Chapter 1

INTRODUCTION

Introduction and Review of Literature

1. General information on *Candida albicans*

Candida albicans is an opportunistic yeast-like fungus which commonly colonizes human mucosal surfaces. It is the principle yeast isolated from the mouth and is frequently present in the oral cavity of healthy adults as a part of normal flora. *C. albicans* remains the most persistent and pervasive yeast pathogen in humans, capable of invading every tissue of the body.(5) Systemic or local changes within the host may promote a proliferation of person's own commensal flora with a possibility of host tissue invasion, although oral candidiasis is uncommon in previously healthy people who have not received prior medical therapy. *Candida* species are in fact ubiquitous human pathogens, causing localized, invasive or disseminated disease in normal or immunocompromised hosts, promoted by such common factors as invasive procedures, catheters, immunosuppressive therapy, malignancy, immaturity, immunodeficiency, granulocytopenia, broad-spectrum antimicrobial agents and intravenous drug abuse.(34)

The genus *Candida* is a collection of some 150 asporogenous yeast species. They are classified among the fungi imperfecti in the class "Deuteromycetes" because of their inability to form sexual stage. However sexual forms of the yeast have recently been identified and this nomenclature awaits clarification. These saprophytic *Candida* species can be isolated from many environmental sources including soil, fresh and salt water, plants, insects and foul. However, pathogenic species are isolated only from humans and other mammals, and only one species, *C. albicans*, has been thoroughly studied. *C. albicans* is isolated most frequently from mucocutaneous tissue, specifically

from the alimentary, gastrointestinal, genital and urinary tracts.[5] Although there are over 100 species of *Candida,* however, only seven species are of major medical importance. Two of these, *C. albicans* and *C. tropicalis*, account for 80% or more of isolated yeast species.[5] The other pathogenic *Candida* species are *C. glabata, C. parapsilosis, C. stellatoidea, C. guilliermondii, C. krusei* and *C. pseudotropicalis*.[97] *C. glabata* and *C. parapsilosis* account for 10 to 15% of isolates.[5]

The prevalence of *C. albicans* in healthy asymptomatic individuals varies from; 10-30% from stools, to 50% from the oral cavity of newborns delivered through *C. albicans*-infected birth canals. *C. albicans* is more frequently isolated from the vaginal cavity of pregnant women than non-pregnant women. Although the organism is rarely isolated from the healthy skin of young people, the rate of such isolation increases with the age of the patients, up to 30% in the elderly.[5] *C. albicans* is frequently considered as a part of normal flora, especially from mucocutaneous specimen sources, unless the organism is recovered in high numbers or in pure culture. On the other hand, since *C. albicans* is seldom an environmental contaminant (particularly of healthy skin), the presence of *C. albicans* in specimens collected from presumably sterile sources is usually considered significant. Therefore, the isolation and identification of *C. albicans* from clinical specimens such as stool, urine or throat culture may indicate colonization and not necessarily infection. *Candida* species are most commonly present in healthy subjects in the mouth, other parts of the gastrointestinal tract and vagina, followed by the skin whist the isolation from eye and urine is much less common. Generally the distribution of *Candida* species in different sites is similar, *C. albicans* is most commonly isolated followed by *C. glabrata, C. tropicalis* and *C. parapsilosis*, [61] with the exception of normal skin where other species, particularly *C. parapsilosis* and *C. guilliermondii*, are common.[61]

The oral carriage rate of *Candida* in healthy individuals varies from 2 - 71%. In addition, the carriage rate in patients during hospitalization for the treatment or diagnosis of diseases other than candidiasis tends to be consistently higher than in normal subjects.[61] However, the frequency of yeast carriage found in the oral cavity is dependent on isolation technique and time of sampling. When samples were taken with swabs, the mean value of isolates was lower when compared with the mean value of isolates from saliva/mouth wash and imprint culture methods.[61] Samaranayake et al.[87] showed that the oral rinse method and imprint method had the same sensitivity.

2. Pathogenicity of *C. albicans*

C. albicans is a notorious opportunistic pathogen and the major factor contributing to its virulence is its ability to persist on the mucosal epithelia of healthy people. The pathogenicity of different biotypes and strains of *C. albicans* varies and most factors which have been related to fungal virulence are in the first place factors contributing to *Candida* persistence. The potential virulent factors of *Candida* are listed in Table 1 .

2.1 Adherence of *Candida*

An important aspect of the pathogenicity of *C. albicans* may be its specific affinity and binding to acrylic resin (*Candida*-associated denture stomatitis) and other plastics (catheter-related candidiasis).[99] The mechanism of attachment is believed to involve the interaction of cell wall components of *C. albicans* with the target surface.[51] The initial contact of yeast to epithelium may be due to non-specific adhesion followed by specific adhesion.[93] It may be that the *Candida* cells “bump” into epithelial cells, initially binding reversibly and then physiological changes strengthen the adhesion. It has also been suggested that the adhesion of *C. albicans* to oral mucosal cells might entail interactions involving divalent cations.[32] The adsorption of macromolecules onto epithelial cells is believed to occur via electrostatic

interactions involving calcium ions and other ionic groups. *Candida* cells might also attach by similar mechanisms.

Table 1 Potential virulent factors of *Candida* species.

