CHAPTER IV

MATERIALS AND METHODS

Yeast isolates

A total of 197 isolates of *Candida* obtained from oral lesion of 98 HIV-infected patients with oral candidiasis were included in this study. All patients were visited the Outpatients Clinic at King Chulalongkorn Memorial Hospital from March, 1996 to April, 1997. HIV-infected patients were divided into two groups, AIDS related complex (ARC) and acquired immunodeficiency syndome (AIDS), according to the clinical criteria of AIDS definition in 1993 (132). Samples were obtained by swabbing the oral mucosa of the subjects with sterile cotton swabs, plated onto Sabouraud Dextrose Agar (SDA) and incubated at 30 °C for 48-72 hours. At least 2 colonies from each positive oral sample were picked up and streaked for purification and isolation on fresh plate of SDA. Isolates were maintained at –70 °C until used.

Identification of yeast isolates

A. Conventional methods

The morphology of yeast isolates was initially confirmed by microscopy. After that, yeast isolates was identified by conventional methods, both morphological and biochemical studies. There were germ tube test, chlamydospore production, carbohydrates assimilation and carbohydrates fermentation.

1. Morphological examination

1.1 Germ tube test

The strains to be tested for germ tube formation were grown on SDA at room temperature (24-28°C) for 24 hours. The yeast colonies were inoculated into 0.5 ml serum and incubated at 37 °C for 3 hours. The cells with germ tubes was determined microscopically at the end of the inoculation period. Most of the isolates are likely to be *C. albicans* if germ tubes form. However, several other species:

C. stellatoidea, *C. utilis*, *C. tropicalis*, and *Schizosaccharomyces fragilis*, are also able to form germ tube but with longer periods. Some exceptional strains of *C. albicans* without germ tube formation are also founded.

1.2 Chlamydospore formation

Chlamydospore formation was determined at room temperature on glutineous rice agar supplemented with 1% (v/v) Tween 80. The tests were performed in accordance with standard procedures. The small amount of yeast colony on SDA was transferred onto the surface of the glutineous rice agars and covered by a flamed coverslip over the streak and incubated at 25 °C for 48 hours. *C. albicans* produces the chlamydospores. Occasionally, *C. stellatoidea* and *C. tropicalis* produce chlamydospores.

2. Biochemical examinations

2.1 Carbohydrate assimilation

A yeast strain was cultured in Sabouraud dextrose broth (SDB) for 18-24 hours at 30 °C under shaking at 150 cycles/minute. After that, the cells was harvested by centrifugation at 1,500 x g and washed twice with sterile distilled water. To prepare the inoculum size, a suspension of yeast cell was equivalent to a McFarland Standard number 4 – 7 using sterile distilled water. This yeast suspension was spreaded onto assimilation media by a swab. Disks impregnated with 10% solutions of the following carbohydrates; glucose, maltose, sucrose, lactose, galactose, trehalose, inositol, mellibiose, cellibiose, raffinose, dulcitol, and xylose, were placed on to the plate. All of these plates were incubated at 24 - 30 °C for 24 - 72 hours. Growth around the disk indicates the utilization of the carbohydrate contained in the disk (positive result). The disk without any carbohydrate but distilled water was included as negative control.

2.2 Carbohydrate fermentation test

The yeast suspension was prepared as same as the inoculum suspension used for assimilation test. Then, 0.2 ml of the suspension was added to each 16x125 mm tube containing 10 ml of broth with 1% sugars; glucose, sucrose, lactose, maltose, galactose, and trehalose with Durham tube and covered the surface

of medium with 2 ml parafin oil. All the tubes were incubated at 37 °C for 48 hours. The indicator of bromcresal purple changed from purple to yellow indicated the acid production. Acid or acid with gas were read as a positive reaction.

