Chapter 2

MATERIALS AND METHODS

1. *C. albicans* strains

A total of 206 isolates: 189 strains from 41 HIV-infected patients with oral candidiasis were studied and 17 strains from six non-HIV subjects without oral candidiasis were included in this study (details shown in Appendix I). These 41 HIV-infected patients were at CDC-stage IV and admitted to Songklanagarind Hospital, Songkhla, Thailand because of their disease. The other six HIV-seronegative healthy blood donors who were asked to be volunteered for this study. *C. albicans* were collected using the oral rinse method of Samaranayake et al.[87] and were identified by the API 20C AUX and chlamydospore test. All yeasts were preserved in Sabouraud's dextrose agar (SDA) at room temperature as stock cultures, and sub-cultured regularly.

2. DNA extraction

All *C. albicans* isolates were subcultured twice on SDA and incubated at 37°C for 24 hours in order to obtain fresh cultures. Cells were harvested and transferred to 10 ml YEPD broth (0.3% yeast extract, 1% mycopeptone, 2% dextrose) for overnight growth at 37°C on an orbital shaker. One and a half milliliter of broth containing yeast cells was centrifuged and the pellet was washed in 1 ml of 1 M sorbitol. To digest the cell wall, cells were re-suspended in 1 ml of 1 M sorbitol, 50 mMKH_2PO_4 (pH 7.5), 3 μl of β-mercaptoethanol and 150 units of lyticase. The mixture was then incubated at 37°C for 90 minutes, and centrifuged to remove the supernatant. Cells were lysed using 0.5 ml GES reagent (see Appendix II) and left for 20 minutes at room temperature. One hundred microliters of 5M potassium acetate and 0.5 ml phenol : chloroform : isoamyl alcohol (25:24:1) were then added, mixed well and centrifuged. The top layer was collected in a new tube without disturbing the

middle white layer (the bottom layer was discarded) and the supernatant added to 300 µl chloroform, centrifuged and collected in a new tube. DNA was precipitated with 0.5 ml 2-propanol and suspended in 50 µl TE (10 mM Tris and 1 mM EDTA pH 7.5). RNA was digested with 1µl RNaseA (5 mg/ml) stock solution at 37°C for 15 minutes. The DNA was re-precipitated using 0.5 ml 2-propanol and washed with 0.5 ml 70% cold ethanol. DNA pellet was air dried or slightly warm temperature (e.g. 37°C). Finally, DNA was dissolved in 60 µl TE (Tris EDTA pH 8) and stored at -20°C until assayed.

3. DNA concentration measurement

After extraction, the presence of DNA was demonstrated on 1.5% agarose gel, stained with ethidium bromide and viewed on UV translumination. The concentration of DNA was calculated from the absorbance measured according to the formula below.

Concentration of sample = Abs_{260} x DF x 50 µg/ml

Abs_{260} = absorbance of the sample at 260 nm

DF = dilution factor

The purity of DNA was calculated using the absorbance ratio (Absorbance ratio = $A_{\lambda 260}$ / $A_{\lambda 280}$) and, a value of equal or greater than 1.8 indicated the required purity.

4. Primers

Seven random primers were tested initially for RAPD analyses. They were selected because they have been successfully used in previous studies for analyses of *Candida* or other yeasts. The details of these primers and references are shown in Table 2.

Table 2 Random primers tested in pilot studies for RAPD analysis of *C. albicans* isolates.

Name of Primer, Sequence (5′-3′)	Reference
1. Primer 1 (5′-ACA ACT GC TC-3′)	- Howell, Anthony and Power 1996,[30] Mazurier, et al. 1992.[48]
2. M 13 (5′-GAGGGTGGCGGTTCT-3′)	- Diaz-Guerra, et al. 1997,[19] Xu, et al. 1999.[132]
3. PA 0 3 (5′-AGTCAGCCAC-3′)	- Xu, et al. 1999,[132] Hannula, et al. 1997.[23]
4. C1 (5′-ACGGTACACT-3′) and C3 (5′-GTTTCCGCCC-3′)	- Holmberg and Feroze 1996,[26] Metha, et al. 1999,[53] Clemons, et al. 1997.[12]
5.RSD11(5′GCATATCAATAAGCGGAGGAAAAG-3′) 6. RSD12 (5′-GGTCCGTGTTTCAAGACG-3′)	-Department of Oral-Biosciences, Faculty of Dentistry, University of Hong Kong

* C1 and C 3 were used in combination for RAPD study.

