CHAPTER VI

DISCUSSION

Candida spp. has become c ommon h uman p athogens c apable c ausing b oth l ocal and systemic infection. A major concern is a disseminated infection, witch occurs with increase prevalence in post operative and immunocompromised patients. Numerous on the prevalence of difference Candida spp. have led to the general consensus that C. albicans is the most commonly isolated species. However there has been a growing trend of recovery of non-C. albicans species. One possible explanation is the increased use of fluconazole and other azole, which has positively selected for some less sensitive Candida species, such as C. glabrata and C. krusei [2, 80, 163]. In contrast others have reported that there were no significant changes in the prevalence of Candida species with fluconazole use [82] and that C. albicans is present at a stable level and is still predominant in systemic infection [23].

At present, yeast infections are usually treated as a general fungal infection and agents such as the polyene amphotericin B or the newer azole drugs, which are intended to control a broad array of fungi, are used. The treatment is usually continued for an extended period of time. These agents are not always successful since the widespread use of these generalized drugs has resulted in the rapid development of antifungal drug resistance. An analysis of clinical isolates indicates that resistance is due not only to resistant strains of C. albicans but also to an increasing number of non-albicans Candida strains. Various yeast species appear to develop resistance to the commonly used drugs at frequencies much higher than that for C. albicans. For instance, C. tropicalis and C. parapsilosis, which are both associated with endocarditis, are inherently resistant or can quickly develop resistance to polyene and azole drugs. C. dubliniensis, which is associated with oral candidiasis, has been shown to develop stable fluconazole resistance at a high rate after exposure to azoles. The MICs of azole drugs for other yeast species such as C. glabrata, which is associated with cancer and bone marrow transplant patients, are significantly higher than those for C. albicans. The present levels of drug doses used can suppress the growth of sensitive strains but allow the growth of the more resistant species. These organisms, which can quickly develop resistance or for which the MICs of the presently used drugs are higher, probably account for a large number of resistant yeast infections in certain populations.

In 2003, Yang *et al.* [164] investigated the prevalence *Candida* species in three hospitals and community clinic in Singapore, the results were 62.1% of *C. albicans*, 17.9% *C. glabrata*, 8.3% *C. tropicalis*, 5.8% *C. parapsilosis*, 2.7% *C. dubliniensis*, 1.0% *C. krusei*, and 0.6% *C. guilliermondii*. The distribution of *Candida* species in our study differs from that reported. In particular, the prevalence of *C. albicans* was comparatively low (45.9% versus 62.1%), although it was still the predominant species. The most prevalence species in our study after *C. albicans* were *C. tropicalis* (21.7 %), *C. parapsilosis* (15.8 %), *C. glabrata* (7.5 %), *C. dubliniensis* (5.0%), *C. guilliermondii* (2.5 %), and *C. krusei* (1.6%). In our reported, a striking finding was that *C. albicans* and *C. dubliniensis* are distinct by our method, whereas their biochemical phenotypes are very similar. *C. dubliniensis* may be underreported in clinical sample because most current used isolation and identification method fail to recognize this yeast. *C. dubliniensis* is an emerging pathogen garnering attention for its ability to develop *in vitro* resistance to fluconazole [137]. The majority of *C. dubliniensis* isolates have also recovered from the oral cavity of HIV infected. However *C. dubliniensis* has also been found as an oral carriage organism and have been implicated as an agent oral candidiasis in HIV-negative patients including normal healthy individual and diabetic.