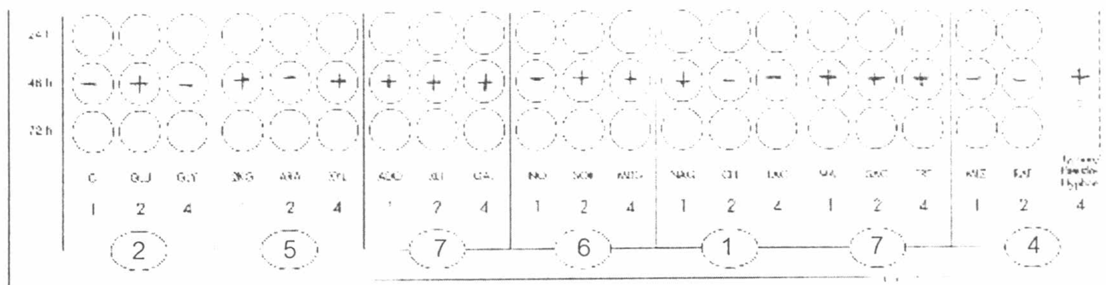
To determine the species of *Candida*, the pattern of carbohydrate assimilation and fermentation were compare with Table 5 (32). The unidentified species were examined by API 20C AUX commercial kit (API 20C AUX, bioM'erieux, France). Also, some of the identified yeast with conventional method were verified by this kit.

2.3 API 20C AUX Yeast identification system (bioM'erieux, France)

API 20C AUX is a comercial kit for the precise identification of the most frequently encountered yeasts in clinical microbiology. The API 20C AUX strip consists of 20 cupules containing dehydrated substrates which enable the performance of semi-solid minimal medium and the yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source. The reactions are read by comparing them to growth controls and identification is obtained by referring to the Analytical Profile Index (API) or using the identification software.

Test names

GLU	Glucose	MDG	alpha-Methyl-D-Glucoside
GLY	Glycerol	NAG	N-Acetyl-D-Glucosamine
2KG	2-Keto-D-Gluconate	CEL	Cellibiose
ARA	L- arabinose	LAC	Lactose
XYL	D-xylose	MAL	Maltose
ADO	Adonitol	SAC	Saccharose/Sucrose
XLT	Xylitol	TRE	Trehalose
GAL	Galactose	MLZ	Melezitose
INO	Inositol	RAF	Raffinose
SOR	Sorbitol	Hyphae/pseudohyphae	



2 576 174 *C. albicans*

Figure 7. The API 20C AUX (bioM'erieux, France) sheet results

Table 5. Cultural and biochemical characteristics of yeasts frequently isolated from clinical specimens[a]

Species	Growth at 37°C	Pellicle in broth	Pseudo- or true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of[b]:												Fermentation of:						Urease	KNO_3 utilization	Phenol oxidase	Ascospores
							Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose				
Candida albicans	+	−	+	+	+	−	+	+	+*	−	+	−	−	−	+	−	+	−	F	F	−	−	F	F	−	−	−	−
C. catenulata	+*	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	F*	−	−	−	−	−	−	−	−	−
C. guilliermondii	+	−	+	−	−	−	+	+	+	−	+	+	+	−	+	+	+	+	F	−	F	−	F*	F	−	−	−	−*
C. kefyr	+	−	+	−	−	−	+	−	+	+	+	−	+*	−	+*	+	−*	−	F	−	F	F*	F	−	−	−	−	−
C. krusei[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	+*	−	−	−*
C. lambica	+*	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	F	−	−	−	−	−	−	−	−	−*
C. lipolytica[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−*
C. lusitaniae[d]	+	−	+	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	F	−	F	F	−	−	−	−*
C. parapsilosis[e]	+	−	+	−	−	−	+	+	+	−	+	−	−	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
C. rugosa	+	−	+	−	−	−	+	−	−	−	+	−	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−	−
C. tropicalis[d,e]	+	+	+	−[f]	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F	F	−	F	F	−	−	−	−
C. zeylanoides	−	−*	+	−	−	−	+	−	−	−	−*	−	−*	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Torulopsis glabrata	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	F	−	−	−	−	F	−	−	−	−
T. candida	+	−	−	−	−	−	+	+	+	+*	+	+	+	−	+	+	+	+*	W	−	W	−	−	W	−	−	−	−*
T. pintolopesii[g]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−	−	−
Cryptococcus neoformans	+	−	R	−	−	+	+	+	+	−	+	−	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	+	−
Cryptococcus albidus	−*	−	−	−	−	+	+	+	+	+*	+*	+	+	+	+	+	+	+*	−	−	−	−	−	−	+	+	−	−
Cryptococcus laurentii	+*	−	−	−	−	+	+	+	+	+	+	+*	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus luteolus	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
Cryptococcus terreus	−*	−	−	−	−	+	+	+*	−	+*	+*	−	+	+	+	−	+	−*	−	−	−	−	−	−	+	+	−	−
Cryptococcus uniguttulatus	−	−	−	−	−	+	+	+	+	−	−*	−	−*	+	+	+*	−*	−	−	−	−	−	−	−	+	−	−	−
Rhodotorula glutinis	+	−	−	−	−	−*	+	+	+	−	+*	−	+	−	+	+	+	−	−	−	−	−	−	−	+	+	−	−
Rhodotorula rubra	+	−	−	−	−	−*	+	+	+	−	+	−	+*	−	+	+	+	−	−	−	−	−	−	−	+	−	−	−
S. cerevisiae	+	−	−*	−	−	−	+	+	+	−	+	−	−	−	−	+	+*	−	F	F	F	−	F	F*	−	−	−	+
H. anomala	+*	−	−	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F*	F	−	F	−	−	+	−	+
Trichosporon beigelii	+*	+	+	−	−	−	+	+	+	+	+*	+*	+*	+	+	+*	+*	+*	−	−	−	−	−	−	+*	−	−	−
T. pullulans	+*	+	+	−	−	−	+	+	+	+*	+	+*	+	+*	+*	+*	+	−	−	−	−	−	−	−	+	+	−	−
G. candidum[h]	−*	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
B. capitatus	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P. wickerhamii[h]	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−

