoplakia)

Candida leukoplakia is chronic, discrete raised lesions that vary from small, palpable, translucent, whitish areas to large, dense, opaque plaques, hard and rough on palpation. These lesions may be homogenous or speckled and do not rub off easily.

Candida leukoplakia occurs usually on the inside surface of one or both cheeks and less often on the tongue (Arendorf, et al., 1983).

1.1.5.4 Candida -associated denture stomatitis (Denture- induced stomatitis, Denture sore mouth, Chronic atrophic candidiasis)

The characteristic presenting feature of denture- induced stomatitis are chronic erythema and oedema of the mucosa that contacts the fitting surface of the denture. Apart from the soreness this condition is usually symptomless.

1.1.5.5 Angular stomatitis (Angular cheilitis)

Angular stomatitis affects the angles of the mouth, characterized by soreness, erythema, and fissuring, and is commonly associated with denture -induced stomatitis (Budtz, 1990). It has been cited that iron deficiency anemia and other vitamin deficiencies may predispose to angular cheilitis (Odds, 1988).

1.1.5.6 Median rhomboid glossitis

Median rhomboid glossitis is characterized by an area of papillary atrophy that is elliptical or rhomboid in shape, symmetrically placed centrally at the middle of the tongue, anterior to the circumvallate papillae (Scully & El-Kabir, 1994). Occasionally it presents with a hyperplastic exophytic or even lobulated appearance.

1.1.6 Morphology and growth of candida.

There is a great heterogeneity in the morphological forms of candida under different environmental conditions. The organism is readily distinguished from bacteria by its greater size. The model form of the individual cell is ovoid in shape and size varies from 3-5µm in length. It is a Gram-positive organism and often shows budding that preferentially takes place, at the polar region of the cell (figure 01). The distinguishing feature of candida

species is their ability to form pseudohyphae (figure 02), the only exception being *C. glabrata* (Scully & Kabir, 1994).

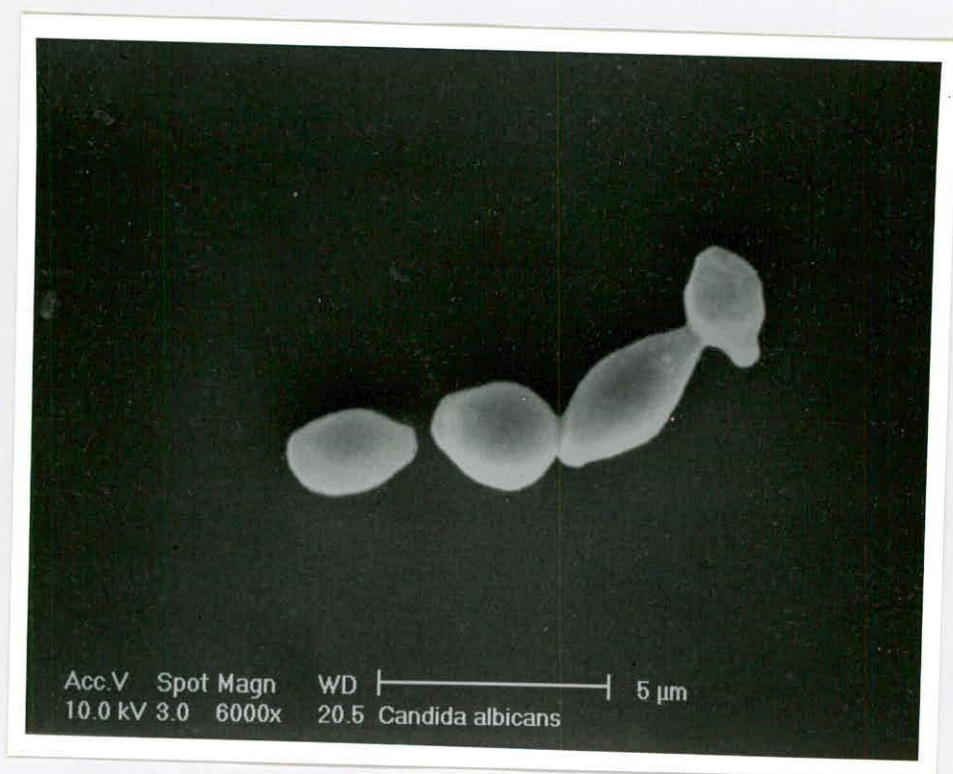


Figure 01-Scanning electronic microscopic appearance of a *C. albicans* blastospore

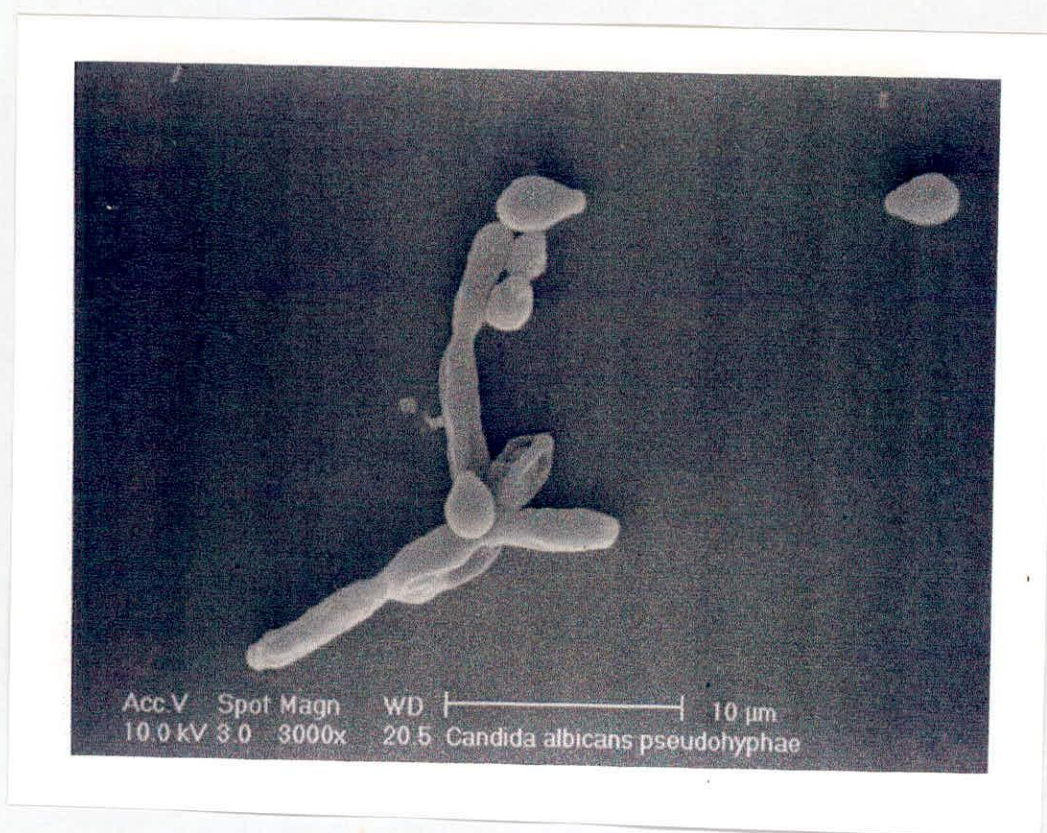


Figure 02- Scanning electronic microscopic appearance of a *C. albicans* pseudohyphae

1.1.7 Morphogenesis of *C. albicans*

C. albicans exhibit two unique cellular forms, blastospore and the true hyphal form.

A blastospore is a unicellular form of a fungus that is frequently referred to as a yeast cell. A specific process of mitotic cell division, termed budding, separates it from the mother cell. A hypha is a microscopic tube. This is divided into multiple fungal cells by septae. The term 'germ tube' is used to describe a cylindrical cellular material, which arises from the blastospore itself without a constriction at the mother cell, but as a continuation of the cell (figure 03). This term is only used to refer to newly evaginating hyphae up to the time of formation of the first septum (Shepherd, 1990). Conversion of the ovoid cell to a hyphal cell takes place via the germ tube formation.

C. albicans also produces pseudohyphae, which arises from a blastospore as a result of budding and remains attached to its parent and get elongated. These end-to-end aggregations of blastospores give rise to pseudohyphae. Increased temperature and the neutral pH favour formation of pseudohyphae. Odds (1985) observed the true hyphal form of *C. albicans* at 37°C, while the yeast form appeared when incubated below 30°C (Odds, 1985). Formation of the chlamydo spores is another important feature of *C. albicans*. These structures can be seen only in vitro (Odds, 1988). They are large, refractile, thick walled cells that are often subtended from hyphae or pseudohyphae by a specialized blastospore like cell called a suspensor cell.

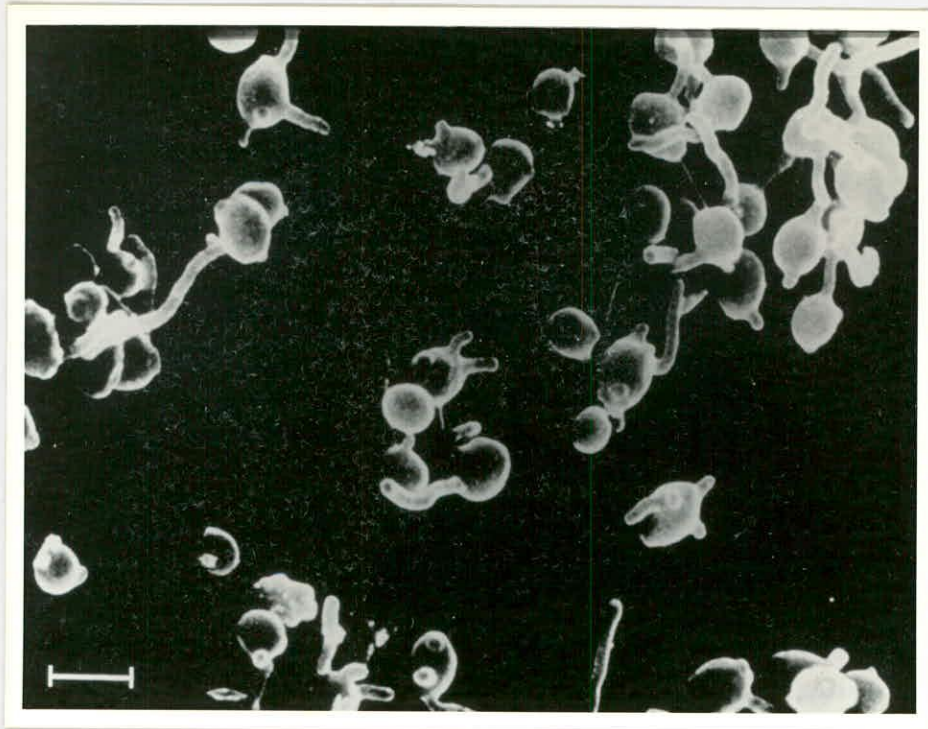


Figure 03- Scanning electronic microscopic appearance of a *C. albicans* germ tube

1.1.8 Laboratory studies of candida species.

1.1.8.1 Sampling techniques

Number of workers have studied the carriage rates of *C. albicans* in the oral cavity. However, it is difficult to compare the results of different studies due to the different sampling techniques used in these studies. The sampling techniques that have been used mainly were oral swabs (Hellstein, et al., 1993; Pfaller, et al., 1994), saliva (Umazume, et al., 1995), oral rinse (Samaranayake, et al., 1986) and imprint technique.

Swabs are of practical use in sampling the very young (Russel & Lay, 1973) or those unable to follow up the instructions, which entail other collection techniques.

Although swabs are useful for assessing the presence or absence of candida, due to the contamination and dilution by saliva, this technique cannot provide a quantitative estimate of oral yeast carriage (Sedgley & Samaranayake, 1994).

As opposed to swabbing, sampling of saliva involves the patient spitting a volume of mixed saliva into a sterile container. Saliva will be inoculated onto appropriate media and incubated. This allows colony-forming units per ml (cfu/ml) of saliva to be determined (Silverman, et al., 1990). Salivary flow rate and time of the collection may affect the accuracy of this technique.

To obviate the errors associated with the above techniques, a third method involving an oral rise with saline has been employed. In this technique the patient is asked to rinse the

mouth with saline for a predetermined time and spit into a sterile container. A known aliquot of the sample is inoculated onto appropriate media to determine the colony count.

Samaranayake et al., (1986) has modified this technique by concentrating the oral rinse to enhance the sensitivity of the oral rinse method.

Arendorf and Walker in 1979 suggested that imprint technique was the best method for in-depth studies, involving different intra oral sites, where candida was the only organism of interest.

When Samaranayake and colleagues (1986) compared the imprint and the oral rise technique, they found oral rinse method was the more effective for longitudinal and cross sectional screening of large number of individuals. They also demonstrated that this technique could be used to detect the overall candida score and also for quantification of other microorganisms found in the oral cavity, at the same time.

1.1.8.2 Isolation methods

Sabouraud's dextrose is the most commonly used isolation medium for candida species. On Sabouraud's dextrose agar (SDA) when incubated at 37°C for 48 -72 hours *C. albicans* appears as cream coloured large convex colonies.

Pagano Levin agar, described by Pagano et al., facilitates the identification of different species of yeast in the same sample. It allows various yeast species to be distinguished through visual differences in coloration, facilitated by a reduction of triphenyl tetrazodium chloride incorporated into the medium. Modified Pagano Levin (MPL) media prepared by

the method of Yamane and Saitoh (1985), contain Pagano Levin base (Difco Laboratories, Detroit, Mich.), 2,3,5 triphenyltetrazodium chloride (0.1 mg/ml; Difco) and gentamycin (50(g/ml). Different candida species elicit varying colour reactions when grown in Pagano- Levin medium. Investigators have found a slightly higher sensitivity of MPL (95%) than SDA (93%).

Blood agar is also used to isolate candida species, which appear as grayish colonies on it.

1.1.8.3 Identification of *C. albicans*

Identification of *C. albicans* isolates, at both medical and research laboratories are traditionally based on the standard techniques such as germ tube formation, biochemical tests and chlamydaspore production. These techniques however, require a pure growth of the yeast. These are time consuming and have their inherent weaknesses, as they are not species specific. Additionally, isolates of the same species can differ in key phenotypic and genotypic characteristics leading to misidentification. Hence there is a need for new methods, which would facilitate rapid and accurate identification of such microorganisms.

1.1.8.3.1 Germ tube test

The presumptive identification of *C. albicans* is mainly based on its ability to produce short, slender tube like structures called germ tubes, when incubated at 37⁰C for 2-3 hours in serum (Beradinelli & Opheim, 1985). In addition to the human pooled serum, a number

of other mixtures induce germ tube formation including plasma, saliva, and egg white. However, the safety issues concerned with their use has led to the production of defined media, which facilitates the germ tube formation (Beheshti, et al., 1975). Several factors have been identified to influence the germ tube production. The rate of germ tube production has been shown to be strictly glucose dependent (Vidotto, et al., 1996), and fructose also plays an important role (Vidotto, et al., 1996).

1.1.8.3.2 Chlamydospore formation

Depending on the environmental conditions and the nutritional conditions *C. albicans* may form large thick walled, spherical spores called chlamydospores. These are also believed to be dormant stages of the fungus (Torosantucci & Cassone, 1983). *C. albicans* can be identified by the chlamydospore formation on cornmeal agar in the presence of Tween 80 (Walsh & Pizzo, 1993).

1.1.8.3.3 Biochemical identification of *C. albicans*.

Biochemical identification of candida is primarily based on its ability to assimilate and ferment different carbohydrates. Assimilation is the process of utilizing the carbon compound as its sole source of energy in the presence of oxygen, while in fermentation it utilizes the carbohydrate as its sole source of energy in the absence of oxygen.

Commercial kits are now available for the assimilation tests. Different manufactures have tried to improve the accuracy of these commercial kits.

API 20CTM was introduced in 1975 as a microtube system for identification of yeast (Analytab products Inc). This system miniaturized the traditional procedures of carbohydrate fermentation and assimilation permitting identification of yeasts within 72 hours after incubation. Recently this has undergone extensive modification. Numerical approach with a computer-assisted system for the identification of clinically important yeast has been introduced. Scientists have evaluated the modified API20C system for its accuracy (Buesching, et al., 1979). They have concluded that identification of yeasts based solely on the assimilation patterns provided by the modified API20C system was not reliable unless used in combination with microscopic morphological features.

AbbottTM yeast identification system (Abbott lab, Diagnostics Division, Irving, Tex) is a 24-hour instrumental method for identifying medically important yeasts. This is based on matrix analysis of 19 biochemical reactions and the germ tube test. This is a rapid test, which can give results within 20-24 hours (Cooper, et al., 1984).

Autobac 1TM is a semiautomated instrument used for rapid determination of antimicrobial susceptibility of bacteria. The technology of this instrument was used to determine the carbon assimilation patterns of yeasts (John, et al., 1978).

The Uni -Yeast Tek systemTM (Corning Medical) is a commercial kit for the rapid identification of medically important yeasts. It is composed of multisectioned dish containing seven carbon assimilation agars, i.e. - urea agar, nitrate assimilation agar, a central well containing corn meal agar with tween 80, a broth containing 0.05 glucose and

2.6% beef extract. Several investigators have evaluated (Cooper, et al., 1978; Bowman & Ahearn, 1975) this system. This system has been found to be time consuming and it has been identified as the main draw back of this technique.

The API Candida systemTM consists of a strip of ten APITM tubes allowing 12 colorimetric tests to be performed: five carbohydrate acidification tests (glucose, galactose, sucrose, trehalose, and raffinose) and seven enzymatic tests (β -maltosidase, α -amylase, β -xylosidase, β -glucuronidase, urea hydrolysis, N-acetyl- β -glucosamidase, and β -galactosidase). This is identified as a system adapted to identify clinically important yeasts in a routine clinical microbiological laboratory (Helene, et al., 1996).

API20CAUXTM system is also an assimilation test used to detect assimilation patterns of 19 different carbohydrates by yeasts. On comparison, this system with the VitekTM YBC assimilation system, API 20CAUX required personnel experience in reading the results (Gale, et al., 1999).

Methods have recently been developed for detecting the production of the enzymes by yeasts isolates grown in cultures. The BactiCardTM Candida (Remel Co., Lenexa, Kans.), MUREX *C. albicans* 50TM (Murex Diagnostics) and Albicans - SureTM (Clinical Standards Laboratories Rancho Dominguez Calif) assays were compared with the standard germ tube method (Heelan, et al., 1996). All three test kits based on the detection of enzymes L-proline aminopeptidase and β -galactosaminidase in yeast cells grown on culture media and are based on the principle that *C. albicans* produces both enzymes whereas other yeasts produce only one or neither of the enzymes. These three kits were found as more sensitive than the germ tube test and also found as specific as the germ tube test for

presumptive identification of *C. albicans* isolated from clinical specimens (Crist, et al., 1996).

1.1.9 Typing and characterization of *C. albicans* isolates

C. albicans is the most frequently isolated yeast from humans. This high frequency of isolation has led to many investigators to study the epidemiological aspects of this organism. However, until recently not many studies concentrated on the pathogenic potential of *C. albicans*. As a result, we know little about the degree of strain variation within species and its relationship to the ability of the organism to colonize or invade a specific site of the body. Differences among the intraspecies strains have now been recognized. These include the ability of strains to adhere to various cellular and plastic surfaces, ability to respond to various host pathophysiological changes and development of resistance to antifungal drugs

The differentiation between exogenous and endogenous acquisition is important for determining appropriate preventive measures to control the nosocomial transmission of candida species. Strain typing studies are required to avoid implicating an environmental source solely on the basis of the presence of a single species of candida. A number of typing systems have been described for strain differentiation within candida species (Merz, 1990; Silverman, et al., 1990). The phenotypic typing systems that have been discussed are based on analysis of phenotypic differences by biotyping (Odds, et al., 1983), enzyme profiles (Boerlin, et al., 1996), susceptibility patterns to antimicrobial agents (Fernando, et

al., 1997; Hernandez, 1992) serological typing (Mercure, et al., 1996) and morphotyping techniques (Phongpaichit, et al., 1987).

The biotyping method described by Williamson, Samaranayake and MacFarlane (Williamson, et al., 1987) have been extensively used. This method was also employed to biotype *C. albicans* in the current study.

1.1.9.1 Serotyping of *C. albicans*

When the serotyping of *C. albicans* was first introduced (Hasenclever, & Mitchell, 1961) isolates of this organism were divided into two different serotypes, called A and B on the basis of the antigenic characteristics of the cell wall. However, due to its low discriminatory power, this method has now been superseded by other simple and better methods.

1.1.9.2 Morphotyping

Brown-Thomson in 1968 described for the first time a typing system (Brown –Thomsen, 1968) based on the different colony morphologies exhibited by *C. albicans* strains when streaked on malt agar. They were able to differentiate 14 types of colony morphologies in their study. But due to the high frequency of switching (Slutsky, et al., 1985) of *C. albicans*, morphotyping was found to be unreliable. It was also reported that morphologic characteristics might be lost on repeated subculture or storage. In spite of these

disadvantages, the simplicity and good discrimination of the strains make it a useful typing scheme for *C. albicans* (Hunter, et al., 1989).

1.1.9.3 Resistogram typing

Resistogram typing method, based on resistance of *C. albicans* to different concentrations of chemicals, was modified to increase the reproducibility and the discriminatory power (Warnock, et al., 1979).

1.1.9.4 Killer typing

Killer typing method is a simple procedure for typing *C. albicans* isolates based on the susceptibility of the strains to the toxic effects of nine different killer yeasts (Polonelli, et al., 1983). Killer factors are extracellular toxins produced by certain yeasts that kill sensitive yeast strains but not the producer strain within the genus or within other genera. One drawback of this method is the lack of a standard panel of toxin-producing yeasts. Several scientists applied this biotyping method for their studies (Bruatto, et al., 1991).

1.1.9.5 Biotyping

Odds and Abbott used the biochemical characteristics for the first time for strain differentiation of *C. albicans* (Odds & Arbbott, 1980). This method has identified 512

biotypes using a set of nine different tests. Subsequently, this was modified by the inclusion of boric acid resistant test (Odds & Arbbott, 1983). Due to the difficulties in interpretation of the results its value is limited as a research tool.

Thereafter a number of tests have been evaluated to improve the simplicity, reproducibility and sensitivity of biotyping.

1.1.9.5.1 APIZYMTM

The APIZYMTM system has been found as a rapid and reproducible method of biotyping *C. albicans* (Roman, et al., 1983; Williamson, et al., 1986). It allows the systematic and rapid study of nineteen enzymatic reactions using a very small quantity of the sample.

1.1.9.5.2 API20CTM

API 20CTM system was also found to be an important biotyping method for *C. albicans* (Williamson, et al., 1986). This is specially useful for precise identification of the most frequently encountered yeasts in clinical microbiology. API 20C AUXTM strip consists of 20 cupules containing dehydrated substrates, which enable the performance of nineteen assimilation tests. The cupules are inoculated with a semisolid minimal medium and the yeast will grow only if they are capable of utilizing each substrate as the sole carbon source. Due to the reproducibility and the simplicity of using commercially available API systems, a number of studies investigating the biotypes of *C. albicans* using API 20 C

system both in infected and healthy individuals (Xu & Samaranayake, 1995) have grown considerably.

