CHAPTER IV

MATERIALS AND METHODS

Screening of the yeast isolate

Six clinical isolates from oral carvity of HIV-infected patients were recruited. Thepatients were treat with antifungal drug (Table 3). Their minimum inhibition concentration (MIC) were detected by using gradient concentration of fluconazole on strip called Epsilometer test (Etest) (ABbiodit, Sweden).

Table 3 . Drugs were treat in recruited patients

Strains of *C. albicans*	Drug and Dose of treatment
K 39.1	Diflucan 400 mg/day
K44.1	Diflucan50 mg/day
K49.1	Nizoral
K 51.1	Diflucan 400 mg/day
K 54.1	Sporal 200 mg/day
K 78.1	Diflucan

A fluconazole susceptible *C. abicans* strain, K44.1, from oral lesion of HIV infected patients with oral candidiasis was used in this study. Its minimum inhitory concentration by Epsilometer test (Etest) was 8 μg/ml. The range of susceptible range is ≤ 8 μg/ml while the susceptible dependent on dose (SDD) range and resistant range of this agent is 16-30 μg/ml and ≥ 64 μg/ml, respectively (73).

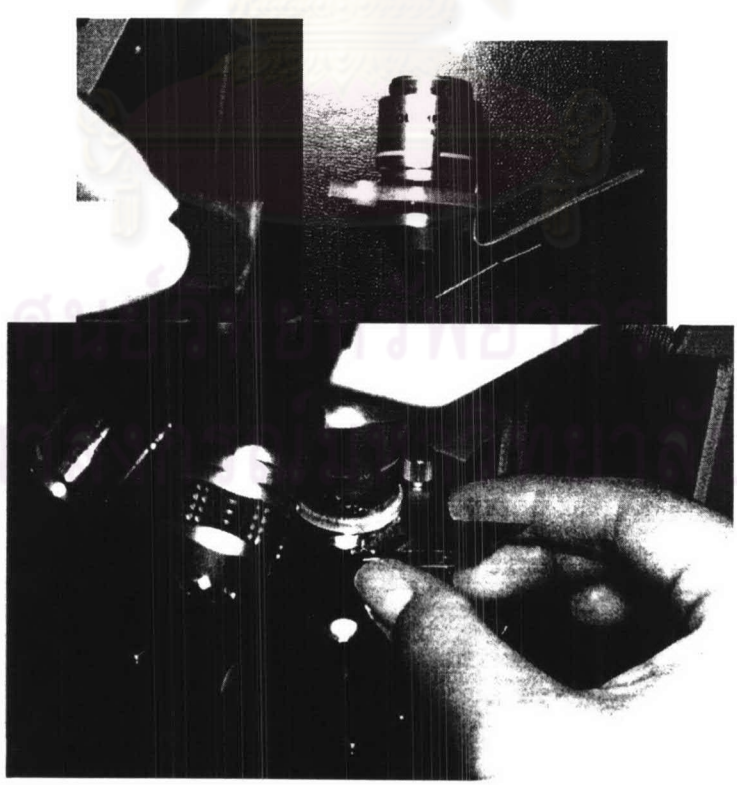
Single cell isolation

To avoid genetic diversity of yeast cells, the single cell isolation was performed. At first, the yeast at the exponential phase, 30°C for 24 hours, in Sabouraud dextrose broth (SDB) was prepared and streaked on a Sabouraud dextrose agar (SDA) plate.

1.The modified single cell isolation cell tool

Using light microscope which the modified single isolation tool (under support of Mr. Boonchuay Eampokalap, Bamrajnaradul Hospital) was attached with the low objective lens, the single cell was picked up and released onto another side of the SDA plate with free-cell area. In detail, the pasture pipette was stretched through the flame to make the string shape. Then, this pipette was attached with magnetic bar which was placed on the stainless steel plate. This plate was trapped with the low objective lens of microscope by rubber ring. (Figure 7)