Mechanisms	Molecular factors
Adherence	Extracellular hydrolase (Enzymes)
Persorption	Proteinase
Dimorphism	Lipases
Germ tubes	Anaphylatoxins
Switching	Killer toxins
Interference with	Nitrosamines
-Phagocytosis	Acid metabolites
-Immune defences	
-Complement	
Synergism with bacteria	

Based on the data from Samaranayake and MacFarlane 1990.[86]

2.2 Persorption

Persorption means the passage of yeast from the intestinal lumen through the intact mucosa into the blood stream. There is evidence of direct passage of *C. albicans* and *C. tropicalis* blastospores through the endothelial surface [35] and the fast penetration of *C. albicans* blastospores through murine intestinal mucosa [13] which indicated that the action of fungal hydrolytic enzymes may effect the sequence of events which allows persorption.

2.3 Dimorphism and germ-tube formation

Most isolates of *C. albicans* form true hyphae at 37° C and blastospores below 30° C, which pseudohyphae may be produced between

these temperatures.[62] Both types of cells (hyphae and blastospore) can be seen in the same mycotic lesion, therefore, both the yeast and the mycelial forms of *C. albicans* adhere, invade and proliferate in an infected host.[86,98]

Germ-tube formation is the onset of hyphal growth of *C. albicans*, is induced by contact with serum and is suspected of being involved in the pathogenesis of candidiasis.[2,81] The rapid formation of germ tubes is generally held as a diagnostic criterion of *C. albicans*; in addition, germ tubes have also been observed with some isolates of *C. tropicalis*.[47] As well, the formation of germ tubes is accompanied by an increased adherence to epithelial cells.[33] The enhanced adherence of germ tubes of *C. albicans* (serotype A) has been related to specific adhesins with proteinaceous constituents of 60,000 or greater in molecular weight.[119] It is tempting to speculate that germ tubes of *C. albicans* serotype A may enhance virulence, whilst blastospores may be more virulent in serotype B cells. Such a pattern of virulence would reflect the differential expression of extracellular proteinase by the two serotypes. Although it is known that the formation of germ tubes is correlated with increased virulence in most strains of *C. albicans*, but no specific factor can be linked with this effect at the molecular level. Recently, fimbrie comprising the glycocalyx and enabling yeast adhesion to epithelial cells were observed in a transmission electromicroscope.[123]

2.4 High-frequency switching

High frequency switching is the other "so-called" phenotypical shuttle system of most strains of *C. albicans* and *C. tropicalis*. It is recognised by differences of colony morphology, and involves the size and shape of blastoconidia. *C. albicans* frequently exhibits variant colonial forms when being grown *in vitro*, and this switching can be triggered by low doses of UV radiation.[103] Therefore, switching is associated with changes in micromorphology and physiologic properties as well as a number of putative virulence traits. Switching represents a true reversible transition system

without recognizable differences among the DNAs,[82] and switching may serve the yeast as a means of evading the host defense system. A switching system, "white-opaque" transition, has been examined for the capabilities of the two phenotypes to adhere to oral epithelial cells. "White" cells were shown to be significantly more adhesive than "opaque" cells.[32] It is thought that the switching mechanisms of *Candida* may help to potentiate its pathogenicity : (1) when invading into different body environments, (2) by eluding the immune system by altering its surface antigenicity, and (3) by escaping the action of antifungals.

2.5 Interference with phagocytosis

The first host defense against candidal invasion consists of phagocytosis; polymorphonuclear neutrophils are the most important. Certain isolates of *C. albicans* produce acidic peptides *in vitro* which can inhibit the attachment of fungal hyphae to phagocytes and inhibit the induction of the respiratory burst of the phagocytes after being stimulated by *Candida* cells.[86] Neutrophilic granulocytes may allow yeast phase *C. albicans* to evade intracellular killing, if the blastospores are internalized in "unsealed" phagosomes.[8] The internal environment of phagosomes is acidic, and therefore competition between acid fungal hydrolases and acid hydrolases of the phagosome (e.g. cathepsin D) may determine the outcome of phagocytosis. It is known that proteolytic candidal strains (acid proteinase) are generally more cytotoxic to phagocytic cells in vitro than are non-proteolytic strains.[44]

2.6 Interference of immune defense

Valdez et al.[121] reported on a dose-dependent immunosuppressive effect in mice produced by viable *C. albicans* cells which involved both humoral and cellular immunity. Also a polysaccharide fraction of *C. albicans* has been shown to inhibit the proliferation of human T-lymphocytes and the production of interleukin 1 and 2.[43]

2.7 Interference with complement

Heidenreich and Dierich[25] reported the binding of human complement proteins C3b and C3d to *C. albicans* and *C. stellotoidea*, but not to other *Candida* species. Others have found that the candidal C3b receptor was non-identical with the corresponding receptor on human neutrophilic granulocytes.[22,40] The fungal receptor binds C3b non-covalently and thus impairs phagocytosis of the fungal cells. Therefore, it can be hypothesized that this mechanism might provide *C. albicans* with a means to evade the host defenses.

2.8 Synergism with bacteria

There appears to be synergism between *C. albicans* and the pathogenic bacterium, *Pseudomonas aeruginosa*, in burned mice.[59] The results suggested a role for microbial proteinases in the establishment of such synergism. It was also noteworthy that *Staphylococcus aureus* and *Candida* species commonly cause synergistic infections, such as angular cheilitis.