1.1.9.5.3 Combined method

Williamson, et al., (1987) have used a combination of three different tests, i.e. the APIZYMTM, API20CTM and the boric acid resistant test for biotyping *C. albicans* isolates. With this method they identified 234 potentially identifiable biotypes. In the present study this combined method is applied for biotyping *C. albicans*.

1.1.9.6 Typing method based on protein analysis

Typing methods based on protein analysis are also used as tools for strain differentiation within the species. These include immunoblot fingerprinting (Lee, et al., 1986), polyacrylamide gel electrophoresis of cellular proteins and multilocus enzyme electrophoresis (Boerlin, et al., 1996).

Multilocus enzyme electrophoresis (MEE) or iso-enzyme typing was applied to study the diversity of *C. albicans* and was identified as a very powerful tool for typing *C. albicans* in epidemiological studies.

1.1.9.7 Molecular typing methods

Over the last several years, molecular methods of studying strain relatedness have proved to be quite useful, allowing investigators to examine retrospective putative outbreaks of candidiasis and assess the epidemiological aspects of these outbreaks (Maffei, et al., 1990; Voss, et al., 1995; Reagan, et al., 1990). These molecular typing methods have been used extensively for the detection and typing of candida strains (Schroppel, et al., 1994) and also, for detection of species (Thanos, et al., 1996).

1.1.9.7.1 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) was applied to identify outbreaks of candidiasis (Matthews & Burnie, 1989). In this method, digestion with a single restriction enzyme yields several fragments present in high enough concentrations to give distinct bands on a stained electrophoretic gel (Scherer & Stervens, 1987). These bands are derived from mitochondrial and ribosomal DNA and they vary sufficiently in size to permit their detection as separate bands on an agarose gel. This is an epidemiologically useful typing system. Number of scientists have applied this RFLP fingerprinting technique in strain typing studies of *C. albicans*. (Ruiz-Diez, et al., 1997; Doi, et al., 1994). Although RFLP has the advantage of being simple and rapid to perform, it has the disadvantage of detecting a limited number of polymorphisms. This is due to the fact that certain patterns

tend to appear too frequently so that some times geographically distinct strains appear identical (Magee, et al., 1992).

1.1.9.7.2 Pulse field gel electrophoresis (PFGE)

Electrophoretic karyotyping by pulse field gel electrophoresis (PFGE) has also shown to achieve more polymorphisms (Vazquez, et al., 1994). When compared to RFLP method the later two techniques have the disadvantage of their greater cost and longer time required. PFGE analysis might be considered by some to be the most difficult of these methods to perform, requiring intact chromosomes and specialized electrophoresis equipment as well as taking the longest period of time to perform (Clemons, et al., 1997), while others have indicated that this is highly sensitive and useful method for strain discrimination (Doi, et al., 1994).

1.1.9.7.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was adapted for genotyping candida using paired primers derived from previously characterized sequences for PCR amplification. It has been stated that this technique has a high discriminatory power, reproducibility, high sensitivity, and been rapid and simple to perform (Xu, et al., 1999; Diaz-Guerra, et al., 1997; McCullough, et al., 1995; Thanos, et al., 1996)

1.1.9.7.4 Randomly amplified polymorphic DNA (RAPD)

A new polymorphism assay has been developed based on this PCR amplification of random DNA fragments with single short primers of arbitrary nucleotide sequence (Welsh & MacCelland, 1990). This method has the advantage of not requiring any prior sequence information. These polymorphisms have been termed as random amplified polymorphic DNA (RAPD) markers (Williams, et al., 1990). Bostock and colleagues in 1993 applied this technique for typing of *C. albicans* (Bostock, et al., 1993) for the first time. RAPD method has several advantages. It is less labour intensive, requires only small amounts of DNA and also relatively easy to standardize among several laboratories. It requires little knowledge of the molecular biology of the species, and no prior knowledge of the sequence is necessary. Unlike PCR typing performed with paired primers from a known sequence, its detection limit is not confined to changes occurring in only a small part of the genome. Each primer gives PCR products of different molecular weights. At the same time, a primer with great potential for detecting polymorphisms within a given species may be poor at discriminating strains of another species. Sources of polymorphism include deletion of a priming site and insertions that either increase the size of the PCR product or render the priming sites too distant to support amplification. It has been shown that the RAPD typing technique to be rapid and economical with high reproducibility and typerbility for *C. albicans*.

RAPD technique has been applied at the species (Hannula, et al., 1997) and the genus (Metzgard, et al., 1998) level of *Candida*. RAPD method has been compared with other

molecular typing methods (Bart-Delabesse, 1995) and was found to be a sensitive technique.

Therefore, in the present study Random amplified polymorphic DNA fingerprinting (RAPD) method was applied for genotyping *C. albicans*.

It had already been found that resolution of RAPD typing of candida strains depends heavily on the choice of primers and the genomic locus selected (Dassanayake & Samaranayake, 2000). Primer RSD12 (5'GCA TAT CAA TAA GCG CAG GAA AAG3') (Fell, 1993) and RSD10 (5'CCG CAG CCA3') was selected in the present study. These have previously employed successfully for the analysis of DNA repeats in *C.albicans*.

1.1.9.8 A new approach to typing

As with many bacterial pathogens, combination of these methods may be more discriminating than any single method (Pfaller, et al., 1990). None of the phenotypic or genotypic typing procedures alone provides a definitive answer to epidemiological questions. Optimal approaches should include a combination of as many as different markers.

Hence in the present study, two typing methods were applied, biotyping using the method of Samaranayake and MacFarlane and RAPD fingerprinting for strain typing of *C. albicans*. Then these two methods were combined to assess the ability to discriminate *C. albicans* strains.

1.2 AIMS AND OBJECTIVES

1.2 AIMS AND OBJECTIVES OF THE STUDY

1. To determine the proportion of people colonized with *C. albicans* in their mouths, among children, adult and elderly volunteers selected from the Colombo district.
2. To determine the phenotypic and genotypic diversity of *C. albicans* isolated from these three age groups.

1.3 MATERIALS AND METHODS

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1.3 MATERIALS AND METHODS

1.3.1 The study group

The study group consisted of 120 Sri Lankan volunteers. They were divided into three defined age groups. Less than 6 years (<6 years), between 30-50 years and more than 70 years (>70 years). Each group consisted of 40 individuals. Informed consent was obtained from each participant before taking the sample for the study.

A questionnaire was used to obtain information on volunteers and included, the name age, gender, address, presence or absence of dentures, use of drugs, alcohol, smoking habit, betel chewing, past medical history, and information on current medication including antibiotic therapy (appendix 1) .

1.3.2 Sampling technique

Each subject included in the study was given a sterile, disposable polystyrene universal container with ten milliliter (10ml) of sterile phosphate buffered saline (PBS).

They were asked to rinse their mouth for 60 seconds (60s) with 10 ml sterile phosphate buffered saline (PBS 0.1M, pH7.3) given in the sterile universal container. After 60s subjects were asked to return the oral rinse to the universal container as described by

Samaranayake, et al., 1987. Samples were taken between 9.00am and 12.00 noon assuming

they have brushed the teeth before that. Dentures, if any, were not removed during the collection of sample.

Following collection the oral rinse samples were kept at 4°C and immediately brought to the microbiological laboratory. In the interim period between collecting and processing, the samples were stored at 4°C and transported to the laboratory in a portable cooler with an internal temperature of 7°C. Temperature of the cooler was measured before and after transport using a thermometer.

1.3.3 Processing of the sample

Oral rinse samples were processed according to the concentrated rinse method previously described by Samaranayake, et al., in 1987.

Each sample was centrifuged for 10 minutes at 1700g and the supernatant was discarded. The pellet was resuspended in 1 ml of sterile PBS and mixed for 30 seconds to make the final concentrated suspension. Twenty-five micro liter (25µl) of this suspension was inoculated on to a Sabouraud's dextrose agar (SDA) plate (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England), and the plates were streaked in three directions using sterile wire loop to obtain a colony count. Plates were incubated at 37°C for 48 hours.

1.3.4 Microbiological identification

After 48 hours incubation at 37°C, all plates were examined for the colony morphology (colour of the colony, size, elevation, odour, and translucency of the colony) and colonies were identified as yeast species, primarily by the Gram's stain. Each different type of yeast colony was subcultured on to SDA plates to obtain pure culture.

1.3.4.1 Preservation of isolates.

Each candida isolate was stored on filter paper strips for further analysis. Sterile filter paper strips measuring 2cm × 1cm were used to stock the cultures. Few colonies from a purified 24 hours old yeast culture on Sabourauds dextrose agar were streaked on to the filter paper strip using a straight sterile wire maintaining sterile conditions. These strips were then placed in labeled sterile disposable polystyrene universal bottles and kept at – 20°C.

Identification of the *C. albicans*

1.3.4.2 Preparation of the germ tube agar medium

Four grams (4g) of rice powder (Oxoid) was incorporated into 400 ml of distilled water and dissolved by boiling. This suspension was filtered using cotton wool. Four grams of

Ox bile (Oxoid), 4ml of tween 80 (Atlas Powder Co.), and 4g of bacteriological agar (Oxoid) were added to the filtrate. Thereafter the volume of the suspension was increased up to 370 ml by adding distilled water. This suspension was dissolved in a boiling water bath. Finally, the pH was adjusted to 6.9 and autoclaved at 121°C for 15 minutes. When the medium was cooled down to 55°C, 20 ml of urea was added before pouring into 96mm petri dishes.

1.3.4.3 Germ tube test

Germ tube test was performed on each yeast isolate using a 24 hour old purified culture on SDA, and using *C. albicans* ATCC 90028 strain as a positive control.

A colony was touched with a sterile wire loop and inoculated on the Germ Tube Agar medium and the streaked lines were covered by a cover slip and incubated at 37 °C for 4-6 hours. After 5 hours the plates were examined for the presence of the germ tube under a microscope.

Germ tube test was also performed using human serum. One colony of yeast from a 24-hour-old culture was inoculated into a 2.5-ml human serum and kept for incubation at 37°C for 2-3 hours. *C. albicans* ATCC 90028 strain was used as a positive control. After 2 1/2 hours a drop of the suspension was placed on a slid and covered with a cover slip and examined under the microscope using the 40x magnification.

A germ tube was identified as a tube about 1- 2 μ M in length and arising from the blastospore without a constriction at the junction of the mother cell.

1.3.5 Biotyping of *C. albicans*

The method described by Williamson, Samaranayake and MacFarlane (1987) was used for biotyping the isolates in this study. This comprised of three tests. These included two commercial tests API ZYM™ (BioMe'rieux, France) and API 20C™ (BioMe'rieux, France) and an in-house boric acid resistant test.

1.3.5.1 API ZYM™ test

The API ZYM system (Analytical Profile Index; Bio Me'rieux SA, France) (Appendix 2), was used to screen the enzyme patterns of microorganisms.

The API ZYM system tests for the presence of an array of enzymes in *C. albicans*. It comprises of 20 different cupules with one control (no substrate added) and nineteen different substrates. These are: 2-naphthyl phosphate, 2-naphthyl buterate, 2-naphthyl caprylate, 2-naphthyl myristate, L-leucyl-2-naphthylamide, L-valyl-2-naphthylamide, L-cystyl-2 naphthylamide, N-benzoyl-DL-arginine-2-naphthylamide, N-glutaryl-phenylalanine-2-naphthylamide, 2-naphtyl phosphate, naphthol-AS-BI-phosphate, 6-Br-2-naphtyl- α D-galactopyranoside, 2-naphtyl- β D-galactopyranoside, naphthol-AS-BI- β D-glucuronide, 2-naphthyl- α D-glucopyranoside, 6Br-2- α D-mannopyranoside, and 2-naphtyl- α L-fucopyranoside. The corresponding enzymes that are assayed: phosphatase alkaline, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, trypsine,

chymotrypsin, phosphatase acid, naphthol-AS-BI-phosphohydrolase, α glucosidase, N-acetyl- β glucosaminidase, α mannosidase, and α fucosidas, respectively.

The inoculum of candida was prepared in sterile distilled water, using a 24 hour culture grown on Saboraud's dextrose agar (SDA). The suspension of candida was adjusted to have a turbidity of McFarland unit 5 to 6, using a double –beam spectrophotometer (Model UV-150-02, Shimadzu Corporation, Kyoto, Japan). Each cupule was then filled with 65 μ l of the suspension and the strips were covered and incubated aerobically in a moist chamber at 37 °C in the dark for four hours. After 4 h, one drop of reagent ZYM A (25g Tri-hydroxymethyl-amino-methane, 11mL hydrochloric acid (37%), 1 auryl sulphate 10g, distilled water 100mL) and one drop of reagent ZYM B (Fast Blue BB 0.35 g, 2-methoxyethanol 100mL) were added to each cupule and the strips were placed under 100 watt light source for 5 minutes to develop the colour. The presence of a particular enzyme was shown by a change in the colour. The development of the colour was compared with the API ZYM colour chart provided by the manufacturer (Analytical Profile Index; Bio Merieux SA, France).

Each API ZYM biotype, thus obtained was designated in alphabetic order as capital letters beginning from 'A'

1.3.5.2 API 20C AUX™ Test

The second test utilizes the API 20C AUX™ yeast identification system (Analytical Profile Index; Bio Merieux SA, France) (Appendix 3) and it evaluates the ability of *C. albicans* to

assimilate different carbohydrates as its sole carbon source for growth. The API 20C AUX™ comprises 20 miniaturized cupules containing different carbohydrates. The carbohydrates are: glucose, glycerole, 2-keto-D-gluconate, L-arabinose, D-xylose, adonitol, xylitol, galactose, inositol, sorbitol, (methyl-D-glucoside, N-acetyl-D glucosamine, celiobiose, lactose, maltose, saccharose, trehalose, melezitose, and raffinose.

Candida suspension of 2 McFarland unit turbidity as measured with a double-beam spectrophotometer (ModelUV-150-02, Shimadzu Corporation, Kyoto, Japan) was prepared using 0.85% NaCl, and 24 hour culture of *C. albicans* grown on SDA plate. A micropipette was then used to transfer 100 µL of the suspension to the medium identified as C medium in the commercial kit. After gentle homogenizing with the pipette, the cupules were filled evenly with the suspension in the C medium, taking care to avoid air bubble formation. The strips were covered and then placed in a moist chamber and incubated at 30°C for 48-72 hours. The utilization of the carbohydrate by *C. albicans* will result in its growth and would increase the opacity of the respective cupule. Increase in the opacity compared to the control was considered as positive. The results obtained were then transferred to a numerical profile. The tests results were recorded on the result sheets provided with the kit and were divided into groups of three according to the manufacturer's instructions. Each test was assigned a value of 1, 2 or 4 for its positive reaction as instructed by the manufacturer. The presence of hyphae or pseudohyphae was the 21st test and was given a value of 4. A final 7-digit numerical profile was obtained by adding together the three numbers within each group. This numerical profile was fed into the computer software

program APILAB Plus V3.3.3 (bioMerieux, France) to get the species identification. Each new biotype of *C. albicans* identified was given a Roman numeral beginning from 1.

1.3.5.3 Boric acid resistant test

The third test was the resistance of *C. albicans* to boric acid. Boric acid (E Merck, Darmstadt, Germany) at a concentration of 1.8mg/mL was incorporated into a basal medium containing: 6.7g yeast nitrogen base (Difco Laboratories, Detroit, Michigan, USA), 1.5g L-asparagine (Sigma Chemical Co., St Louis, MO, USA), 10.0 g glucose (Difco), 15.0g bacto-agar (Difco), and 1000mL of distilled water.

1.3.5.3.1 Preparation of the inoculum for boric acid resistant test

The *C.albicans* inoculum was prepared by using a 24 hours old culture grown on a SDA plate. A *C.albicans* suspension at a concentration of 1×10^6 cells per ml was prepared by microscopic counting in an improved Neubauer haemocytometer (Hawksley, England).

The optical density of this suspension was measured using a double –beam spectrophotometer (Model UV-150-02, Shimadzu Corporation, Kyoto, Japan) at 520 nm. Subsequent preparations of the *C.albicans* suspension were simplified by adjusting to the same optical density. A standard suspension of 5µl was inoculated onto the plate and incubated at 37 °C aerobically for 48 hours. Control plates containing the same basal medium without boric acid, was also inoculated with the same quantity of the *C.albicans* suspension simultaneously and incubated at 37° C aerobically for 48 hours. The *C.albicans*

strains were identified as R (resistant to boric acid) if confluent growth was found and as S (sensitive to boric acid) if no growth was found. The control plate was checked for growth of *C. albicans*.

1.3.5.4 Identification of the biotyping profile

A final biotype code was assigned to each isolate based on the combination of these three test results (ie; APIZYM™, API20CAUX™ and Boric acid resistant test).

This biotype code is designated by using two capital letters and one, central number (e.g J1S) the first letter represents the result of the biotype profile of API ZYM™ assay, the central number represents the result of the API 20C AUX™ and the last letter denotes the resistant (R) or sensitivity (S) of the *C. albicans* to boric acid.

1.3.6 Genotyping of *C. albicans*

1.3.6.1 Preparation of DNA for Random Amplified Polymorphic DNA (RAPD)

C. albicans isolates were grown on Sabouraud's Dextrose agar (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) medium for 24 hours at 37°C. Using the flat side of a sterile toothpick, cells were scraped off and resuspended in 1 ml of 1 M sterile sorbitol. The cells were centrifuged at 13000 rpm for 5 minutes and then supernatant was discarded and the pellet was washed again with 1 ml of 1M sorbitol. Cells were harvested by centrifugation at 13000rpm for 5 minutes at room temperature and resuspended in 1 ml of

sterile SE buffer (1.2M sorbitol, 0.1M EDTA, pH7.5), 2µl of sterile β mercaptoethanol and 15µl of 20mg/ml sterile lyticase (Lyticase, Sigma, USA) and incubated at 37°C in a water bath for one hour until the spheroplasts were formed due to lysis of cell walls. The spheroplasts were next, harvested by centrifugation at 10,000rpm for 1 minute at room temperature. These spheroplasts were washed once with SE buffer and resuspend in 0.4 ml of 0.15M NaCl / 0.1M EDTA pH 8.00. To this suspension 10µl protenase K (20mg/ml) and 50µl SDS (sodium dodecyl sulfate) (10%) were added, followed by 40µl RNAase (10mg/ml). The suspension was then incubated at 55°C for 1 hour in order to lyse the spheroplasts with SDS and digest the proteins and RNA by protenase K and RNAase. This mixture after incubation was next centrifuged at 13,000 rpm for 15 minutes at room temperature. The resultant supernatant obtained following centrifugation was used for the extraction of DNA. The DNA was extracted, once with equal volume of sterile phenol:chloroform:isoamyl alcohol (400µl) and, once with equal volume of sterile chloroform (400µl). 300µl of upper aqueous layer was transferred to a new sterile 1.5ml microfuge tube and DNA was precipitated by adding 0.6 volume of isopropanol and allowed to stand for 30 minutes at room temperature. This was next centrifuged at 13,000rpm for 15 minutes, at room temperature and the resultant pellet was washed with 0.5ml of 70% sterile ethanol. Supernatant was removed after centrifugation at 13,000rpm for 5 minutes and the DNA pellet was dried in the tube for 5 minutes at 55°C. Finally the DNA thus prepared was dissolved in 50µl sterile TE buffer (10 mM Tris, 0.1mM EDTA pH 8.0) and stored at 4°C. Only one sample was handled at a time. Strict aseptic precautions were taken to avoid cross contamination.

1.3.6.2 Random Amplified Polymorphic DNA (RAPD) analysis.

Randomly amplified polymorphic DNA fingerprinting was done only for the extracted DNA from the strains of *C. albicans* using the method described in 1993 (Bostock, et al., 1993). Two random primers were selected for the RAPD. Primer source was SEQ WEB-CGC-Prime. These were inversely oriented repetitive primers found in the genome of candida. These primers act both as the forward and the backward primer.

Thermocycling was performed in a PTC-100 MiniCycler machine (MJ Research, MS, USA) and RoboCycler 40 (Stratagene, LJ, California, USA).

1.3.6.2.1 Preparation of the master mixture

PCR master mixture was prepared in a separate room and precautions were taken to avoid cross contamination of sample and PCR products. A negative control with out the template DNA was carried out with PCR.

The 50 µl PCR master mixture contained 5µl of 10x PCR buffer (200mM Tris-HCl (pH8.4) and 500 mM KCl), 0.2mM dNTPs, 1.5 mM MgCl, 1µM of primer RSD 12 (5'GCA TAT CAA TAA GCG CAG GAA AAG3') and RSD10 (5'CCG CAG CCA3'), 2µl template DNA and 2.5U of Taq polymerase (Life technologies, USA). The tubes were overlaid with 50µl mineral oil.

1.3.6.2.2 The RAPD cycle parameter

The cycle parameter were, 30 seconds of denaturation at 94°C, 2 minutes of annealing at 52°C (primer RSD 12) or 27°C (RSD 10) and 2 minutes primer extension at 72°C, followed by 45 cycles of 30 seconds denaturation at 94°C, 2 minutes annealing at 57°C (primer RSD12) or 32°C (primer RSD10) and 2 minutes primer extension at 72°C. A final extension was performed at 72°C for 15 minutes, and the RAPD products were at 4°C until further analysis.

1.3.6.2.3 Gel electrophoresis

1.3.6.2.3.1 Casting the gel

0.8%- 1% TBE agarose gel was used for the gel electrophoresis. Appropriate amount of agarose was weighed and mixed with half the quantity of 0.5x TBE buffer and dissolved in a microwave oven. The rest of the TBE buffer was added to the mixture while mixing it using the magnetic stirrer. Liquid gel was allowed to cool and was then poured into the gel cast device (190 ml) and allowed to solidify at room temperature. The gel was then kept in the gel tank filled with the TBE buffer (89mM Tris, 89mM-boric acid, 2.5mM EDTA, pH8). When the gel was totally submerged the comb was removed to obtain the wells.

1.3.6.2.3.2 Sample loading

The RAPD products (5 μ l) were mixed with the loading dye (1 μ l) before loading into the wells. The loading dye used was 6 $\times$ concentrate, containing 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) sucrose water. Three wells were loaded with the marker (Gene rulerTM MBI Fermentas) to compare the positions of the bands and one negative control (without template DNA) was run with each time.

1.3.6.2.3.3 Gel running conditions

Gel was run for two hours at 100V in the 190 ml gel tank at ambient temperature.

1.3.6.2.3.4 Staining with ethidium bromide

Gel was stained using ethidium bromide (0.5 μ g/ml) for 45 minutes and visualized with UV light.