5. PCR assays

Firstly, the primers were tested using PCR according to the instructions from previous studies (Table 2). If a successful result was not achieved then adjustments to the reactants and/or the temperature profile was made until a satisfactory result was obtained. The relevant details including the concentration primers and $MgCl_2$ used, and PCR reaction condition are shown in Table 3.

6. Gel electrophoresis and gel images

The amplicons were electrophoresed on 1.5% agarose gel electrophoresis in 1 x TBE buffer. A 1kb DNA ladder was used as a size marker. Gels were internally stained with ethidium bromide (concentration of 4.5 μg/100 ml) and visualized on UV transluminator. The gel images of all the isolates were captured by a digital camera (Nikon® Pronea 6I DCS 330) and then digitized into the Dendron database to develop the dendrogram.[104]

7. Biotyping

One hundred and six isolates were biotyped using the method of Williamson et al.[131] Two commercial kits, API ZYM and API 20C AUX (Bio Merieux), were used together with a boric acid resistance test.

The API ZYM system was used to define the enzyme patterns. It consists of an array of miniaturised tests in plastic cupules able to detect the positive presence of nineteen enzymes: alkaline phosphatase, esterase, lipase esterase, lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. After inoculation of a standard suspension of the organism and incubation for 4 h at 37 °C, the colour reaction in each cupule was read according to the manufacturer's instruction.

The API 20C AUX is based on the ability to assimilate nineteen carbon sources: glucose, glycerol, 2-keto-D-gluconate, L-arabinose, xylose, adonitol, xylitol, galactose, inositol, sorbitol, methyl-D-glucoside, N-acethyl-D-glucosamine, cellobiose, lactose, maltose, sucrose, trehalose, melezitose and raffinose. These carbon sources and the inocula were prepared according to the manufacturer's instruction. The results were read by comparison of opacity with the control.

Boric acid resistance of the isolates was assessed by showing colony growth at a concentration of 1.8 mg/ml of boric acid incorporated into an agar plate.

8. Antifungal susceptibility testing

A total of 94 strains were tested in this study. The minimum inhibitory concentration (MIC) for ketoconazole of each isolate was determined using macrodilution method of National Committee for Clinical Laboratory Standards (NCCLS).[58] The broth dilution method was performed following

the NCCLS instruction. A working suspension of each inoculum was made by a 1:100 dilution of the 0.5 McFarland standard yeast suspension in 0.85% saline followed by a 1:20 dilution in RPMI broth. Two-fold dilutions of ketoconazole from 64 to 0.0075 μg/ml were prepared and inoculated with the working suspension. The mixtures were incubated at 37°C for 48 hours. The MIC was read at the concentration that produced an 80% reduction of turbidity compared to a drug-free control.

9. Genetic analysis

To study the genetic relatedness of *C. albicans*, Dendron® 3.0 software program (Technology Innovation Center, Oakdale, IA) was used according to Soll.[104] The RAPD fingerprinting patterns of each strain was compared and a similarity value (S_{AB}) was calculated based on the matching of fragment positions. The similarity values were then clustered arithmetically by the unweighed pair group method, and dendrograms were generated to visualize the relationships among isolates. The threshold of an S_{AB} at 0.80 is the most arbitrary one for clustering the similar strains because it is approximately midway between the measure of unrelatedness (0.69) and the threshold for high relatedness (0.90).[104]

Table 3 The summary of the concentrations of 7 random primers, $MgCl_2$ and the temperature profiles used in PCR analyses.