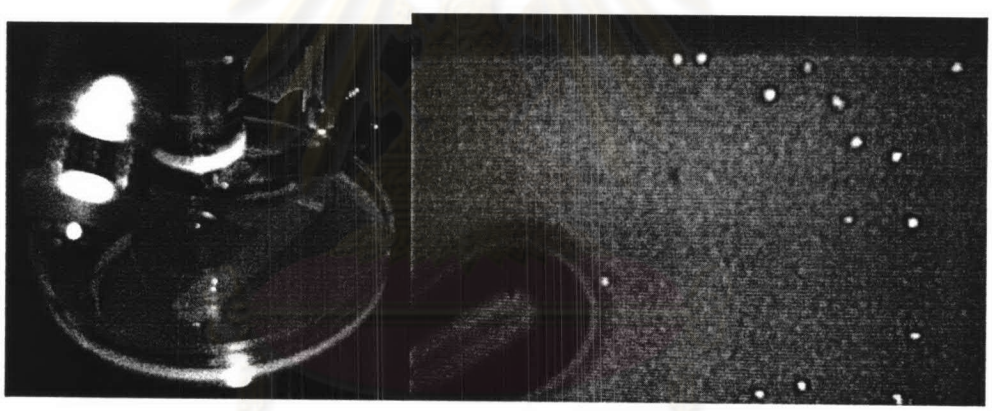
Figure 7 Demonstrated the tools of single cell isolation.



2. Single cell isolation

The diluted suspension of this *C. albicans* was streaked on SDA. Under microscope, the single cell on the streak line was pulled out by the tip of the pipette (Figure 8) and then placed on another area of SDA. The plate was incubated at 30°C for 24 hours. Each of the twenty-five single cells was grown overnight in SDB in separated tube. These cells were immediately used to determine its MIC against fluconazole again by broth dilution test and to perform the induction experiment. These cells were kept at -70°C

Figure 8 Demonstrate the single cell isolation method.



Fluconazole powder

Pure powder of fluconazole with the potency of 99.8 % provided by Siam Pharmaceutical Limitted. The drug was kept at -20°C until used.

Minimum Inhibitory concentration (MIC) Determination

1. Epsilometer test (Etest)

To determine the MIC of fluconazole, the exponential phase of each separated single cell at the concentration of 1-5 x 10^6 was prepared in SDB. This culture was spreaded on the surface of RPMI 1640 (Sigma, USA) plate. After that, the Etest strip

(ABiodisk, Sweden) with gradient concentration fluconazole from 0.016 μg/ml to 256 μg/ml was placed on the plate and the plate was incubated at 30 °C for 48 hours. The MIC was read at 80% of fungal inhibition growth

Reference strains, *C. parapsilosis* ATCC22019 was used as experimental control, medium and the strip test.

2. Broth Microdiluton test

The broth microdilution test in this study was followed the standard method from National Committee of Clinical Laboratory Standard (NCCLS) volume 17 number 9 in 1997: Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard (M27-A) (73)

Fluconazole stock solution preparation

The stock solution of fluconazole at the concentration of 640 μg/ml in sterile water was prepared. The amount of drug powder was determined as below.

$$\text{Weight (mg)} = \frac{\text{Volume (ml) X Desired concentration (μg/ml)}}{\text{Assay potency (μg/mg)}}$$

To obtain 640 μg/ml.stock solution, 0.026 mg of fluconazole powder was used to suspend in 4.0 ml

Working solution

The fluconazole stock solution was diluted 1:5 with RPMI 1640 medium to achieve 2 times strength needed for the broth microdilution test.

Broth dilution test

The range of fluconazole concentration to be tested were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 μg/ml . To perform the test, one hundred microliters of 2x concentration of each tested concentration were dispensed into the 96 sterile well plate (Nunc, Sweden). For example, in the plate row 3 contains the highest drug concentration and row 12 contains the lowest drug concentration and row 1 contains RPMI 1640 medium drug free

medium 200 µl for media sterility test. While row 2 contains 100 µl of RPMI 1640 drug free medium was added the yeast cell for growth control. Reading method or MIC point at 80% of fungal growth inhibition when compared with the reference strain *C. parapsilosis* ATCC22019.