1.3.6.2.3.5 Gel photographs

A polaroid 'gun camera' was used with UV filter and polaroid type 667 high- speed film to take the gel photograph. The aperture was set to f/8 and the shutter on 1 second.

The band produced by each isolate on the gel was photographed and the band patterns were used to identify the different genotype. Only the clearly visual bands were scored and the molecular weights of the bands were compared with the marker.

1.3.7. Statistical analysis

Chi square test, Yates corrected test and the Z test were performed in the analysis of the results. (appendix 4)

Due to the small number of the results some data were considered as a single group in a few analysis.

1.4RESULTS

1.4 Results

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1.4 RESULTS

1.4.1. Characteristics of the study population

Twenty male and 20 female volunteers were included from each age group. In less than 6 year age group the mean age was 5.2 years (range 4.1 yrs to 6 yrs), 30-50 year age group it was 38 years (range 30.2 yrs to 50 yrs), while above 70 year age group the mean age was found as 77.6 years (range 70 yrs to 91.6 yrs).

1.4.1.1 Effect of Sex, betel chewing, smoking and the presence of dentures for the colonization of yeast

In >70 year age group

This age group had the betel chewing and smoking habit predominantly. Also four people had artificial teeth in their mouth. Among the 20 males included in the study 11 carried yeast in the mouth whereas 8 carried yeast among the 20 females included. Among them 6 males and 4 females had *C.albicans*. There is no statistically significant association with the sex for the colonization of yeast and *C.albicans* in the oral cavities in the >70 years age group.

In this age group 33 out of 40 people chewed betel and 18 of them carried yeast in the mouth. Among them 9 carried *C.albicans*. 15 out of 40 smoked cigarettes and among them 12 carried yeast (6 carried *C.albicans*). Betel chewing and the smoking were

significantly associated for colonization of yeast in this age group (table 1.1). Betel chewing also showed a statistically significant association for the colonization of *C.albicans* in this age group ($p<0.0001$). However, smoking, and sex did not show statistically significant association for colonization with *C.albicans*

Four people had artificial teeth but among those only one carried *C.albicans*.

Less than 6 year age group and 30-50 year age group

In the less than 6 age group among the 20 boys included in the study 11 carried yeast in the mouth whereas 12 carried yeast among the 20 girls included. Among them 6 boys and 4 girls had *C.albicans*. There is no statistically significant association with the sex for the colonization of yeast and *C.albicans* in the oral cavities in the less than 6 years age group.

In 30-50 age group among the 20 males included in the study 3 carried *C.albicans* in the mouth whereas 5 carried *C.albicans* among the 20 females included. Only *C.albicans* was isolated from this age group. There is no statistically significant association with the sex for the colonization of yeast and *C.albicans* in the oral cavities in 30-50 year age group.

Smoking, betel chewing and the presence of dentures were not found to be predominant factors in these two age groups in the study.

Table 1.1 Effect of sex, betel chewing, and smoking for the carriage of yeast in the oral cavities of above 70 age group.

	Sex				Betel chewing				Smoking			
	Male n=20	Female n=20	Z _{cal}	P value	Betel chewing Yes n=33	Betel chewing No n=7	Z _{cal}	P value	Smoking Yes n=15	Smoking No n=25	Z _{cal}	P value
Yeast	11	8	1.1	NS	18	01	2.54	0.004	12	07	3.35	P<0.001
<i>C.albicans</i>	06	04	0.72	NS	09	01	3.79	P<0.0001	06	04	1.64	NS

Total number volunteers in .70 yrs age group isolated yeast in the mouth =19

Total number of *C. albicans* isolated =10

n = number included in each category

NS = not significant

1.4.2 Microbiological Identification

1.4.2.1 Colony morphology

Candida species showed characteristic colony morphology on Sabouraud's dextrose agar after 24 to 48 hours incubation at 37°C. The colonies appeared as pasty, cream to white with mostly regular edges with a typical yeast odour. Some colonies had irregular edges. Size of the colonies differed. Some were very small and had the diameter of 1-3mm (figure 04). Different colony morphologies observed from different yeast isolates and their Gram stained appearance are given in table 1.2.

Table 1.2 Colony morphology and gram stain characters of the different yeast species isolated in the study

Candida species	Colony morphology	Gram stain
<i>C. albicans</i> :	White to cream coloured colonies with regular edge	Gram positive budding cells with pseudohyphae
<i>C. parapsilosis</i>	Small white colonies with irregular edge.	Small oval blastospores arranged like a flower
<i>C. krusei</i>	Compared to the <i>C. albicans</i> colonies these colonies are larger in size, semi- glossy, white and surface is wrinkled.	Cylindrical blastospores with pseudohyphae
<i>C. tropicalis</i>	Cream coloured flat colonies	Oval blastospores and pseudohyphae
<i>C. guilliermondii</i>	Flat colonies. White in colour	Oval blastospores with pseudohyphae
<i>C. glabrata</i>	Small white colonies	Very small blastospores
<i>Pichia ohmeri</i>	Cream colour colonies with an irregular edges (flower like appearance)	Cells were irregular edged and no pseudohyphae seen.

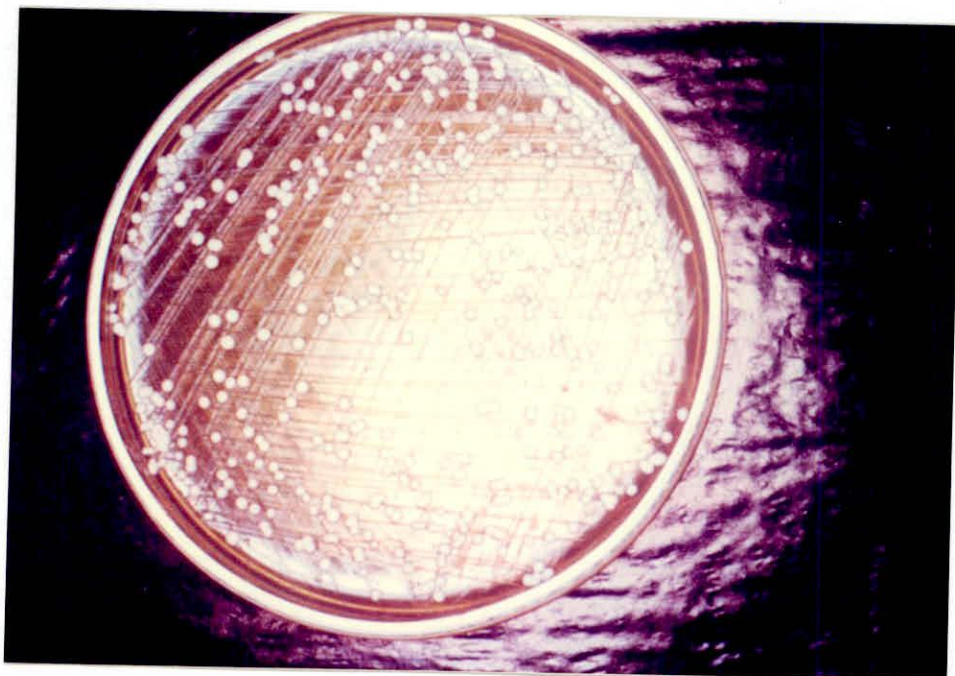


Figure 04- The characteristic colony appearance of *C. albicans* on SDA plate. Cream to white pasty colonies with regular edges.

1.4.2.2 Grams stain

Gram positive round to oval cells with characteristic budding cells were seen in smears stained by Gram's method (figure 05).

Some strains showed gram positively stained pseudohyphae.

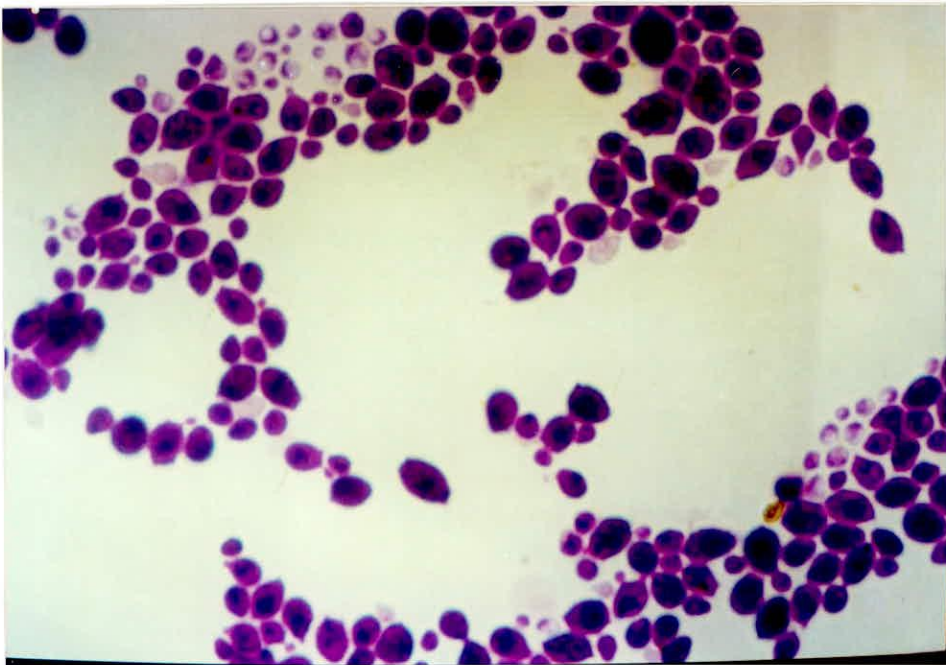


Figure 05- Gram +ve candida blastospores with the budding cells (10×100 magnification)

1.4.2.3 Germ tube test

All yeast isolates were tested by the germ tube test to differentiate *C. albicans* from other species. The germ tube test was performed by two methods, using human serum and the germ tube agar medium. Of the 61 strains of yeast isolated, 29 gave positive results with the germ tube test. All 29 were positive for germ tube formation by both methods.

A 24-hour-old pure culture of *C. albicans* ATCC 90028 on SDA was used as the positive control for both methods.

Germ tube formation was noted within 2-3 hours at 37°C using human serum compared to the germ tube agar, which required 4-6 hours incubation at 37°C (figure 06).

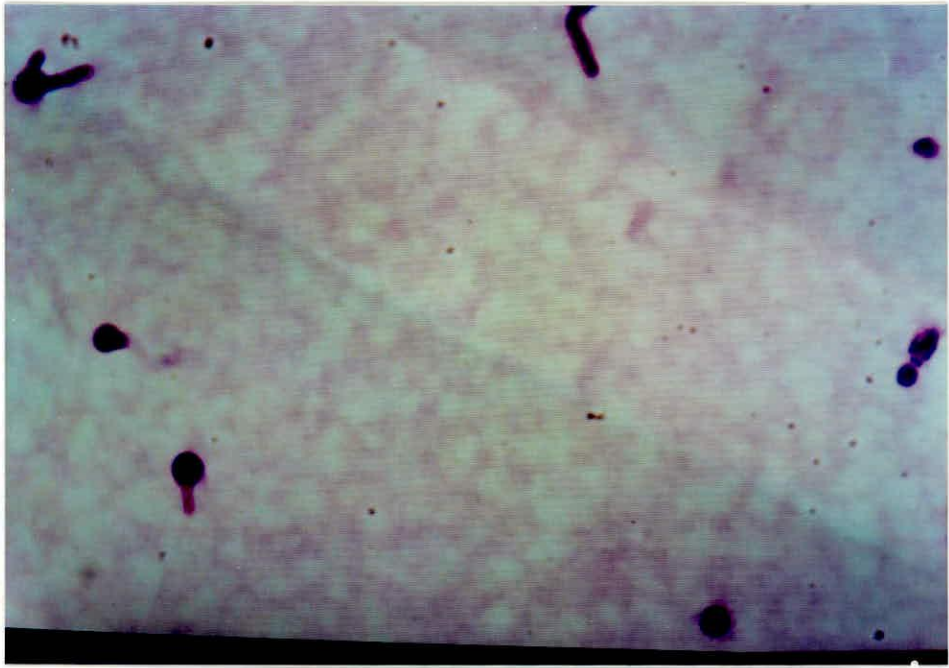


Figure 06- *C. albicans* germ tube using human serum. (Grams stained) 10x100 magnification.

1.4.2.4 API 20 C AUX™ test

All yeasts isolated from the volunteers were tested by API 20C AUX™ test for their carbohydrate assimilation pattern. The test results were analyzed using computer program (APILAB Plus V3.3.3) (BioMerieux, France) for final identification. *C. albicans* identification was confirmed by this test.

The yeast isolates were identified as follows with the API20CAUX™ test:

Species	Number of isolates
<i>Candida albicans</i>	29
<i>Candida parapsilosis</i>	15
<i>Candida tropicalis</i>	05
<i>Candida krusei</i>	01
<i>Candida glabrata</i>	04
<i>Candida guilliermondii</i>	02
<i>Pichia ohmeri</i>	10

1.4.3 Recovery of yeast

Yeasts were recovered from 49 of 120 individuals (40.8%). Twenty-eight subjects had *C. albicans*, while the others had candida species other than *C. albicans* including *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. glabrata*, *C. guilliermondi*, and *Pichia ohmeri*. (Table 1.3) Appendix 5 shows a scanned electron microscopic appearance and the colony morphology of so-called *Pichia ohmeri*

1.4.3.1 From the age group above 70 years

Among the forty (40) volunteers, 10 carried *C. albicans* in their mouths (25%) (Table 1.3). Multiple species were found in six subjects. Among these two shared *C. albicans* with other candida species. Altogether, nineteen adults who were above seventy-year-old (47.5%) carried yeast species in their mouths. *C. parapsilosis* was the second most common species (15%) recovered from the mouths in this age group.

1.4.3.2 From the age group below 6 years.

Among the 40 children under six years of age, 10 carried *C. albicans* in their mouths. Recovery rate of *C. albicans* alone was 25 percent (25%) (Table 1.3). Twelve children carried other candida species. Among these six had *C. parapsilosis* while the others had *C.*

tropicalis, and *Pichia ohmeri*. Six children shared multiple strains. One child had two different strains of *C. albicans* in the mouth. Three shared *C. albicans* along with *C. parapsilosis*. A total of twenty-three children below 6 years of age (57.5%) carried yeast in their mouths.

1.4.3.3 From the age group 30-50 years.

Among the 40 volunteers only eight carried *C. albicans* in their mouths (20%). It is noteworthy that *C. albicans* was the only yeast isolated from this age group. Multiple species of yeasts were not isolated from the same individual as in other age groups.

1.4.3.4. Candida species isolated in different age groups

Seven species of candida were isolated from 50 individuals. Isolation rate of *C. albicans* ranged from 20-27.5% among the three different age groups. The isolation rate was least (20%) in the 30-50 years age group. In this group no multiple species were isolated unlike in the other two age groups. In the under 6 years old age group seven non-albican candida species were isolated while in the over 70 years age group 15 (out of 28) non-albicans candida strains were isolated. The second common species to be isolated was *C. parapsilosis* followed by *C. tropicalis*.

1.4.3.5 Statistical analysis

C.albicans was isolated with similar frequency (range 20-27.5%) in all three age groups. However *C. parapsilosis*, *C. tropicalis* and *Pichia ohmeri* were recovered exclusively from the groups at extremes of age; ie <6 years and >70 years old. Similarly, the total isolation of yeasts were significantly more in the age groups at extremes of age (62.5-70%) than in the 30-50 years old age group (20%). Isolation of *C. guilliermondii*, *C. krusei* and *C. glabrata* were confined to the >70 years old group of the study population.

Statistically significant association was found between the age group and the presence of *C. albicans* (chi square 10.52, df=2, P=0.005). Also a statistically significant association was observed between the other yeast species and the age group (chi square 10.52, df=2, p=0.005) (Table 1.3)

When *C. parapsilosis* was considered which was the second most common species isolated from the study there was no significant association with an age group. Similarly there was no association shown by the third common organism ie; *Pichia ohmeri* with a particular age group.

Table 1.3. Different species of yeasts recovered from the different age groups.

Species	Frequency of different species of yeast isolated from each age group			
	<6 years age group (n*=40)	30-50 years age group (n=40)	>70 years age group (n=40)	Total (n=120)
<i>Candida albicans</i>	11 (27.5%)	08 (20%)	10 (25%)	29 (24.16%)
<i>Candida parapsilosis</i>	06 (15%)	00 (0%)	07 (17.5%)	13 (10.83%)
<i>Candida tropicalis</i>	01 (2.5%)	00 (0%)	04 (10%)	05 (4.16%)
<i>Candida guilliermondii</i>	00 (0%)	00 (0%)	02 (5%)	02 (1.66%)
<i>Candida krusei</i>	00 (0%)	00 (0%)	01 (2.5%)	01 (0.83%)
<i>Candida glabrata</i>	00 (0%)	00 (0%)	01 (2.5%)	01 (0.83%)
<i>Pichia ohmeri</i>	07 (17.5%)	00 (0%)	03 (7.5%)	10 (8.33%)
Total	25 (62.5%)	08 (20%)	28 (70%)	61 (50.83%)

* Number of persons included in the study in each age group

Figure 7

Different species of yeasts isolated from the study

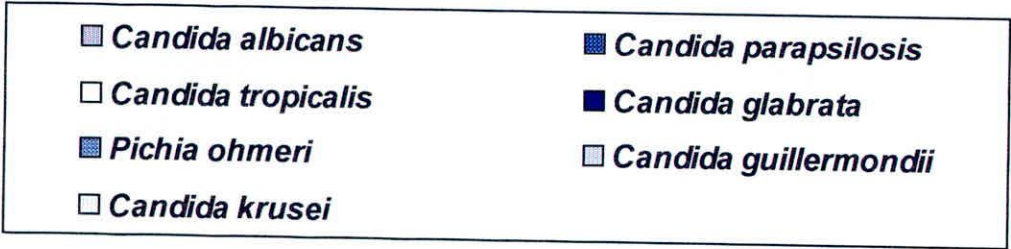
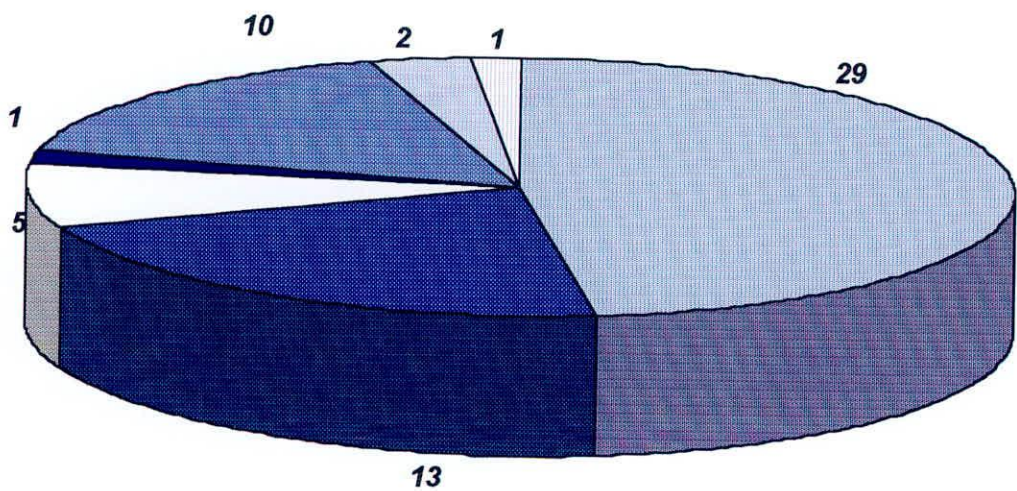
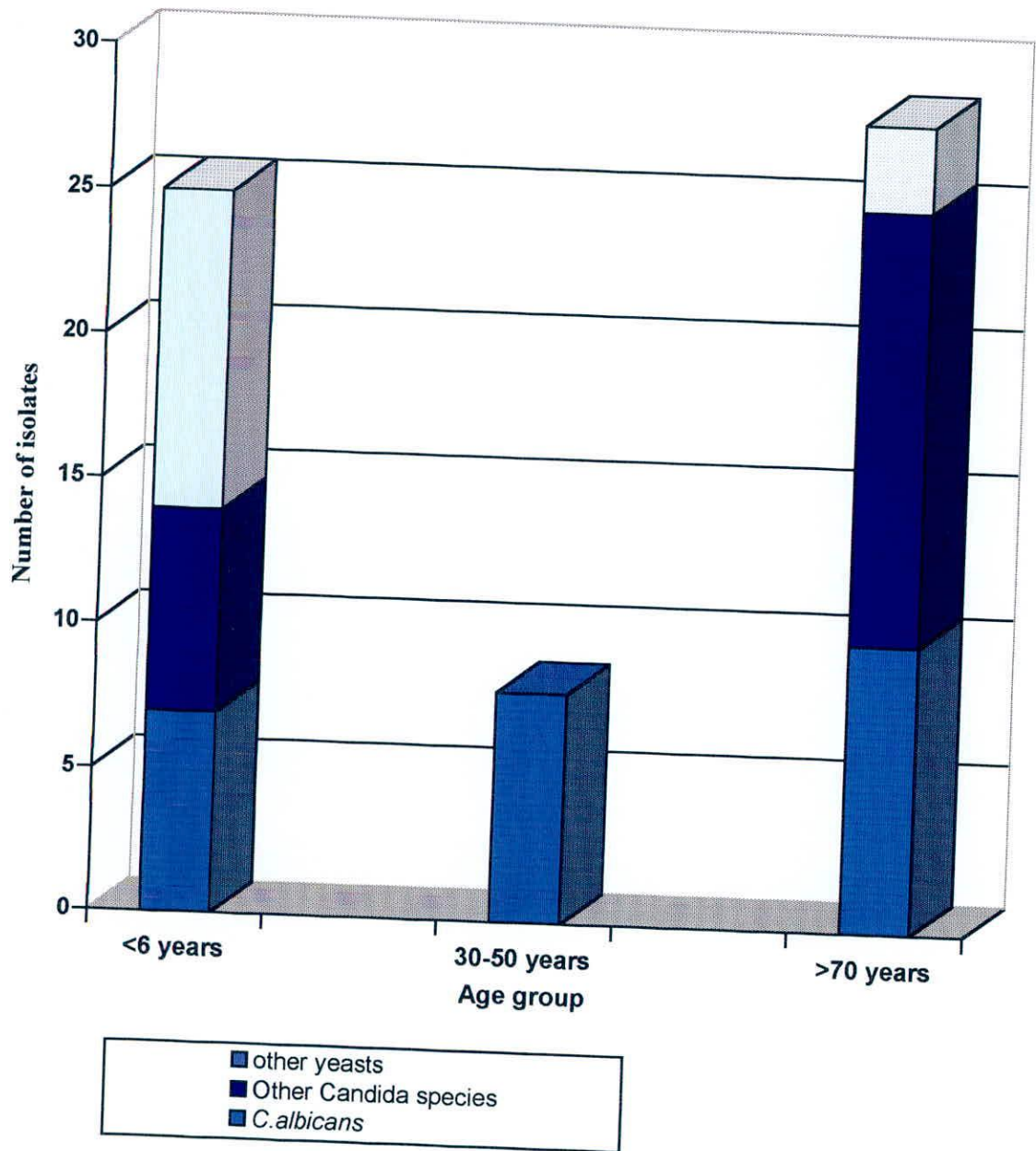


Figure 8

Isolation of *Candida albicans* and other yeast species from the different age groups



1.4.4. Results of biotyping

1.4.4.1 API 20 C AUX TMtest

API 20C AUX TM assimilation profile for *C. albicans* was matched with the table provided by the manufacturer and the biotype was identified as a numerical value. Among the 29 *C. albicans* strains 27 had a similar assimilation profile (2576174), which categorized them in biotype 1. Other two strains had two different assimilation profiles. The profile of one strain (2566174) was identified within the biotype 14, while the other strain that had an assimilation profile of 2576134 was identified as biotype 23.