2.9 Enzymes of *Candida*

Extracellular proteinases are glycoproteins and have been involved in the pathogenesis of candidiasis. *Candida* proteinase acts as a keratinase *in vitro* [60] and hence may be involved in fungal invasion through orthokeratinized mucosa. In mucosal tissue, the fungus encounters blood vessels which are readily invaded. At this stage of infection, certain strains of *C. albicans* and *C. tropicalis* may cause blood coagulation. The enzymatic effects of *Candida* proteinases also require a lowered pH, therefore salivary proteins including IgA can be almost completely degraded by the acidic proteinase of *Candida* especially under low pH conditions.[91] It has been shown that parotid saliva is more resistant to the proteolytic action of *Candida* proteinase when compared with mixed saliva. Acid proteinase or aspartyl proteinase production was increased by *C. albicans* isolated from later stages of HIV infection and may contribute to candidiasis.[91] *Candida* also can produce phospholipases which are concentrated at the tips of fungal hyphae and

localized in the vicinity of host cellular compartments where active invasion is occurring. These enzyme activities were found in most *C. albicans* strain but not in organisms known to be less virulent than *C. albicans*, such as *C. glabrata, C. tropicalis* and *C. parapsilosis*.[88]

2.10 Toxic substances produced by *C. albicans*

C. albicans has been found to produce an "endotoxin" or "canditoxin" which was secreted by yeast cells *in vivo* and acts as an anaphylactic agent,[31] but another study showed that the levels of endoxin found *in vivo* might not be sufficient to produce a toxic effect.[16] Other types of proteinaceous substances, killer toxins, can act rather like antibiotics of the bacteriocin type. Killer toxins are secreted *in vitro* by a number of yeasts including species of the genus *Candida*. They cause a microbiocidal effect on other fungus and on a variety of bacteria.[74] However, there are no confirmatory observations on the action of killer toxins on mammalian cells, nor have traces of substances been monitored in tissues infected with *Candida*.

2.11 Nitrosamines

There is evidence of the production of carcinogenic nitrosamines by *C. albicans*, which often colonizes oral leukoplakias. *C. albicans* isolates from such precancerous lesions had a particularly high nitrosation potential.[86]

2.12 Acidic metabolites of *Candida*

Samaranayake[85] found a pH drop from 7 to 3 within 48 hours when *C. albicans* and *C. glabrata* were grown in saliva supplemented with glucose, indicating the remarkable acidogenic potential of *Candida* species in the presence of a carbohydrate such as glucose. The short-chain carboxylic acids produced by *Candida* may potentiate its virulence. There is evidence to indicate the acidic metabolites play a role in the pathogenesis of chronic atrophic candidiasis, as very low pH levels (about 4.0) are observed beneath upper dentures *in vivo*.[89]

3. Oral Candidiasis and HIV Infection

Candida infection, with oral thrush and esophagitis as frequent manifestations, is the most common opportunistic infection encountered in AIDS.[27,90] Ever since the first clinical definition of AIDS (1981), the WHO has recognized candidosis or candidiasis of the mouth, esophagus, trachea, bronchi and lungs as "major" opportunistic infections and important indicator diseases. The strong association of oral candidiasis with AIDS is well known and encompasses several unorthodox presentations. Oral candidiasis is a common sign of HIV infection in children as well as adults,[29] and most of those with perinatal HIV infection manifested mucocutaneous candidiasis in the first year of life. In young HIV-infected infants, mucocutaneous candidiasis may early presage the severe morbidity.

The worldwide increased frequency of infections with human immunodeficiency (HIV), and technological advances in organ transplantation and chemotherapy have dramatically increased the incidence of candidiasis.[132] The frequency of isolation of *Candida* species and the clinical signs of oral candidiasis increased with advancing human immunodeficiency virus (HIV) infection.[36,118] The profound immunodeficiency, particularly affecting T-helper cells during HIV infection precipitates a number of secondary infections, and candidiasis particularly affecting the oral mucosa is widely prevalent.[84] A study reported a prevalence at 36-88 % of all patients suffered from oral candidiasis during the course of HIV infection.[36] In the review of Samaranayake and Holmstrup,[84] the frequency of oral candidiasis in adults and children with HIV ranged from 11-96 %, but the majority of reports were of the frequencies greater than 50 %. The study of Korting et al.[36] also showed the prevalence of isolates of *C. albicans* and clinical signs of oral candidiasis increasing with advancing HIV infection, 57.7 % microbiological recovery of *C. albicans* from oral cavities of stage 1 patients, 76.5 % from stage 2 patients, and 87.5 % from stage 3 patients (stage 1: seropositive

latency; stage 2: persistent generalized lymphadenopathy; stage 3: full-blown AIDS).

The prevalence of *Candida* species in a group of Thai AIDS patients was reported by Teanpaisan and Nittayananta[115] at 66.66 % in AIDS patients whereas 10.81 % (8/74) were found in normal subjects. They also showed that *C. albicans* was the most common species recovered from AIDS patients (96.66%). In addition the study of Torssander et al.[118] reported that the carriage of *Candida* was prevalent (77.8%) among HIV seropositive homosexual men, and one half of patients with positive smears had no clinical signs of oral candidiasis. The foci of oral candidiasis may act as potential reservoirs of organism for local spread of the disease in the compromised host; the study of 20 patients with AIDS and oral candidiasis by Tavition, Raufman and Rosenthal[113] showed that oral candidiasis might be an indicator of the esophageal candidiasis. Klein et al.[34] studied the frequency with which unexplained oral candidiasis leading to unequivocal acquired immunodeficiency syndrome (AIDS) in patients at risk (intravenous drug abuser, homosexual or bisexual men, or both). They found that AIDS developed in 80 % (12 of 15) patients with oral candidiasis and T4/T8 ratio < 0.51 as compared with none (no candidiasis) of four patients with ratio > 0.60. Also, of the patient with oral candidiasis, those with lowest T4/T8 ratios had the poorest prognosis.