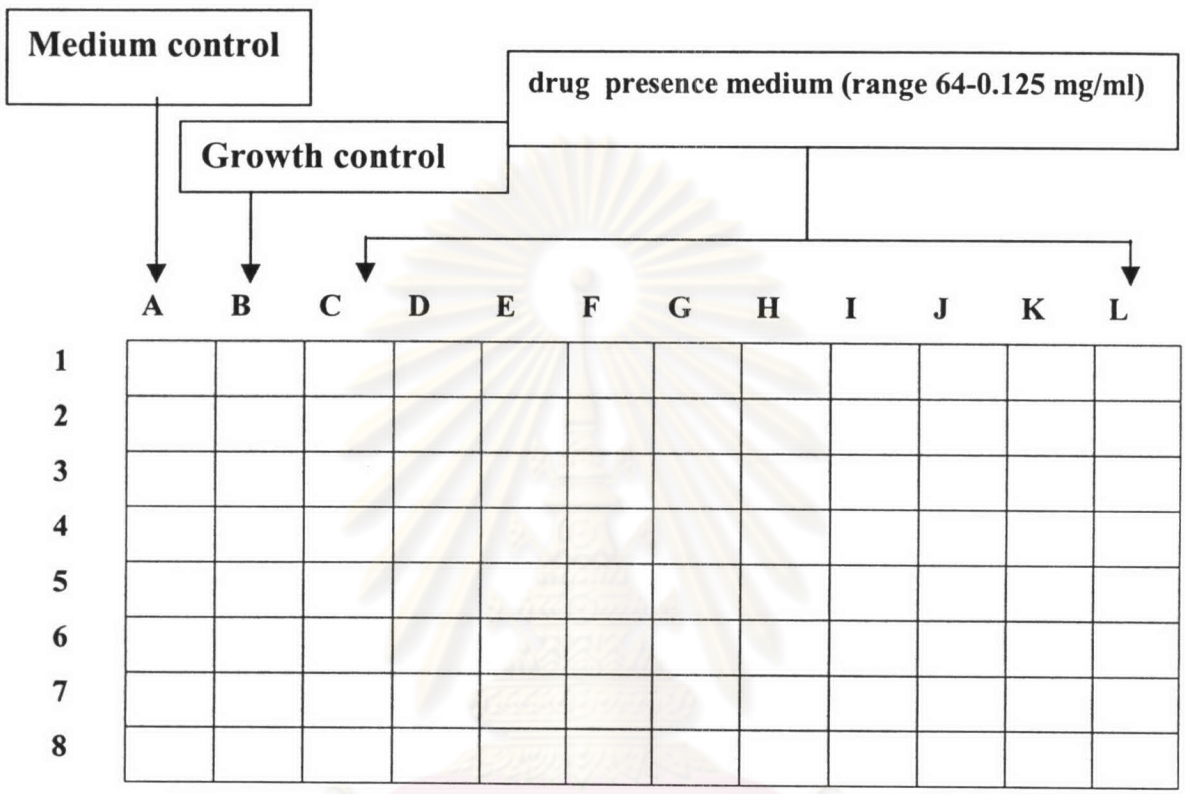
These trays were sealed, kept in plastic bag and stored frozen at –70 °C for up to 6 month without deterioration of drug potency.

Inoculum size Preparation

The exponential phase culture derived from each single cell from SDB was centrifuged to pack the cell, then washed with distilled water at least two times after that the cell density was adjusted to 0.5 McFarland in 0.85% sterile normal saline. This procedure will yield a yeat stock suspension of $1x10^6$ -$5x10^6$ cells/ml. The stock yeast suspension is mixed for 15 seconds with a vortex, diluted 1:50 and furthur diluted 1:20 with medium to obtain the 2 time test inoculum.

The inoculum is diluted 1: 2when the wells are incubated and the desired final inoculum size is acheive.The quality control, *C. parapsilosis* ATCC22019, are tested in the same manner and are included each time an isolated.The microdilution plate are incubated at 35 °C for 48 hours for the presence or absence of visible growth. The growth in each well is composed with that of the growth control (drug free medium) well. The minimum concentration inhibition (MIC) for azole as the lowest concentration which prominent decrease in turbidity is observed.

Figure 9 Preparation of broth microdilution plate



Drug resistant induction

After single cell of K44.1 strain was isolated and grown in SDB, MIC of each of 25 cells was determined again by Microdilution test paralleled with reference strains.

1. Resistant Induction

To induce drug resistance, two, three and four times concentration of that of the original MIC (8 μg/ml) was used in this experiment. Five single cells as five repeats were performed in each group.