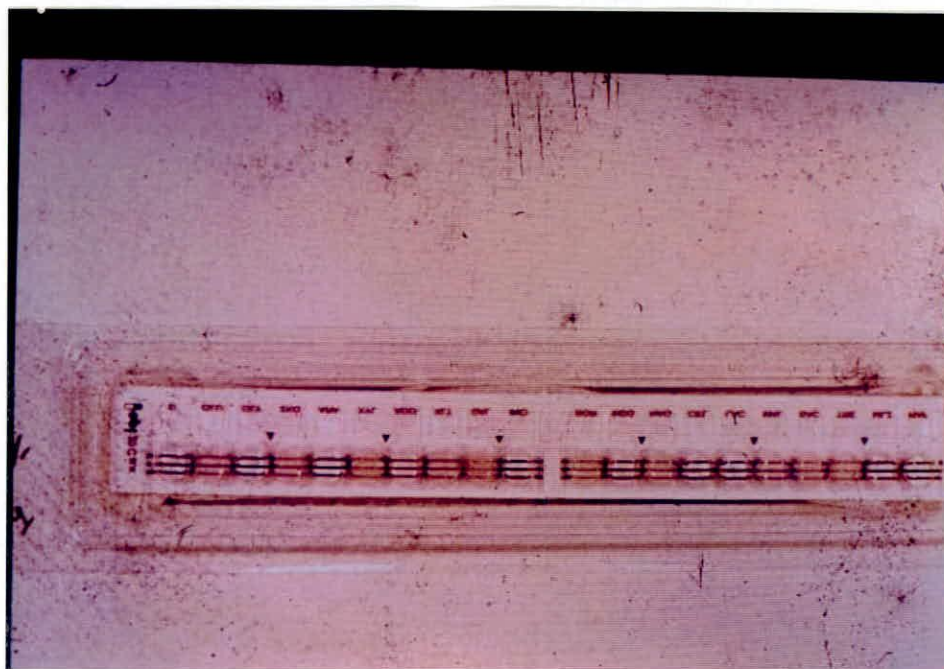


Figure 9- Photograph of an API 20 CAUX TM test strip for a *C. albicans* isolate. Turbidity is seen in the following wells respectively: glucose, 2-keto-D-gluconate, D-xylose, adonitol, xylitol, galactose, sorbitol, α -methyl- D-glucosidase, N-acetyl-D glucosamine, maltose, saccharose, trehalose,

The numerical profile is 2576174 based on these results. APILAB computer programme identified this isolate as *C. albicans* strain biotype 1.

Appendix 03 shows the biotyping profiles identified for *C. albicans* using API20CAUX TMtest.

1.4.4.2 API ZYM TMtest

All *C. albicans* strains were tested by the APIZYMTM test. It identifies the enzyme profiles of a given strain. Results were matched with the chart provided by the kit manufacturer to identify the biotype. Profiles were identified by an upper case letter of the English alphabet. Among the twenty nine (29) isolates twenty seven (27) were identified as the J type. Other two strains showed two different profiles. One was identified as the P type while the other gave completely a new type, which, was not mentioned in the chart provided by the manufacturer. It gave a positive result with α manosidase. We identified and named the new biotype as type BB (table 1.4).

Table 1.4 Different biochemical patterns obtained with the API ZYM™ test and the biotypes identified among the 29

C. albicans isolates

Biochemi cal pattern	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	X
J	-	+	+	+	-	+	+	+	-	-	+	+	-	-	-	+	-	+	-	-	27
P	-	+	+	+	-	+	+	+	-	-	+	+	-	-	-	+	+	+	-	-	1
BB	-	+	+	+	-	+	+	+	-	-	+	+	-	-	-	+	+	-	+	-	1

- 1 : control
2 : phosphatase alkaline
3 : esterase
4 : esterase lipase
5 : lipase
6 : leucine arylamidase
18 : N-acetyl- β glucosaminidase
19 : α manosidase
- 7 : valine arylamidase
8 : cystine arylamidase
9 : trypsin
10 : chymotrypsine
11 : phosphatase acid
12 : naphthol- AS-BI-phosphohydrolase
20 : α fucosidase
- 13 : α galactosidase
14 : β galactosidase
15 : β glucoronidase
16 : α glucosidase
17 : β glucosidase
- X : no. of isolates
- negative reaction
+ positive reaction

BB –new biotype which has not been reported before

1.4.4.3 Boric acid resistant test

All the *C. albicans* strains were evaluated for their resistance or the sensitivity to boric acid. Twenty-one strains were resistant to boric acid while the other eight strains were sensitive to the boric acid (figure 11). Depending on the sensitivity or resistant to boric acid these 29 isolates were grouped in a third biotype profile that identified each strain as S (sensitive) or R (resistant).



Figure 11- An inoculated plate showing the boric acid resistant test. Plate A was the control which did not contain boric acid (ie. only the basal medium). The plates were divided into two parts to inoculate two strains of *C. albicans*. Both strains of *C. albicans* grew well on the control plate. Plate B was the test, which contained boric acid. Only one *C. albicans* strain shows growth, which identified as a resistant strain (R). The other strain was sensitive (S) to boric acid and did not grow in the presence of boric acid.

1.4.4.4. Combined results of three biotyping tests (API ZYM™, API20CAUX™ and boric acid resistant test)

A final biotype was obtained by combining the results of above three API ZYM™, API20CAUX™ and boric acid test. Results of the APIZYM™ test was identified by a capital English alphabet letter starting from A, whereas for the results of API20CAUX was allocated a number starting from 1 and the results of boric acid test was given either R (resistant) or S (sensitive) letters.

Based on biotyping system seven (7) different subtypes were identified among the 29 *C. albicans* isolates.

Eighty two percent of the isolates were included in two subtypes, subtype JIR (65%) subtype JIS (17%). Biotype JIR was the predominant biotype (65%) (table 1.5). The *C. albicans* strain with biotype BB1R which has not been mentioned in any of the previous studies, gave a unique enzyme assimilation pattern in the APIZYM™ test was isolated from less than 6 years old age group.

Table 1.5 Distribution of biotypes in different age groups

Age group	Biotype						
	J1R	J1S	J11R	J14S	J23S	BB1R	P1S
30-50 years	05	01	01		01		
<6 years	07	02				01	01
>70 years	07	02		01			

 Biotype not described by previous researcher

Phenotypic variation of *C.albicans* with the age group

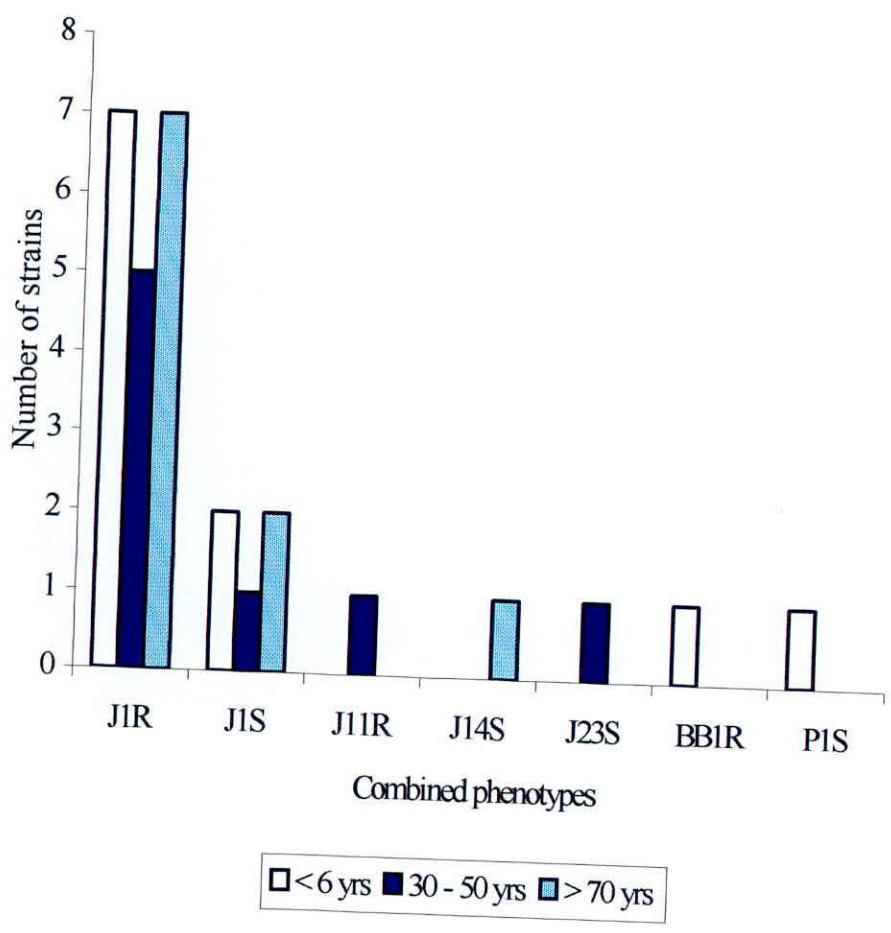


Figure 13 shows the phenotypic variation of *C.albicans* in different age groups

1.4.5. Results of genotyping

The genotyping of *C. albicans* was done by RAPD fingerprinting using two sets of primers (RSD10 and RSD12). Bands were scored visually and only the clearly visible bands were taken into consideration.

1.4.5.1 *C.albicans* genotypes isolated from 30-50 years age group

Eight *C. albicans* strains isolated from adults, in 30-50 years age group were separated into two genotypes using RSD 10 (5'CCG CAG CCA3') primer (figure 13), as well as the RSD12 (5'GCA TAT CAA TAA GCG CAG GAA AAG3') primer (figure 14). Results of the genotyping using RSD10 and RSD12 primer for this age group is tabulated in the table 1.6.

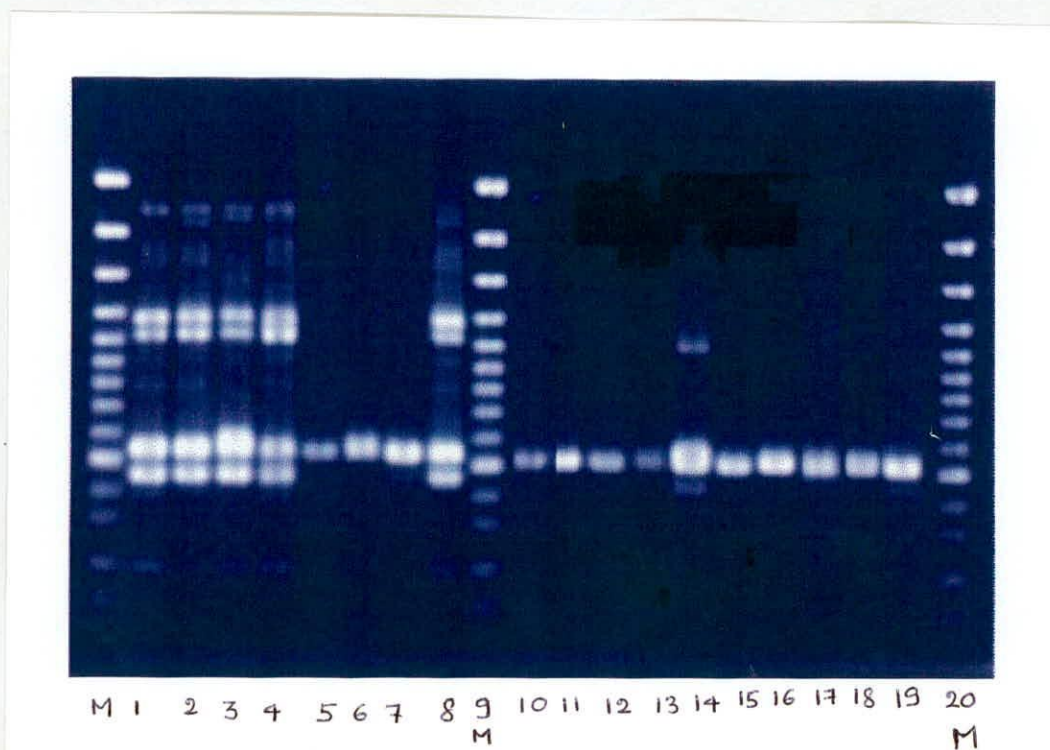


Figure 13- Gel electrophoresis of the RAPD fingerprints using RSD 10 primer for *C. albicans* isolates obtained from 30-50 year group and of age and >70 years old group.

The molecular weight profiles of 18 isolates with the RSD 10 primer are shown in figure 14. M represents the molecular weight marker (GeneRule™ 100bp DNA Ladder Plus).

The first eight lanes represent the RAPD fingerprinting results of isolates obtained from the 30-50 years old healthy adults. First four isolates represent the same genotype (α) having four bands with molecular weights of 300, 400, 900 and 1031 Kb. Isolate 5,6 and 7 are fell into another genotype (β) yielded only a single band with the molecular weight 400kb; 8th isolate again belongs to the genotype α

Lane number 09 again represents the molecular weight marker. Lane number 10 to 18 represent the RAPD results of >70 years old adults. The band patterns of all these isolates with showing a single band at 600 kb position were similar and hence categorized into the genotype β .

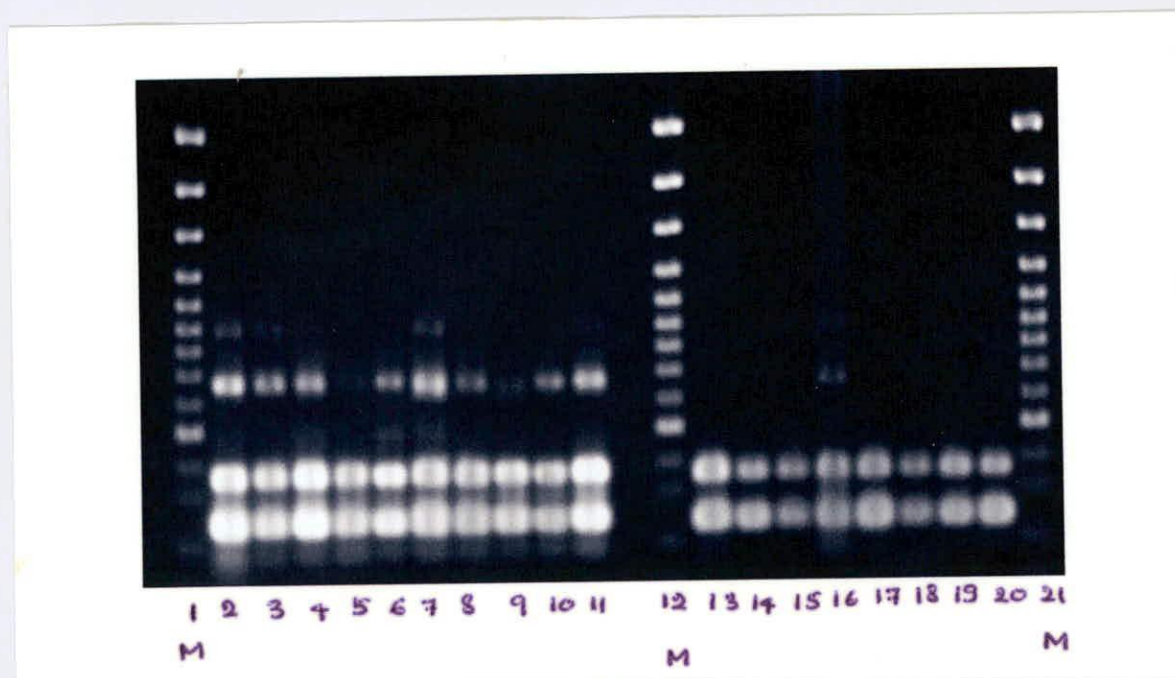


Figure 14 - RAPD Gel electrophoresis profiles using RSD 12 primer for the *C. albicans* isolates obtained from 30-50 years adults and, >70 years old group.

The molecular weight profiles of 18 isolates with the RSD 12 primer are shown in the figure 14. First lane represents the molecular weight marker (GeneRuler™100bp DNA Ladder Plus). Isolates from the lane 2 to 11 show the RAPD fingerprinting results of *C. albicans* isolates obtained from the adults over 70 years. Isolates in the second, third lanes and the isolate in the 7th lane having the banding positions at 100, 200, 600 and 800 kb belongs to one genotype (μ). Isolates representing lane number 4, 6, 8, 10 and 11 fell into one genotype (π) contained three band with the molecular weights 100, 200, and 600kb. Lane number 5 and 9 shows only two bands with the molecular weight 100 and 200kb (genotype σ).

Lane 12 represents the marker. Lanes 13 to 20 represents the RAPD fingerprinting results of the isolates obtained from the 30-50 years old adults. Except the isolate in the lane number 4, all others fell into one genotype (σ), which gave two common bands at the 100 and 200 kb positions. The three bands corresponding to 100, 200 and 500 kb seen in the 4th lane, identified that particular isolate as genotype π .

Table 1.6. Results of the genotyping using RSD10 and RSD12 primer for the age group 30-50 years.

RSD 12 \ Genotype with RSD 10	α	β
	π	σ
	01	
	04	03

1.4.5.2 *C.albicans* genotypes isolated from >70 year age group

Genotypes of ten strains of *C. albicans* isolated from the older adults in above 70 years of age were determined with RSD 10 and RSD 12 primers. In this group when RSD 10 primer was used only one genotype (figure 13) emerged while three genotypes were obtained with RSD 12 primer (figure 14). The results of genotyping with RSD 10 and RSD 12 primer for the above 70 year age group is tabulated in table 1.7.

Table 1.7.Results of the genotyping using RSD10 and RSD12 primer for the age group more than 70 years.

Genotype with RSD 10 RSD 12	β
μ	03
σ	02
π	05

1.4.5.3. *C.albicans* genotypes isolated from age group <6 years

Eleven *C. albicans* isolates from the children below <6 years of age, were fingerprinted using both the primers RSD 10 and RSD12. Primer RSD 10 identified six genotypes (figure 15) while RSD 12 identified only one genotype (figure16).

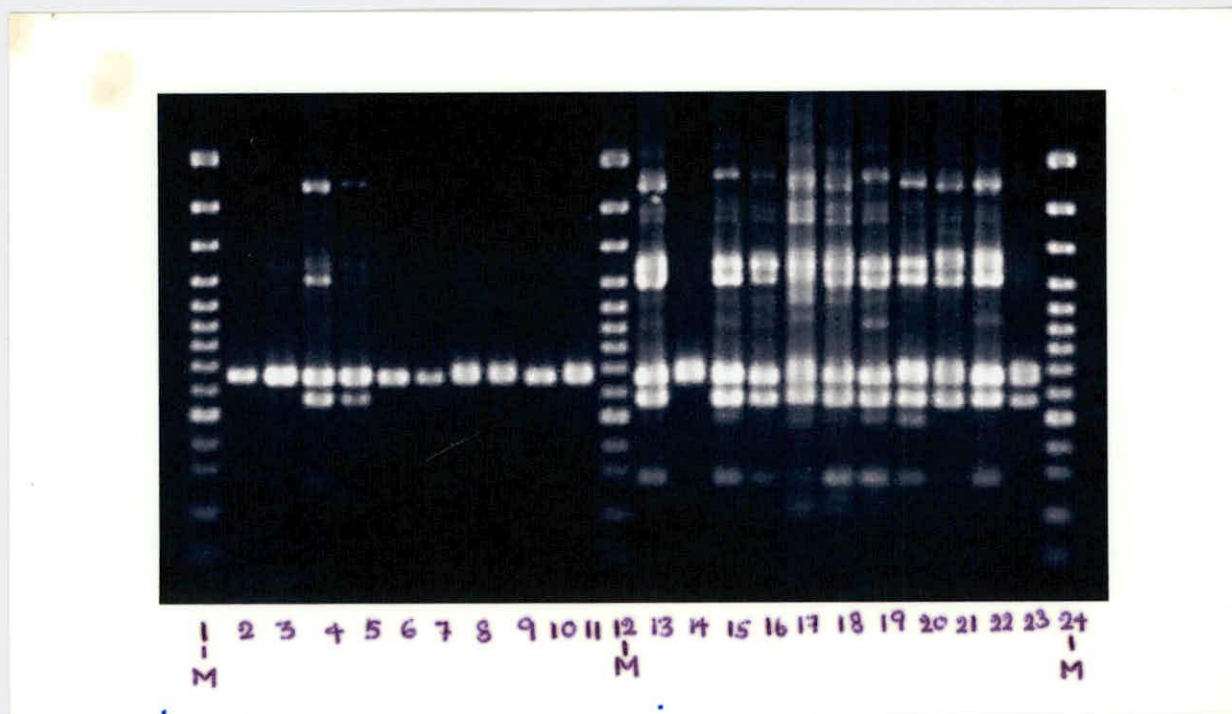


Figure 15. Gel electrophoresis of the RAPD fingerprints using RSD 10 primer for the *C. albicans* isolates obtained from aged children <6 years old, and isolates of premature babies

From the lane number 13 to lane number 23 represents the RAPD genotyping results of the *C. albicans* isolate obtained from the <6 year old children. Lane number 12 is the molecular weight marker (GeneRuler™ 100bp DNA Ladder Plus). Lane 13, 15, 20, 21 and 22 are belongs to one genotype containing four bands with the molecular weight 500, 600, 1200 and 3000kb. These five isolates were recognized as genotype γ , while the isolate in the lane number 14 has only one band with 600kb and belongs to the genotype β isolate in the lane 23 belongs to the genotype λ having only two bands with the molecular weight 500 and 600kb, while the isolate in the lane number 16 having four clear bands with the weight 500, 600, 1013 and 1200 kb and fallen into the genotype α . Isolate in the lane number 19 shows an extra band with the molecular weight 800kb and another band with 400 kb and be recognized as genotype θ . Isolates representing the lane numbers 17 and 18 belong to the same genotype ϕ giving a more complex banding pattern.