3.1 Pathogenicity of *C. albicans* in HIV infection

Among the HIV-infected patients, the progression of immune-suppression status is expressed by an increase in the severity and prevalence of oral candidiasis. The mechanisms whereby hypervirulent *C. albicans* is selected in HIV-infected patients are likely to be multifactorial, associated both with the pathological effect of HIV infection and the increased candidal proliferation in these patients. The transition of *C. albicans* from harmless commensal to virulent pathogen in the susceptible host includes hyphal

formation, thigmotropism, enzyme production (protease secretion), adherence and phenotypic switching interfered with defense system and other molecular factors of *C. albicans*.[111] Oral langerhans cells are infected by HIV and may also play a role in candidiasis. *Candida* itself may induce immunosupression and this can influence the prognosis of HIV infection.[61] There are possible mechanisms by which HIV infection may eventually result in the selection of *Candida* strains with altered phenotypic and genotypic characteristics within the susceptible hosts (Figure 1).

The simple selection of *Candida* with altered phenotypes or the selection of genetically distinct strains may cause alterations in candidal populations in HIV infection. The phenotypic selection to alter the strains may be enhanced by cell surface changes and may be associated with adherence which allows the *Candida* to agglutinate with a monoclonal antibody directed at *Candida* surface antigen.[7] A study with AIDS patients, half of whom developed candidiasis during the study and were treated with antifungals, noted that the same *C. albicans* genotypes tended to persist during the course of disease progression.[52] Indeed, antifungal prophylaxis may be a major cause of candidal selection involving increased candidal adherence and protease secretion which occurred not only in strains isolated from healthy but asymptomatic HIV-infected subjects.[112]

Switching is a phenotypic alteration often seen especially in response to stress. Switching is under genetic control and may occur at relatively high frequencies of between 10^{-2} and 10^{-3} per cell division.[103] The selective advantage of switching was demonstrated by a study of the genetic relatedness of oral and vaginal *Candida* isolates. In the women who carried *Candida* species in both sites, the two strains were genetically unrelated in approximately half of the cases. In the other half, the two strains were genetically similar but non-identical. It was suggested that in the latter half the infecting strains were derived from a single ancestor that utilized switching to

alter their phenotype to adapt to alternative environmental niches.[103] Such an adaptive ability may assist in survival and colonization of the host. In addition it may lead to genetic selection which further complicates the study of the pathogenesis of candidiasis.

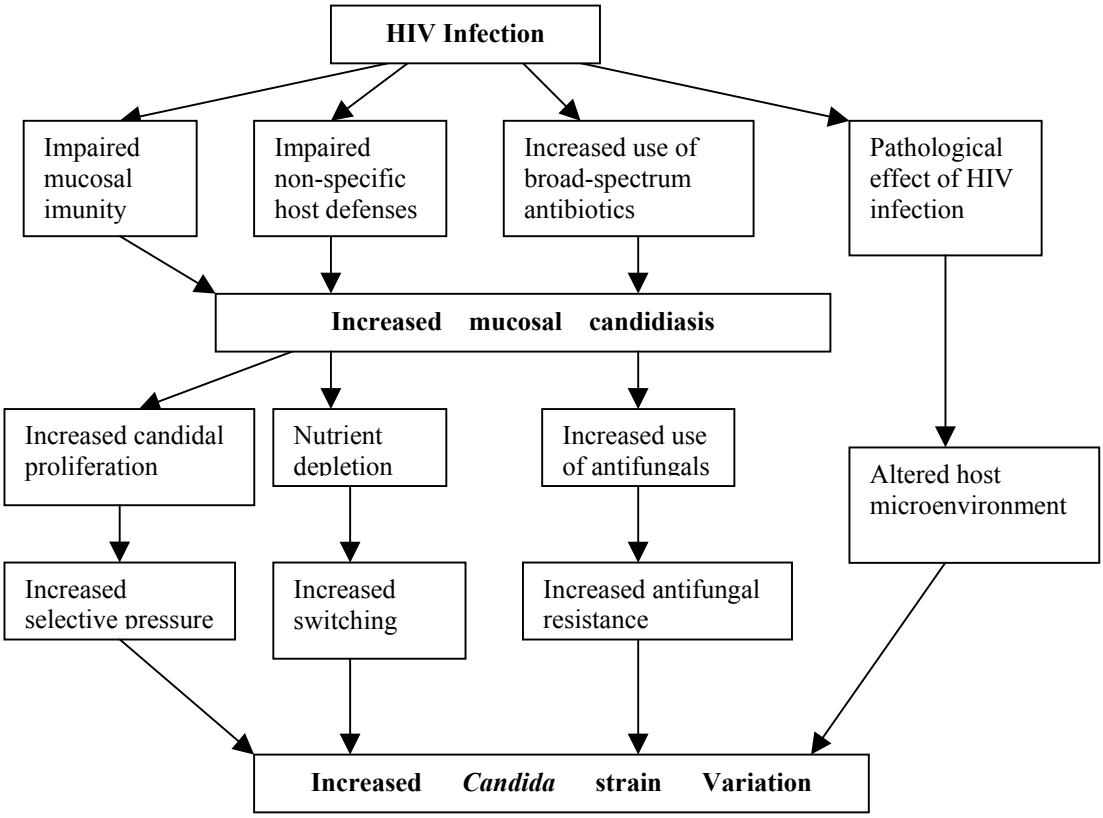

Figure 1 Schematic diagram illustrating the possible theoretical considerations which may contribute to the selection of *Candida* strains.[111]