1. Group 1 (No. 1-5) : Control group:
   - only RPMI 1640 broth without fluconazole.
2. Group 2 (No. 6-10) : Original MIC group:
   - RPMI 1640 broth with 8 μg/ml concentration of fluconazole.

3. Group 3 (No.11-15) : 2x of the original MIC
    - RPMI 1640 broth with 16 μg/ml concentration of fluconazole.
4. Group 4 (No.16-20) : 3x of the original MIC
    - RPMI 1640 broth with 24 μg/ml concentration of fluconazole
5. Group 5 (No.21-25) : 4x of the original MIC
    - RPMI 1640 broth with 32 μg/ml concentration of fluconazole

The same culture for MIC testing was also immediately used for the induction experiment.

To prepare the control group or group 1, 4.5 ml RPMI 1640 broth with 0.5 ml culture at the concentration of 0.5 McFarland (1-5 $x10^6$ cell per ml) was shaked at the speed of 150 round per minute, 30°C for 16 -18 hours. The second day, 0.5 ml of the first day culture was pipette to 4.5 ml fresh RPMI 1640 broth and cultures with the same condition. The same experiment as the second day was performed until the day of 60. The culture was picked up for determination of MIC, the resistant gene and the resistant genes' functions at 14, 29, 50 and 60 days.

To prepare the experiment for group 2, 4 ml of RPMI 1640 broth with 0.5 ml of 10x concentration of 8 μg/ml (80 μg/ml) as the base medium plus 0.5 ml culture at the concentration of 0.5 McFarland was well mixed and shaked at the speed of 150 rounds per minute (rpm), 30°C for 16 -18 hours. The second day, 0.5 ml of the first day culture was pipetted to 4.5 ml fresh base medium and cultured with the same condition. Everyday from the second day until the day of 60, the experiment was performed as the second day. The culture was picked up for determination of MIC, the resistant gene and the resistant genes' functions at 14, 29, 50 and 60 days.

The remain groups, group 3, 4, and 5 were also prepared the same as group 2 but with different fluconazole concentrations (Table 4) .

Table 4 Preparation of experiment for Fluconazole induction test

	RPMI 1640 broth (ml)	Culture* (ml)	Stock fluconazole (μg/ml)/ Amount (ml)	Final drug concentration (μg/ml)	Total volume
Group 1 (No.1 - 5)	4.5	0.5	--	--	5.0
Group 2 (No. 6 – 10)	4.0	0.5	80 / 0.5	8	5.0
Group 3 (No. 11 – 15)	4.0	0.5	160 / 0.5	16	5.0
Group 4 (No. 16 – 20)	4.0	0.5	240 / 0.5	24	5.0
Group 5 (No. 21 – 25)	4.0	0.5	320 / 0.5	32	5.0

* 0.5 McFarland turbidity

The induction experiment, from was grown overnight on SDB and streaked on SDA and incubated at 30°C for 48 hours. The induction experiment was started by stock was grown in RPMI 1640 broth with 8 μg/ml concentration of fluconazole by shaking at 150 rpm at 30°C for 16-18 hours. The second day, The induction procedure was performed by transferring 500 μl of the culture from that of the previous day to the 4500 μl of new RPMI 1640 broth everyday for 60 days. To examine the induction ability of the yeast, the culture was collected at each interval time, 14, 29, 49, and 59 day and kept at -70°C for investigate the MIC and determine the resistant genes.

***ERG 11* gene sequencing**

1. Genomic DNA preparation by Wizard™ Genomic DNA Purification Kits (Promega, USA)

Candida strains were grown overnight in 8 ml SDB at 30 °C on rotary shaker. Of each cell suspension, 0.5 ml was pelleted, washed three times in DW and resuspended in 300 µl cell lysis buffer with glass beads and then incubated at room temperature for 50 min on a shaker. After incubation, the mixture was centrifuged at 5,000 rpm for 0.5 min. The supernatant was transferred to a new tube and was centrifuged at 13,000 rpm for 3 min. After the supernatant was removed, 300 µl of nucleic acid lysis buffer was added to the sample and mixed. Protein precipitate buffer was added to the suspension at 100 µl mixed, and centrifuged at 13,000 for 3 min. The supernatant was transferred to a new tube, 500 µl of isopropanol was added, and the suspension was incubated at –20 °C for 30 min. The supernatant was then decanted, the extracted DNA was rinsed with 70% ethanol, dried at room temperature, and dissolved in 50 µl of sterile distilled water.