Chapter 2 will discuss the results of premature babies (lane number 1-11 are the results of RAPD analysis for the *C.albicans* isolates obtained from premature babies)

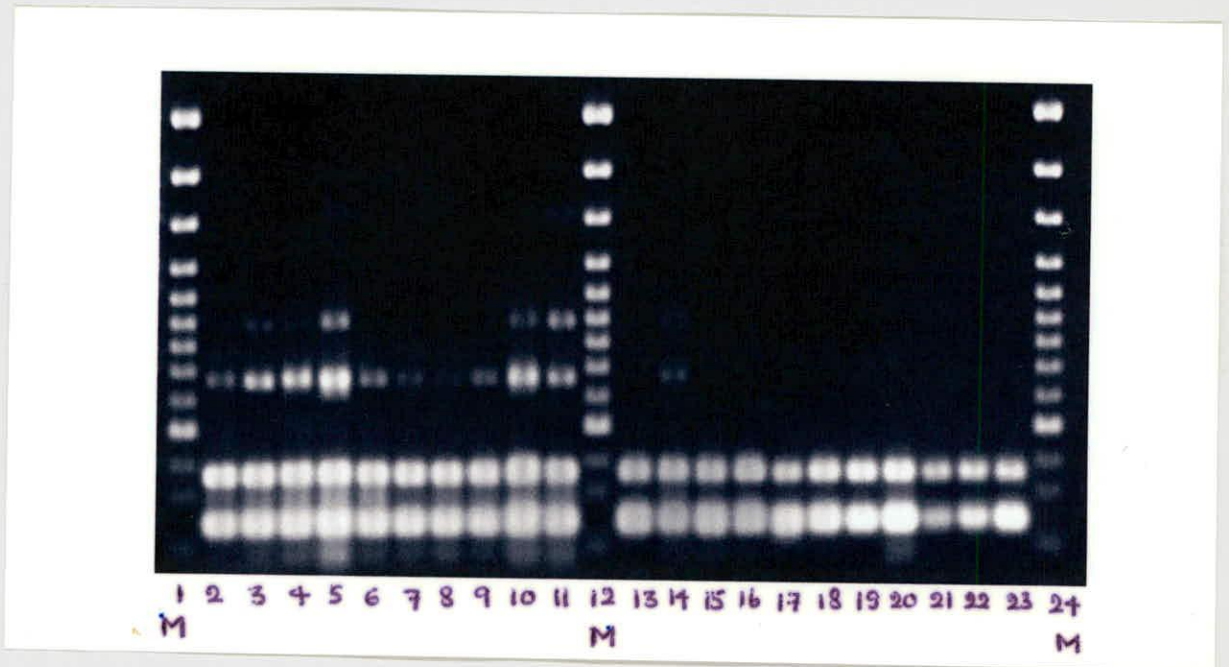


Figure 16. Gel electrophoresis of the RAPD fingerprints using RSD 12 primer for the *C. albicans* isolates obtained from aged <6 years children.

Lane numbers 13 to 23 of this figure 16 represent the results of RAPD genotyping of the *C. albicans* isolates obtained from the under 6 years old children. Lane numbers 1-11 are the results of RAPD analysis for the *C. albicans* obtained from premature babies. This will be discussed in chapter 2

Lane number 12 is the molecular weight marker (GeneRuler™100bp DNA Ladder Plus). Except the isolate in the 14th lane other isolates belonged to a single genotype (π). Isolate in the 14th lane exhibits two extra bands and fallen into the genotype σ .

The results of genotyping with RSD 10 and RSD 12 primer for the less than 6 years age group is tabulated in table 1.8

Table 1.8.Results of the genotyping using RSD10 and RSD12 primer for the age group <6 years.

RSD 10 RSD 12	β	γ	α	θ	ϕ	λ
	μ	01				
π		05	01	01	02	01

1.4.5.4 The combined genotypes

The genotypes were identified by combining the results of both sets of primers. DNA fingerprinting identified a total of 10 genotypes of *C.albicans* in the study (Table 1.9). Highest discrimination was obtained with the primer RSD 10 (5'CCG CAG CCA3'), which resolved the 29 *C. albicans* isolates into 6 distinct genotypes. RSD12 (5'GCA TAT CAA TAA GCG CAG GAA AAG3') showed moderate resolving potential, eliciting only 4 genotypes. Although the banding patterns were complex the results were reproducible and the different strains were delineated. Thirty four percent of the isolates were contained in three genotypes ($\beta\mu$, $\beta\pi$, $\alpha\pi$)

1.4.6 The composite typing scheme

Combination of the biotyping and genotyping methods resulted in a composite typing method, which delineated 18 different composite types of *C. albicans* (Table 1.13). As a result, this composite typing system separated the largest biotype group (Subtype J1R) into 10 smaller groups. Conversely, the biotyping system separated each of the larger genotypes into 1 to 3 smaller composite types (table1.11)

Among the *C.albicans* isolates of this study three clusters of composite type patterns have emerged. Table 1.13 shows these three clusters and the distribution of *C.albicans* strains isolated from different age groups. We identified these clusters as group 1, 2 and 3.

C.albicans strains isolated from the <6 old were grouped together in composite type group1. Similarly, *C.albicans* strains isolated from the age group 30-50 years and >70 years were grouped into the composite type group 2 and 3.

Table 1.9 Different combined genotypes (based on RAPD with RSD10 and RSD12 primers) of the *Calbicans* isolated from the study population

Genotype	Number of <i>Calbicans</i> strains with the genotype
$\alpha\sigma$	4
$\alpha\pi$	2
$\beta\sigma$	5
$\beta\mu$	4
$\beta\pi$	5
$\gamma\pi$	5
$\phi\pi$	2
$\theta\pi$	1
$\lambda\pi$	1

Table 1.10 Genotypic variations of *C.albicans* among the different age groups

Age group	Genotypes								
	$\gamma\pi$	$\beta\mu$	$\alpha\pi$	$\alpha\sigma$	$\beta\sigma$	$\beta\pi$	$\phi\pi$	$\theta\pi$	$\lambda\pi$
<6 years	05	01	01				02	01	01
30-50 years			01	04	03				
>70 years		03			02	05			
Total	05	04	02	04	05	05	02	01	01

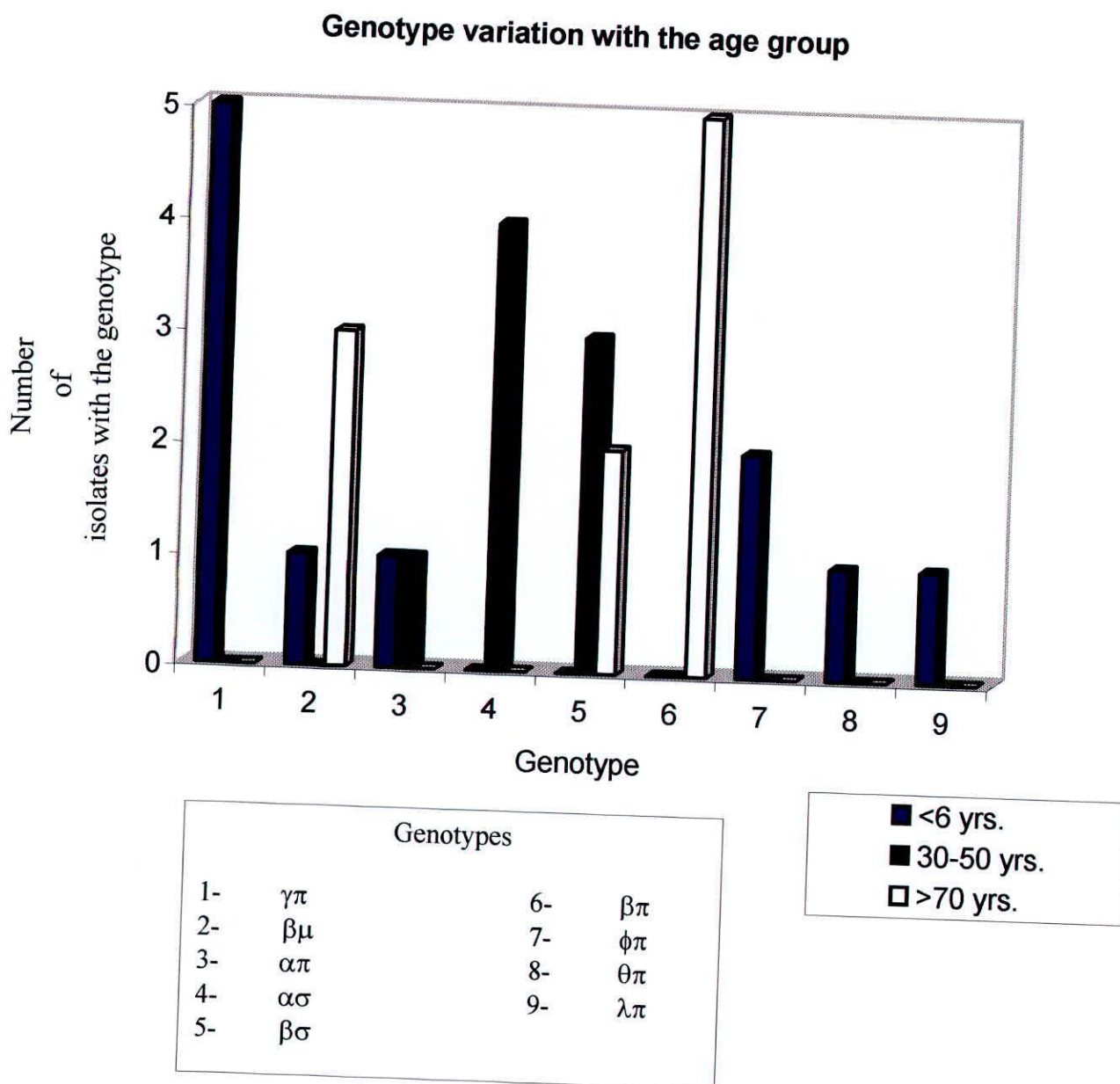


Figure 17. Showing genotypic variation of *C. albicans* isolated from different age groups

Table 1.11 Comparison of the distribution of biotypes and genotypes of 29 *Candida albicans* isolates

Number of <i>C. albicans</i> isolates with genotype ^a										
Biotype code ^b	No. of <i>C. albicans</i> isolates with biotype	$\beta\mu$	$\beta\pi$	$\beta\sigma$	$\gamma\pi$	$\theta\pi$	$\alpha\pi$	$\phi\pi$	$\lambda\pi$	$\alpha\sigma$
J1R	19	2	4	3	4	1	1	1	1	2
J1S	5	2	1	0	1	0	0	0	0	1
J14S	1	0	0	1	0	0	0	0	0	0
P1S	1	0	0	0	0	0	0	1	0	0
J11R	1	0	0	0	0	0	0	0	0	1
J23S	1	0	0	1	0	0	0	0	0	0
BB1R	1	0	0	0	0	0	1	0	0	0

^a genotypes obtained by using two primers.

^b Biotypes obtained by the three biotyping tests.

Table 1.12 An overview of discrimination of *C.albicans* strains with different typing systems used in the study

	< 6 years	30-50 years	>70 years	Total
Total no. strains isolated from 40 volunteers in each age group	11	08	10	29
Biotyping method				
No. of types with APIZYM	03	01	01	03
No. of types with API20CAUX	01	03	02	03
No. of types with boric acid resistant test	02	02	02	02
No. of combined biotypes	04	04	03	07
Genotyping method				
No. of types with RSD 10 primer	06	02	01	07
No. of types with RSD 12 primer	02	02	03	03
No. of combined genotypes	06	03	03	09
Composite types	08	06	06	18

Table 1.13 Distribution of composite types with the age group

Age Group (Years)		Composite Type									
<6	04	J1R $\gamma\pi$	Group 01								
	01	J1S $\gamma\pi$									
	01	J1S $\beta\mu$									
	01	BB1R $\alpha\pi$									
	01	J1R $\phi\pi$									
	01	P1S $\phi\pi$									
	01	J1R $\theta\pi$									
	01	J1R $\lambda\pi$									
30-50	02	J1R $\alpha\sigma$	Group 02								
	01	J11R $\alpha\sigma$									
	01	J1S $\alpha\sigma$									
	01	J1R $\alpha\pi$									
	02	J1R $\beta\sigma$									
	01	J23S $\beta\sigma$									
> 70	02	J1R $\beta\mu$	Group 03								
	01	J1S $\beta\pi$									
	04	J1R $\beta\pi$									
	01	J14S $\beta\sigma$									

1.5DISCUSSION

1.5 DISCUSSION

Saline oral rinse samples were obtained from 120 community-dwelling Sri Lankans living in Colombo district. In our study we used the concentrated oral rinse sampling method because it is more sensitive, more versatile and importantly, this method generates universally comparable data (Samaranayake, et al., 1986). This technique could be used to detect the overall candida score and also for quantification of other microorganisms found in the oral cavity. Also oral rinse technique was found to be more effective for longitudinal and cross sectional screening of large number of individuals.

Identification of *C. albicans* was done by looking at the colony morphology, Gram stain, API 20C AUXTM test and also by germ tube test. For germ tube test two methods were performed ie. The method using human serum and the method using germ tube agar medium. Though both produced identical results in the present study each has its own drawbacks. When these two methods are compared, the use of germ tube agar medium for the identification of germ tubes in *C. albicans* is more reliable than the use of serum. Human serum may have antibodies to candida and has a possibility to inhibit the germ tube formation. Therefore, fetal calf serum is recommended for the test and but it is very expensive. When one compares the required time for these tests, the serum method takes requires less time (2 to 3 hours) than the use of germ tube agar medium (4 to 5 hours).

API 20C AUXTM used in the study is specially useful for precise identification of the most frequently encountered yeasts in clinical microbiology. Due to the reproducibility and the simplicity of using commercially available API systems, a number of studies investigating

the biotypes of *C. albicans* both in infected and healthy individuals using API 20 C™ system (Xu & Samaranayake, 1995) has grown considerably. Furthermore, use of a commercial kit widely used among researches allows comparison of data obtained from different studies.

Proportion of people colonized with *C.albicans* in oral cavity

The overall prevalence of yeast in the oral cavity was 41% while *C. albicans* alone accounted for 23% of the isolates. *C. albicans* was the most predominant yeast isolated from the study population.

Samaranayake (1990) had documented the carriage rate of candida in the oral cavities of healthy volunteers ranging between 2-71% with a median of 34%.

Although our findings fall into this range, it is difficult to compare the results of these studies due to the differences in the sampling techniques and other variables.

In a previous study done in the UK (Samaranayake, et al., 1989), on the out patients attending the dental clinics and the vegetarians, using the concentrated oral rinse sampling method, a yeast prevalence of 21% and 24% was identified. Similarly using an identical method yeast prevalence of 33.3% was reported in Hong Kong. (Sedgley,1994).

Colonization with *C.albicans* in different age groups.

Subjects over 70 years had a higher rate of colonization of yeasts than those aged 30-50 years.

Similarly, subjects below six years also showed a higher colonization rate of yeast compared to the age group 30-50 years. In the absence of comparable studies in the literature, these results could be viewed as base line data for this particular population group.

In this study, healthy volunteers aged between 30 -50 years had the lowest colonization rate in oral cavity as compared to that of children aged below 6 years and adults above 70 years. This indicates that those in the extremes of age have a greater likelihood to be colonized with *C.albicans* when compared to young adults. There is a statistically significant variation of colonization with *C. albicans* among the three different age groups in this study ($P= 0.005$).

Colonization with yeast

When considered the non albicans candida species a statistically significant variation was noted among the three age groups ($P=0.005$). *C. parapsilosis* was not statistically related to a particular age group. Isolation of *Pichia ohmeri* was restricted to >70 yr and <6 yr age groups. Though there is no statistical association among these two species with these two

age groups, a high colonization of these species in the extreme age groups, ie >70 years and <6 years is noted. Interestingly we could only isolate *C. albicans* in the 30-50 age group. Moreover *Pichia ohmeri* and *C. parapsilosis* and *C. tropicalis* that are considered as emerging pathogens (Hazen, 1995) were isolated only from the >70 years old age group.

These data support other reports that elderly (David Hay, 1993) and children (Levy, 1998) may create conditions that favour the oral colonization of yeast, regardless of health. It is tempting to speculate that decreased salivary flow, poor oral hygiene, and denture wearing status, less number of natural teeth, debility, observed in >70 age group compared with younger healthy subjects, may contribute to increased colonization amongst the elderly.

In the present study volunteers were inquired about the habit of smoking, betel chewing with tobacco leaves and drugs. 54% of persons who carried yeast in the oral cavities among the >70 age group, were betel chewers.

Most of the >70 year aged study population were edentulous.

In the above 70 age group the habit of betel chewing had a significant association ($p=0.004$) with the colonization of the oral cavity by yeast. Also it showed a significant association for the colonization of *C.albicans* in this age group ($p<0.0001$). Smoking also played a statistically significant role for the colonization of yeast in the present study ($p<0.001$). There was no statistically significant association with the sex for the colonization of oral cavity with yeast.

Cigarette smoke may contain a factor which is soluble in saliva and that may lead to localize epithelial alterations, facilitating colonization with candida (Arendorf and Walker, 1980). This also might contribute to the high colonization of yeast in the >70 age group.

However this would not explain the increased yeast colonization in the <6 years age group. It would be interesting to study the effect of deciduous dentition in colonization of the oral cavity with yeast.

In 30-50 age group, we came across three drug users but they did not carry yeast in their mouth.

Typing of *C.albicans*

One of the important considerations regarding the prevention and control of nosocomial candidiasis is whether the infection is endogenous or exogenous (Pfaller, 1994). Our understanding of this area is still evolving; however the use of molecular typing methods to fingerprint Candida species has been very useful in epidemiologic studies designed to address these issues (Voss, et al., 1995; Reagan, 1990).

The major tenet of epidemiologic typing is that although one or more typing methods may be used to demonstrate convincingly that two nosocomial pathogens are different from one another; it is very difficult to prove that they are the same strain. As such epidemiological typing systems are used in combination to enhance the discrimination among different bacterial strains.

Biotyping

In the current study biotyping system developed by Samaranayake et al was used to type *C. albicans* strains. This was a combination of three tests including API ZYM, API 20CAUX[™] and Boric acid resistant test. All these three tests have been previously used as single tests (Korting, et al., 1988), and also as combined tests (Peter, 1997), for biotyping *C. albicans*. However, each method by itself was far less discriminatory than the combination of the three methods for typing *C. albicans* strains. Hence a combination of these methods was used in the present study.

Biotyping system, which applied to type the strains of *C. albicans* in the study, was proved to be extremely reproducible, and the use of standardized commercial kits helped to overcome irregularities in media preparation and interpretation of data, thus generating universally comparable data.

In the current study, biotype 1 (JIR) was the major biotype isolated in all three different age groups. Also in the previous studies done in the UK (Franks, 1990) and South Africa (Hauman, 1993) biotype 1 was found to be the commonest type.

Only the type J was obtained from the API ZYM assimilation test in our study.

This is in contrast to the results of previous findings from different regions in the world, where A type was the most predominant (Peter, 1997). Table 1.15 summarizes different biotypes identified from a healthy group in Hong Kong and the biotypes identified in the present study population in Sri Lanka. There was a statistically significant difference in the

prevalence of Biotype J1R between these two geographical locales (Sri Lanka 65% and Hong Kong 3.7%) (Peter, 1997).

In studies carried out among healthy Hong Kong Chinese, biotype A1S of *C.albicans* was more frequent in the oral cavity. On the contrary the Caucasians had a significant number of strains that were resistant to boric acid (Peter, 1996).

In the present study 72.4% strains showed resistance to boric acid and 27.6 % of the strains were sensitive to boric acid (Table 1.14). A highly significant difference was noted with the results of boric acid test (as shown in table 1.14), among the data from the present study and the documented data from Hong Kong ($p<0.001$) and also with the data from England ($p=0.01$) (Peter., 1996).

In the current study, J1R was the major biotype isolated in all three different age groups. There was a highly significant difference ($P=0.000005$) observed in this predominately found biotype in the current study with documented data from Hong Kong isolates (Table 1.15).

Biotype variations of *C. albicans*, were mainly found between Hong Kong and Australia in previous studies. Nonetheless, A1R and A1S biotypes were reported from the four different geographical areas ie; Australia, England. Germany and HongKong (Peter, 1997).

Therefore the available data from the present study and the other studies quoted indicates that the prevalence of *C.albicans* strains differ in different geographical locations.

The reason for this is yet unclear.

Biotyping variation with the age group

In all the three age groups *C.albicans* biotype J1R was more frequent than the other biotypes. As mentioned before J type was the most predominant type (93%) obtained from the AP1ZYM™ assimilation test. A new subtype (BB) was identified in the <6 age group by the AP1ZYM™ assimilation test. This has not been documented in previous studies.

Statistically significant association was not found in the three different age groups with regard to the biotypes of *C.albicans*.

Table.1.14 Comparison of *Candida albicans* biotypes resistant or sensitive to boric acid isolated from different geographical areas.

Country	Resistant number	Percentage	Sensitive number	Percentage	P value
Sri Lanka	21	72.4%	8	27.6%	X ² =12.93 df=1 P= 0.0003
Hong Kong	18	31.6%	39	68.4%	
Sri Lanka	21	72.4%	8	27.6%	X ² =0.77 df=1 NS
Australia	23	62.1%	14	37.9%	
Sri Lanka	21	72.4%	8	27.6%	X ² =0.56 df=1 NS,
Germany	19	63.3%	11	36.7%	
Sri Lanka	21	72.4%	8	27.6%	X ² =6.09 df=1 P=0.01
England	6	35.3%	11	64.7%	

Reference- Peter,1997

Table 1.15. A comparison of *Candida albicans* biotypes isolated from healthy individuals in Sri Lanka and Hong Kong

Biotype	Sri Lanka		Hong Kong		P value
	Number	%	Number	%	
J1R	19	65.5	1	3.7	$X^2=20.65$
Others	10	34.5	26	96.3	$P=0.000005$
J1S	5	17.2	1	3.7	$X^2=1.45$
Others	24	82.8	26	96.3	NS
J11R	1	3.4	0	0	$X^2=0$
Others	28	96.6	27	100	NS
J14S	1	3.4	0	0	$X^2=0$
Others	28	96.6	27	100	NS
J23S	1	3.4	0	0	$X^2=0$
Others	28	96.6	27	100	NS
BB1R	1	3.4	0	0	$X^2=0$
Others	28	96.6	27	100	NS

Hong Kong data from reference Peter ,1997

Genotyping system

Randomly amplified polymorphic DNA fingerprinting, which we applied for the study, was reproducible, sensitive and easy to perform. With the strict adherence to physical containment of procedures and agents, this could yield highly specific and reproducible results.

RAPD fingerprinting identified 9 different genotypes among the 29 isolates of *C. albicans*.

More genetic variation was observed in <6-year age group. Eleven isolates of *C.albicans* were categorized into six different genotypes. Only four biotypes were obtained for this group by biotyping the isolates.

