Chapter 4

DISCUSSION

Several typing methods have been used for strain delineation of *C. albicans*. These include typing system based on (i) factors associated with virulence or pathogenesis, which have not been able to predict the pathogenic potential of strain;[55] (ii) phenotypic characteristics, for example the resistance to toxins in the "killer system", biotyping methods, or the colonial morphology of the strains; (iii) typing system based on genetic techniques i.e. genotyping. There is a general consensus on the necessity to determine whether recurrent oral candidiasis is due to relapse, caused by persistence of the yeast in the oral cavity, or due to re-population from an endogenous source (eg. vagina, skin), exogenously or, completely new infection. The above typing methods for strain differentiation in terms of phenotypes and genotypes have therefore been used as an important tool in the attempt to resolve this doubt. Although recent advances in molecular biology have permitted classification of *C. albicans* into subtypes, the combination of phenotypic and genotypic typing methods may be of taxonomic and epidemiological relevance. Here a genotyping and a biotyping system together with antifungal susceptibility testing was used to elicit a relationship between the foregoing features of *C. albicans* from a group of HIV-infected and healthy individuals.

Biotyping systems based on phenotypic characteristics have been useful for diagnostic and epidemiological investigation since this simple method allows differentiation of *C. albicans* strains.[131] From this study of 112 *C. albicans* strains obtained from HIV-infected individuals, 57 biotypes were generated indicating a high level of discrimination of the system and the phenotypic diversity among the strains.

However, possible errors in biotyping method arise due to poor quality control of laboratory media leading to unsatisfactory interlaboratory

reproducibility.[11,67] Hence, the biotyping method of Williamson et al.[131] was applied, where quality controlled, commercially available systems (API 20C, API ZYM system) made the technique extremely reproducible. Boric acid resistance has been previously used and was found to be equally reproducible.[66,127,131] Also, the *in vitro* antifungal susceptibility testing according to NCCLS system leads to standardization and uniformly comparable data.[14,126]

Moreover, phenotypic switching in a minority of strains may lead to high frequency variations and unstable results. Genetic typing is therefore called for since the DNA sequences are unlikely to be affected with minor environmental changes.[42,92,114]

One of the frequently used genotyping methods or *Candida* is based on the generation of visible bands due to separation of DNA fragments produced by restriction enzymes (RFLP). Subsequent hybridization of previously digested DNA fragments with specific DNA probes or using moderately repetitive sequence have been used to discriminate *C. albicans* strains. Other methods employed are karyotyping, or analysis of chromosome bands obtained by pulse field gel electrophoresis (PFGE) and, the Random Amplified Polymorphic DNA (RAPD-PCR) obtained by PCR amplification. Among these molecular approaches for genotyping *C. albicans*, RAPD is the least labor-intensive, rapid in application, and is the technique of choice for large-scale clinical epidemiological studies.[18,23,38,117] Although RAPD is very effective method for evaluating genetic profiles of clones of *C. albicans*, the subjective interpretation of band profiles becomes unwieldy when a large number of isolates are investigated. Hence in order to compare these genetic profiles of up to 40 isolates, an automated computer program, Dendron, was utilized.[104]

Many RAPD studies have employed previously reported primers for their investigations.[12,19,53,77,107,132] Here, a number of primers were also selected from previously reported studies and molecular profile differentiation was greatest with primer RSD11. Hence the latter was used for the main

experiment with 189 strains of *C. albicans* HIV-infected patients and 17 strains from healthy individuals.

The cell wall of *C. albicans* is a multilayered structure which composed mainly of glucan and mannan polysaccharides with lesser amount of chitin, protein and lipid. For extraction procedure, it must be designed to remove these macromolecular components from the wall by degrading their native structure.[61] Therefore *Candida* cell wall can be removed by pretreatment of the cells with sulhydryl-reducing agents, to disrupt disulphide bonds in wall proteins and, a mixture of enzymes, including polysaccharides, proteinase and chitinase to effect complete wall removal.[10,21] In the present study, cell wall lysis was accomplished using the chemical agents and enzyme (lyticase). Most workers have used this procedure for extracting DNA from the cells.