2.Polymerase chain reaction (PCR) amplification

PCR amplification was performed on total DNA with primers as described by Marichal *et al* (58)

CaERG2 5' TCATAACTCAATATGGCTATTGTTG 3'

CaERG3 5' GAAAGTTGCCGTTTTATTAAAACATAC3'

The 1.6 kb of DNA fragment amplified with these primers is the *ERG11* : cytochrome P450 14-αdemethylation gene. Reaction volume of 20 µl contained 2 µl of 10xPCR buffer, 5 µl of a deoxynucleoside triphosphate mixture (2 mM each dNTP), 1 µl of each primer (5 pmol of each primer), 0.5 µl (5.0U) of Taq DNA polymerase (Promega,USA) , and 3 microlitres of template DNA, with the remaining volume consisting of distilled water. Negative controls were performed with sterile distilled water in place of the template DNA. Amplification reaction were carried out in a Hybaid Expresss (Hybaid, , USA) with initial denaturation for 1 min at 95 °C, followed by 35

cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final extension for 5 min at 72 °C. The size and purity of the PCR products were confirmed by 1% agarose gel electrophoresis.

3. Purification of PCR products

The PCR products of *C. albicans* were purified by QIAquick PCR purification kit as described by the manufacturer (QIAGEN, Germany). The QIAquick system is a combination of spin column technology with the selective binding properties of a uniquely designed silica-gel membrane. DNA was absorbed to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities were efficiently washed away and the pure DNA was then eluted with Tris buffer. The concentration of DNA was measured by spectrophotometer (Bio-Rad, U.S.A) and approximately adjusted to 100 ng µl for preparation of sequencing reaction.

4. Sequencing reaction preparation

Approximately 100-150 ng of DNA sample was sequenced using CaERG1 5' TCAATATGGTATTTGTTG 3' and CaERG4 5' GAGCAAATGAACGGTC 3'TCCGTAGGTGAACCTGCGG-3' with ABI prism Big Dye terminator cycle sequencing ready reaction kit (Perkin Elmer Corporation, USA). The sequence reaction required 4 µl of BigDye terminator as described by manufacturer, with 3.2 pmole of primer and 100 ng of purified PCR product template in a total volume of 10 µl The sequencing cycle was performed using 25 cycles of denaturing step: 10 sec at 96 °C, annealing step: 5 sec at 50 °C, and extension step: 4 min at 60 °C.

5.Ethanol-Sodium Acetate Precipitation

The PCR products were precipitated using 2 µl of 3 M sodium acetate (NaOAc), pH 4.6 and 50 µl of 95% ethanol (EtOH) for each sequencing reaction as described by manufacturer 6. The precipitated DNA was stored at –20 °C until used. The precipitated

DNA was subjected to automated sequence analysis on ABI prism 310 automated sequencer (Perkin Elmer Corporation, USA).

6.Analysis

The nucleotide sequences were compared with *C. albicans* reference strain by using ClustalX program.

mRNA expression

1.Isolation of total RNA from yeast cells (74).

An overnight culture is diluted in the medium and measured an optical density at 600 nm of 1-3. The cultured cells are collected by centrifugation (2500g, 5 min at 4 °C) and resuspended in 10 ml sodium acetate buffer and transferred to 50 ml plastic tubes. One-tenth volume of 10% SDS and 1.2 volumes phenol (65 °C) are added to the cell suspension and immediately transferred to the water bath shaker set at maximum speed for 4 min at 65 °C and cooled down quickly to room temperature in dry-ice ethanol water bath. The organic and aqueous phase are separated by 10 min centrifugation at room temperature. The remaining aqueous phase the same volume of prewarmed phenol is added and centrifugation is repeated as describe above. The upper phase is transferred to new plastic tube and one volume of chloropane is added , the sample is vortexed for 2 min and centrifugation again. The aqueous phase is transferred to a new tube and extracted once with 1 volume chloroform-isoamylalcohol (24:1). The upper phase is transferred to new tube, 1/10 volume of 3 M sodium acetate and 3 volume ethanol are added after that briefly vortexed. The RNA is precipitate at –20 °C for several hours.