The genetic selection which occurred in the HIV-infected patients and some studies showed the replacement in the original commensal strains. If this replacement of genotypes occurs only once and occurs early in the course of HIV infection, then the replaced genotypes of infecting *C. albicans* will be stable even though the phenotypic change has happened during the time of HIV infection.[96,52] This was supported by DNA fingerprinting studies with moderately repetitive probes, which indicated that HIV-infected patients tend

to be colonized by a single endogenous strain of *Candida* that persists throughout recurrent bouts of oral candidiasis.[76,96] In other study of a serial analysis of 17 isolates collected over a period of 2 years, the patients were treated with increasing doses of fluconazole during recurrences of oral candidiasis. The resistance to fluconazole increased in subsequent isolates but they were found to be genotypically identical.[79,129]

3.2 Clinical manifestations

There are at least four distinct clinical variants of oral candidiasis in HIV-infected patients [72] : pseudomembranous (thrush), erythematous (atrophic), hyperplastic and angular cheilitis. Erythematous candidiasis affects about one in two HIV-positive individuals with oral candidiasis, pseudomembranous candidiasis and angular cheilitis are the next most common entities, while hyperplastic candidiasis is the least common. A feature of oral candidiasis in HIV infection is the presentation of the disease in multiple oral sites (in HIV-negative individuals this would be called chronic-multifocal oral candidiasis).[28] About 60% of those with HIV and erythematous candidiasis have multifocal lesions, most frequently on the palate and dorsum of the tongue, which is depapillated.[29] Pseudomembranous candidiasis presents as whitish-yellow, soft and creamy, drop-like or sometimes confluent membranes. Removal of the confluent membranes with gauze leaves a red and sometimes slightly bleeding surface. Pseudomembranous candidiasis lesions involve most frequently the tongue, hard and soft palate and buccal mucosa but may be seen anywhere in the mouth. The hyperplastic form of oral candidiasis in HIV-infected patients is most commonly seen bilaterally on the buccal mucosa and seldom in the retrocommissural area which is, in contrast, the classic site in normal individuals. The lesions are irremovable whitish yellow patches, which must be distinguished from hairy leukoplakia.[71]

4. Diversity of *C. albicans*

In a study of 130 isolates of *C. albicans*, 33 biotypes were found showing the phenotypic diversity in human *C. albicans*.[131] Also, some studies showed a genetic diversity either in different anatomical locations of the same person,[106,17,132] or in the same body sites of different subjects.[132] In addition, different geographic locales influence the genetic variability of *C. albicans*; for example in the study of Clemons et al.[12] showed a high degree of genetic diversity between *C. albicans* isolates from Southeast Asia and those from the United States and Europe.

Phenotyping or genotyping methods can be utilized to demonstrate the great diversity among the strains of *C. albicans*. It is hypothesized that this adaptive strategy of *C. albicans* may assist in survival and colonization of the host that lead to genetic selection and increased *Candida* strain variation. Furthermore, such variation may relate to the pathogenicity of *C. albicans.* For example, it has been shown that **c**hanges in the phenotypic characteristics of *C. albicans* potentiated the advancement of oral candidiasis and, the resistance to antifungal therapy.[3,129] However, phenotypic changes may not accompany the changes in genotypic strains. Marffei et al.[46] studied the phenotypes and genotypes of *C. albicans* strains which were isolated from pregnant women with recurrent vaginitis and showed that alteration of morphotype and antifungal type was observed in 50 % of the patients, but the genotype of the strains isolated from the same patients at different times was identical in all subjects. It was suggested that the recurrent vaginal candidiasis could be caused by the persistence of a single genotype. Some studies assumed that most cases of candidiasis originate from the commensal strain inhabiting the vaginal cavity, oral cavity or gastrointestinal tracts prior to infection. However only limited population genetic surveys support this concept.[19,46] On the other hand, there is evidence of reduced genetic diversity among *C. albicans* which were isolated from oral cavities of HIV-infected patients, suggesting the

possibility that a commensal strain(s) was replaced by a genetically more uniform strain before the inception of oral candidiasis in immunocompromised patients.[7,96] However, the latter data have not been reconformed as yet due to the limited number of isolates required for the genetic studies. In addition, an analysis of *C. albicans* strains from various anatomical locations of the same body in healthy women suggested that different body sites might select for genotypes.[106]

5. Techniques for typing of *C. albicans*

Two major typing methods are used in the study of *C. albicans* : phenotyping and genotyping.

5.1 Phenotyping

Phenotyping has been widely used for epidemiological and taxonomical studies. It includes morphotyping,[105] antifungal typing,[20,46] patterns of sugar assimilation,[36] sensitivity to killer factor,[73] enzyme profiles, [83] serotyping [6] and biotyping method.[65,131] Of these, the most commonly used method is biotyping.

Morphotyping refers to the switching system distinguished by colony morphology on the proper agar substratum or environment. Soll et al.[105] found that there was high frequency switching at the site of infection in 11 patients with acute *C. albicans* vaginitis. The multiple phenotypes (morphotypic change) of a single vaginal isolate at the site of a single infection represented the switched phenotypes of the same strain. Another study [46] found the alteration of morphotype in 50% of the patients with recurrent vaginitis, while the genotype of isolates from the same patient at different times was identical in all cases. Morphotyping is a simple technique with good reproducibility depending on the experience of the examiner and, the results are considered to be reliable.