[a]*, Strain variation; R, rare; F, sugar is fermented (i.e., gas is produced); W, weak fermentation. Based on data from Barnett et al. (6) and Kreger-van Rij (44).

[b]In these columns, + indicates growth greater than that of the negative control.

[c]*C. lipolytica* assimilates erythritol; *C. krusei* does not. Maximum growth temperatures are 43 to 45°C for *C. krusei* and 33 to 37°C for *C. lipolytica*.

[d]*C. lusitaniae* assimilates rhamnose; *C. tropicalis* usually does not.

[e]*C. parapsilosis* assimilates L-arabinose; *C. tropicalis* usually does not.

[f]Rare strains of *C. tropicalis* produce teardrop-shaped chlamydospores.

[g]*Torulopsis pintolopesii* is a thermophilic yeast capable of growth at 40 to 42°C.

[h]Not yeasts but may be confused with several yeast genera.

B. Molecular techniques

1. Electrophoretic karyotyping

1.1 Preparation of yeast chromosomal DNA for Pulsed Field Gel Electrophoresis (PFGE)

The sample plug containing yeast chromosomal DNAs for PFGE were prepared by the method of Iwaguchi et al. (30) with the following modifications. A yeast strain was grown for 18-24 hours at 30 °C under shaking at 150 cycles/min in 4 ml Sabouraud dextrose broth (SDB). Early stationary phase cells were collected by centrifugation at 1,500 x g for 2 min at room temperature. The cells were washed twice with 1 ml 50 mM sodium EDTA (pH 7.5) and collected by centrifugation as described above. Yeast cells were suspended in 0.5 ml 50mM Tris-HCl (pH 7.5). A 0.08 ml portion of the suspension was mixed with 0.03 ml lysing enzyme solution (5 mg ml^{-1}; Sigma, USA) in 50 mM Tris-HCl (pH 7.5) and then 0.3 ml 1% (w/v) low melting temperature agarose (USB, Spain) dissolved in 0.125 M sodium EDTA (pH 7.5) was added. After gentle mixing, the solution was pipetted into a mould chamber (Bio-Rad, Canada) and allowed to solidify at 4 °C for 1 hours. The solidified agarose plugs were placed in a microtube containing 1 ml LET buffer (450 mM EDTA (pH 7.5), 45 mM Tris-HCl (pH 7.5), 650 mM 2-mercaptoethanol) and incubated for 18-24 hours at 37 °C, and then transferred agarose plugs to NDS buffer (480 mM EDTA (pH 9.0), 48 mM Tris-HCl (pH 8.3), 0.57 mg/ml sodium dodecyl sulphate, 0.16 mg/ml proteinase K). After 18-24 hours incubation at 55 °C, the NDS buffer was discarded and the plugs were washed three times with 50 mM sodium EDTA (pH 9.0). The agarose plugs were either analyzed by PFGE or stored at 4 °C in 50 mM sodium EDTA (pH 9.0) until used.