Our study found *C. albicans* in sputum (25.4%), oral swab (18.2%), BAL (18.2%), body fluid (14.5%), blood (12.7%), skin (3.6%), and other (7.2%). *C. tropicalis* was found in blood (34.6%), urine (19.2%), body fluid (11.5%), corneal swab (7.7 %), BAL (7.7 %), sputum (7.7 %), and others (11.5 %). *C. parapsilosis* was found in nail (47.4 %), skin (10.5 %) corneal swab (10.5%), blood (10.5%), and others (21.1%). *C. glabrata* was found in BAL (33.3 %), blood (22.2%), sputum (22.2%) and others (22.2%). *C. guilliermondii* found in blood (66.7%) and body fluid (33.3%). Two isolates of *C. krusei* was found in nail and body fluid. *C. dubliniensis* was found in sputum (83.3%) and pus (16.7%). In blood specimens we found that *C. tropicalis* (40.9%) was higher than *C. albicans* (31.8%). It differs from previous reported that showed *C. albicans* is predominant in candidaemia [164-166]. The same as previous reported in oral swab, sputum, BAL and body fluid, *C. albicans* were also predominant. In nail, we found that *C. parapsilosis* was the dominant organisms (81.8%) whereas *C. albicans* was found only 9.1 %, it differs from Dorko *et al.* and Jautova *et al.* study that *C. albicans* was found mostly in this specimen [167, 168].

The comparative of identified *Candida* species results between our conventional results and routine work showed uncorresponding species, our study found *C. albicans*

higher than in routine work (50.8% versus 35.8%). The reason was in routine of Mycology Unit, Chulalongkorn Hospital used only chlamydoconidia for identified *Candida* spp. whereas our study used more other testes such as germ tube production, carbohydrate assimilation and fermentation in identified *Candida* spices. In our conventional method, 38 isolates o f 1 20 i solates g ave t he u ncorrespond p atterns i n carbohydrate assimilation and fermentation. However, we used the *Candida* commercial kit (API 20C AUX) for confirmed these isolates. Interestingly, we found the one isolate that germ tube was negative and carbohydrate assimilation and fermentation uncorrespond to any *Candida* species, but positive in chlamydoconidia production. The API 20C AUX result of this isolate was *C. albicans* with 97.3 % identity. This result suggests that the genotypic base approaches may show advantages over phenotypic. Identification of the various species of *Candida* spp. is a daunting problem. Thus, currently used methods are mainly base on phenotypic characteristics and therefore lead to inconsistent results. The aim of this study was to evaluate a new PCR-RFLP base approach for the identification of seven species of *Candida* on the RFLP patterns of the rDNA internal spacer region.

Various molecular techniques using the ITS regions as molecular targets for identification of fungi have been evaluated. These methods include direct sequence analysis of amplified DNA [140, 160, 169], utilization of genus- or species-specific primer and oligonucleotide probe [148, 153] and PCR-RFLP [55]. This study used the ITS1 and ITS4 primers to amplified the ITS region of rDNA. For the assay optimization, the lower limit of detection using *Candida* DNA extracted (all reference strains) for this assay with ITS1 and ITS4 primers was 400 fg of DNA/ sample. Assuming the DNA content of 39.7 fg (range, 37.1 – 41.4 fg) per *Candia* organism [170], this sensitivity may be capable of ten *Candida* yeast/sample. This limit of detection of *Candida* DNA was very close to previous report by Wahyuningsih *et al.* [146], and Virginia *et al* [154].

This study showed that seven medically important *Candida* species could be distinguished on the PCR products and RFLP of ITS region using ITS1 and ITS4 primers. *C. glabrata* and *C. guilliermondii* could be identified on the basis of PCR product size. The identification of the remaining species, *C. albicans, C. tropicalis, C. parapsilosis, C. krusei* and *C. dubliniensis* were necessary used the characteristic profiles of the restriction digestion (*Hae* III, *Dde* I and *Tru9* I) of PCR products. Characteristic profiles, obtained following restriction digestion of PCR products, allowed the discrimination of the remaining isolates studied, with the restriction enzyme *Tru9* I providing the greatest level of species discrimination. Digestion of the amplified PCR products with a combination of