In the >70 age group the 10 isolates which belonged to 3 biotypes were categorized onto 4 genotypes.

Similarly the 8 isolates of 30-50 age group which had 4 biotypes were fallen into 4 genotypes.

In summary 29 isolates of *C.albicans* was divided into nine different genotypes by genotyping. No statistically significant difference of the genotypes were found with respect to the age groups.

Combined typing methods

Scientists from many countries have made an effort to type *C. albicans* using several typing methods. They also found that combination of more than one typing system provides a higher typability. None of the scientists have used the three biotyping methods as used in this study in combination with the RAPD fingerprinting method for characterization of *C. albicans*.

This combined approach has been used infrequently for candida species. However previous researchers have shown an enhanced discriminatory power by the combination of two or more typing systems for the typing of *C. albicans* strains. In 1989 Hunter and Fraser demonstrated enhanced discriminatory power by combining resistotyping and morphotyping and suggested that more than one method may be necessary for better epidemiological studies of *C. albicans*.

The present study has documented a establishment of a typing method for *C. albicans* using biotyping and genotyping as a means of delineating strains of *C. albicans*.

Previous workers have not used this approach of typing system for studying *C. albicans*. Importantly, we have also found that the combination of genotyping and biotyping provided enhanced discrimination among the strains.

New typing system- composite approach

The ideal epidemiological typing method should be simple, rapid, sensitive, and highly discriminatory. In addition the use of standardized methods and reagents is desirable to improve reproducibility and the comparability of results among laboratories.

However the phenotypic characteristics of the yeasts may be variable and hence the genotype could be considered as the "gold standard" typing system for candida.

Although traditionally phenotypes and genotypes are not combined in phenetic taxonomic system this approach has been adapted by some investigators (Pfaller, et al 1990).

By combining the biotyping and the genotyping technique the likelihood of grouping genetically distinct strains into one type is minimized.

In the present study specifically, the *C.albicans* strains are further divided each of 07 different biotypes by genotyping into 1 to 9 unique composite types. Likewise, the biotyping results further divided the most common genotypes ($\beta\mu$, $\beta\pi$, $\alpha\pi$) into 3 to 4 unique composite types. Such diversity suggests that this combined approach may be useful in epidemiological studies.

When the biotyping and the genotyping results were combined, eighteen different composite types were identified. These composite types were grouped into three clusters that were identified as 1, 2 and 3. Table 1.13 shows the different composite types and the groups and the existence of three clusters of composite types.

Statistically significant association was identified in the composite types with the age group. Statistical analysis was done using the chi square method. *C.albicans* strains isolated from the <6 years age group showed a statistically significant association ($P=0.0000012$) to the group 1 composite types. Similarly, *C.albicans* strains isolated from the age group 30-50 years and >70 years a showed a statistically significant association with the composite type group 2 and 3 giving the P value as 0.000015 and 0.0011. Finally the hypothesis of the study was proved by showing a phenotypic and genotypic variation of *C.albicans* strains with respect to the age group. This may also suggest the validity of a combined typing system.

This method will also assist to evolve a typing system for *C. albicans* with good discriminatory power. This typing system may be applicable to other epidemiologic and taxonomic studies and could be used to investigate nosocomial outbreaks of candida infections particularly in neonatal units, as discussed in chapter 2.

Limitations of the present study

The main drawback of the present study was the small number of samples included in the study. This was however, a preliminary study and due to financial constraints the study was confined to a smaller number of study population.

In this present study the term 'healthy individual' was used to those who did not suffer from tuberculosis, cancer or any malignant condition, AIDS, asthmatics, clinically diagnosed diabetes and those on antibiotic therapy. It is possible to have included some individuals especially in >70 year age group with some underlying diseases.

Another drawback was overlooking the oral hygiene of volunteers. The oral hygiene could affect the colonization with yeast.

When looking back, numerous factors that were overlooked in the study could have also affected the colonization of mouth with yeast. These include number of dentures present, any cavities and bad teeth with the frequency of changing the toothbrush, number of brushing times per day, any antiseptic mouth washes used to clean the mouth

The commercial kits used for biotyping *C.albicans* in the present study are costly. API20CAUX™ test needs highly technical experience in reading the results. Also it takes two days (48 hrs) for biotyping. This should be borne in mind when one plans a large scale study.

Highly aseptic conditions with physical containment were ensured for genotyping. But the specificity should be assessed using a probe. Moreover it would be interesting to use several primers for genotyping and evaluate the discriminating powers of each primers and

their combined result. But in the present study due to the limited time and the financial constraint only two primers were used for genotyping. If more primers have been tested a better discrimination could have been found.

Also in genotyping high personal experience is needed for the procedures, specially when reading and analyzing the bands.

Due to the small number of samples and the less information's on the health status of the volunteers in this study could not facilitate to find a link to the pathogenesis of oral candidiasis and also to predict the strains, which are "more virulent".

Contribution of this work to scientific community

This was a preliminary study done with a limited number of volunteers. These results need to be confirmed by a larger study.

Despite the aforementioned shortcomings in the study it has many beneficial outcomes.

As far as known, the foregoing data represent the first survey of oral colonization with yeast in children (age below 6 years), adults (aged between 30-50 years) and elderly (aged above 70 years), in Sri Lanka. Furthermore, this study provides, for the first time, baseline information for those working on the oral carriage of candida specially *C. albicans* in Sri Lankan population. Phenotypic and genotypic diversity of *C. albicans* have not been studied in Sri Lanka before. This study has also generated a new composite typing system for *C. albicans* with better discrimination among strains.

Future Directions

It would be interesting to study the prevalent types of *C.albicans* strains in-patients with oral thrush and compare those isolated from healthy people. Especially it will be useful to find out the prevalent strains in patients with HIV infection, leukemia and other malignancies and the patients on steroid inhalers because these are the major predisposing factors for candida infection. This would definitely shed some light on virulence and pathogenesis of different *C.albicans* strains. Moreover it would help one to identify the colonization with virulent *C.albicans* strains and institute prophylaxis measures early in the immunocompromized patients

Also this method could be used to identify any strain variation with the anotomical site of the infection. ie. Strains of *C.albicans* in vaginal candidiasis, oral thrush with skin candidiasis.

This typing method will also be useful to investigate the source of infection especially in nosocomial outbreaks and also will help to identify the differentiation between therapeutic failures and reinfection.

However the cost of the present typing system is high and would remain a research tool for a considerable time. This method needs high technical skills and is time consuming. These factors should be taken into consideration when improved typing systems are investigating in the future.

1.6.CONCLUSIONS

1.6 CONCLUSIONS

1. *C. albicans* was the most common yeast isolated from the oral cavities of healthy Sri Lankans.
2. Carriage rate of *C. albicans* and non-*albicans* species showed significant association with the age group.
3. Both the biotyping and genotyping techniques were sensitive, reproducible and their combination, improved strain discrimination compared to either method alone.
4. Using a composite typing method based on biotyping and genotyping 18 distinct types were identified among 29 *C. albicans* strains isolated from the study population.
5. Age related variation was observed among *C. albicans* strains by the composite typing method.
6. The combined typing system could be useful to clarify the source and spread of nosocomial *C. albicans* infections. However, the various typing methods must be used with the understanding that more than one approach may be necessary to achieve optimal strain discrimination.

7. Further studies with a large number of isolates are warranted to assess the usefulness of this typing system for epidemiological and pathogenesis studies of *C albicans* and to ascertain and define such relationships between genotypic and phenotypic attributes of *C albicans*.

CHAPTER 2

PHENOTYPIC AND GENOTYPIC

CHARACTERIZATION OF ORAL

***Candida albicans* STRAINS ISOLATED**

FROM PREMATURE BABIES

2.1 INTRODUCTION AND LITERATURE REVIEW

2.1 INTRODUCTION AND LITERATURE REVIEW

Nosocomial *C. albicans* infections are important cause of morbidity and mortality in neonates in neonatal intensive care units (NICUs) (Reef et al 1988). Premature babies (birth weight <1,500 g) are at high-risk of neonatal candidiasis with reports of serious infections from many geographical locations (Lee B.E et al. 1992).

There are several risk factors for acquisition of fungal infections in NICUs. These include prematurity (Nielsen. 2000), which leads to an age-dependent immune deficiency state, colonization with yeast, postnatal steroids, hyperalimentation, use of fat emulsions, and antibiotic therapy.

The most common manifestations of candida infections in neonates are thrush and cutaneous lesions. Candidemia in neonates, although rare, carries a high morbidity and mortality (Saxen et al 1995). But the mortality associated with candidemia is lower among children than adults (Stamos and Rowley 1995).

Candida infections in the newborn period may be congenital, local or disseminated.

Vaginal colonization by *C. albicans* has been shown to occur more frequently in pregnant women. Babies acquire the organism during delivery through the birth canal. *C. albicans* was most commonly transmitted vertically (Waggoner et al 1996). Decreased CD 4⁺ cell lymphocyte level (Vazquez-Torres and Balish, 1997), neutrophil function (Ashman and Papadimitriou, 1995) and use of peripheral venous catheters (Leibovitz et al 1992), prolong hospitalization (Baley et al 1986) also lead to increase in colonization with *C.*

albicans. Use of broad spectrum antibiotics, low birth weights (Harms et al 1992), use of central, arterial, and venous catheters (Fukayama, 1998) and prolonged parenteral nutrition have also been identified as risk factors for colonization by *C. albicans* in neonates (Ruiz-Diez et al 1997). *C. albicans* accounts for the vast majority of fungal infections in NICU, followed by other species of candida (Hostetter, 1994).

Neonatal candidiasis is a nosocomial infection usually acquired from the neonatal care unit. In one study seventy five per cent (75%) of nurses in a newborn unit carried yeasts on their handsⁱ. In documented outbreak of *C. albicans* fungemia in a neonatal intensive care unit it was suggested that the outbreak was due to spread of a particular strain possibly via cross infection (Huang et al 1998). In an extensive study carried out over a 15 year period (1981-1995) to determine the changes in the incidence of candidemia in a neonatal intensive care unit (and to compare the prevalence and case fatality rates of *C. albicans* and *C. parapsilosis* infections, it was found that candidemia increased more than 11- fold over the 15 years. Furthermore, the prevalence of candida species shifted from *C. albicans* to *C. parapsilosis*; and candidemia associated with *C. albicans* had significantly higher mortality than with *C. parapsilosis* (Kossoff, et al 1998). In another study carried on the epidemiology of systemic candidiasis in a tertiary care neonatal unit it was found that of all the deaths, two-thirds were candida related (Narang, et al 1998).

Acquisition of *C. albicans* may be vertical, from the mother or horizontal from mother or hospital staff or fomites.

Such vertical / horizontal transmission can only be proved by typing the isolates using a reliable and discriminatory typing method. Researchers worked on the different typing systems to get more discriminatory results and found that only combined analysis of the results with various methods allowed the source of the outbreak to be identified (Betremieux, et al 1994).

A search of the literature revealed no reports on the prevalence of *C. albicans* in oral cavities of newborns in Sri Lanka. Moreover, so far no study has looked at the typing patterns of *C. albicans* among premature babies in Sri Lanka.

In view of the absence of such data, present study was undertaken to investigate the prevalence of oral *C. albicans* in preterm babies and to type the isolates using biotyping and PCR fingerprinting to genotype *C. albicans*. We also attempted to assess the clinical and epidemiological value of a phenotypic and genotypic typing system developed by us (chapter 01) for *C. albicans* isolated from premature babies.

2.2AIMS AND OBJECTIVES

AIMS AND OBJECTIVS OF THE STUDY

1. To evaluate the prevalence of oral candida in a group of premature babies in Sri Lanka
2. To speciate the yeasts and then to biotype and genotype the *C. albicans* isolates.
3. To assess the clinical and epidemiological value of the phenotypic and genotypic typing system developed by us for *C. albicans* isolated from premature babies.

2.3 MATERIALS AND METHODS

2.3 MATERIALS AND METHODS

2.3.1 The study group

Fifteen (15) premature babies (born before 36 weeks) in special baby care unit at Colombo South Teaching Hospital, Colombo were subjected for the study. The birth weight of all babies included in the study was below 1500g and they babies were less than seven days old when sampled for our study. Sample collection was done over a period of three months starting from June 1998.

2.3.2 Sampling procedure

Samples were taken by gently rubbing a sterile cotton swab over the sublingual mucosa and from the buccal pouch on the side on which the baby was lying. The swabs were placed in a sterile test tube with the cotton plug and kept at 4 °C and immediately brought to the microbiological laboratory. In the interim period between collecting and processing, the samples were stored at 4°C and transferred in portable cooler with an internal temperature of 7°C.

2.3.3 Microbiological identification

The swabs were then streaked on Sabourauds dextrose agar plates and incubated at 37°C for 48 hours.

After incubation, all plates were examined for the colony morphology (colour, size of the colony, elevation, and their translucency) and colonies were identified as Yeast species, primarily by the Gram's stain.

Each different type of yeast colony was subcultured on SDA to obtain pure culture. Germ tube test was performed as a preliminary identification test for *C. albicans*. All positive isolates were stocked on filter paper strips measuring 2cmx1cm and kept at -20 °C.

2.3.3.1 Germ tube test

Germ tube test was performed on each yeast isolate using a 24 hour old purified culture on SDA, and using *C. albicans* ATCC 90028 strain as a positive control.

A colony was touched with a sterile wire loop and inoculated on the Germ Tube Agar medium and the streaked lines were covered by a cover slip and incubated at 37 °C for 4-6. After 5 hours the plates were examined for the presence of the germ tube under a

microscope. A germ tube was identified as a tube about 1- 2 μ M in length and arising from the blastospore without a constriction at the junction of the mother cell.

2.3.3.2 Biotyping of *C. albicans*

The method described by Williamson, Samaranayake and MacFarlane (1987) was used for biotyping the isolates in this study. This comprised of three tests. These included two commercial tests APIZYM (BioMe'rieux, France) and API 20C (BioMe'rieux, France) and an in-house boric acid resistant test. The procedures of APIZYM (BioMe'rieux, France) and API 20C (BioMe'rieux, France) and an in-house boric acid resistant test are the same mentioned under the materials and method in the chapter 01.

2.3.3.3 Genotyping of *C. albicans*

2.3.3.3.1. Preparation of DNA for Random Amplified Polymorphic DNA (RAPD)

C. albicans isolates were grown on Sabouraud's Dextrose agar (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) medium for 24 hours at 37°C. Using the flat side of a sterile toothpick, cells were scraped off and resuspended in 1 ml of 1 M sterile sorbitol. The cells were centrifuged at 13000 rpm for 5 minutes and then supernatant was discarded

and the pellet was washed again with 1 ml of 1M sorbitol. Cells were harvested by centrifugation at 13000rpm for 5 minutes at room temperature and resuspended in 1 ml of sterile SE buffer (1.2M sorbitol, 0.1M EDTA, pH7.5), 2 μ l of sterile β mercaptoethanol and 15 μ l of 20mg/ml sterile lyticase (Lyticase, Sigma, USA) and incubated at 37°C in a water bath for one hour until the spheroplasts were formed due to lysis of cell walls. The spheroplasts were next, harvested by centrifugation at 10,000rpm for 1 minute at room temperature. These spheroplasts were washed once with SE buffer and resuspend in 0.4 ml of 0.15M NaCl / 0.1M EDTA pH 8.00. To this suspension 10 μ l protenase K (20mg/ml) and 50 μ l SDS (10%) were added, followed by 40 μ l RNAase (10mg/ml). The suspension was then incubated at 55°C for 1 hour in order to lyse the spheroplasts with SDS and digest the proteins and RNA by protenase K and RNAase. This mixture after incubation was next centrifuged at 13,000 rpm for 15 minutes at room temperature. The resultant supernatant obtained following centrifugation was use for the extraction of DNA.

The DNA was extracted, once with equal volume of sterile phenol:chloroform:isoamyl alcohol (400 μ l) and, once with equal volume of sterile chloroform (400 μ l). 300 μ l of upper aqueous layer was transferred to a new sterile 1.5ml microfuge tube and DNA was precipitated by adding 0.6 volume of isopropanol and allowed to stand for 30 minutes at room temperature. This was next centrifuged at 13,000rpm for 15 minutes, at room temperature and the resultant pellet was washed with 0.5ml of 70% sterile ethanol. Supernatant was removed after centrifugation at 13,000rpm for 5 minutes and the DNA pellet was dried in the tube for 5 minutes at 55°C. Finally the DNA thus prepared was dissolved in 50 μ l sterile TE buffer (10 mM Tris, 0.1mM EDTA pH 8.0) and stored at 4°C.

Only one sample was handled at a time. Strict aseptic precautions were taken to avoid cross contamination.

2.3.3.2 Random Amplified Polymorphic DNA (RAPD) analysis.

Randomly amplified polymorphic DNA fingerprinting was done only for the extracted DNA from the strains of *C. albicans*. Two random primers were selected for the RAPD. Primer source was SEQ WEB- CGC-Prime. These were inversely oriented repetitive primers found in the genome of *Candida*. These primers act both as the forward and the backward primer.

Thermocycling was performed in a PTC-100 MiniCycler machine (MJ Research, MS, USA) and RoboCycler 40 (Stratagene, LJ, California, USA).

2.3.3.3 Preparation of the master mixture

PCR master mixture was prepared in a separate room and precautions were taken to avoid cross contamination of sample and PCR products.

The 50 µl PCR master mixture contained 5µl of 10× PCR buffer (200mM Tris-HCl (pH8.4) and 500 mM KCl), 0.2mM dNTPs, 1.5 mM MgCl₂, 1µM of primer RSD12(5'GCA TAT CAA TAA GCG CAG GAA AAG3') and RSD10(5'CCG CAG CCA3'),

2µl template DNA and 2.5U of Taq polymerase (Life technologies, USA). The tubes were overlaid with 50µl mineral oil. A negative control with out the template DNA was performed with each PCR run .

2.3.3.4 The RAPD cycle parameter

The cycle parameter were, 30 seconds of denaturation at 94°C, 2 minutes of annealing at 52°C (primer RSD 12) or 27°C (RSD 10) and 2 minutes primer extension at 72°C, followed by 45 cycles of 30 seconds denaturation at 94°C, 2 minutes annealing at 57°C (primer RSD12) or 32°C (primer RSD10) and 2 minutes primer extension at 72°C. A final extension was performed at 72°C for 15 minutes, and the RAPD products were at 4°C until further analysis.

2.3.3.5 Gel electrophoresis

2.3.3.5.1 Casting the gel

0.8%- 1% TBE agarose gel was used for the gel electrophoresis. Appropriate amount of agarose was weighed and mixed with half the quantity of 0.5x TBE buffer dissolved in a microwave oven. The rest of the TBE buffer was added to the mixture while mixing it

using the magnetic stirrer. When the gel got cooled it was poured into the gel cast device (190 ml) and allowed the gel to solidify at room temperature. The gel was then kept in the gel tank which filled with the TBE buffer (89mM Tris, 89mM-boric acid, 2.5mM EDTA, pH8). When the gel submerged the comb was removed to get the wells.

2.3.3.5.2 Sample loading

The RAPD products (5 µl) were mixed with loading dye (1µl) before loading into the wells. The loading dye used was 6× concentrate, containing 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) sucrose water. Three wells were loaded with the marker (Gene rulerTM MBI Fermentas) to compare the positions of the bands and one negative control with out template DNA was run with each time.

2.3.3.5.3 Gel running conditions

Gel was run for two hours at 100V in the 190 ml gel tank.

2.3.3.5.4 Staining with ethidium bromide

Gel was stained using ethidium bromide (0.5 μ g/ml) for 45 minutes and visualized with UV light.

2.3.3.5.5 Photographing gel

A polaroid 'gun camera' was used with UV filter and polaroid type 667 high- speed film to take the gel photograph. The aperture was set to f/8 and the shutter on 1 second.

The band produced by each isolate on the gel was photographed and the band patterns were used to identify the different genotype. Only the clearly visual bands were scored and the weight was compared with the marker.

2.4 RESULTS

2.4 RESULTS

2.4.1 Isolation and speciation of candida

All fifteen babies included in the study were less than seven days old and they had a birth weight less than 1500g. All these babies were in the baby incubators in the premature baby unit at the Colombo South Teaching Hospital.

Among the 15 babies chosen for the study only one baby showed clinical evidence of oral thrush.

Ten of the fifteen babies yielded candida species from their oral samples giving the incidence as 67 per cent. When the candida isolates were speciated using germ tube test and API20 C™ test all isolates were identified as *C.albicans*. No other species of yeast was isolated from the oral cavities of these babies.

2.4.2.Results of germ tube test

All the *C. albicans* isolates gave positive result for the germ tube test by the both methods.

2.4.3 Biotyping Results

When the strains were biotyped using API ZYM™, API20CAUX™ and Boric acid resistant the isolates fell into three different biotypes. Table 2.2 shows the different biotypes and the genotypes. Except for one isolate (p1) others assigned to type J with the APIZYM™ results. Majority of the isolates (80%) were resistant to boric acid; two isolates were sensitive to boric acid.

Interestingly, we were able to isolate one biotype, which has not been mentioned in previous studies. This isolate showed a positive result for 6-Br-2-naphty- α D-mannopyranoside in APIZYM™ assimilation test. This isolate was classified as CC in the APIZYM™ classification and biotype was designated as CC1S.

The API 20 C AUX™ profiles of 10 *C.albicans* yielded two types. Finally the combination of the three-biotyping results yielded three different biotypes amongst 10 isolates. Except p1 isolate others could be assigned to the biotyping system developed by us.

2.4.4 Results of genotyping

Five different genotypes were obtained by genotyping. All ten isolates demonstrated some similarity, having common bands with both primers. Table 2.1 shows the results of genotyping using RSD 10 and RSD12 primers.