1.2 Pulsed Field Gel Electrophoresis (PFGE)

Yeast chromosomal DNA was separated by electrophoresis on the CHEF (Contour Clamped Homogeneous Electric Field) DRIII instrument (Bio-Rad, Canada) with a hexagonal electrode array. Electrophoresis was carried out by using 0.5 X TBE (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA) as running buffer that maintained at 14 °C. DNA samples in the sample plugs were applied to 0.8 % (w/v) agarose gels (Gibco BRL, USA) in running buffer. The running condition

included a 300 s switching time at 140 V for 24 hours followed by a 1200 s switching time at 80 V for 48 hours as described previously (30). *C. albicans* FC18 and *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad, Canada) was used as the size reference markers. After electrophoresis, gels were stained with 0.5 µg of ethidium bromide per ml for 30 minutes, destained with distilled water for 30 minutes, observed under UV light (302 nm) on UV transilluminator (Gel doc 2000, Bio-Rad, Canada) and photographed using thermal printer (Mitsubishi model P91, Japan). The electrophoretic karyotype patterns were compared by Quantity One sorfwere (Bio-Rad, Canada). To distinguish the different strains of *C. albicans*, restriction fragment length polymorphism with *Sma*I followed by Southern hybridization with repeating sequence, RPS, as a probe were performed. These method was confirm the strains with same karyotype from each individual patients.

2. Restriction fragment length polymorphism (RFLP)

This was performed as described previously (133). Total chromosomal DNA of *C. albicans* was digested with the restriction endonuclease *Sma* I. The sample plugs for PFGE were cut into small pieces, 3 x 10 mm, and washed three time with sterile distilled water. This gel was equilibrated in the 1x concentration of restriction digestion buffer (150 µl) for 30 minutes at room temperature. Then the plugs was transferred to 150 µl of fresh restriction buffer that contained 30U *Sma* I (Promega, USA) and incubated overnight at 37 °C. The digested plugs were washed twice with sterile distilled water and this digested DNA was fractionated by PFGE. Electrophoresis of DNA fragments was carried out on 1% (w/v) agarose gels in 0.5 X TBE with a ramp of 20 to 100 s switch time at 180 V (5.2 V/cm) for 14 h at 14 °C. *Hin*d III digested of bacteriophage lambda DNA (Gibco BRL, USA) and *S. cerevisiae* chromosomal DNA (Bio-Rad, Canada) were included into each gel as molecular size standard. The gel were stained with 0.5 µg of ethidium bromide per ml for 30 minutes, destained with distilled water for 30 minutes, observed under UV light (302 nm) on UV transilluminator (Gel doc 2000, Bio-Rad, Canada). Thermal printer (Mitsubishi model P91, Japan) was used to assess loading and separation.

3. Southern blot and Southern hybridization

3.1 Probe preparation for Southern hybridization

The specific DNA for strain differentiation of *C. albicans*, repeat sequence or RPS, was used for hybridization.

a). Extraction and purification of plasmid DNA

E. coli DH5α carrying plasmid pRPS102, containing RPS102 gene was used. The extraction and purification of plasmid DNA was performed according to Maniatis *et al* with the following modification. Briefly, to prepare the plasmid, a single *E. coli* DH5α transformant colony was inoculated into 50 ml of LB (Luria-Bertani) medium containing ampicilin (50 μg/ml). The culture was incubated overnight at 37 °C with vigorous shaking (300 cycles/minute on rotary shaker). The bacterial cells were harvested from a 50 ml culture by centrifugation at 6,000 x g for 15 minutes at 4 °C. The bacterial cells were lysed by Alkali lysis method. The bacterial pellets were resuspened in 4 ml of ice cold solution I (25 mM Tris-HCl (pH 8.0), 10 mM sodium EDTA (pH 8.0), 50 mM glucose) by vigorous vortexing. After that, 8 ml of a freshly prepared solution II (0.2 N NaOH, 1% SDS) was added. The contents were mixed gently by inverting the tube several times and stored the tube on ice. Six ml of ice cold solution III (3 M sodium acetate, pH 5.2) was added and mix by shaking several times and stored the tube on ice for 3-5 min. The material lysate was centrifuged at 10,000 x g for 10 minutes, 4 °C in refrigerator centrifuge (Kubota, Japan). The supernatant was transferred to a fresh tube. The supernatant was extracted with an equal volume of phenol:chloroform (1:1) and the contents of the tube was mixed until an emulsion forms. After centrifugation at 12,000 x g for 2 minutes at 4 °C, the upper aqueous phase was transferred to a fresh polypropane tube. The interface and lower organic phase was discarded. The double stranded DNA was precipitated by adding 2 volumes of ice cold isopropanol at room temperature, vortexing and allowing the mixture to stand for 2 minutes at room temperature. After that, the tube was centrifuged at 12,000 x g for 5 minutes at 4 °C and the supernatant was discarded. The tube was standed in an inverted position on a paper towel to allow all of the fluid to drain away. The pellet of double-stranded DNA was rinsed with 70% ethanol at 4 °C and the pellet of nucleic acid was dried in the air. The nucleic acid was redissolved in 50 μl of distilled water. Four ml of Dnase-free