Antifungal typing is shown by the sensitivity pattern to antifungal agents. It not only permits the distinction of a strain, but also provides important information about the most suitable treatment.[46] Amphotericin B, flucytocine and azole-derivatives, fluconazole, itraconazole and ketoconazole, are the only drugs of value in the treatment of systemic mycoses currently available.[63] So far, most studies have used antifungal susceptibility testing in combination with other typing methods in epidemiological studies.

The pattern of sugar assimilation can be measured by the carbohydrate assimilation ability of *C. albicans*. The commercial available carbohydrate assimilation system for yeast identification is widely used in clinical laboratories for biotyping *C. albicans* [36] and because of its good reproducibility, sensitivity and specificity is commonly used as a reference system for yeast identification, although its discriminatory power is limited. [67,1,12,115]

The killer system makes use of phenomenon that yeast strains can produce a substance lethal to other strains of the same species. This has been a convenient, sensitive and reproducible method used as an epidemiological marker for differentiating strains of *C. albicans.* Polonelli et al.[73] found that all of the *C. albicans* cultures were sensitive to one or more killer yeasts when using this system. This system has the potential to differentiate up to 512 strain types of *C. albicans* according to their susceptibility to the killer effect of nine different killer yeasts.

Enzyme profiling is used for preliminary investigation of *C. albicans* by assessing the enzymatic characteristics of *C. albicans* isolates in the commercially available enzyme profile system, API 20 ZYM.[83]

Serotyping is one the first biotyping methods used to discriminate among *C. albicans* strains using agglutination tests. *C. albicans* isolates were compared for their ability to be agglutinated by a monoclonal antibody [24] and separated into two serotypes A and B. This method is still commonly used to

type strains.[7,108] The shortcoming of serotyping was realized when it was found that antigen expression could be affected by the phase of growth and that serotype B cells could produce serotype A antigen.[75] For the serotyping of *C. albicans* , antigenicity was based on the polysaccharide moities of the phosphomono-protein complex causing a problem with variability.[100]

The commonly used biotyping method was firstly developed by Odds and Abbott[65] for distinguishing *Candida* species and strains of a species.[64,65] The typing method included nine assays involving the growth of cells on tested agar media with different compositions and four additional tests. The system proved effective in a number of epidemiological studies when intralaboratory standardization was achieved.[36,67,68,133] However, it was found to have poor interlaboratory reproducibility.[66,67] At the present, there is a widely used complex biotyping system developed which used combined methods of enzyme profiles (API ZYM system), carbohydrate assimilation patterns (API 20C system) and sensitivity to boric acid.[131] It is reproducible, discriminatory, reasonably fast, easy to perform and needs no special equipment. Using such method, 33 biotypes were found of 130 isolates [131] and 38 biotypes of 218 oral *C. albicans* isolates were found in another study.[116]

All the phenotyping methods have the unavoidable disadvantage of phenotypic change. The same genotype may exhibit different phenotypes, as cellular physiological factors which are expressed depend upon the selective pressure or ecological niche. For that reason, it may not always provide information of genetic relatedness. In early studies, the biotyping method was based on phenotype rather than genotype and therefore runs the risk of grouping isolates which are phenotypically similar but genotypically dissimilar and separating isolates which are phenotypically dissimilar but genotypically similar. This problem is compounded by high-frequency switching, which can reversibly alter several biotyping parameters, including pattern of sugar assimilation, susceptibility to antifungal drugs, and the environmental

constraints on hyphal formation.[36,70,105] A report showed the selection of strains with more resistant DNA subtype was not uncommon during the course of fluconazole therapy in the majority (62%) of the AIDS patients with oropharyngeal candidiasis.[70]

The phenotypic study is therefore not a "gold standard" method by which to determine strain relatedness. Over the last decade, the myriad of molecular biology-based methods for DNA typing or genotyping has greatly simplified the analysis of diversity of *Candida* species.

5.2 Genotyping

In the development of genotyping methods for clinical and epidemiological analysis, three commonly used methods have been widely applied for genotypic analysis of *Candida albicans* : (1) restriction endonuclease analysis (REA),[1,9,12,19,20,45,46] (2) randomly amplified polymorphic DNA (RAPD) [12,17,19,30,53,124,132]and (3) pulsed-field gel electrophoresis (PFGE).[12,53, 70]

5.2.1 Restriction endonuclease analysis (REA) uses endonucleases that cut the DNA molecule at a limited number of specific locations. These enzymes called "restriction enzymes" were discovered in bacteria in the late 1960s. In nature, these enzymes protect the bacteria against intruding DNA from other organisms, such as virus or other bacterial cells. They work by cutting up the foreign DNA, a process called "restriction". Most restriction enzymes are very specific, recognizing short nucleotide sequences in DNA molecules (restriction site) and cutting at specific points within these sequences.[39]

Restriction endonucleases cut covalent phosphodiester bonds of both strands. Since the target sequence usually occurs (by chance) many times in strand of DNA molecule, an enzyme will make many cuts. Copies of a DNA molecule always yield the same set of "restriction fragments" when exposed to that enzyme. In other word, a restriction enzyme cuts a DNA molecule in a

reproducible way. After eletrophoresis, the DNA fragments which contain different molecular weights are shown as a banding pattern under UV light (Figure2).