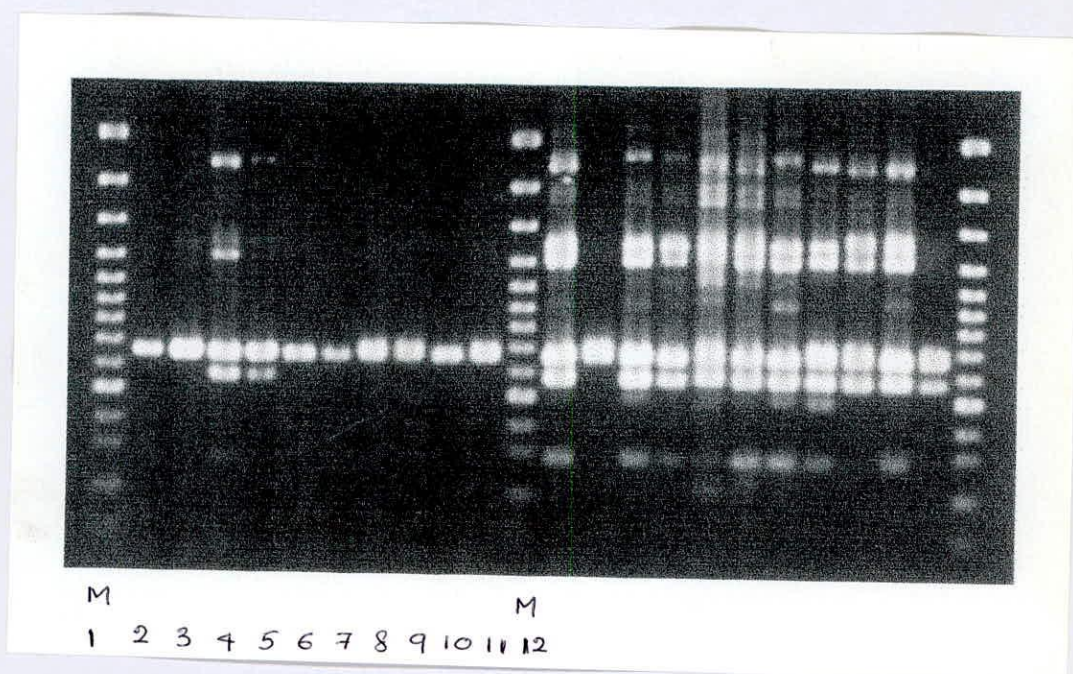


Figure-18 Gel electrophoresis of the RAPD fingerprints using RSD 10 primer for the *C. albicans* isolates obtained from premature babies. Lane number 1-11 represents the results of the isolates from premature babies, while lane number 13-23 represents the results of less than 6 yrs age group (discussed on chapter 1).

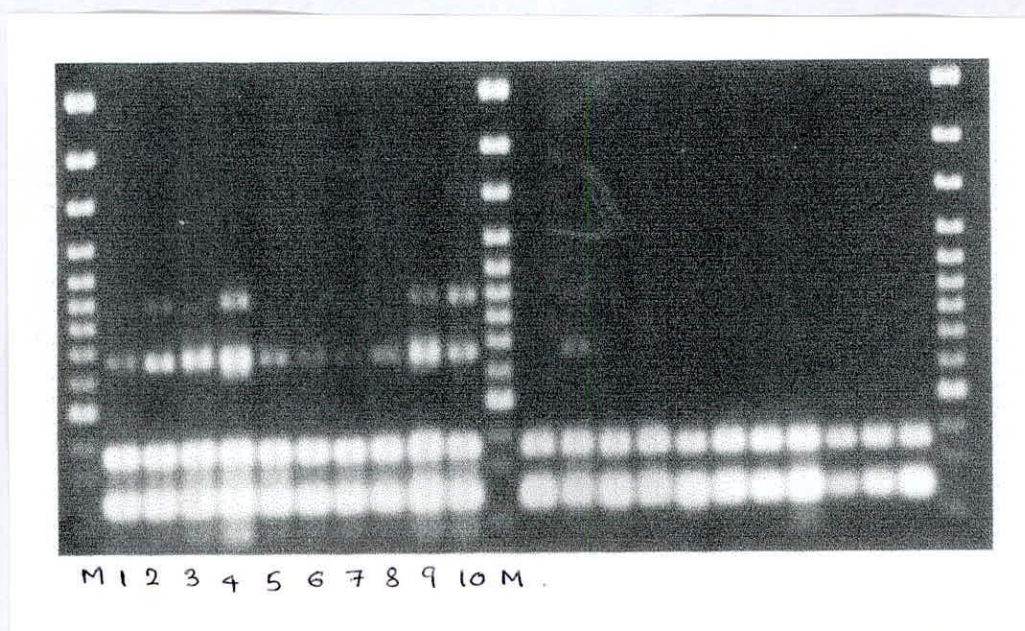


Figure-19 Gel electrophoresis of the RAPD fingerprints using RSD 12 primer for the *C. albicans* isolates obtained from premature babies

2.1 Results of the genotyping using RSD10 and RSD12 primer for the *Calbicans* strains isolated from premature babies

RSD 10 RSD 12	β	η	λ
σ	03	01	
μ	03		01
π	02		

2.4.5 Results of combined typing system

Three biotypes were obtained by phenotyping and, RAPD analyses revealed five different genotypes. All ten isolates demonstrated some similarity, with RSD 10 primer having a common band at ~600 bp as opposed to two isolates (P3, P4) having extra bands. Also with RSD 12 primer all the ten isolates showed a similarity having three common bands in ~200, ~300 and ~600 positions. However, when these results were compared with the corresponding phenotypes no similarity emerged.

When the two typing systems were combined in order to obtain the composite types, a total of five composite types were obtained for 10 *C. albicans* isolates. Except for two (p1 and

p3) isolates all other isolates could be accommodated into the typing system developed by us. We were able to find a difference in the sample number P3 both with genotyping and biotyping (composite type of sample number p3 was CCλμ).

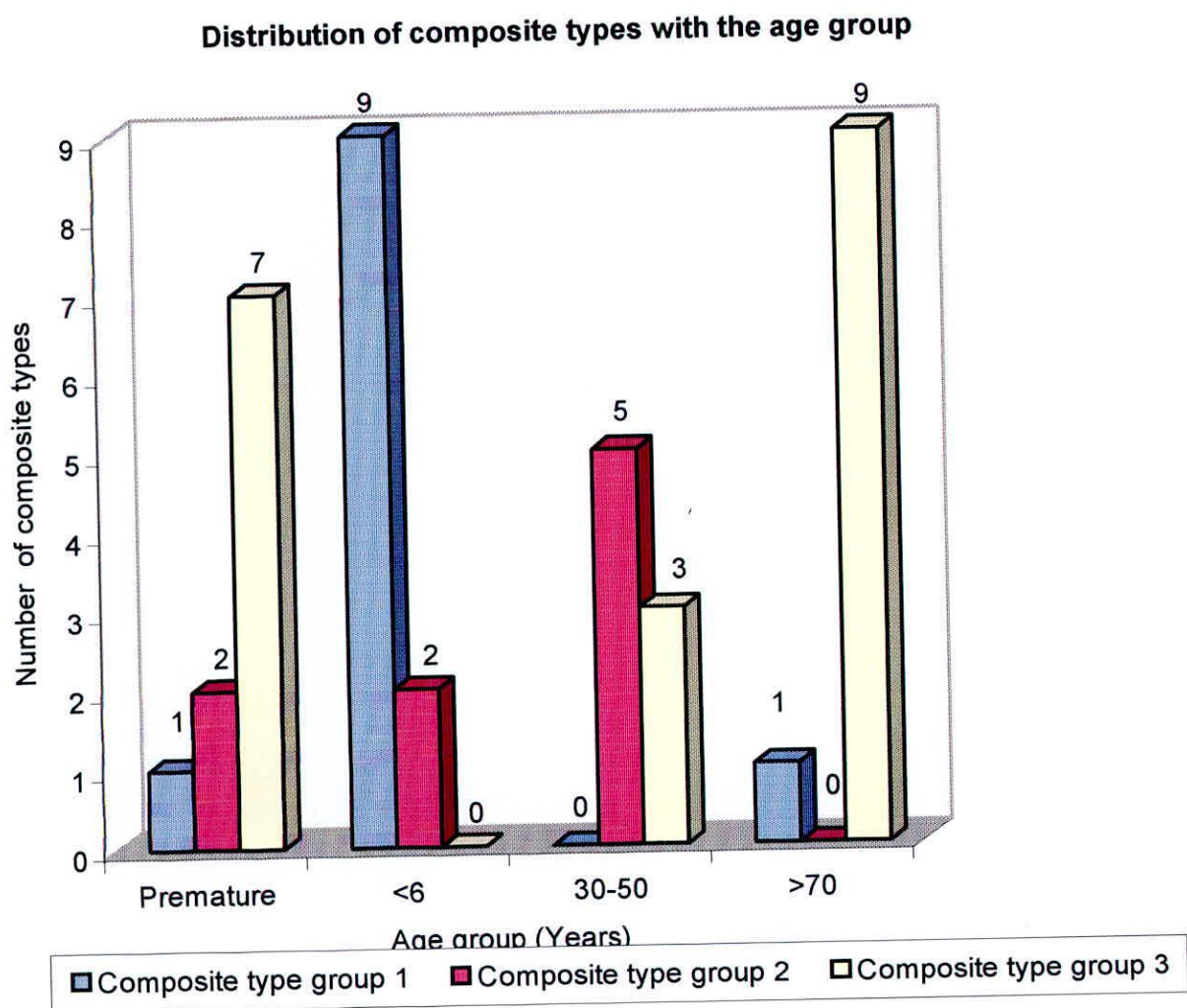
Statistical analysis was done using the chi square method. A statistically significant association ($p=0.001$) was observed between composite types of *C.albicans* strains isolated from age group >70 years and premature babies.

Table 2.2. Comparison of distribution of biotypes and genotypes of 10 *C.albicans* isolates

No. of <i>C.albicans</i> isolated with genotype						
Biotype code	No. of <i>C.albicans</i> isolates with biotype	$\lambda\mu$	$\beta\sigma$	$\beta\pi$	$\beta\mu$	$\eta\sigma$
J1R	06		02		03	01
J1S	03			03		
CC11R	01	01				

Table 2.3 Distribution of Composite types with the Age Group

Age group (Years)	Composite type																			
	Group 01					Group 02					Group 03									
	J1R $\gamma\pi$	J1S $\gamma\pi$	J1S $\beta\mu$	BB1R $\alpha\pi$	J1R $\phi\pi$	P1S $\phi\pi$	J1R $\theta\pi$	J1R $\lambda\pi$	J1R $\alpha\sigma$	J11R $\alpha\sigma$	J1S $\alpha\sigma$	J1R $\alpha\pi$	J1R $\beta\sigma$	J23S $\beta\sigma$	J1R $\beta\mu$	J1S $\beta\pi$	J1R $\beta\pi$	J14S $\beta\sigma$	CC1R $\lambda\mu$	J1R $\eta\sigma$
<6	04	01	01	01	01	01	01	01												
30-50									02	01				02	01					
> 70			01										01		02	01	04	01		
Premature babies			01										02		02	03			01	01



Statistical analysis showed there is a statistically significant association between composite types of *C.albicans* strains isolated from age group >70 years and premature babies.

2.5 DISCUSSION

2.5 DISCUSSION

The overall incidence of colonization of the mouth by candida species among 15 premature babies in a special baby care unit at Colombo South Teaching Hospital was 67 %. *C. albicans* was the only yeast we could isolate from this study population.

We used mouth rinses when sampling oral cavities of older children and adults to minimize the false negatives. However it was not possible to collect oral rinses from neonates, and sterile swabs were used instead to collect the samples from this group. This obviously can reduce the isolation rate and is less satisfactory. As such these results may under estimate the true prevalence of candida. Five different strains were noted among 10 isolates indicating that these colonizing strains were non-identical. Colonization of neonates with *C. albicans* could occur primarily from the birth canal and from non-maternal sources, possibly via cross contamination through hands of health care workers or parents (Sharp, et al 1992).

Nevertheless, the finding that 67 per cent of premature babies harbour *C. albicans* in the mouths within the first seven days of life represents a substantially higher figure than previously reported (Martius, et al 1998).

It is of interest to note that, although 10 out of 15 babies harboured *C. albicans* in their mouths, only one had clinical candidiasis at the time of sampling.

This may perhaps reflect a high rate of colonization and low incidence of thrush in these babies.

Despite the high colonization by candida species observed in neonates, few prospective studies have evaluated the source or the mode of transmission either by phenotyping or genotyping methods.

In the present study 5 different composite types of *C.albicans* were identified by phenotyping and genotyping. These composite types correlated well with our typing system except for two isolates. Interestingly most of these composite types were those assigned to group 3. No statistically significant variation was observed with the composite types of premature babies and the composite types of >70 year age group.

This shows a statistically significant association ($p=0.001$) with these two age groups (premature and >70 year age group) with respect to the composite types of *C.albicans*

These data demonstrate the utility of genotypic-phenotypic typing method for enhancing our understanding of epidemiology and pathogenesis of *C. albicans* infections. These methods including the application of biotyping and RAPD analysis with the primer RSD 10 and RSD 12, will undoubtedly be useful for further studies including to identify the potentially virulent strains, risk factors for invasive diseases, and developing and assessing interventions that will prevent transmission.

However further work with a large number of strains and other epidemiological surveillance data are also required to confirm the reliability and sensitivity of these methods.

Determination of the source exogenous infection and mode of transmission requires further investigations.

This study re-emphasized the importance of a reliable typing system in epidemiological studies of *C. albicans*.

2.6 CONCLUSIONS

2.6 CONCLUSIONS

1. The rate of colonization of candida in the oral cavies of the premature babies was 67%. *C. albicans* was the only yeast isolated from the premature babies.
2. Genotyping and biotyping systems developed by us characterized the ten *C. albicans* strains into 5 different composite types.
3. These composite types except for two were well correlated with the group 3 of the composite typing system established by us.
4. These composite types demonstrated an association with the composite types of *C.albicans* strains isolated from age group >70 years..
5. Further work with a large number of strains and other epidemiological surveillance data are required to confirm the accuracy and reliability of these results.

2.7 REFERENCES

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2.8 APPENDICES

Appendix 1

QUESTIONNAIRE

1. NAME :
2. ADDRESS :
.....
3. RESIDENCE :
.....
4. AGE : (a) <6 yrs -
(b) 30-50 yrs. -
(c) >70 yrs. -
5. OCCUPATION :

6. POSSIBLE PREDISPOSING FACTORS FOR SUPERFICIAL CANDIDIASIS

- (a) Known diabetics:-
- (b) General debility (specify):-
- (c) Dentures :-
- (d) Bottle fed babies:-
- (e) Pregnancy :-
- (f) Habits :-
 - i. Drug addicts:-
 - ii. Alcohol :-
 - iii. Smoking:-
 - iv. Beetle chewing :-

(g) Immunosuppression

i. Drug:-

* Steroids - Oral:-

Inhaled:-

* Broad Spectrum antibiotics - Yes/No

Duration:-

* Cytotoxic Drugs:-

* Progesteron therapy:-

* Oral contraceptives:-

ii. Diseases:-

* AIDS

* Leukaemia

* Other
Malignancies

8. PAST MEDICAL HISTORY :

(a) P/H of TB:-

(b) Bronchiectasis:-

(c) Cancer:-

Appendix 2

APIZYM



ENGLISH

2 520 0

API ZYM

FOR THE RESEARCH OF
ENZYMATIC ACTIVITIES

Instruction manual
Version A

API ZYM is a semi-quantitative micromethod designed for the research of enzymatic activities. The technique is applicable to all specimens (tissues, cells, biological fluids, microorganisms, washings, soil, oil, etc.). It allows the systematic and rapid study of 19 enzymatic reactions using very small sample quantities.

API ZYM has not been developed in view of obtaining the precision of spectrophotometric or electrophoretic procedures but has been mainly developed to permit the performance of enzymatic determinations from a complex sample which has not been purified. It can be used to screen specimens, thus providing a spectrum of enzymatic determinations which can be further tested by spectrophotometric and/ or electrophoretic procedures.

Principle

The **API ZYM** strip is composed of 20 microtubes, the bottom of which forms a support especially designed to contain the enzymatic substrate and buffer. This support allows contact between the enzyme and the generally insoluble substrate.

- The **API ZYM** kit enables the performance of 25 tests. It consists of :
 - 25 **API ZYM** strips
 - 25 incubation trays
 - 25 report sheets
 - 1 instruction manual

- In order to use **API ZYM**, it is necessary to have available :

- pipettes (# 9 756 0) or
PSI pipettes (# 7 025 0)
- Ampoule rack (# 7 020 0)

- **Suspension Medium** (# 2 011 0) or
sterile distilled water
- **ZYM A** and **ZYM B** reagents (# 7 047 0 and 7 048 0)
- **McFarland Standard** (# 7 090 0)

Plus standard laboratory equipment :

Incubator, refrigerator, Bunsen burner, marker pen

Storage

API ZYM strips should be stored at 2-8°C. The expiry date is printed on each pack.

The reagents should also be stored at 2-8°C, except for **ZYM A** which forms a precipitate at this temperature. The precipitate does not affect any of the properties of the reagent and dissolves again at 60°C. It is better to store the reagent at room temperature.

ZYM B is light-sensitive and its deterioration is marked by the development of a pink colour. The reagent should be kept in the dark ; it is best to wrap the ampoule in aluminium foil and keep it in the refrigerator.

Composition of reagents

• ZYM A :

Tri-hydroxymethyl-amino-methane	25	g
Hydrochloric acid (37 %)	11	ml
Lauryl sulphate	10	g
Distilled water	qsp 100	ml

• ZYM B :

Fast Blue BB	0.35	g
2-methoxyethanol	qsp 100	ml

Instructions for use

1. Preparation of the specimen

Dilute the specimen in a minimum volume of 2 ml of sterile distilled water or in another diluter such as normal saline without any buffer.

• Microorganisms :

Prepare a suspension with a turbidity between a McFarland No 5 and No 6 standard. Pure growth from an agar slant or sediment from a centrifuged broth culture can be used to prepare the suspension. In order to obtain reproducible results, it is important that the organisms to be compared be initially grown on the same isolation medium, the diluter be the same and the suspension be of the same optical density. This technique assays for constitutive enzymes. Inducible enzymes can be detected by adding the corresponding inducer(s) to the culture medium. In view of the absence of growth during the test, no particular precaution concerning sterility or incubation need be taken - even for anaerobes.

• Cell Suspensions : (Cell cultures, spermatozoa) :

With sterile distilled water or saline, the specimen is centrifuged and the sediment is adjusted to contain between 10^5 - 10^7 cells / ml. The concentration will depend on the enzymatic activity of the specimen.

• Tissues :

The specimen should be ground with a Potter tube (few mg) and suspended in 2 ml of distilled water.

• Biological fluids :

Undiluted fluids may be used directly or if the enzymatic activity of the raw liquid is too strong, it may be diluted. If the volume of the specimen is insufficient (less than 2 ml), bring it up to 2 ml with distilled water.

2. Preparation of strips

- Set up an incubation tray and lid.
- Record the specimen number on the elongated flap of the tray.
- Dispense approximately 5 ml of tap water into the incubation tray to provide a humid atmosphere during incubation. A plastic squeeze bottle may be used for this.
- Remove the API ZYM strip from the sealed envelope and place it in the incubation tray.

3. Inoculation of the strip

With a Pasteur pipette, inoculate two drops of specimen in each cupule of the strip (65 microliters).

4. Incubation of the strip

After inoculation, place the plastic lid on the tray and incubate generally for 4 hours at 37°C . The time of incubation and temperature may vary depending on the sample to be tested. However, when samples are being compared all test conditions (time, temperature, growth media, density of suspension) must be the same. The inoculated strip should not be placed in bright light.

5. Reading the strip

- After incubation, add one drop of ZYM R and one drop of ZYM B reagents.
- Let the colour develop for five minutes. If possible, put the strip under a powerful light source (1.000 watt bulb) for about 10 seconds with the bulb being placed about 4" above the cupules. The procedure will eliminate any yellow colour which may appear in the cupules due to any excess of Fast Blue which has not reacted. After light exposure, negative reactions become colourless. Placing the strip in daylight for a few minutes will produce comparable results.

6. Recording the reactions

A value ranging from 0-5 can be assigned corresponding to the colours developed as per the colour chart herewith enclosed. Zero corresponds to a negative reaction ; 5 to a reaction of maximum intensity. Values 1 through 4 are intermediate reactions depending on the level of intensity. The approximate number of free nanomoles may be known from the colour strength : 1 corresponds to the liberation of 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles and 5 to 40 or more nanomoles.

7. Disposal

The colours remain stable for several hours after the strip has been inoculated with the reagents. After reading, the strips can be dried at room temperature and stored in a dry atmosphere. All hazardous or pathogenic material (strip and incubation box) should be autoclaved, incinerated or immersed in a disinfectant for decontamination prior to disposal.

Quality control

The media, strips and reagents are subject to systematic quality control checks at various stages of manufacture. A further bacteriological and biochemical check may be carried out in the laboratory by using the following strains or enzymes:

1. *Bacteroides thetaiotaomicron*, ATCC 8492
2. β -Glucosidase, Sigma 8625
3. α -Chymotrypsin, Sigma C 4129

which give the following results :

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	-	+	V	+	-	V	-	-	-	+	V	+	+	+	+	V	+	-	+	
2.	-	V	V	-	+	V	-	V	-	+	V	+	-	+	+	V	-			
3.	-	-	+	+	-	V	-	V	+	-	-	-	-	-	-	-	-	-	-	

SUMMARY OF RESULTS

No	ENZYME ASSAYED FOR	SUBSTRATE	pH	RESULT	
				Positive	Negative
1	Control			No colour or colour of the sample if it has an intense coloration	
2	Phosphatase alkaline	2-naphthyl phosphate	8.5	Violet	No colour or colour of the control if the strip has been exposed to an intense light source after addition of the reagents. Very pale yellow if the strip has not been exposed to an intense light.
3	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
5	Lipase (C 14)	2-naphthyl myristate	*	Violet	
6	Leucine arylamidase	L-leucyl-2-naphthylamide	*	Orange	
7	Valine arylamidase	L-valyl-2-naphthylamide	*	Orange	
8	Cystine arylamidase	L-cystyl-2-naphthylamide	*	Orange	
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11	Phosphatase acid	2-naphthyl phosphate	5.4	Violet	
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	*	Blue	
13	α galactosidase	6-Br-2-naphthyl- α D-galactopyranoside	*	Violet	
14	β galactosidase	2-naphthyl- β D-galactopyranoside	*	Violet	
15	β glucuronidase	Naphthol-AS-BI- β D-glucuronide	*	Blue	
16	α glucosidase	2-naphthyl- α D-glucopyranoside	*	Violet	
17	β glucosidase	6-Br-2-naphthyl- β D-glucopyranoside	*	Violet	
18	N-acetyl- β glucosaminidase	1-naphthyl-N-acetyl- β D-glucosaminide	*	Brown	
19	α mannosidase	6-Br-2-naphthyl- α D-mannopyranoside	*	Violet	
20	α fucosidase	2-naphthyl- α L-fucopyranoside	*	Violet	

API Z/A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A		*	*	*		*	*				*	*				*		*		
B		*	*	*		*	*				*	*				*	*	*		
C		*	*	*		*	*				*	*				*		*		
D		*	*	*		*	*				*	*				*		*		
E		*	*	*		*	*				*	*				*		*		
F		*	*	*		*	*				*	*				*		*		
G		*	*	*		*	*				*	*				*		*		
H		*	*	*		*	*				*	*				*	*	*		
I		*	*	*		*	*				*	*				*	*	*		
J		*	*	*		*	*	*			*	*				*	*	*		
K		*	*	*		*	*	*			*	*				*	*	*		
L		*	*	*	*	*	*	*			*	*				*	*	*		
M		*	*	*		*	*	*			*	*				*	*	*		
N		*	*	*		*	*	*			*	*				*	*	*		
O		*	*	*		*	*	*			*	*				*	*	*		
P		*	*	*		*	*	*			*	*				*	*	*		
Q		*	*	*		*	*	*			*	*			*	*	*	*		
R		*	*	*		*	*	*			*	*			*	*	*	*		
S		*	*	*	*	*	*	*			*	*				*	*	*		
T		*	*	*		*	*	*			*	*				*	*	*		
U		*	*	*		*	*	*			*	*				*	*	*		
V		*	*	*	*	*	*	*			*	*				*	*	*		
W		*	*	*	*	*	*	*			*	*				*	*	*		
X		*	*	*	*	*	*	*			*	*				*	*	*		
Y		*	*	*	*	*	*	*			*	*				*	*	*		
Z		*	*	*	*	*	*	*			*	*				*	*	*		
AA		*	*	*	*	*	*	*			*	*				*	*	*		

47.9

Appendix 3

API20CAUX™ test

20 210

07628 C - 10/98

api 20 C AUX

Yeast identification system

For *in vitro* diagnostic use

API 20 C AUX is a system for the precise identification of the most frequently encountered yeasts in clinical microbiology. The complete list of those yeasts that it is possible to identify with this system is given in the Identification Table at the end of this package insert.