Rnase A (10 mg/ml) was added to the nucleic acid and the tube was incubated for 30 minutes at 25 °C. After that, DNA solution was extracted with phenol:chloroform (1:1) according to the above methods. The nucleic acid of plasmid RPS102, was redissolved in 50 μl of distilled water and stored the DNA at –20 °C.

b). Probe (RPS102) purification

The plasmid pRPS102 was digested with *Pst* I restriction enzyme (Gibco BRL, USA) and incubate at 37 °C for 4 hours. DNA fragments were separated by agarose gel electrophoresis. The gel was stained with 0.5 μg of ethidium bromide per ml for 30 minutes, destained with distilled water for 30 minutes, observed under UV light (302 nm) on UV transilluminator (Gel doc 2000, Bio-Rad, Canada). The probe DNA was purified from gel bands by GFX™ PCR DNA and Gel Band Purification Kit (Amersham pharmacia biotech, USA). Briefly, the slice of agarose containing the RPS102 DNA band (2.1 kb) was excised by using a clean razor blade. Then, the slice was cut into several smaller pieces and transferred to the 1.5 ml microcentrifuge tube. Ten μl of Capture Buffer was added for each 10 mg of gelslice (maximum colume capacity is 300 μl of Capture Buffer added to a 300 mg gel slice) and the tube was mixed by vortexing vigorously. The mixture was incubated at 60 °C until the agarose is completely dissolved (5-15 minutes). After the agarose was completely dissolved, centrifuged briefly to collect the sample at the bottom of the tube. The sample was transferred to the GFX Column placing in a collection tube and incubated at room temperature for 1 minute. The microcentrifuge was centrifuged at full speed (10,000 to 16,000 x g) for 30 seconds. The flow-through was discarded by emptying the Collection Tube and the GFX Column was placed back inside the Collection Tube. Five hundreads microliter of Wash Buffer was added to the column and their was centrifuged at full speed for 30 seconds. The Collection Tube was discarded and the GFX Column was transferred to a new 1.5 ml microcentrifuge tube. Fifty microliter of sterile double distilled water was applied directly to the top of the glass fiber matrix in the GFX Colume. The sample was incubated at room temperature for 1 minute and centrifuged at full speed for 1 minute to recover the purified DNA.

c). Probe labeling

DNA probe (RPS102) is random primed labeling with Digoxigenin-11-dUTP using DIG-High Prime (Roche, Germany). One microgram RPS102 probe was added to the sterile double distilled water to reach a final volume of 16 μl in a reaction vial. The DNA was denatured by heating in a boiling water bath for 10 minutes and quickly chilled in an ice water bath. The DIG-High Prime was mixed thoroughly and added 4 μl to the denatured 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 (pH 8.0). The concentration of DIG-labeled DNA was evaluated by DIG Quantification Teststrips and DIG Control Teststrips (Roche, Germany).

3.2 Southern blot

The gel from restriction digestion of total chromosomal DNA were washed twice with distilled water. Then, the gel was gently shaked in 0.25 N HCl for 15 minutes to depurinate the digested DNA. Before denaturing the DNA in 0.5 N NaOH, the gel was rinsed twice in deionized water. Then, the DNA was blotted to nylon membrane (Hybond-N^+, Amersham pharmacia biotech, USA) in 10x SSC for 90 minutes at 5 inches Hg by a vacuum blotter model 785 (Bio-Rad, Canada). The blotted membrane was soaked in 2x SSC for 5 minutes and dried at room temperature. The membrane was irradiated with UV light (302 nm) for 4 minutes to fix DNA. The blotted membrane is now ready for hybridization or stored at 4 °C until used. To verify the blotting system, the gel was restained with ethidium bromide for 30 minutes and destained with distilled water for 30 minutes, observed under UV light (302 nm) on UV transilluminator (Gel doc 2000, Bio-Rad, Canada).