The REA method has shown to be extremely reproducible and highly discriminatory. However, it requires a greater amount of DNA and is time consuming.[26,45,94] A study reported that the resolving power of REA was adequate for discerning strain differences,[53] although some studies showed that the results of analysis by REA and RAPD are the same.[12,38]

5.2.2 Randomly amplified polymorphic DNA (RAPD) is a technique based on polymerase chain reaction (PCR) principles using random primers. Any piece of DNA can be quickly amplified by these random primers and easily visualized as a discrete band of specific size when submitted to agarose gel electrophoresis (Figure 3).

RAPD analysis is a faster and less technically demanding methodology;[38,45] smaller amounts of purified DNA (i.e., < 25 ng) are required compared with the amounts needed to do REA or PGFE. The reproducibility of RAPD analysis is dependent upon the careful standardization of the PCR conditions. The overall RAPD patterns are stable in repetitive experiments, even though differences in band intensity do occur.[26] Some studies used only RAPD analysis for genetic studies because of the advantages of high discriminatory power, reproducibility, requiring little starting material, giving rapidity and ease of performance.[1,38,124,132]

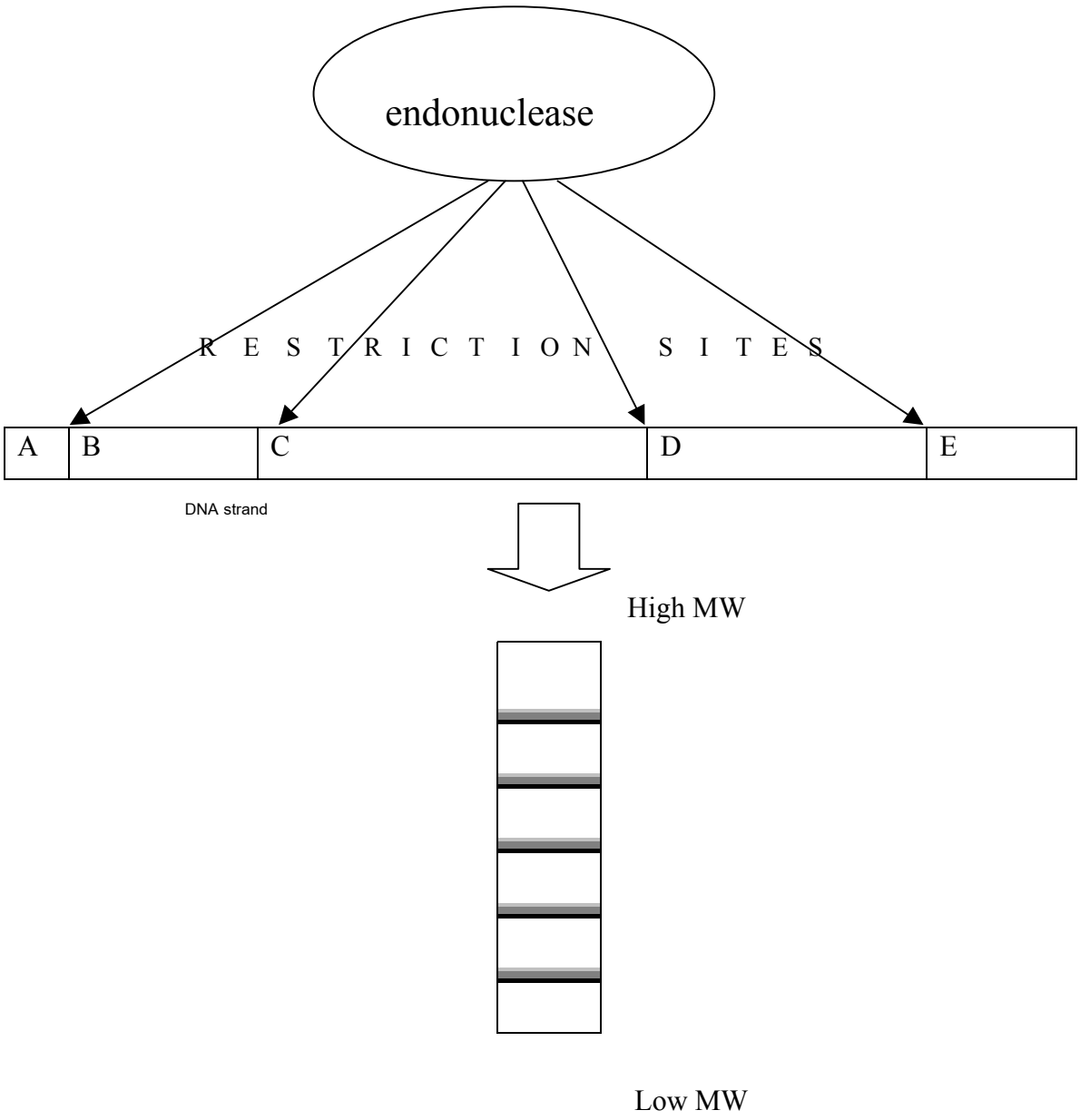

Figure 2 Restriction endonuclease cuts DNA strand at the specific restriction sites and DNA fragments are electrophoresed in a banding pattern running from high molecular weight to low molecular weight: fragment C, D, B, E, A orderly.