2. Probe preparation

The probe for Northern hybridization was performed by PCR amplification from genomic DNA.

ERG11 gene

ERG11.1 ATG GGT GGT CAA CAT ACT TC

ERG11.2 CTT CAT CAG AAG AGT TAA ATC

CDR1 gene

CDR1.0 5'-TTA TGT CCA ACA ACA AGA TGT TC-3'

CDR1.1 5'-CTG TAC ATG AAA ATC CAA AAT-3'

MDR1 gene

BENRN 5'-AAA AGC TTA TGG ATT ACA GAT TTT TAA GAG-3'

BENRC 5'-AAA AGC TTC TAA TTA GCA TAC TTA GAT CTT-3'

CDR2 gene

CDR2.1 5'-GGT ATA TAA ACT GGA CAA CA-3'

CDR2.2 5'-CGG AAT CTG GGT CTA ATT GT-3'

3.Probe labeling

DNA probe is random primed labeling with DiGOXIGENIN-11dUTP using DIG-High Prime (Roche, Germany). One microgram probe was added to the sterile double distilled water to reach final volume of 16 µl in reaction vial. The DNA was denatured by heating in boiling water bath for 10 minutes and quickly chilled in an ice water bath. The DIG-High Prime was mixed throughly and added 4 µl to the denature DNA, mixed and centrifuged briefly. After that, the reaction vial was incubated for 20 hours at 37 °C. The reaction was stopped by adding 2 µl 0.2 M EDTA (pH8.0). The concentration of DIG-labeled DNA was evaluated by DIG Quantification Teststrips (roche, Germany).

4. Northern dot blot

10 µg of total RNA were dotted on the nylon membrane (Hybond-N+, Amersham pharmasia biotech, USA) and dried at room temperature. The membrane was irritated with UV light (302 nm) for 4 minutes for fix RNA. The blotted membrane is now ready for hybridization or stored at 4 °C until used.

5. Northern hybridization

The hybridization was performed according to the instruction manual of DIG high prime labeling and detection starter kit I (Roach, Germany). DIG Easy Hyb was preheated to hybridization temperature (50 °C). The membrane was prehybridized with preheated DIG Easy Hyb at 50 °C for 30 minutes in hybridization bottle with gentle agitation in hybridization oven (Thermo Hybaid, USA). The DIG-labeled probe was denatured by boiling for 5 minuted and rapidly cooling in ice/water, then added to preheated DIG Easy Hyb (3.5 ml/100 cm^2 membrane) and mixed well but avoid foaming. The preehybridization solution was pored off and the probe/hybridization mixture was added to the bottle and the hybridization was performed overnight with gentle agitation in hybridizatioon oven (Thermo Hybaid,USA) at 50 °C. The membrane was removed from the bottle to a box. The membrane was washed twice in ample 2X SSC containing 0.1% SDS at 15-25 °C under constant agitation. For low stringency condition, the membrane was washed twice in ample0.5X SSC containing 0.1% SDS at 68 °C under constant agitation. After hybridization and stringency washes, the membrane-bound labeled probe was detected by color detection with NBT/BCIP. The color was detected by immunological detection that all incubations should be performed at 15-25 °C with gentle agitation. Briefly, the membrane was rinsed briefly in Washing Buffer. The membrane was incubated for 30 minutes in Blocking Solution (100ml/100 cm^2 membrane) and incubated in Antibody Solution (150mU/ml, 20 ml/100 cm^2 membrane)for 30 minutes. After that, the membrane was washed in Washing Buffer (100ml/100 cm^2 membrane) for 15 minutes and equilibrated in Detection Buffer (20ml/100 cm^2 membrane) for 2-5 minutes. Incubate membrane in freshly prepared color substrate solution (NBT/BCIP) in appropriate container in the dark for overnight (16h). The reaction was stopped when desired spot or band intensities was achieved, by washing the membrane for 5 minutes with sterile double distilled water (50ml/100 cm^2 membrane) . The presence of mRNA was detected by color development.