PRINCIPLE

The API 20 C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of 19 assimilation tests. The cupules are inoculated with a semi-solid minimal medium and the yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source.

The reactions are read by comparing them to growth controls and identification is obtained by referring to the Analytical Profile Index or using the identification software.

REAGENTS

Kit contents (25 tests) :

- 25 API 20 C AUX strips
- 25 incubation boxes
- 25 ampoules of C Medium
- 25 result sheets
- 1 package insert

Additional products (not included in the kit) :

- Suspension Medium, 2 ml (ref. 70 600 or 70 700) or NaCl 0.85 % Medium, 2 ml (ref. 20 060 or 20 070)
- RAT Medium [Rice Agar Tween] (ref. 41 794)
- Sabouraud Medium (ref. 42 026 or 43 171)
- Pipettes or PSlipettes (ref. 70 250)
- McFarland Standard (ref. 70 900) No. 2
- API 20 C AUX Analytical Profile Index (ref. 20 290) or identification software (consult bioMérieux)
- Ampoule rack (ref. 70 200)

Required laboratory equipment :

- 30°C incubator
- Refrigerator
- Bunsen burner
- Marker pen

TEST NAMES

GLU GLUcose	MDG α-Methyl-D-Glucoside
GLY GLYcerol	NAG N-Acetyl-D-Glucosamine
2KG 2-Keto-D-Gluconate	CEL CELlobiose
ARA L-ARabinose	LAC LACTose
XYL D-XYlose	MAL MALTose
ADO ADOnitol	SAC SACcharose/Sucrose
XLT XyLiToI	TRE TREhalose
GAL GALactose	MLZ MeLeZitose
INO INOsitol	RAF RAFfinose
SOR SORbitol	

COMPOSITION OF THE MEDIA

Suspension Medium 2 ml	Demineralized water	
NaCl 0.85 % Medium 2 ml	Sodium chloride	8.5 g
	Demineralized water	1000 ml
C Medium 7 ml	Ammonium sulphate	5 g
	Monopotassium phosphate	0.31 g
	Dipotassium phosphate	0.45 g
	Disodium phosphate	0.92 g
	Sodium chloride	0.1 g
	Calcium chloride	0.05 g
	Magnesium sulphate	0.2 g
	Histidine	0.005 g
	Tryptophane	0.02 g
	Methionine	0.02 g
	Agar	0.5 g
	Vitamin solution	1 ml
	Trace elements	10 ml
	Water	qsp 1000 ml
	final pH : 6.4-6.8	

Although C Medium contains agar, it requires no prior heating and may be as easily pipetted as a liquid medium. It is preferable to warm it at room temperature a few hours before use. Do not shake.

STORAGE OF THE STRIPS AND MEDIA

The strips and media should be stored at 2-8°C until the expiration date indicated on the packaging.

WARNINGS AND PRECAUTIONS

- For *in vitro* diagnostic use only.
- Qualified laboratory personnel should use aseptic technique and established precautions for infectious agents.
- Do not pipette specimens or reagents by mouth.
- Do not use reagents past the expiration date.
- Upon removal from refrigerator, allow reagents to come to room temperature (20-30°C).
- Open ampoules carefully as follows :



- Hold the ampoule in one hand in a vertical position (white plastic cap uppermost).
- Press the cap down as far as possible.
- Cover the flattened part of the cap with the upper part of the thumb.
- Apply thumb pressure in an outward motion to the flattened part of the cap to snap off the top of the ampoule inside the cap.

- * *For ampoule with no dropper-cap :*
 - Carefully remove the cap.
- * *For ampoule with dropper-cap :*
 - Turn the ampoule upside down and maintain it in a vertical position.
 - Squeeze on the cap to transfer all the reagent into the dropper-bottle.
- All inoculated products should be considered infectious and handled appropriately.
- All patient specimens and microbial cultures are potentially infectious and should be treated according to the recommended precautions (NCCLS M29-A, *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline* - December 1997).
- After completing test, reading and interpretation, all specimens, spills and inoculated products must be autoclaved, incinerated or immersed in a germicide prior to disposal.
- Interpretation of the test results should be made by a competent microbiologist who should also take into consideration the patient history, the source of the specimen, colonial and microscopic morphology and, if necessary, the results of any other tests performed, particularly the antimicrobial susceptibility patterns.

INSTRUCTIONS FOR USE

Specimens and yeast cultures should be considered infectious and handled appropriately by trained and competent technicians.

Aseptic technique and usual handling precautions for yeasts should be observed throughout this procedure ; refer to "NCCLS M29-A, *Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue*; Approved Guideline - December 1997".

For additional handling precautions, refer to Biosafety in Microbiological and Biomedical Laboratories, HHS Publication No. (CDC) 93-8395, 3rd Edition (May 1993), or to the regulations of each country.

Preparation of the strip

- Prepare the incubation box (tray and lid) and distribute about 5 ml of distilled water or demineralized water [or any water without additives or chemicals which may release gases (e.g. Cl₂, CO₂, etc.)] into the honey-combed wells of the tray to create a humid atmosphere.
- Record the strain reference on the elongated flap of the tray. (Do not record the reference on the lid as it may be misplaced during the procedure.)
- Remove the strip from its individual packaging and place it in the incubation tray.

Preparation of the inoculum

- Open an ampoule of Suspension Medium or NaCl 0.85 % Medium as indicated in the paragraph "Warnings and Precautions" (ampoule with no dropper-cap) or use any tube containing 2 ml of the same solution without additives.

- Using a pipette, pick up a portion of a yeast colony either by suction or by successive touches and make a suspension with a turbidity equal to **2 McFarland**.
- Dispense 1 drop of the yeast suspension onto appropriate morphology media (e.g. RAT Medium [Rice Agar Tween]).
- Open an ampoule of C Medium as indicated in the paragraph "Warnings and Precautions" (ampoule with no dropper-cap) and transfer 100 µl (2-4 drops) of the previous suspension into it. Gently homogenize with the pipette, avoiding the formation of bubbles.

Inoculation of the strip

- Fill the cupules with the suspension obtained in the ampoule of C Medium. Avoid the formation of bubbles by placing the tip of the pipette against the side of the cupule. Care should be taken not to overfill or underfill the cupules (the surface should be flat or slightly convex, but never concave), otherwise incorrect results may be obtained.
- Place the lid on the tray and incubate at 30°C for 48-72 hours.

Reading the strip

After 48 hours of incubation, or 72 hours (if the tests, in particular glucose, are not clearcut after 48 hours), compare growth in each cupule to the 0 cupule, which is used as a negative control. A cupule **more turbid than the control** indicates a **positive** reaction to be recorded on the result sheet.

In order to minimize the risks of contamination when reincubation is necessary, remove the lid only when reading the strip and replace immediately.

Identification

Identification can be obtained :

- using the Analytical Profile Index : the pattern of the reactions obtained must be coded into a numerical profile

On the result sheet, the tests are separated into groups of 3 and a number 1, 2 or 4 is indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number is obtained which constitutes the numerical profile.

NOTE : The presence of hyphae (mycelium) or pseudohyphae (pseudomycelium) constitutes the 21st test and has a value of 4 when positive.

- using the identification software by manually entering the 7-digit numerical profile via the keyboard.

Figure 1 shows a schematic diagram of a 12x12 grid of nodes. The grid is divided into four 6x6 quadrants. The top-left quadrant contains a 6x6 grid of nodes with alternating '+' and '-' signs. The top-right quadrant contains a 6x6 grid of nodes with alternating '+' and '-' signs. The bottom-left quadrant contains a 6x6 grid of nodes with alternating '+' and '-' signs. The bottom-right quadrant contains a 6x6 grid of nodes with alternating '+' and '-' signs. The grid is labeled with 'x' and 'y' coordinates on the left and right sides. The top-left node is labeled 'x=0, y=0'. The bottom-right node is labeled 'x=11, y=11'. The grid is labeled 'x' and 'y' on the left and right sides. The grid is labeled 'x' and 'y' on the left and right sides.

2 764 774 *Trichosporon asahii*

QUALITY CONTROL

The strips and media are systematically quality controlled at various stages of their manufacture. For those who wish to perform their own quality control tests with the strips, it is recommended that the following stock cultures be used, to obtain the results below :

	0	GLU	GLY	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF
1.	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
2.	-	+	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
3.	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+

Profiles obtained after 48 hours of incubation after culture on Sabouraud agar

1. <i>Cryptococcus laurentii</i>	ATCC	18803	ATCC : American Type Culture Collection,
2. <i>Candida glabrata</i>	ATCC	15126	10801 University Boulevard,
3. <i>Candida guilliermondii</i>	ATCC	6260	Manassas, VA 20110-2209, USA.

DISPOSAL OF USED MATERIAL

After use, all ampoules, pipettes, strips and incubation boxes should be autoclaved, incinerated or immersed in a disinfectant for decontamination prior to disposal.

LIMITATIONS

The API 20 C AUX system is intended uniquely for the identification of yeasts included in the database (see Identification Table at the end of this package insert). It cannot be used to identify any other microorganisms or to exclude their presence.

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PROCEDURE p. I
IDENTIFICATION TABLE p. II

TABLEAU D'IDENTIFICATION / IDENTIFICATION TABLE / PROZENTTABELLE /
TABLA DE IDENTIFICACION / TABELLA DI IDENTIFICAZIONE

% de réactions positives après 48-72 h à 30°C / % of positive reactions after 48-72 hrs. at 30°C /

% der positiven Reaktionen nach 48-72 Std. bei 30°C /

% de las reacciones positivas después de 48-72 H a 30°C / % di reazioni positive dopo 48-72 ore a 30°C

API 20 C AUX	V3.0	0	GLU	GLY	ZKG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	HYPH
<i>Candida albicans 1</i>	0	100	14	99	2	88	94	90	99	0	94	85	99	0	0	99	97	97	5	0	99	
<i>Candida albicans 2</i>	0	100	1	99	1	90	1	75	99	0	70	1	99	0	0	90	1	5	1	0	99	
<i>Candida boidinii</i>	0	100	55	1	0	89	70	89	25	0	95	1	55	0	0	1	1	1	0	0	100	
<i>Candida ciferrii</i>	0	100	80	80	100	100	71	60	100	100	43	0	99	60	0	99	100	99	0	99	100	
<i>Candida colliculosa</i>	0	100	96	100	0	0	0	5	13	0	60	1	0	0	0	3	99	60	0	0	99	
<i>Candida dubliniensis</i>	0	100	96	99	0	0	99	60	100	3	99	0	40	0	0	100	60	0	0	0	99	
<i>Candida famata</i>	0	100	96	98	60	60	98	75	99	0	100	99	99	89	70	100	100	96	78	75	1	
<i>Candida glabrata</i>	0	100	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	0	0	1	
<i>Candida guilliermondii</i>	0	100	99	97	79	85	97	92	99	0	97	88	99	95	0	94	100	99	90	95	46	
<i>Candida kefyr</i>	0	100	27	0	1	18	1	25	100	0	34	0	0	1	95	1	100	1	1	96	75	
<i>Candida krusei/inconspicua</i>	0	99	73	0	0	0	0	0	6	0	2	0	64	0	0	0	0	0	0	0	79	
<i>Candida lusitanae</i>	0	100	90	95	1	65	95	20	30	0	99	60	95	80	0	100	99	100	99	0	75	
<i>Candida magnoliae</i>	0	100	32	50	0	0	0	0	10	0	60	0	0	0	0	2	97	10	1	75	1	
<i>Candida norvegensis</i>	0	100	85	0	0	0	0	0	2	0	0	0	0	0	25	0	0	0	0	0	93	
<i>Candida parapsilosis</i>	0	100	94	88	89	89	93	3	99	0	99	89	99	0	0	100	100	93	99	1	99	
<i>Candida pelliculosa</i>	0	100	99	0	0	67	1	1	56	0	70	95	1	70	0	97	99	87	96	30	70	
<i>Candida rugosa</i>	0	100	74	0	1	70	1	26	99	0	94	0	69	0	0	0	0	0	0	0	99	
<i>Candida sphaerica 1</i>	0	100	31	2	0	2	0	62	99	0	99	68	0	35	1	95	100	99	29	76	99	
<i>Candida sphaerica 2</i>	0	100	88	1	0	1	0	36	94	0	99	60	0	31	99	80	100	63	80	64	1	
<i>Candida tropicalis</i>	0	100	9	99	1	96	99	12	99	0	99	69	99	17	1	99	73	100	72	1	99	
<i>Candida utilis</i>	0	100	99	0	0	60	0	1	5	0	1	3	0	37	0	98	96	16	72	79	69	
<i>Candida zeylanoides</i>	0	100	100	87	0	0	1	0	1	0	99	0	99	0	0	0	0	74	0	0	75	
<i>Cryptococcus albidus</i>	0	100	0	98	80	81	0	0	6	30	60	65	0	99	10	98	100	82	81	51	1	
<i>Cryptococcus humicola</i>	0	100	82	100	100	100	36	64	100	100	95	100	100	98	100	100	99	99	95	99	99	
<i>Cryptococcus laurentii</i>	0	100	6	92	99	99	69	76	99	84	63	76	92	96	99	92	99	92	96	99	25	
<i>Cryptococcus neoformans</i>	0	100	0	100	14	91	71	1	93	97	100	99	88	10	0	99	99	75	97	88	25	
<i>Cryptococcus terreus</i>	0	100	0	100	87	100	0	0	45	50	99	0	96	96	36	0	0	64	0	0	1	
<i>Cryptococcus unguiculatus</i>	0	100	3	99	99	99	3	0	1	99	50	99	100	0	0	100	100	75	100	7	25	
<i>Geotrichum capitatum</i>	0	95	92	0	0	0	0	0	25	0	10	0	2	0	0	0	0	0	0	0	95	
<i>Geotrichum penicillatum</i>	0	100	100	0	0	92	0	0	75	0	88	0	0	0	0	0	0	0	0	0	92	
<i>Hansenula polymorpha</i>	0	100	84	0	1	1	66	36	0	0	90	1	1	20	0	94	90	46	97	0	2	
<i>Kloeckera spp</i>	0	100	0	50	1	0	0	0	0	0	0	0	0	0	96	0	0	0	0	0	1	
<i>Pichia ohmeri</i>	0	100	99	96	0	0	66	0	84	0	93	98	99	56	0	99	99	93	0	80	84	
<i>Prototheca wickerhamii</i>	0	100	100	0	0	0	0	0	65	0	0	0	0	0	0	0	0	0	100	0	1	
<i>Rhodotorula glutinis</i>	0	100	15	91	0	0	8	0	60	0	84	3	0	1	0	91	100	69	84	96	1	
<i>Rhodotorula mucilaginosa 1</i>	0	100	5	4	15	33	92	81	10	0	5	0	0	0	0	33	100	5	1	87	25	
<i>Rhodotorula mucilaginosa 2</i>	0	100	60	1	80	80	64	62	80	0	60	1	0	1	0	98	100	95	86	98	25	
<i>Rhodotorula minuta</i>	0	100	100	100	98	95	3	0	0	0	5	0	85	60	1	0	95	95	95	0	1	
<i>Saccharomyces cerevisiae 1</i>	0	100	8	0	0	0	0	0	78	0	1	13	0	0	0	75	90	2	1	62	30	
<i>Saccharomyces cerevisiae 2</i>	0	100	1	0	0	0	0	0	99	0	1	29	0	0	0	99	99	99	85	81	25	
<i>Sporobolomyces salmonicolor</i>	0	100	1	0	0	0	0	0	5	0	80	0	0	0	0	0	100	85	0	70	90	
<i>Trichosporon asahii</i>	0	100	20	100	100	100	0	5	100	0	1	94	100	100	100	100	98	66	20	0	95	
<i>Trichosporon inkin</i>	0	100	4	100	0	98	0	0	95	98	0	100	57	100	95	100	100	95	89	0	95	
<i>Trichosporon mucoides</i>	0	100	40	99	74	100	63	65	100	92	78	100	94	100	100	100	100	78	82	99	95	

Appendix 4

STATISTICAL ANALYSIS

Statistical analysis was done using chi square test and Z test.

Z test

$$Z_{cal} = \frac{P_1 - P_2}{\sqrt{\frac{P_1 q_1}{n_1} + \frac{P_2 q_2}{n_2}}}$$

Compare Z_{cal} with 1.96

If $Z_{cal} > 1.96$

Difference is significant at 5%

Chi squared test (X^2)

$$X^2 = \sum \frac{(O-E)^2}{E}$$

O=observed values

E=Expected values

Yates Correction

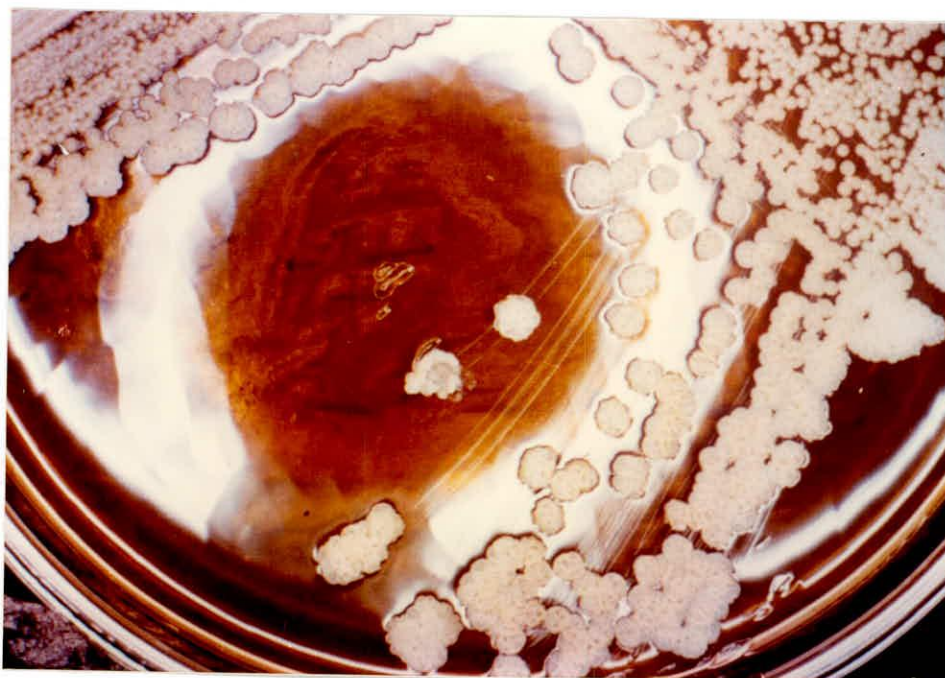
If total number of observations is less than 100 it is advisable to use Yates continuity Correction

With Yates correction

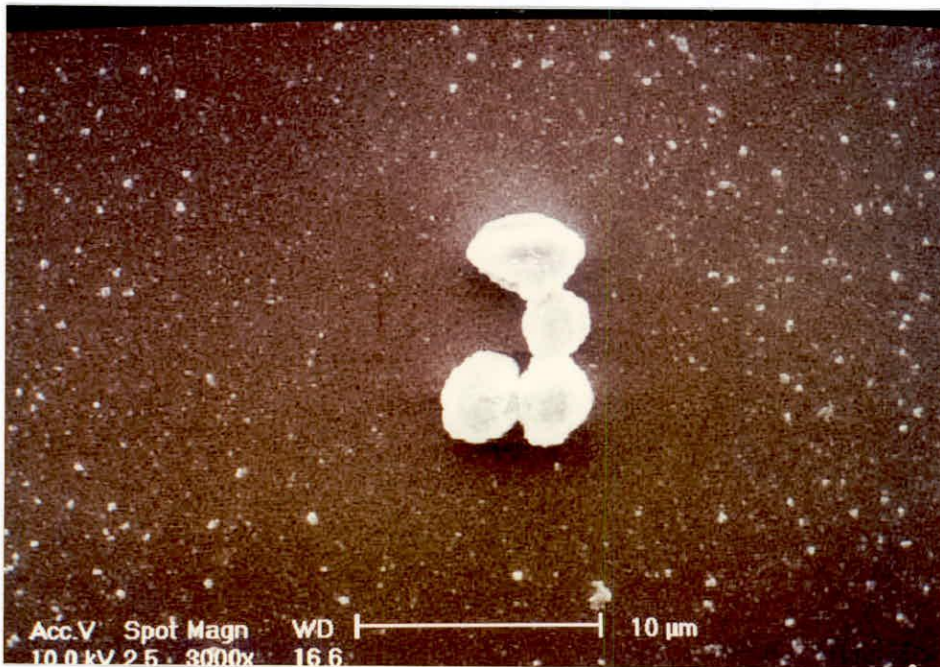
$$X^2 = \frac{\sum \left[(O-E) - 0.5 \right]^2}{E}$$

Appendix 5

Pichia ohmeri



Colony morphology of *Pichia ohmeri*- Colonies showed irregular edges. Flower like appearance. Cream coloured



Pichia ohmeri- Scanned Electron Microscopic appearance

Appendix 6

ETHICAL CLEARNCE



FACULTY OF MEDICAL SCIENCES
UNIVERSITY OF SRI JAYEWARDENEPURA, SRI LANKA

My No.

Your No.

Nugegoda,
Sri Lanka.

23rd/July/01

Dear Ms M M weerasekera

Application NO:-074

Title- **The phenotypic and genotypic diversity of oral Candida albicans isolates in Sri Lankan cohorts of varying age groups**

I am pleased to inform you that the ethical committee confirmed the approval for the above research proposal at its meeting held on 6th July 01.

A handwritten signature in black ink, appearing to read 'Sivayogan'.

Dr S Sivayogan
Chairman ERC/FMS

A handwritten signature in black ink, appearing to read 'S A Jayaratne'.

Dr S A Jayaratne
Sec/ERC/FMS