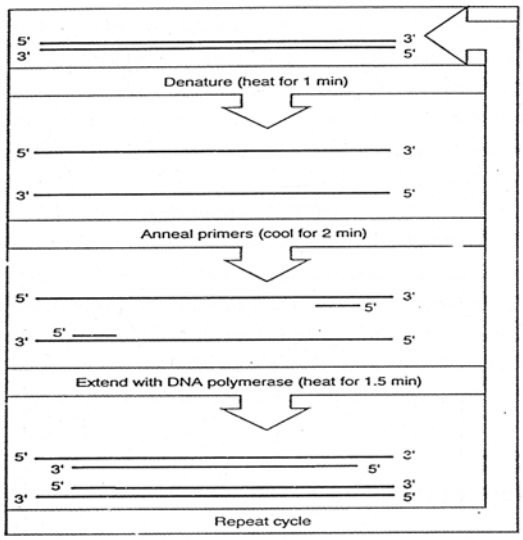

Figure 3 A single cycle of PCR produces a number of copies of a target sequence and can be performed in 5 minutes.

5.2.3 Pulsed – field gel electrophoresis (PFGE) is a genotyping technique where chromosomal separations are made by contour–clamped homogenous electric field (CHEF) electrophoresis. Plugs containing chromosomal DNA are loaded into agarose gel in TBE buffer and resolved in an electric field. The resulting chromosomal bands are visualized by UV transillumination after ethidium bromide staining and photographed to analyze the genotypes.[26]

PFGE karyotyping analysis has been found to be less discriminatory than either REA or RAPD.[12,19] This technique may be considered to be the most difficult method to perform because it requires intact chromosomes, needs specialized eletrophoresis equipment and takes time (over 48 h. per run).[12,26,45] However, some authors have indicated that it is a highly sensitive and a useful method of strain discrimination.[53,122,125]

Rationale for the Study

Oral candidiasis is a major cause of chronic discomfort and irritation, and may be life-threatening in HIV-infected patients. Besides, the factors resulting in candidial strain variation (Figure 1), typing or identification of the causative strains of *C. albicans* is essential in clinical investigation for finding the pathogen of the disease. Studies in these areas could directly lead to the successful prevention and treatment of candidiasis. These typing studies can result in finding the route of infection whether it comes from exogenous or endogenous source.

If the infection comes from exogenous source, as proposed by the "replacement" hypothesis, the commensal strains are replaced by new certain genotypes which are more virulent, then identifying the routes of transmission of these potentially more virulent genotypes could lead to measures that limit their spread.[132] The study of *C. albicans* strain analysis between partners in HIV-infected couples [20] suggested that *C. albicans* of oral isolates could be transmitted between sexual partners and that replacement of the oral flora, especially by a resistant clone (fluconazole-resistant isolates), could cause oropharyngeal candidiasis in HIV-infected patients. In another study, Schmid et al.[96] showed that the genotypic diversity among the AIDS-group strains was significantly reduced when compared with that of strains from non-HIV-infected control population. In the majority of patients with AIDS, the original commensal strains were replaced, with replacement occurring early in the disease process, and occurring only once. Mehta et al.[53] showed that there is an intrafamily genotypic identity (i.e., each member within the family harbored the same strain) in six of twelve families, and two or more members of a family commonly shared the same strain. They showed the ability of replacing some strains of the original strain in the same host group, and it has been suggested that HIV infection might be associated with the selection of *C. albicans* strains

replacing the original commensal strains. Therefore, this hypothesis would express homogenous genetic strains, and show a high degree of genetic similarity among strains associated with specific body sites and/or certain types of hosts.

If the infection is from endogenoud commensal strains, proposed by the "persistence" hypothesis, the process of recurrent episodes of oral candidiasis is caused by original commensal strains which then persist during the course of HIV infection. This would predict that strains isolated from patients during HIV infection would display genetic diversity as similarly distributed as in normal subjects. Many reports have supported this hypothesis. Whelan et al.[128] determined the genotypes of *C. albicans* in patients with AIDS and found that there was a variety of strains associated with oral and esophageal candidiasis. Anthony et al.[1] reported that the genetic patterns of *C. albicans* isolated from HIV infected patients were distinct in different patients, and by observations in the following months found that the same patterns of genetic strains persisted over time. Other genotypic studies also found that there was genetic diversity in *C. albicans* isolated from the oral cavity of HIV-infected patients [9,19] and indicated the genotypes of *C. albicans* tended to remain stable, that colonizing strains developed resistance and phenotypic change in the longitudinal period.[46,52,56,92] All the studies above have shown that the causative genetic strains have persisted along the time of HIV infection even though the phenotypes had been changed with time.

Even the most recent studies have not yet clarified the nature of *C. albicans* infecting HIV patients whether it is caused exogenously or endogenously. In order to prove these hypotheses, the distribution of genotypes has to be studied. And to find out whether the exogenous or endogenous strains are truly virulent or causative for candidal infection, the combination of genotyping and phenotyping that can represent virulent property such as antifungal susceptibility should be studied. Therefore this can identify the

causing pathogen as well as establish the more specific treatment for these patients.

From reviewing the literature, previous clinical epidemiological studies in typing of *C. albicans*, genotyping or phenotyping has been mostly used. However, the study in their association is very few. The study in such way may lead to the finding of appropriate typing method. Therefore a possible association between the phenotype with the genotype and the progression of HIV disease would be interesting to study.

In Thailand, there is no report in genotypic study. Only the data of phenotype of *C. albicans* isolated from a group of AIDS patients using standard biotyping method has been reported.

Therefore, this investigation should be studied in phenotype as well as their genotypes and, whether there is any relation between the phenotype and genotype.

Aims of the Study

The aims of this study were:

1. To investigate the phenotypes and genotypes of *C. albicans* in a group of Thai HIV-infected patients with oral candidiasis using a standard biotyping method and an RAPD technique, respectively.
2. To determine the association between the genotypes and biotypes of *C. albicans* and their antifungal susceptibility.