Several early studies of *C. albicans*, primarily using serotyping and biotyping techniques have suggested that a variable number of *C. albicans* strains causes oropharygeal disease in HIV-infected patients.[7,36] However, other studies using molecular techniques have shown that in fact most patients are infected with a unique strain during the long-term follow-up period by indicating the less predominant strains were not more drug resistance.[3,57,76,128] In a recent longitudinal study of oral *C. albicans* isolated from HIV-positive patients, Samaranayake et el.[92] reported that some patients maintained the identical *C. albicans* isotype on contiguous sequential visits, while in others, this was found irregularly. They explained that the failure to isolate the genetically identical strains during successive visits may be due to the random selection of only five yeast colonies from the primary culture. Because of the limited number of colonies randomly chosen at any one sampling, this may not represent the population in oral cavity. These studies leave many questions unanswered about the causative strains that whether the disease caused from the majority (predominant) or minority (less predominant) of *C. albicans*

strains recovered. However, it could be generalized from these previous studies and the current observations the persistence of an identical strain may occur during HIV disease progression.

In this study, RAPD-PCR genotypic study indicated that a large number of strains are loosely connected when observed through the dendrogram data. This suggests that genetic shuffling may have taken place during HIV infection. Here, high degree of loosely connected *C. albicans* strains occurred in the vast majority (accounting at 76% i.e. S_{AB} less than 0.80). From this data it was noted that the variation of genotypes are not only between persons but also within a single individual as shown by different genotypes (2-15 genotypes) discovered in a single person (see Appendix IV). Several previous studies also showed the genotypic shuffling in groups of HIV-infected patients on long-term observation.[15,37,52,57,76,79,92] They reported that the identical strain colonized on a majority of times while a small number of different strains were only present less frequently.

To study the relationship between genotype and phenotype, the previously reported data of biotyping and antifugal susceptibility testing in our laboratory was used.[116] The biotyping results showed 57 biotypes with the major biotype A1S accounting for 30.4%. Other previous study of biotyping in HIV-infected group from diverse geographic locations, Hong Kong, Australia, Germany and England, demonstrated that the major biotpype was A1R (17.9%) and the second commonest biotype was A1S accounting for 11.1%.[120] Similarly, another previous study in healthy individuals in Britain found that A1S and A1R were also the commonest biotypes, accounting for 26% and 23% of all isolates, respectively.[131] The predominant biotypes that have been found in Chinese and Tanzanian population were A1S (21% of isolates) and J1S (19.5%).[49,133]

In a previous report, it was found that there was no difference in biotypic profile among HIV-infected and HIV-uninfected individuals with and

without oral candidiasis in a Thai population.[116] However, the sample size was quite small for statistical analysis. Another similar but unrelated study has reported no significant difference between the groups of HIV-uninfected patients with and without chronic hyperplastic candidiasis,[130] a condition uncommon amongst Thai patients.

The association between biotype, genotype and, antifungal susceptibility has not been described before. This study showed no association among these three parameters. The A1S biotype, for example, belonged to many different genotypes. In addition, it was found that most of the "low" antifungal susceptible strains belonged to minor biotypes.

One way to ascertain the pathogenic *C. albicans* strains may be to study their antifugal susceptibility. The strains with a high MIC could be considered more virulent than those with lower MIC. The level of susceptibility may also depend on the resistance to antifungal agents *in vivo.* Other studies have demonstrated the true emergence of *C. albicans* strains resistant to antifungal drug.[3,46,56,79] However similar phenomenon has not been described for ketoconazole. In this data on ketoconazole susceptibility testing, we found diverse levels of MIC associated with strains isolated from different individuals was found. Hence, it is tempting to postulate that several different genotypes in the same oral niche may act differently with regard to antifungal sensitivity.

The results of ketoconazole susceptibility testing yielded diverse MICs ranging from 0.0075 - ≥ 4.0 μg/ml (Table 9). When the "high" and "low" susceptible strains were sub-divided, we found 27% (25/94) low-susceptible and the remainder highly susceptible. This indicates that most of the *C. albicans* strains in this study has not developed resistance to ketoconazole. In addition, when the MIC ≥ 4.0 μg/ml was considered, only 9.57% (9/94) of *C. albicans* strains filled in to this category. Another recent longitudinal study in oropharyngeal candidiasis has shown that *C. albicans* developed fluconazole resistance (MIC ≥ 64 μg/ml) over a time in 29% (12/42) of all isolates.[50]

Furthermore, other observation showed some instances of a high prevalence of non-*C. albicans* replacement, with a higher drug resistance species in late-stages of HIV infection especially *C. dubliniensis*.[37,50,80,102] The trend in presence of non-*albicans* is more frequently associated with severe symptoms of oropharyngeal candidasis. This calls for further investigations on candidal species and changes in their antifungal resistance in the context of opportunistic infections in HIV-infected patients.