Hae III, *Dde*I and *Tru9* I restriction enzymes would allow identification of *C. albicans, C. dubliniensis, C. parapsilosis, C krusei, C. tropicalis, C. glabrata* and *C. guilliermondii.* Several studies using a combination of PCR of regions of the rDNA repeated follow by restriction digestion as a mean of species specific identification have been published. Niesters *et a l.* [51] a mplified t he s mall s ubunit r DNA a nd, u sing a c ombination of five enzymes, could distinguish four *Candida* species. Maiwald *et al.* [171] used a similar procedure to identified presumptively eight *Candida* species with PCR products of the small subunit rDNA and digestion with six restriction enzymes. Williams *et al.* [55] used PCR to amplified the internally transcribed spacer region of the rDNA repeat and digested the amplified fragments with three restriction enzymes. They conclude that eight *Candida* species could be identified on the basis of size and sequence variation. In addition to six of seven s pecies i ncluded i n o ur s tudy e xcept *C. d ubliniensis.* T hey i dentified 2 a dditional species; *C. pseudotropicalis* and *C. stellatoidea.* In their study, it is hardly to interpret of *Candida* species because of no *Candida* reference strain patterns whereas our study has a *Candida* reference strain patterns for interpretation. From our data show this method is a suitable method for identification of *Candida* species, particularly, for indicating the incidence of *C. dubliniensis.*

In our study, both the reference and clinical strains gave exact the same RFLP patterns except with *C. albicans.* We detected some differences in the *Tru9* I RFLP profiles of clinical *C. albicans* that show in Figure 21, (CA2-CA7). In contrast with *Hae* III and *Dde* I restriction enzymes (Fig.22) did not detect any genetic difference between reference and clinical *C. albicans* strains. All atypical *Tru9* I RFLP isolates were confirmed with *Mbo* I, and the result of this enzyme showed the same pattern as *C. albicans* reference strain (Fig.23). This might be due to polymorphism or mutations that are present in the strain analyzed, which changes the restriction site thereby, resulting in a fragment of difference molecular weight and size. In 2002, Millon *et al.* [160] studied in sequence analysis of the PCR–amplification ITS region of rDNA using ITS1 and ITS4 primers showed six variable sites in the base order yielding 10 type of sequences from 39 *C. albicans* isolates (Table 7). Adding of *Tru9* I restriction site (T/TAA) will be found in mutation at position 106 (nucleotide change C to T) and position 130 insertion of T. It was resulting in change a pattern of *Tru9* I of *C. albicans* clinical isolates (Fig 21). Three distinct patterns were observed from clinical isolate (CA5, CA6, and CA7), and other patterns (CA2, CA3 and CA4) showed a prominent band that could be attributed to the heterozygous at the rDNA locus. From the sequencing analysis, we confirmed that it has

been the insertion mutation at the same position of previous report and created a new *Tru9* I restriction site (Fig 25B).

The restriction profile of the ITS rDNA gene suggests that the difference (taking *Dde* I enzyme as an example) is due to a single *Dde* I restriction site change in *C. dubliniensis* and *C. albicans*. We optimized a molecular method for identification of seven *Candida* species using to amplify a rDNA segment, and RFLP to further differentiate among the species. Seven *Candida* reference strains and 120 clinical isolates have been successfully analyzed with three-difference cutter restriction enzymes in this identification technique. An additional advantage of this genotypic approach to the identification to species level of *Candida* isolates is its rapidity. Other workers have reported the identification of certain *Candida* species based on molecular methods, but often these studies have included lengthy procedures such as hybridization steps requiring overnight incubations. Furthermore, other studies have not tended to examine large numbers of isolates that include all the recognized pathogenic *Candida* species. Identification of these *Candida* species can be achieved within a working day by this procedure, although overnight digestion of PCR products with restriction enzymes does increase the time required to identify other *Candida* species. In summary, a genotypic method has been evaluated for the identification of *Candida* species. The reliability of the technique has been demonstrated by the examination of a large number of *Candida* isolates from a range of sources. From a study we recommend that PCR products size is the first step for analysis if the product size around 600-800 bp, it should be cut only with *Tru9* I for confirm species and if it around 500 bp, it should be cut with two enzyme; *Tru 9* I and *Mbo* I or *Tru9* I and *Dde* I. This strategy could be applied to routine work for identification of *Candida* spp. The future studies of this thesis are sequencing analysis of the atypical patterns of *C. albicans* cutting with *Tru9* I for confirm the hypothesis of the mutation in resulted the *Tru9* I cutting site change.