3.3 Southern 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 (10ml/100cm^2 filter) was preheated to hybridization temperature (39 ^{o}C). The membrane was prehybridized with preheated DIG Easy Hyb at 39 ^{o}C for 30 minutes in hybridization bottle with gentle agitation in hybridization oven (Thermo Hybaid, USA). The DIG-labeled RPS102 probe (about 25 ng/ml) was denatured by

boiling for 5 minutes 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 forming. The prehybridization solution was poured off and the probe/hybridization mixture was added to the bottle and the hybridization was performed overnight with gentle agitation in hybridization oven (Thermo Hybaid, USA) at 39 °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 conditions, the membrane was washed twice in ample 0.5X SSC containing 0.1% SDS at 65 °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 (1-5 minutes) in Washing Buffer. The membrane was incubated for 30 minutes in Blocking Solution (100 ml/100 cm^2 membrane) and incubated in Antibody Solution (150 mU/ml, 20 ml/100 cm^2 membrane) for 30 minutes. After that, the membrane was washed twice in Washing Buffer (100ml/100 cm^2 membrane) for 15 minutes and equilibrated in Detection Buffer (20 ml/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 (16 h). The reaction was stopped when desired spot or band intensities were achieved, by washing the membrane for 5 minutes with sterile double distilled water (50 ml/100 cm^2 membrane).

Etest susceptibility testing for yeasts

Susceptibility of *Candida* spp. was testing with 5 antifungal drugs (amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole). The Etest was performed according to the manufacturer's instructions. The test medium was RPMI-1640 (Angus Biochemicals, USA) agar (1.5%) buffered to pH 7.0 with morpholine propanesulfonic acid (MOPS) and supplemented with glucose (2%). The inoculum was prepared from 24 hours cultures of *Candida* spp. in 3 ml SDB. Cell inoculums were suspended in 0.85% sterile normal saline and adjusted to a concentration corresponding to a 0.5 McFarland standard. The inoculum was diluted 1:5 in sterile 0.85% NaCl, and the surface of medium was flooded with the suspension. The excess was poured off, and the plate was let to dry before the Etest

strips (AB Biodisk, Sweden) were applied. The plates were incubated at 35 °C and read at 48 hours. The Etest MIC was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal strip followed Etest Reading Guide for Yeasts (Etest technical guide 4, AB Biodisk)

C. krusei ATCC 6258, *C. parapsilosis* ATCC 22019, and *C. albicans* ATCC 90028 were included as quality controls. The MICs of quality control strains were within the control ranges for Amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole as established by AB Biodisk (Table 6). The criteria for interprete the results of antifungal susceptibility testing were according to the NCCLS document M27-A (Table 7).

Table 6. Etest quality control specifications for antifungal susceptibility testing

Antifungal agents	**MIC (µg/ml) for:**		
	***C. krusei* ATCC 6258**	***C. parapsilosis* ATCC 22019**	***C. albicans* ATCC 90028**
Amphotericin B (AP)	0.5 – 2	0.25 – 2	0.25 – 2
Flucytosine (FC)	≥32	0.125 – 0.5	0.25 - 2
Fluconazole (FL)	≥256	2 - 16	0.125 – 1
Itraconazole (IT)	0.125 – 0.5	0.064 – 0.25	0.064 – 0.25
Ketoconazole (KE)	0.25 - 1	0.032 – 0.125	0.008 – 0.064

Table 7. NCCLS M27-A MIC interpretive criteria

Antifungal agents	**MIC (µg/ml) for:**			
	Susceptible	**Susceptible-Dose Dependent (S-DD)**	**Intermediate**	**Resistant**
Amphotericin B (AP)	≤0.5	-	-	-
Flucytosine (FC)	≤4	-	8 – 16	≥32
Fluconazole (FL)	≤8	16 – 32	-	≥64
Itraconazole (IT)	≤0.125	0.25 – 0.5	-	≥1
Ketoconazole (KE)	≤0.5	-	1	≥2