The concept that a microorganism can alter its phenotype by changing its gene expression in response to changes in its environment is part of central dogma of biology. However, changes in expressed phenotype can be classified under three headings according to the frequency and reversibility of the change. The most usual change of expressed phenotype is “mass conversion”, in which alteration in the environment trigger reversible changes in gene expression in almost all cells of a population. “Point mutation” is a different type of phenotypic variation in which random DNA mutation lead to changed phenotypes in the individual cell. Such a phenomenon is irreversible but its frequency of occurrence within a population is generally low. The last heading is “phenotypic switching”, as defined by Soll,[103] is the result of spontaneous alterations in gene expression that arise at frequencies higher than in point mutation but lower than in mass conversion. This phenotypic switching is also reversible.

Such phenotypic switching has now been recognized among many microorganism and it is regarded as a possible microbial strategy to escape host defenses. Evidence is now accumulating that phenotypic switching may serve as an attribute of virulence or resistance to host defense in at least one pathogenic fungal species. The extent to which switching behavior plays a role in the disease process may even extend to the development of resistance to antifungal agents in the course of treatment.[64]

In this study, the phenotypic switching phenomenon was not evaluated. But the fact that ketoconazole resistant and sensitive strains of *Candida* were having the same genotype may indicate the possibility of such switching that may occur *in vivo*, intra-orally. Further studies are however warranted to confirm or refute this hypothesis.

HIV-infected patients have frequent episodes of oral candidiasis. Acute and recurrent infections are common in these patients because of immunosuppression who may be on antifungal prophylaxis to prevent reinfection. Thus in clinical terms, regular antifungal susceptibility tests may be necessary since there are frequent reports of treatment failure.

It is also likely that long-term antifungal prophylaxis increases the risk of developing drug resistant strains.[54] Therefore, use of antifungal drug should be limited to treatment and not prophylaxis. Concurrently, the physicians who are faced with a dilemma selecting an antifungal agent appropriate to treat this condition might choose susceptibility test as indicator for therapy. Azoles are widely used in the treatment of candidal infections in HIV disease. Here, ketoconazole was used for the study of susceptibility testing as a surrogate for an azole drug but no clinical data were obtained from these patients. In future studies clinical and laboratory data should be correlated to derive useful information.

As for the second objective of the study, the relationship among genotype, biotype and antifungal susceptibility testing was analyzed amongst 106 and 94 isolates respectively. There was no association among them when using Chi-square test, $p < 0.01$ (Table 9). There are few studies in assessing the relationship of three typing methods. One study indicated no correlation between genotype and antifungal resistance.[15] In addition, the absence of any meaningful association between genotype and drug resistance imply that the wide variation in antifungal resistance is not due to inherent differences in the

ability of yeasts to develop drug resistance. This phenomenon may be due to the exposure to the selective pressure imposed by the presence of the drug.

Genotyping of *C. albicans* in Thai HIV-infected patients may be further expanded by correlating data from other geographic locations. There is a previous study indicating a high degree of genetic diversity in between *C. albicans* isolates from Southeast Asia and, from the United States and Europe. [12] Hannula et al.[23] reported no distinct genotypic differences between Finnish and American subjects. Other previous studies revealed that the isolates from Europe included European-specific clade [78] while South Africans obtained its specific clade.[4]

This study also compared genotypes of HIV-infected and healthy HIV-uninfected individuals. The number of samples from the latter group was not adequate for statistical analysis. Further, to achieve this aim, not only the sample size but also the number of strains per person should also be similar. According to some number of strains per person per visit should be as much as five whereas others suggest higher number. In this study, due to technical problems with stored isolates drying or getting contaminated, the number of strains from each subject was irregular (1-15 strains). Future workers should include a comparative number of control and test strains from healthy and diseased subjects, respectively to obtain useful data.