Primer and concentration	$MgCl_2$	Initial denaturation	Denaturation	Annealing	Extension	No.of cycles	Final extension
5′-ACA ACT CCTC-3′ (1μM)	1.5 mM	-	94°C 5 sec	36°C 30 sec	72°C 1 min	30	-
C1 (5′-ACG GTA CAC T-3′) (0.8 μM)	6 mM	-	94°C 1 min	36°C 1 min	72°C 2 min	45	72°C 10 min
C3 (5′-GTT TCC GCC C-3′ (0.8 μM)							
M13 (5′-GAG GGT GGC GGT TCT-3′) (10 ng/25 ml)	1.5 mM	97°C 3 min	93°C 20 sec	50°C 1 min	72°C 20 sec	40	72°C 5 min
PA03 (5′-AGT CAG CCAC-3′) (10 ng/25ml)	1.5 mM	97°C 3 min	93°C 1 min	36°C 1 min	72°C 2 min	45	72°C 5 min

Primer and concentration	$MgCl_2$	Initial thermal cycles				Following thermal cycles				Final extension
		Denaturation	Annealing	Extension	No. of cycles	Denaturation	Annealing	Extension	No. of cycles	
RSD11 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) (1 μM)	1.5 mM	94°C 30 sec	41°C 2 min	72°C 2 min	5	94°C 30 sec	47°C 2 min	72°C 2 min	45	72°C 10 min
RSD12 (5′-GGT CCG TGT TTC AAG ACG-3′) (1 μM)	1.5 mM									

For the dendrogram generation, a similarity coefficient based simply on band positions is firstly calculated. The data for two banding pattern (lanes A and B) can be synopsized by the binary value 0 and 1, where 0 indicates no band at a position and 1 indicates a band at that position. The value n_{AB} is the number of bands common in lane A and B (coded 1,1), *a* is the total number of bands in lane A not present in lane B (coded 1, 0), and *b* is the total number of bands in lane B not present in lane A (coded 0,1). The sample size (total number of band positions) in this case is n_{AB} + a + *b*. The total number of mismatchs is *a* + *b*. The coefficient of Jaccard (Sj) which has been widely used was applied in this study. Sj is computed by the formula:

$$Sj = \frac{n_{AB}}{(n_{AB} + a + b)}$$

It varies between 0 and 1.00. A measure of 0 reflects no common bands, while a measure of 1.00 reflects all common bands. Measures of 0.01 to 0.99 represent increasing degrees of commonness. All bands are treated equally in the computation regardless of differing patterns that may not contain the same number of bands. An increasing pattern complexity should provide increasing resolution. Therefore, the possible number of different Sj value increases as the patterns increase in complexity. For example of the Sj values in which pattern A contains one band and patter B contains one band, the possible Sj values in this case are either 1.00 (bands are common) or 0 (bands are not common). When pattern A contains two bands and pattern B contains two bands, the possible Sj values are 1.00 (both bands are common), 0.33 (one band is common), or 0 (no bands are common). To consider three bands for both pattern A and pattern B, the possible values in this case are 1.00 (all three bonds are common), 0.50 (two bands are common), 0.20 (one band is common), and 0 (no bands are common).

Thus, in the dendrogram, the two strains with the highest S_{AB} are grouped, with a branch-point corresponding to the S_{AB}. The program then

searches for the strain-strain or strain-unit pair with the next highest S_{AB} and groups them, with a branch-point corresponding to that S_{AB}. The process continues to include all strains. A unit can be two or more strains, and a branch point for a unit-strain or unit-unit is determined by the mean S_{AB} between each member of the unit and another strain or unit. Therefore, branch-points involving a unit are not as accurate as S_{AB} values calculated for two strains.

10. Relationship analysis

The relationship of genotype, biotype and antifungal susceptibility were assessed by mapping these phenotypes with the dendrogram which referred to genotypes. And the association these three typing methods was then statistically analyzed by Chi-square test.